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Biochemical characterization of the human RECQ1 helicase, a molecule that physically interacts with human replication protein A.

Sheng CUI

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Supervisors:

Dr. Alessandro VINDIGNI

Prof. Arturo FALASCHI

External examiner:

Prof. Karl-Peter HOPFNER

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SISSA – Via Beirut 2-4 – 34014 TRIESTE – ITALY

To my dearest Dan Dan, Miss DAI Yangguang
I love you forever

To Prof. JIN Qi
Without your help, I wouldn't have what I have today.

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1. Introduction

1.1 Overview of DNA helicases

About 50 years ago, James Watson and Francis Crick revealed that the double helical structure of DNA consists of two right-handed polydeoxynucleotide chains that are coiled around the same axis, and the heterocyclic amine base of one strand pairs with a base of the other strand through hydrogen bonds [1]. In many aspects of DNA metabolism, such as replication, recombination, repair, and transcription, the DNA duplex must be transiently unwound and the complementary strands of DNA must be separated, inducing the breaking of the Watson-Crick base pairs. In all these processes, the DNA unwinding reaction is catalysed by a class of enzymes called DNA helicases. DNA helicases are characterized by their capacity to translocate along the single stranded DNA strand and unwind the DNA duplex. The reaction is generally coupled to NTP hydrolysis, which is believed to provide the energy source for the unwinding reaction. Therefore, most of DNA helicases contain conserved NTPase domains. These NTPase motifs along with the other conserved motifs form the seven conserved helicase domains present in several known helicases (Figure 1). According to amino acid sequence similarity, Gorbalenya and Koonin proposed a general classification that divides helicases into five super families, from SF1 to SF5[2]. Among these, SF1 and SF2 are the two families that contain most of

the DNA helicases discovered so far. A large number of putative DNA helicases have been identified in prokaryotes and eukaryotes as well as in bacteriophages and viruses. To date, 12 different helicases have been identified in *Escherichia coli* [3], whereas 65 putative helicases have been identified in humans.

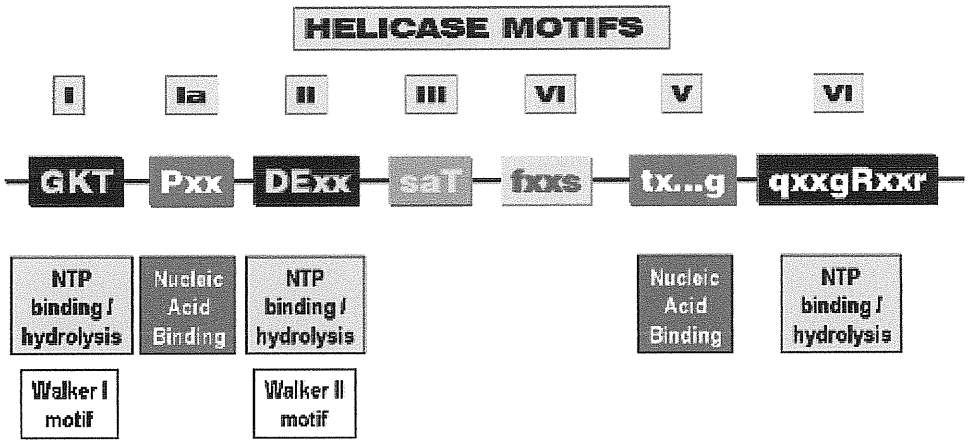

Figure 1 Conserved "helicase domains". Capital letters indicate highly conserved amino acids, small letters more frequent conservative substitutions. x represents variable residues but indicates important spacings between conserved positions. The sequence in the Walker II motif provided the name for DEAD, DExH, and DEAH subgroups. The boxes underneath the sequence indicate the functional role of the motifs derived from X-ray structures and mutational analysis.

DNA helicases have three main activities: DNA binding, NTP hydrolysis and DNA unwinding. Most of known helicases bind ssDNA in a sequence independent manner with high affinity. The equilibrium dissociation constants vary from the picomolar to the low nanomolar range[4]. Many helicases can also bind dsDNA with an affinity that is much lower than that for ssDNA [4]. The NTP hydrolysing activity of helicases converts NTP to NDP and phosphate, and Mg^{2+} is an essential cofactor for

this reaction. In addition, nucleic acids stimulate NTP hydrolysis and increase the hydrolysis rate up to 100 fold. The energy obtained from NTP hydrolysis is probably used either for the translocation of the enzyme along the nucleic acid chain or for the unwinding of the double stranded region of the substrates or both. Most helicases do not show a strict specificity for NTPs and they hydrolyze more than one kind of ribonucleoside and deoxyribonucleoside triphosphate (rNTPs and dNTPs). However, not all nucleotides support DNA unwinding, and ATP is the preferred nucleotide for most helicases [5].

These enzymes are characterized by different DNA unwinding polarities and substrate specificities. Some helicases can only unwind partial duplexes in a 5'- 3'direction with respect to the bound strand, such as T7 gene 4-ring helicase [6] and *E. coli* DnaB helicase[7]. On the other hand, the SV40 large T antigen[8], *E. coli* PcrA[9], RepA [10], UvrD [11] and RecQ helicases are enzymes [12] that translocate and unwind DNA with a 3'- 5' polarity. Other helicases can work in both directions, and are able to unwind blunt-ended DNA duplexes. In addition, the structure of the substrates unwound can vary from linear duplexes to fork like structures and 4-way junctions. The characterization of the substrate specificity of helicases is of fundamental importance for the understanding of their biological function. Another parameter that differentiates these enzymes is their processivity that corresponds to the maximum length of base pairs that can be unwound by a single helicase molecule. The processivity can vary from less than 100 base

pairs to more than 10,000 base pairs and is also highly dependent from the structure of the substrate used. In addition, the unwinding activity of many helicases is regulated by other DNA binding proteins. For example, the human replication protein A is a ssDNA binding protein that can greatly improve the processivity of human RecQ helicases [13-15].

1.2 RecQ helicases

The name RecQ derives from the first helicase of the family discovered in *Escherichia coli*. The *E. coli* RecQ is one of the component of the RecF pathway required for the repair of UV-induced DNA damage. Successively, members of the RecQ helicase family have been found in organisms that range from bacteria, to plants and animals [16, 17]. In micro-organisms like *E. coli*, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* only one representative per species is present, while higher eukaryotes contain more than one RecQ helicase. For example, five members of the RecQ family have been found so far in human cells, namely RECQ1, WRN, BLM, RECQ4 and RECQ5 [18]. All of them share a common central domain of approximately 450 amino acids containing seven highly conserved motifs also present in several helicases from other families [19]. Among these motifs are an ATP binding sequence (Walker A box) and the DExH box, which is instead characteristic of the RecQ family. The RecQ helicases are divided in two classes according to the length of the amino (N)- and carboxy (C)-terminal

domains. The *E. coli* RecQ, and human RECQ1 and RECQ5 form the first group of RecQ helicases. They are characterized by short N- and C-terminal domains, and their sequence is between 400 and 650 amino acids long. The WRN, BLM, RECQ4, Sgs1 (from budding yeast) and Rqh1 (from fission yeast) helicases, are part of the second group since they have extended N- and C-terminal tails and are all between 1300 and 1500 amino acids long. The function of these extended tails is still under investigation. One possibility is that the additional portions mediate the interaction of these helicases with other proteins. In fact, several proteins have been shown to interact with these longer helicases such as replication protein A [20, 21], proliferating cell nuclear antigen [22], DNA topoisomerase I [22], Ku heterodimer [21, 23, 24], DNA polymerase δ [25] and p53 [19, 26, 27]. A common feature of all the RecQ helicases studied so far is that they unwind DNA with a 3′ to 5′ polarity. On the other hand, only the DNA unwinding activity and substrate specificity of the human BLM and WRN helicases have been thoroughly investigated [26, 28-31], while little or no information is available so far on the catalytic properties of the other three human helicases, namely RECQ1, RECQ4 and RECQ5 [32, 33].

1.2.1 Human RecQ helicases and diseases

Bloom's syndrome

Bloom's syndrome is a rare, autosomal recessive genetic disorder in humans. The BS patients display proportional dwarfism and their skin shows erythema, mostly on the face induced by sun exposure. They have an intelligence level lower than normal, both sexes show impaired fertility, and most importantly, they suffer from immune deficiency. The very high incidence of cancers is another striking feature of BS patients. They have high tendency to develop almost all type of cancers, leukemia, lymphomas, carcinomas, Wilms tumor, etc [34]. The established BS cell lines show the same type of chromosomal aberration, as do normal cells. However, the hallmark of BS cell line is the 10-fold increase of SCE (sister-chromatid exchange)[35] that is generally believed to be happening in HR (homologous recombination) events in S or G2 phases.

Werner's syndrome

Werner's syndrome is an autosomal recessive disorder with higher frequency than Bloom's syndrome, 1-22 cases per 1 million people. Otto Werner first described WS in 1904 [36]. The WS patients are typically of short stature. At age 20-30, the WS patients change the face, including thinning and sharpening of the nose, which assume the so-called "bird like" appearance. Premature-aging is one of the main manifestations of WS, like greying, thinning and losing hair in early ages. WS is also associated with diabetes and an excess of cancers of rare types, such as sarcomas, melanomas, etc. Consistently, WS cell lines display replicative senescence much more quickly

than normal cells. The WS cells require double time to grow and have average life span that is 27% of that of the normal cells[37]. WS cells are hypersensitive to a genotoxic agent, 4NQO, and this has become a hallmark feature of WS cell lines[38]. They are also sensitive to DNA cross-linking reagents and UV, x-ray or γ-irradiation[38]. Differently from BS cells, WS cells don't show the increased frequency in SCEs[39]. However, these cells show evidence of unregulated homologous recombination (HR)[40] and particularly of abnormal non-homologous end-joining (NHEJ), which are responsible for the large accumulation of DNA double-strand breaks (DSBs) in WS cells[41].

Rothmund–Thomson syndrome

Rothmund–Thomson syndrome (RTS) has been known for over a century. Very recently this syndrome has been linked to RECQ4, a member of RecQ helicases. The patients show diverse skeleton abnormalities, like low bone density and pathological fracture. Radial ray defect is the most striking feature for RTS. Similar to all RecQ helicase disorders, RTS patients are also proportionally small and with skin abnormalities. UV sensitive-rush and thin hair and eyebrows are also common in RTS patients. Different from the other two disorders, RTS associates particularly with one type of cancer, osteosarcoma. RTS cell lines show strong evidence for genomic instabilities, such as high frequency of chromosomal rearrangement. Nevertheless these cells do not show the high frequency of SCEs observed in BS cells[42]. Interestingly, some RTS patients do not possess any mutation of the RECQ4

gene, implying that other human RecQ helicases may be also responsible for this pathogenesis.

1.2.2 Properties of RecQ helicases

Common features

The signature domain of RecQ helicase family is the central conserved helicase domain of about 450 amino acids (Figure 2). The central domain contains all the classical seven helicase motifs. The DExH box in motif II classifies the RecQ helicase family into the SF2 super helicase family. In addition, most RecQ helicases also possess the so-called RecQ C-terminal domain (RQC) that is highly homologous among the RecQ family members. The RQC domain appears to mediate protein-protein interactions[43]. For example, the flap endonuclease 1 (FEN-1), a DNA structure-specific nuclease required during Okazaki fragment processing [44], was found to interact with WRN through RQC domain[45]. The BLM and WRN helicases contain an additional conserved motif in their C-terminal tail called the Helicase-and-RNase-D-C-terminal (HRDC) domain that is involved in ssDNA binding[46]. In addition, the Werner's syndrome helicase and its orthologues, such as FFA-1 in *Xenopus laevis*, contain an exonuclease domain in their N-terminus [47, 48]. This domain functions as a 3'-5'exonuclease independently from the helicase domain.

Recently, the crystal structure of the catalytic core of the *E. coli* RecQ helicase has been solved in its DNA unbound form [49]. The molecule forms a

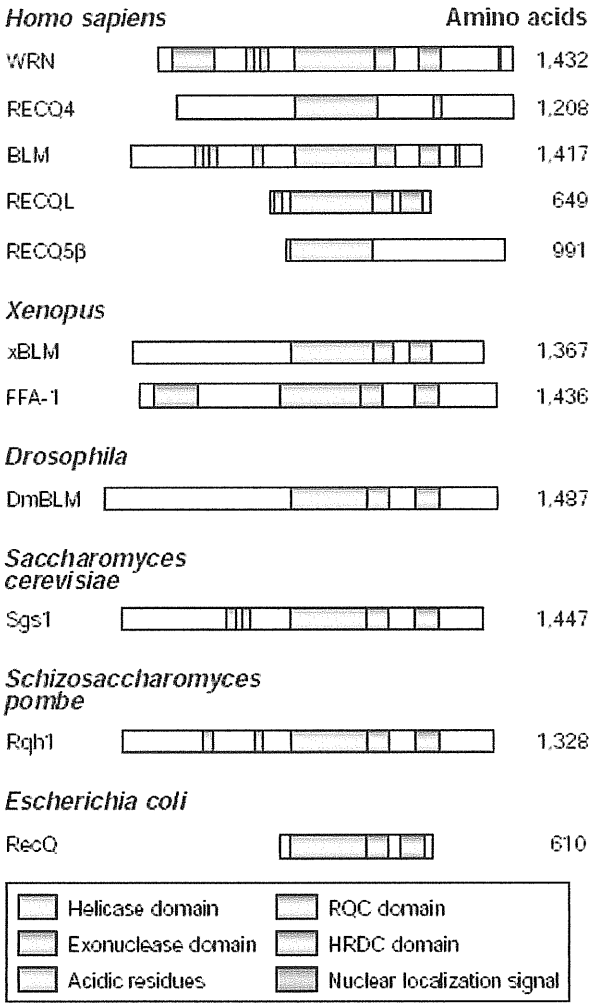

Figure 2 The RecQ helicase family. Schematic representation of selected members of the RecQ family of DNA helicases. Family members have been identified in *E.coli* (RecQ), fission yeast (Rqh1), budding yeast (Sgs1), flies (DmBLM), amphibians (xBLM, FFA-1) and humans (WRN, RECQ4, BLM, RECQL, RECQ5β), as indicated on the left. Proteins are aligned by their conserved helicase domain, which is shown as a green box. The conserved RQC and HRDC domains are shown as orange and purple boxes, respectively. The exonuclease domain in the amino-terminal region of WRN and its orthologues is shown as a blue box. Regions containing patches of acidic residues are shown as violet boxes. The nuclear localization signal sequences identified at the extreme carboxyl terminus of certain family members is shown as a black bar. The remaining pale yellow portions of each protein represent regions that are poorly conserved. At least three splice variants of the human RECQ5 protein are expressed, only one of which is shown. The size of each protein (in amino acids) is indicated on the right.

Y-shaped structure with a major cleft on its surface and is composed by four subdomains. The two N-terminal subdomains form the helicase region, while

the remaining two form the RQC domain. Interestingly, one of the two RQC subdomains folds as a platform of helices that binds Zn^{2+} through four conserved cysteine residues while the other forms a so called winged-helix (WH) subdomain that shares significant structural similarities with other DNA binding proteins. The structure of *E. coli* RecQ is more closely related to helicases of the SF2 family such as the hepatitis C virus NS3 helicase [50] than to SF1 members such as PcrA [51]. Nevertheless, the mode of ATP binding seems to be quite similar to that observed for PcrA and a conserved patch of aromatic and charged residues used in PcrA for ssDNA binding is also present in *E.coli* RecQ, suggesting that these enzymes may use a similar mechanism to bind and unwind DNA. The resolution of the crystal structure provided key indications on the mechanism that *E. coli* RecQ may adopt to unwind DNA. Nonetheless, several aspects still demand further investigation. For example, although the structure shows that the core domain of *E. coli* RecQ is monomeric, it cannot be ruled out that the full-length molecule forms oligomers either free in solution or when bound to DNA [52].

Substrate specificity

The characterization of the substrate specificity of RecQ helicases provides valuable information of the functions of these enzymes. Examples of DNA substrates commonly used for the DNA helicase assay are shown in Figure 3. The common feature of all RecQ helicases is that they can unwind 3'tailed, fork like substrates and M13 based partial DNA duplex substrates.

The Sgs1 and *E. coli* RecQ can unwind long DNA duplexes of a few hundred base pairs, while WRN, BLM and dmRECQ5 can only unwind approximately 100 base pairs[13, 14]. Nevertheless, the processivity of WRN and BLM is greatly improved in the presence of human replication protein A (hRPA) [13, 14]. The *E. coli* RecQ has broad DNA substrate specificity and acts on DNA duplexes containing blunt or forked termini, duplexes with 3' or 5' single strand (ss) tails, D-loops, and 3- or 4-way (Holliday) junctions [53].

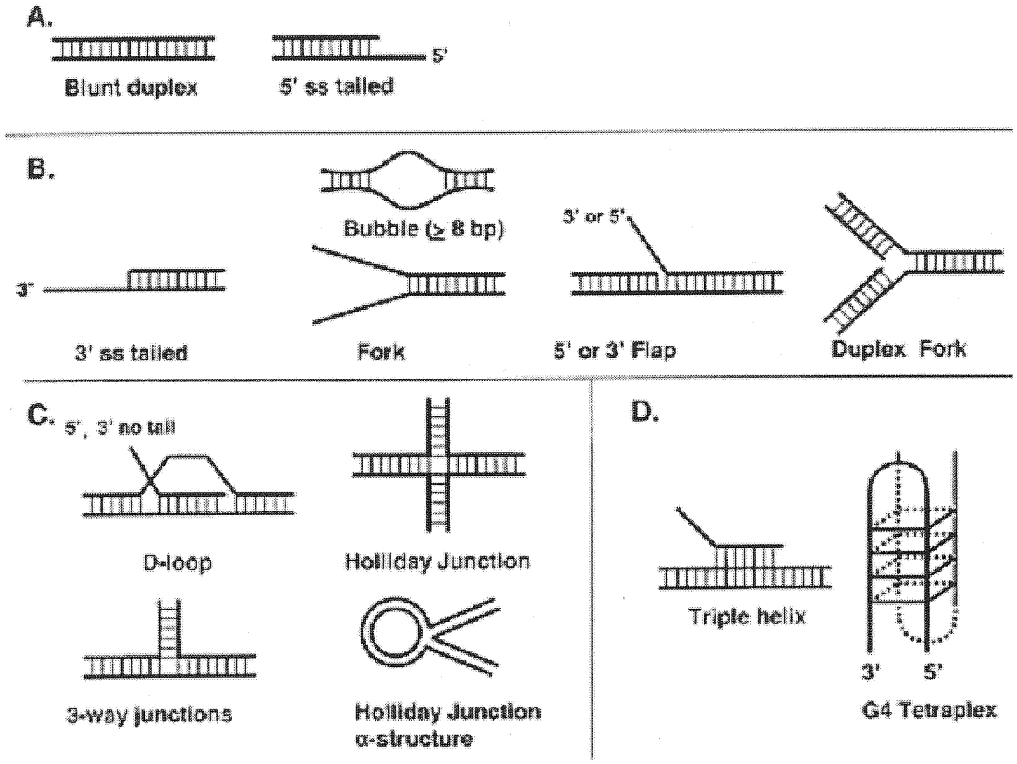

Figure 3. *A*, substrates for *E. coli* RecQ but not eukaryotic RecQ helicases. *B*, B-form duplex DNA intermediates in replication and repair. These substrates are moderately preferred by WRN, BLM, and Sgs1. *C*, DNA intermediates in recombination. WRN and BLM interact preferentially with these substrates relative to those in *B*. *D*, alternate DNA structures formed in triplet and G-rich repeat sequences (telomeric DNA). G-quadruplex DNA is a highly preferred substrate for WRN, BLM, and Sgs1.

The human RecQ helicases cannot melt blunt ended dsDNA substrates. They require at least a 3′ tailed partial dsDNA substrate to perform the unwinding. However, BLM and WRN can unwind a sort of blunt ended DNA substrates

called the "bubbled" substrate that contains a non-complementary region in the middle [15, 54]. The size of the bubble has to be greater than 8 base pairs. Furthermore, BLM and WRN bind with high affinity to D-loops, the intermediates of HR, and release the invaded strand. They also easily unwind a Holliday junction (HJ) substrate with short arms and promote extensive branch migration of Holliday Junction α-structure. Finally, BLM, WRN, and Sgs1 have been shown to unwind G-quadruplex and triplex DNA structures with a 3' ssDNA tail even more efficiently than 3′ ssDNA tailed duplexes [43]. These G-quadruplex and triplex structures are formed by particular DNA sequences and may block DNA metabolic pathways. In addition, G-quadruplex and triplex DNA have been implicated in DNA rearrangements including deletions, sister chromatid exchange, and homologous and illegitimate recombination.

Oligomerization state of RecQ helicases

The results obtained so far indicate that RecQ helicases have different oligomeric structures. Some are hexameric, whereas others are trimeric or monomeric. On the other hand, there are contradictory evidences regarding the oligomerization state of these proteins. The BLM helicase has been shown to exist as hexamer and the structure resembles the typical ring-shaped structure of many hexameric helicases form bacteria and viruses[7, 55]. On the other hand, recent studies with the fragment 642-1290 of BLM revealed that $BLM^{642-1290}$ runs as a monomer on a gel filtration column both in solution and

in its ssDNA bound form[56]. Similar gel filtration experiments suggested that the 333-amino acid N-terminal fragment of WRN as well as the full-length recombinant protein elute as a trimer[57]. Successively, atomic force microscopy analysis of the 171-amino acid fragment of WRN, responsible for the exonuclease activity of this enzyme, revealed that this fragment is in a trimer-hexamer equilibrium in the absence of DNA and that this equilibrium is significantly shifted toward the hexamer in the presence of DNA[58]. Also in the case of the *E. coli* RecQ there are still conflicting evidences regarding the oligomerization state of the full-length protein, although the RecQ structure shows that the core domain is monomeric[49]. In fact, kinetic studies indicated that *E. coli* RecQ forms a multimer of at least three subunits in solution[52], whereas gel filtration experiments suggested that *E. coli* RecQ runs as a monomer and recent biophysical studies provided evidences that this enzyme stays in its monomeric form also when bound to single-stranded DNA chains and when unwinding DNA duplexes[59].

Physical and functional interactions of RecQ helicases with other protein partners

The investigation of the substrate specificities of a helicase and the identification the proteins or protein complexes with which a particular helicase interacts is crucial for the understanding of its function. The interactions between helicases and other proteins might not only be important to modulate the substrate preference, but might also help to localize the

helicase at the position where a duplex needs to be unwound in a particular moment of the cell cycle. In humans, a wide range of proteins involved in DNA replication, in homologous (HR) and non-homologous (NHEJ) recombination, and in telomere maintenance, have been found to interact with RecQ helicases. For example, human replication protein A (hRPA) and p53 interact both with BLM and WRN[13, 14, 60, 61]. Topoisomerase III interacts only with BLM[62], while WRN binds and stimulates topoisomerase I[63]. BLM co-localizes with PML bodies and interacts with Rad51, suggesting a possible role of BLM in HR. Further more, BLM is the substrate of the ATM kinase after γ-irradiations and is able to interact with MLH1 [64], a component of the DNA mismatch repair complex. On the other hand, WRN has been shown to interact with many DNA replication proteins. In fact, WRN can interact with hRPA, PCNA, FEN-1 and DNA polymerase-δ. In addition, WRN participates in DNA repair events. In fact, WRN interacts with the DNA-dependent protein kinase (DNA-PKcs) and Ku, which are two crucial components of the NHEJ mechanism. Interestingly, BLM and WRN can also interact with each other [65].

1.2.3 Human RECQ1 helicase

RECQ1 (also known as RECQL or RECQL1) is the first RecQ helicase discovered in humans. It was initially purified from HeLa cells and described as a 73kDa DNA-dependent ATPase Q1. The cDNA of RECQ1 was first

cloned in 1994 and the gene has been localized in chromosome 12p12 [12, 66], a single copy gene placed under a promoter that gives an extremely high level of transcription. RECQ1 is expressed very abundantly almost everywhere in the body, including heart, lung, skeletal muscle and kidney, etc, but poorly in the brain. The gene covers 31,831 bps of genomic DNA containing 15 exons. The 2831 nt mRNA transcript expresses 649 amino acids long polypeptides. There is also report about the alternative mRNA splicing that generates two smaller variants of RECQ1, RECQ1a and RECQ1b [67].

The present work is focused on the characterization of the biochemical properties of RECQ1. Native RECQ1 was purified from HeLa nuclear extracts to homogeneity following an improved procedure established in our laboratory. The substrate specificity of native RECQ1 was determined and its native molecular mass was examined. Interestingly, unwinding experiments performed in the presence of hRPA showed that this protein specifically stimulates the helicase activity of RECQ1 as was previously observed for WRN and BLM. Successively, the cDNA of RECQ1 was cloned and the recombinant RECQ1 helicase was expressed in the baculovirus expression system with high yield. The activity of the recombinant protein was similar to that of the native protein. The oligomerization state of RECQ1 was characterized with both size-exclusion chromatography and dynamic light scattering. Its processivity of unwinding was tested using M13 based substrates with increasing DNA duplex length. A series of experiments were

performed to investigate further the contribution of hRPA to the unwinding reaction. The physical interaction between RECQ1 and hRPA was demonstrated by using co-immunoprecipitation, far-western and ELISA techniques.

2. Materials and Methods

2.1 Reagents

All salts, bovine serum albumin, dithiothreitol, phenylmethylsulfonyl fluoride, were from Sigma (Cambs, UK). The M13mp18 single-stranded DNA (ssDNA) plasmid, T4 DNA ligase and XbaI, NheI, XhoI restriction enzymes were from New England BioLabs. The serums for growing the HeLa cells, glutamine and gentamicin are from Gibco-BRL Corp. (Gaithersburg, MD, USA). The SuperScript™ II RNase H- reverse Transcriptase, Platinum® P*fx* DNA Polymerase, pFastBac1 Donor plasmid, kanamycin, gentamicin, tetracycline, Bluo-gal, IPTG, Sf-900 II SFM medium and fetal bovine serum were from Invitrogen. The protease inhibitor cocktail was from Roche Molecular Biochemicals. All the resins and the pre-packed columns, MONO-S, MOMO-Q and Superdex™ 200 10/30 GL for chromatography were from Amersham Biosciences (Uppsala, Sweden). The Affi-Gel blue resin and hydroxylapatite resin were from Bio-Rad. The TSK-GEL G3000SW column (60 cm × 7.5 mm) and TSK-GEL G3000SWXL column (30 cm × 7.8 mm) were

from TOSOH BIOSEP (Stuttgart, Germany). Most of the chromatographic analyses were carried out using an AKTA FPLC system (Amersham Biosciences). All ssDNA oligonucleotides used to make the DNA substrates were purchased from Sigma (Cambs, UK). The radioactive nucleoside triphosphates were obtained from Amersham Biosciences (Buckinghamshire, UK). The T4 polynucleotide kinase and sequencing grade porcine trypsin for protein digestion were from Promega (Madison, WI).

2.2 HeLa cell culture media and buffers

HeLa cells were grown in Joklin minimal essential medium supplemented with 10% fetal calf serum, 50 μg/ml gentamicin, and 2 mM glutamine and harvested as described previously[68]. All buffers used during the purification of RECQ1 contained 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride as protease inhibitor. Buffer A contained 20 mM HEPES (pH 8.0), 0.1 M NaCl, 1 mM EDTA, and 20% glycerol. Buffer B contained 50 mM Tris-HCl (pH 7.5), 70 mM KCl, 1 mM EDTA, and 10% glycerol. The concentration of NaCl or KCl in all buffers was increased up to 1.0 M for eluting the proteins from the different columns.

2.3 Purification of RECQ1 from HeLa cell nuclear extract

The RECQ1 helicase was purified from 300 g of frozen HeLa cells. The cell nuclei were isolated and salt-extracted with 0.4 M NaCl following the procedure described by Dignam et al [69]. Successively, an additional salt

extraction with 1.0 M NaCl was done to select specifically for proteins that bind tightly to DNA. The extracted proteins were precipitated by slowly adding ammonium sulfate (0.35g/ml), collected by centrifugation at 25,000g for 30 min in a Sorvall SS34 rotor, dialyzed in buffer A, and applied to a Bio-Rex column (2.5 × 33 cm) equilibrated with buffer A [70]. Active fractions eluting at ~ 0.4 M NaCl in buffer A were pooled (Fraction I, 78 ml). All purification steps were carried out at 4 °C, and the unwinding activity after each step of purification was monitored with a 5′-labeled DNA substrate as described [71]. Fraction I was first dialyzed in buffer B and then loaded onto a 10 ml Q-Sepharose column equilibrated with buffer B. The proteins bound to the column were eluted using a linear gradient of 0.07–1.0 M KCl in buffer B. All active fractions eluting at the very beginning of the gradient were pooled (Fraction II, 85 ml) and loaded onto a 1-ml Mono S column. Elution was carried out with the same linear gradient used for the Q-Sepharose column, and the helicase eluted between 0.2 and 0.3 M KCl (Fraction III, 14 ml). Fraction III was dialyzed in buffer B and loaded onto a 4-ml ssDNA-cellulose column (1.6 X 2.5 cm) for the last step of purification. Elution was carried out with a linear gradient of 5 column volumes of 0.07–1.0 M KCl in buffer B. A major peak eluted at 0.24 M KCl. The active fractions were pooled together (Fraction IV, 8 ml, 44.800 units) and stored at -80 °C with 50% glycerol.

2.4 Expression and purification of recombinant RECQ1

The human RECQ1 cDNA encoding 649 amino acids was amplified from lymphocytes prepared from human peripheral blood by using reverse transcriptional (RT)-PCR and was cloned into the XheI and XhoI restriction sites of the pET-28a(+) vector (Novagen). The sequence of RECQ1 cDNA was verified by DNA sequencing. A 2.1 kb DNA fragment, containing the coding sequences for 6 histidines, a thrombin cleavage site and the full length human RECQ1, was obtained with XbaI and XhoI digestion. The DNA fragment was then inserted into the pFastBac1 vector. Recombinant bacmids were prepared according to the manufacture's instructions using the pFastBac1 transfer vector (Invitrogen). SF9 cells were transfected with the recombinant bacmid DNA. In this way, the recombinant baculovirus expressing histidine-tagged RECQ1 protein was produced. The amino acid sequence preceding the 649 amino acids of RECQ1 protein is the following: MGSSHHHHHHSSGLVPRGSHMAS and contains 6 histidines, a linker region, and a thrombin cleavage site. The baculovirus was used to infect Sf9 cells cultured in suspension (suspension culture ~10^6 cells/ml). Seventy-two hours after infection, cells were harvested by centrifugation, and were resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 400mM NaCl, 5 mM β-mercaptoethanol, 1% Nonidet P-40) supplemented with protease inhibitor cocktail (Roche Molecular Biochemicals, Molecular Biochemicals). 12 ml of lysis buffer were used for 150 ml of suspension culture ($1.5x10^8$ cells) and the incubation was for 15 min at 4 °C with gentle agitation. An alternative way to

lyse the cell was using sonication. The cells were harvested by centrifugation and were suspended in sonication buffer (50 mM Tris-HCl pH 8.0, 150mM NaCl, 5 mM β-mercaptoethanol). The suspension was placed on ice and sonicated for 3×15 seconds with 1 minute intervals. Recombinant RECQ1 was identified in the cell lysate by SDS-PAGE. Successively, the lysate was cleared by centrifugation (15,000 rev/min at 4 °C, rotor: Sorvall SA600) and was incubated with TALON metal affinity resin (CLONTECH 1 ml of resin/5 mg of protein) for 2 hr at 4 °C. The resin was washed with buffer containing 20 mM Tris-HCl, pH 8.0, 5 mM β-mercaptoethanol, 12.5 mM imidazole and 500 mM NaCl (two washes) and the same buffer with 100 mM NaCl (two washes). The hexa-histidine-tagged RECQ1 was eluted in buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM β-mercaptoethanol, 120 mM imidazole. The purity of the preparation was verified by SDS-polyacrylamide gel stained with silver. The identity of the purified product was verified both by mass spectrometry sequencing and immunoblotting with specific monoclonal antibody against the hexa-histidine-tag. The recombinant RECQ1 was concentrated and stored in a buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1mM DTT or a buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1mM DTT and 5% glycerol at – 80 °C. The concentration of the RECQ1 was determined by UV absorption measurements using an extinction coefficient at 280 nm of 67790 M^{-1} cm^{-1} estimated from the amino acid sequence (ProtParama, available at www.expasy.org). Approximately 0.75 mg of RECQ1 were obtained from 1.5×10^8 infected Sf9 cells.

2.5 Expression and purification of recombinant hRPA

The expression and purification of hRPA was carried out as described previously [72]. The plasmid p11d-tRPA, an hRPA expression vector containing all cDNAs encoding 3 subunits of hRPA, was transformed into *E. coli* expression strain BL21 (DE3). A single recombinant colony was used to inoculate 10 ml of TB medium with 100μg/ml ampicillin. The culture was grown overnight at 37 °C and 5ml of the culture medium was transferred to 1 liter fresh TB medium supplied with 100μg/ml ampicillin and placed on a shaker at 37 °C until the absorbance at 600 nm reached 0.6. The culture was induced by adding isopropyl-l-thio-P-D-gdactopyranoside (IPTG) to 0.3 mM final concentration and incubated at 30 °C for another 2 hours. The cells were collected by centrifugation at 3000 rpm for 20 min in a Beckman JS4.2 rotor and resuspended in 5 ml of HI buffer (30 mM HEPES pH 7.8, 1 mM dithiothreitol, 0.25 mM EDTA, 0.25% (w/v) inositol, and 0.01% (v/v) Nonidet P-40) with 1 mM phenylmethylsulfonyl fluoride. The resuspended cells were frozen at -80 °C, thawed, and lysed by sonication (3 × 15 second). The lysate was centrifuged in a Sorvall SS34 rotor at 12,000 rpm for 30 min at 4 °C. The supernatant was applied to a 10 ml Affi-Gel blue column (1.5 x 5.6 cm; Bio-Rad) equilibrated with HI buffer containing 50 mM KCl. The column was washed sequentially with 50 ml each of HI buffer containing 50 mM KCl, 0.8 M KCl, 0.5 M NaSCN, or 1.5 M NaSCN. The 1.5 M NaSCN elution was applied directly to a 5 ml hydroxylapatite column equilibrated with HI buffer

and then eluted with 4 column volumes of HI buffer containing 80 mM potassium phosphate. The elution was loaded onto a MONO-Q column equilibrated with HI buffer containing 100 mM KCl. The column was washed with 5 ml of HI buffer containing 100 mM KCl and then developed with a 10-ml linear salt gradient of 100-400 mM KCl. The peak of hRPA eluted at ~300 mM KCl and contained ~ 300 µg of hRPA. The elution was finally dialysed against HI buffer with 50 mM KCl and stored at –80°C in small aliquots. The purity of hRPA was tested by SDS-PAGE. The activity of hRPA was tested by EMSA assay with 5′ γ-^{32}P labeled 30 nt ssDNA.

2.6 Determination of the native molecular mass of native RECQ1

The native molecular mass was determined by glycerol gradient centrifugation and gel filtration analysis following the procedure described previously [71, 73, 74] . More precisely, for the glycerol gradient study, 100 µl of 50 nM RECQ1 were layered on a 15-35% glycerol gradient in buffer B and centrifuged at 320,000 × g for 20 h at 4 °C. The standard protein markers were also run under the same conditions. The markers were catalase (240 kDa, 11.3 S), aldolase (158 kDa, 7.4 S), BSA (66 kDa, 4.22 S), and ovalbumin (45 kDa, 3.5 S). Fractions of 0.2 ml were collected from the top of the tube using an HSI Auto Densi-Flow IIC apparatus (Buchler Instruments) and assayed for helicase activity. For gel filtration, a TSK-GEL G3000SW column (60 cm × 7.5 mm) was used in the AKTA FPLC system equilibrated with buffer B. The solution of RECQ1 was first concentrated from 50 to 500 nM and

then loaded onto the column. The column was run at a flow rate of 1 ml/min. Fractions of 0.25 ml were collected and assayed for helicase activity. The column was pre-calibrated using gel filtration molecular mass markers under the same conditions. The markers were thyroglobulin (670 kDa, Stokes radius of 85 Å), γ-globulin (158 kDa, 48.1 Å), BSA (66 kDa, 35.5 Å), ovalbumin (45 kDa, 30.5 Å), myoglobin (17 kDa, 21.2 Å), and vitamin B12 (1.35 kDa). The partition coefficient Kav is equal to (Ve − V0)/(Vt − V0), where Ve is the elution volume of the sample, V0 is the void volume, and Vt is the total volume of the gel bed. The Stokes radius of RECQ1 was derived from the linear plot of (−log Kav)1/2 versus the Stokes radius of the standard proteins. The molecular mass of native RECQ1 was calculated from the Stokes radius and the sedimentation coefficient using the equation previously described [74].

2.7 Gel filtration experiments for recombinant RECQ1

The TSK-GEL G3000SW$_{XL}$ column (30 cm x 7.8 mm) was equilibrated at a flow rate of 1 ml/min with 50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM DTT. The column was then calibrated using gel filtration molecular mass markers containing thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (45 kDa), myoglobin (17 kDa) and vitamin B_{12} (1.35 kDa). BSA (66 kDa) was also used as a standard. A solution of 2.5 μM RECQ1 (37.5 μg, 200 μl) was loaded onto the column.

2.8 Dynamic light scattering (DLS)

DLS measurements were performed using a DynaPro-MS/X instrument (Protein Solution, Charlottesville, VA) with 12µl quartz cuvette and the scattered light was collected at an angle of 90°. The time-dependent autocorrelation function (ACF) of the photon current was acquired with a built-in software correlator (based on a DSP unit). Samples were gently injected into the cell through a Whatman filter with 0.02 µm porosity. The protein concentration was in the range of 2.0 to 3.0 µM and the buffer used for measurements was 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM DTT (5 mM $MgCl_2$ and 5 mM ATP were also added in separate experiments). The 50-mer ssDNA substrate was added to RECQ1 solutions in slight excess over the protein concentration ([DNA]/[RECQ1] = 1.1–3.0) and the solution was incubated for 30 min at room temperature before collecting data. ACF were incrementally stored every 10 s at a temperature of 23 ± 0.1 °C. ACF were analysed with the provided Dynamics v6.0 software provided, which contains proprietary algorithms to filter out unavoidable (since the small cuvette volume remains open to air during measurements) dust contamination of the ACF at longer times.

2.9 EMSA assay

The recombinant RECQ1 (1µM) was incubated with 5′ γ-^{32}P labeled single strand DNA of 50 T (0.4 nM) in a 20µl reaction mixture containing 50mM Tris-HCl pH 7.5, 100mM NaCl and 1mM DTT. The incubation was 20 min at room temperature. The resulting mixture was then resolved by 6%

non-denaturing polyacrylamide gel electrophoresis and visualized by autoradiography. Gels were run at 100 V and 4 °C in TBE buffer. Labeled DNA fragments were detected in the dried gel by autoradiography (Instant Imager, Packard Corp., Meriden, CT).

2.10 Preparation of DNA helicase substrates

The DNA substrates used in the helicase assay consist of different ^{32}P-labelled oligonucleotides annealed either to M13mp18 phage ssDNA or to ssDNA fragments of different length to create a partial duplexes as described previously [15]. The sequences of all the polynucleotides are complementary to nucleotides of M13mp18 phage ssDNA. The substrates with double-stranded regions of 25 and 50 bp were made using polynucleotides 5'-CTCTAGAGGATCCCCGGGTACCGAG-3' (25 bp) and 5'-GCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGA ATT-3' (50 bp), complementary to nucleotides (nt) 6239-6263, 6231-6280 of M13mp18 phage ssDNA respectively. The substrates with double-stranded regions of 110 bp, 207 bp, 301 bp, 416 bp, 501 bp, 603 bp, and 807 bp were made by PCR amplification of a M13mp18 fragment of the proper length. The forward primer for the PCR was annealed to region 28-47 of M13mp18, whereas the reverse primers were annealed to regions 28-137 for 110 bp, 28-243 for 216 bp, 28-328 for 301 bp, 28-443 for 416, 28-528 501 bp, 28-630 for 603, and 28-834 for 807 bp. The sequence of the 99bp oligonucleotide used for the determination of the direction of unwinding is the same described earlier [75]

(Figure. 7 A and B) as well as the sequences of three oligonucleotides annealed to the M13mp18 phage ssDNA (Figure. 7 D, E, F and G) [71, 75]. The substrate in Figure 7H was made using the oligonucleotides 5′-CTCTAGAGGATCCCCGGGTACCGAGCTCGAATT-3′ (33bp) complementary to nts 6231-6263 of M13mp19 phage ssDNA. The blunt-end 25bp duplex (Figure. 7C) was made annealing the oligonucleotide 5′-GATCTCGCATCACGTGACGAAGATC-3′ with its complementary sequence. The substrate in Figure. 7I was made annealing the oligonucleotide 5′-GATCTCGCATCACGTGACGATTTTTTTTTTTTTTTTTTTTTTTTGATCTC GCATCACGTGACGA-3′ with the complementary ssDNA fragment except for the 25T in the middle. The linear substrates with polyT tails of different length were made using a ^{32}P- labeled 29bp oligonucleotide (5′-ACAAAATAAAAAATCCCGAGGGAATATTAC-3)′ annealed to complementary ssDNA fragments having 3′ tails of 10, 25, 50 and 75 T (Figure. 7L, M, N and O). 25 ng of each oligonucleotide, labelled at the 5' end with T4 polynucleotide Kinase and 0.9 MBq of [γ-^{32}P]ATP, were subsequently annealed to M13mp18 phage ssDNA (4µg) in 40 mM Tris-HCl (PH8.0), 10mM $MgCl_2$, 1mM DTT and 50 mM NaCl. The mixture was heated at 95 ^{o}C for 2 minutes, and slowly cooled to room temperature. Each substrate was purified by gel filtration through a 5 ml sepharose 4B column.

2.11 Preparation of RNA/RNA and RNA/DNA substrates

The RNA/RNA and RNA/DNA substrates were obtained as follows: pDEST17 vector (Invitrogen) containing the Annexin II gene was linearized by Sph I and transcribed in vitro with the T7 RNA polymerase from the specific promoter yielding a 2.2 Kb RNA [76]. A 16 bp DNA or RNA oligonucleotide complementary to the same region of Annexin II (nts 52 to 68) was synthesized and labelled in the 5' end by T4 polynucleotide Kinase with 0.9 MBq [γ-^{32}P]ATP. About 8 pmol of labeled oligonucleotide were mixed with 13 pmol of synthesized annexin II RNA, heated at 95 ^{o}C for 2 minutes and allowed to anneal by slow cooling at room temperature. The substrates were then purified by gel filtration through a 5 ml Sepharose 4B column.

2.12 DNA helicase assay

The helicase assay measures the unwinding of a ^{32}P-labeled DNA fragment from a partial duplex DNA molecule. The 20 µl reaction mixture contained 20 mM Tris-HCl (pH 7.5), 8 mM DTT, 5 mM $MgCl_2$, 5 mM ATP, 10 mM KCl, 4% (w/v) sucrose, 80 µg/ml bovine serum albumin (BSA), and ^{32}P-labeled helicase substrate (0.4 nM). The recombinant RECQ1 was added to the mixture and incubated at 37 °C for the times specified in the figure legends. The reaction was terminated by the addition of 0.3% SDS, 10 mM EDTA, 5% glycerol, and 0.1% bromphenol blue. The products of the reaction were fractionated by electrophoresis on a 12% or 6% non-denaturing

polyacrylamide gel. The gel was dried, and the extent of DNA unwinding was quantitated by electronic autoradiography (Instant Imager, Packard Instrument Co.).

2.13 ATPase assay

Standard ATPase assay reaction mixtures (50 µl) contained 20 mM Tris-HCl pH 7.5, 8 mM dithiothreitol, 5 mM $MgCl_2$, 10 mM KCl, 4% (w/v) sucrose, 80 µg/ml bovine serum albumin (BSA), the specified ssDNA effector (32 µM nucleotide), trace amount of [γ-^{32}P]ATP (~2 nM) mixed with indicated amount of cold ATP, 20 nM RECQ1, and the indicated amount of hRPA. The reactions were initiated by the addition of RECQ1 and incubated at 37 °C. Samples (1 µl) were removed at 3 min intervals and evaluated by thin layer chromatography as described previously [77]. Less than 10% of the ATP substrate was consumed in the reaction over the entire time course of the experiment. The k_{cat} values were expressed as the mean of at least five independent determinations.

2.14 Far western assay

Far Western assay was conducted essentially as described by Brosh *et al.* [21]. Briefly, 0.36 µg of recombinant RECQ1, 20 µg of BSA, 1.5 µg of hRPA, and 1.6 µg of Ku were loaded on a SDS-PAGE electrophoresis and transferred to Hybond-P PVDF membrane (Amersham Pharmacia Biotech). All

subsequent steps were performed at 4 °C. The membrane was immersed 7twice in denaturation buffer (6 M guanidine HCl in PBS) for 10 min followed by 6 times for 10 min in serial dilutions (1:1) of denaturation buffer supplemented with 1 mM dithiothreitol. The membrane was blocked in PBS containing 5% powdered milk, 0.3% Tween 20 for 30 min before being incubated overnight with RECQ1 (0.8 µg/ml) in PBS supplemented with 0.25% powdered milk, 0.3% Tween 20, 1 mM dithiothreitol. The membrane was washed for 4 times for 10 min in PBS containing 0.3% Tween-20, 0.25% powdered milk. The second wash contained 0.0001% glutaraldehyde. Western analysis was then performed to detect the presence of RECQ1 using hexa-histidine monoclonal antibody (Clontech) as primary antibody at 1:5,000 dilution. Anti-mouse IgG/horseradish peroxidase conjugate (Sigma) was used as secondary antibody at a 1:15,000 dilution and detected using ECL (Amersham Pharmacia Biotech) following the manufacturer's instructions. Similar results were obtained without using glutaraldehyde in the second wash. The only difference is that the intensity of the final RECQ1 band is slightly weaker.

2.15 ELISA assay

Purified recombinant RECQ1 was diluted to a concentration of 18 nM in carbonate buffer (0.016 M Na_2CO_3, 0.034 M $NaHCO_3$, pH 9.6), and was added to appropriate wells of a 96-well microtiter plate (50 µl/well), which was incubated at 4 °C. Bovine serum albumin (BSA) was used in the coating

step for control reactions. The samples were aspirated, and the wells were blocked for 2 h at 30 °C with blocking buffer (phosphate-buffered saline, 0.5% Tween 20 and 3% BSA). The procedure was repeated. hRPA was diluted to 144 nM in blocking buffer, and was added to the appropriate wells of the ELISA plate (50 µl/well), which was incubated for 1 h at 30 °C. For ethidium bromide (EtBr) treatment, 50 µg/ml EtBr was included in the incubation with hRPA during the binding step in the corresponding wells. The samples were aspirated, and the wells were washed five times before addition of anti-hRPA (Ab-1) mouse monoclonal antibody (Oncogene Research Products) diluted 1:100 in Blocking buffer and incubated at 30 °C for 1 h. Following three washings, horse-radish peroxidase-conjugated anti-mouse secondary antibody (1:2500) was added to the wells, and the samples were incubated for 30 min at 30 °C. After washing five times, any hRPA bound to the RECQ1 was detected using OPD substrate (Sigma). The reaction was terminated after 5 min with 3 N H_2SO_4, and absorbance readings were taken at 490 nm. Data analysis for determination of the apparent dissociation constant (K_d) was performed as previously described [14].

2.16 Anti-RECQ1 antibody production

Custom polyclonal anti-RECQ1 antibody was raised against a 20 amino acid peptide corresponding to residues 644-662 (GNFQKKAANMLQQSGSKNT) in the carboxyl-terminal region of RECQ1 with an N-terminal cysteine conjugated to Sepharose 4-B for affinity

purification (Alpha Diagnostic International, San Antonio, TX). The peptide was conjugated to KLH carrier protein and raised in rabbit. An IgG fraction from the antiserum was purified using the peptide coupled to Sepharose-4B via Cysteine group. Affinity pure antibody was checked by ELISA using free peptide.

2.17 Co-immunoprecipitation experiment

Nuclear extracts were prepared from exponentially growing HeLa cells as described previously [78]. For co-immunoprecipitation experiments, nuclear extract (1.36 mg protein) was incubated with rabbit polyclonal anti-RECQ1 antibody (1:100) in buffer D (50 mM HEPES pH 7.5, 100 mM KCl, 10% glycerol) for 4 h at 4°C. The mixture was subsequently tumbled with 60 µl of protein-G agarose (Roche Molecular Biochemicals) at 4°C overnight. The beads were then washed three times with buffer D supplemented with 0.1% Tween-20. Proteins were eluted by boiling in SDS sample buffer and the eluate was resolved on 10% polyacrylamide Tris-glycine SDS gels, and transferred to PVDF membranes (Amersham Biosciences). The membranes were blocked with 5% nonfat dry milk in PBS containing 0.1% Tween-20, and probed for hRPA and RECQ1 using anti-RECQ1 (1:20) rabbit polyclonal antibody and anti-RPA (Ab-1) mouse monoclonal antibody (1:20) (Oncogene Research Products) respectively, followed by detection with donkey-anti-rabbit IgG (Santa Cruz Biotech) or horse-anti-mouse secondary (Vector Laboratories Inc.) antibodies conjugated to horseradish peroxidase (HRP).

RECQ1 and RPA on immunoblots were detected using ECL Plus (Amersham Biosciences).

2.18 Microsequence analysis

The Coomassie Blue stained band containing RECQ1 was cut out and digested with sequencing grade bovine trypsin (Promega). The digestion products were separated by micro-high pressure liquid chromatography and analyzed by electrospray ionization mass spectrometry (Finnigan LCQ DECA, Thermo-Finnigan Corp., San Jose, CA).

3. Results

3.1 Purification and characterization of native RECQ1

3.1.1 Purification of native RECQ1

Human helicase RECQ1 was purified from the nuclear extract of HeLa cells following the purification steps described in the "Materials and Methods" section. The final product from ssDNA-cellulose column was loaded on a 10% SDS polyacrylamide gel for an analysis of its purity. The gel was stained with Silver and showed only a single band with MW~70 kDa (Figure 4). In order to confirm the purity of the final product, the solution containing RECQ1 was concentrated 20-fold by acetone precipitation. Also in this case, after loading approximately 2 μg of protein on the SDS-gel only the 70 kDa band was visible (data not shown). Successively, the band was excised

from the gel, digested with trypsin and analyzed by mass spectrometry for protein identification. Ten peptides pertaining to the human DNA helicase RECQ1 (649 aa, Mw 75kDa) were found in the sample (Table 1). The five helicases part of the RecQ family that have been found in human cells are characterized by a conserved central domain of about 450 amino acids. On the other hand, the ten peptides found by mass spectrometry have sequences that are unique for RECQ1 allowing us to be certain about the identity of the protein. Six peptides correspond to sequences located in the central domain of the protein, two in the N-terminal domain and two in the C-terminal tail.

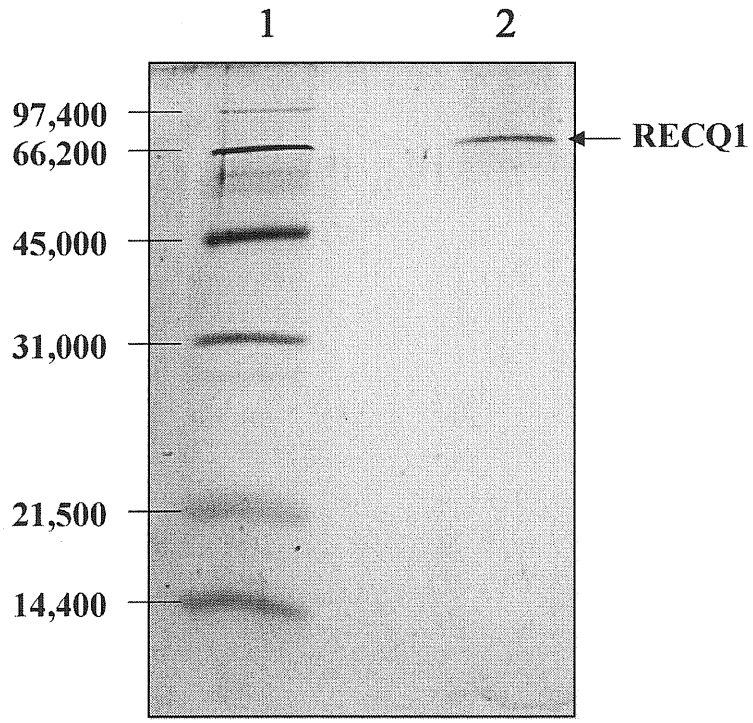

Figure 4. **SDS-polyacrylamide gel of purified RECQ1.** *Lane 1*, molecular mass markers (in daltons); *lane 2*, purified RECQ1 (100 ng). The 10% acrylamide gel was stained with silver for better detection of eventual impurities. No additional bands were detected after loading 2 µg of RECQ1 on the gel.

Table 1. Amino acid sequence of the peptides identified by mass spectrometry present in RECQ1.

Position	*Sequence*
32-38	QQELIQK
79-88	VKDILQNVFK
92-107	FRPLQLETINVTMAGK
186-193	LIYVTPEK
237-242	ALGILK
244-267	QFPNASLIGLTATATNHVLTDAQK
292-306	QKPSNTEDFIEDIVK
326-353	DSEQVTVSLQNLGIHAGAYHANLEPEDK
502-509	QAEELNEK
529-544	VAGVVAPTLPREDLEK

3.1.2 Determination of native molecular weight of RECQ1

We determined the native molecular weight of RECQ1 with both glycerol gradient and size-exclusion chromatography following the procedures previously described [74, 77]. The result indicated that RECQ1 has a sedimentation coefficient of 7.3 ±1.7 S and a Stokes radius as 49.5±10.5 Å correponding to a native molecular weight of ~ 160 kDa, thus suggesting that the protein exists as a dimer in solution (Figure 5). On the other hand this

result does not rule out the possibility that more complex oligomeric structures might be formed in the presence of nucleic acids.

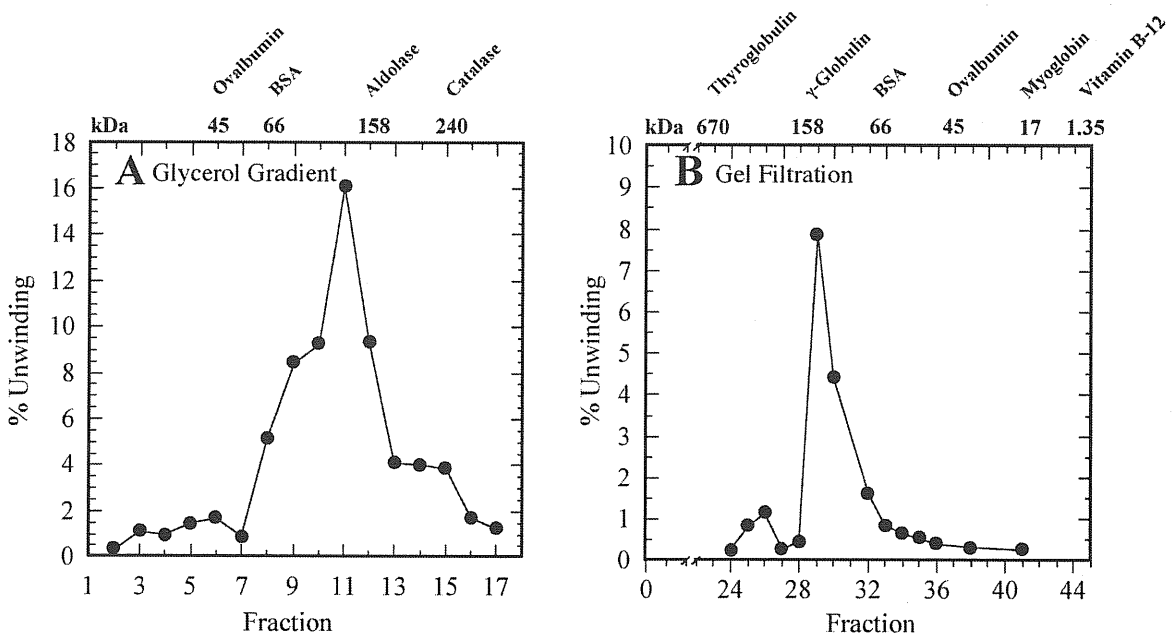

Figure 5. **Glycerol gradient (15–35%) sedimentation and gel filtration analysis.** *A*, the experiment was performed using 100 µl of 50 nM RECQ1, and centrifugation was performed at 320,000 X *g* for 20 h at 4 °C. The distribution of the helicase activity, the positions of the sedimentation coefficients, and molecular mass markers are shown. The markers were catalase (240 kDa, 11.3 S), aldolase (158 kDa, 7.4 S), BSA (66 kDa, 4.22 S), and ovalbumin (45 kDa, 3.5 S). *B*, gel filtration was carried out using of 100 _l of RECQ1 (500 nM) on a TSK-GEL G300SW column (60 cm x 7.5 mm). The distribution of the helicase activity and the positions of the molecular mass markers are shown. The markers were thyroglobulin (670 kDa), γ-globulin (158 kDa), BSA (66 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (1.35 kDa).

3.1.3 Characterization of DNA unwinding activity of native RECQ1

Preliminary studies varying ions type and concentration as well as pH and temperature were performed to optimize the reaction conditions for the unwinding studies (data not shown). Successively, concentration dependence studies under optimal assay conditions showed a maximum value of ~100%

unwinding in 30 min with 4 nM enzyme (Figure 6A). The sigmoidal shape that can be observed at the very beginning of the titration curve is indicative of a cooperative behavior suggesting that more than one dimer of RECQ1 could be involved in DNA unwinding as seen in the case of other helicases [5]. On the other hand, kinetic measurements carried out in the presence of 1 nM (740 pg) enzyme showed that the unwinding rate was linear up to 10 min and deviated from linearity with longer incubation times (Figure 6B). The helicase assays indicated that ATP and Mg^{2+} are indispensable for DNA unwinding. In addition, ATP dependence studies indicated that the optimal ATP concentration for DNA unwinding is between 4 and 5 mM (data not shown). Interestingly, we also observed that the addition of 80 µg/ml of BSA increases the unwinding activity of RECQ1. This observation is in agreement with previous studies showing that the presence of BSA increases the affinity of some proteins for DNA [79, 80].

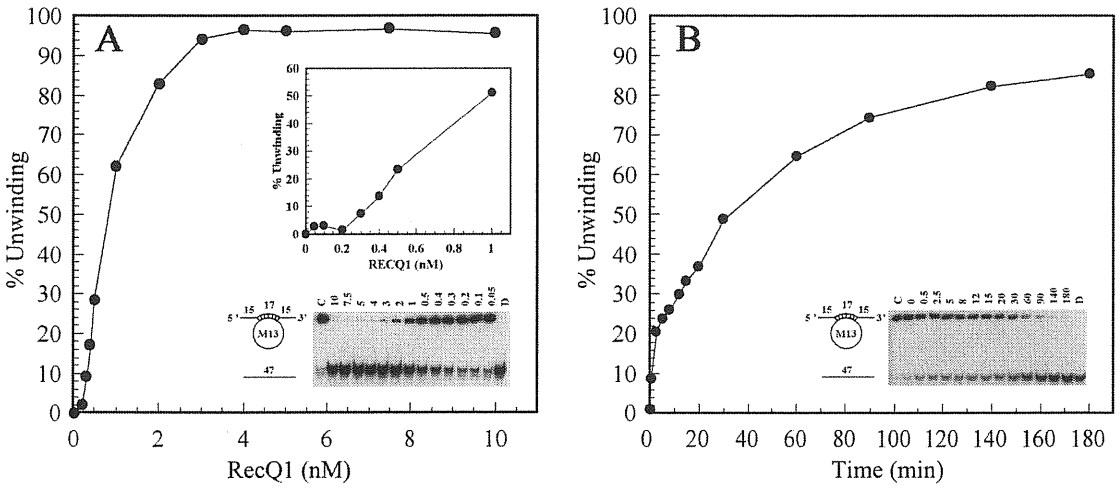

Figure 6. **Concentration and time dependence of RECQ1 activity.** *A*, increasing amounts of RECQ1 were incubated in the standard 10 µl reaction mixture for 30 min at 37 °C. The concentration of enzyme (nanomolar) is indicated above each lane in the autoradiogram, whereas the substrate concentration was constant in all experiments (0.4 nM). *B*, the kinetics

of unwinding was performed using 1 nM RECQ1 and the same reaction conditions used for the concentration dependence experiments. The reaction times (in minutes) are indicated above each lane in the autoradiogram. The *C* and *D lanes* are control assays without enzyme and heat-denatured substrate, respectively.

The only information available on the helicase activity of human RECQ1 is that it unwinds DNA with 3′ to 5′ polarity as the other members of the RecQ family characterized so far [12]. This polarity of unwinding was confirmed by our experiments showing that RECQ1 needs a 3′ ssDNA tail to unwind the substrate (Figure 7 A, B and C). Following this observation, a set of substrates with different structures and with various lengths of the double strand regions was made to obtain novel information on the substrate specificity of RECQ1 (Figure 7). Substrates with a 17 bp oligonucleotide annealed to M13 ssDNA are fully unwound by RECQ1, regardless of the presence or absence of mismatched hanging tails at either the 5′ end, the 3′ end or both (Figure 7 D, E, F and G). However, if the duplex region was increased to 33bp the substrate could not be unwound (Figure 7 H). Similarly to what has been already observed for the BLM and WRN helicase [54], RECQ1 was able to unwind a substrate with "bubble" of 25 nts located in the center (Figure 7 I). Other helicases previously purified in our laboratory have shown to be able to unwind DNA-DNA as well as RNA-RNA and RNA-DNA duplexes [71], on the contrary RECQ1 could not unwind RNA substrates indicating that it can only work as DNA helicase(Figure 7 J and K). Finally, a series of linear substrates with 3′ single strand tails of 10, 25, 50 and 75 T was also made to study the effect of the tail length on the unwinding activity of

RECQ1 (Figure 7 L, N, M and O). Kinetic studies done with this series of substrates clearly showed how only the substrates with tails longer than 10 nt

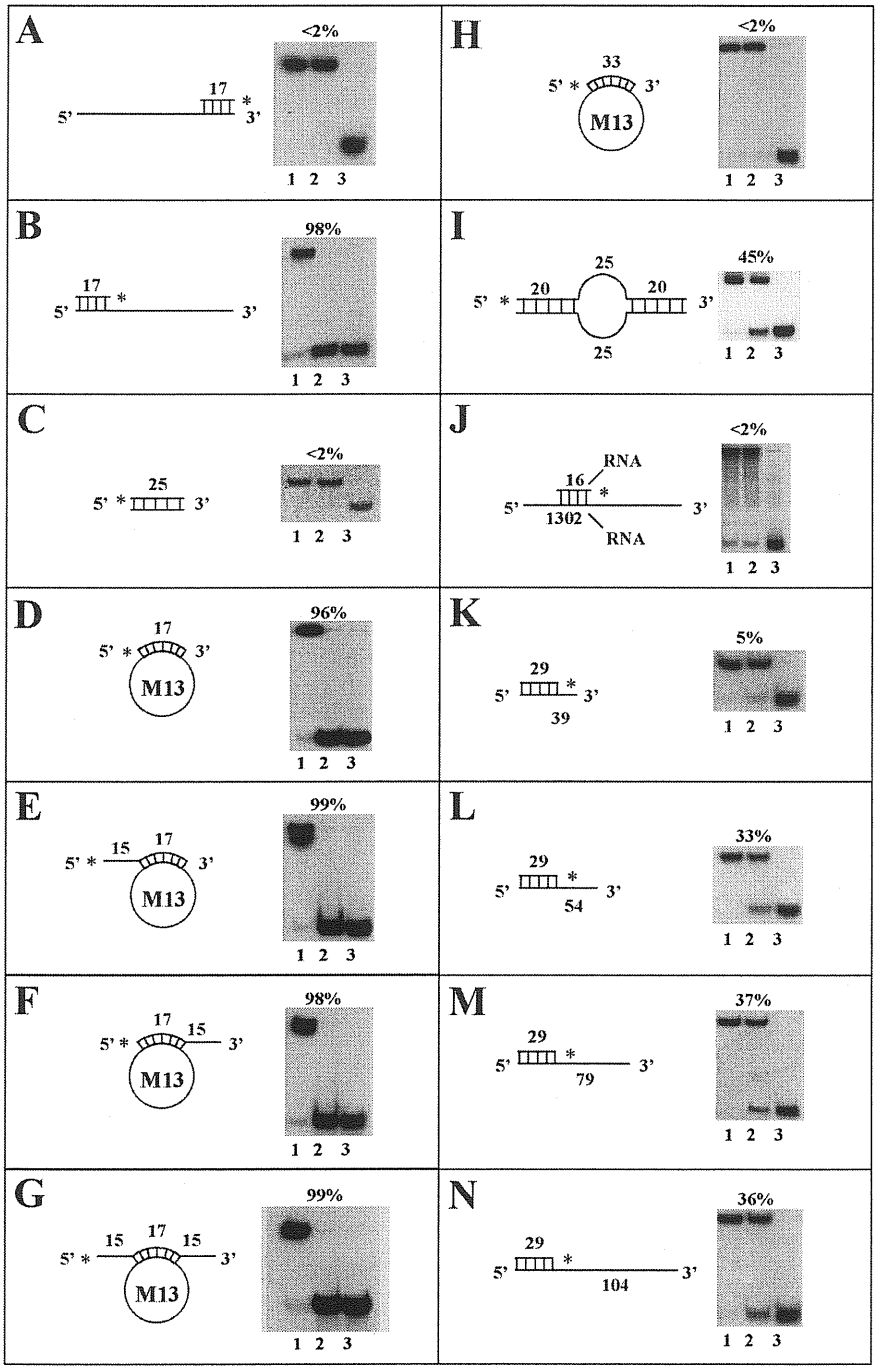

Figure 7. **Helicase activity with various substrates.** Each panel shows the structure of the substrate used and an autoradiogram of the gel. *Asterisks* denote the 32P-labeled end. Percent unwinding is shown above each autoradiogram. *Lanes 1* and *3* correspond to control reactions without enzyme and heat-denatured substrate, respectively. *Lane 2* corresponds to the reaction with pure RECQ1 (_1.0 nM). The direction of unwinding is analyzed in *A* and *B*.

can be efficiently unwound by RECQ1 (Figure 8). A possible explanation is that RECQ1 requires a ssDNA tail longer than 10 nt to efficiently bind the

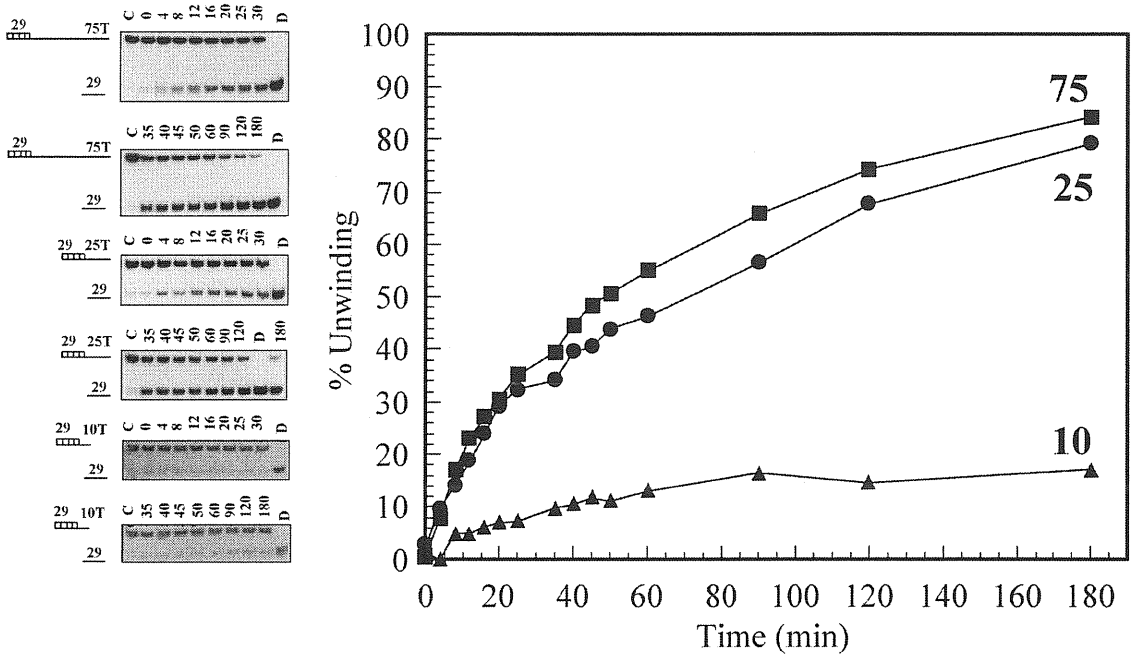

Figure 8. **Tail length dependence studies.** The kinetics of unwinding were determined with three DNA substrates having poly(T) tails of 10 (▲), 25 (●), and 75 (■) nt. The concentration of RECQ1 used in all kinetic studies was 1 nM. The reaction times (in minutes) are indicated above each lane in the autoradiograms. The *C* and *D lanes* are control assays without enzyme and heat-denatured substrate, respectively.

substrate. In order to test this hypothesis, we performed EMSA assay with the linear ssDNA substrates of 10T, 25T and 75 T. The results clearly showed that RECQ1 is able to bind only the substrates with ssDNA tails of 25 and 75 nt (Figure 9).

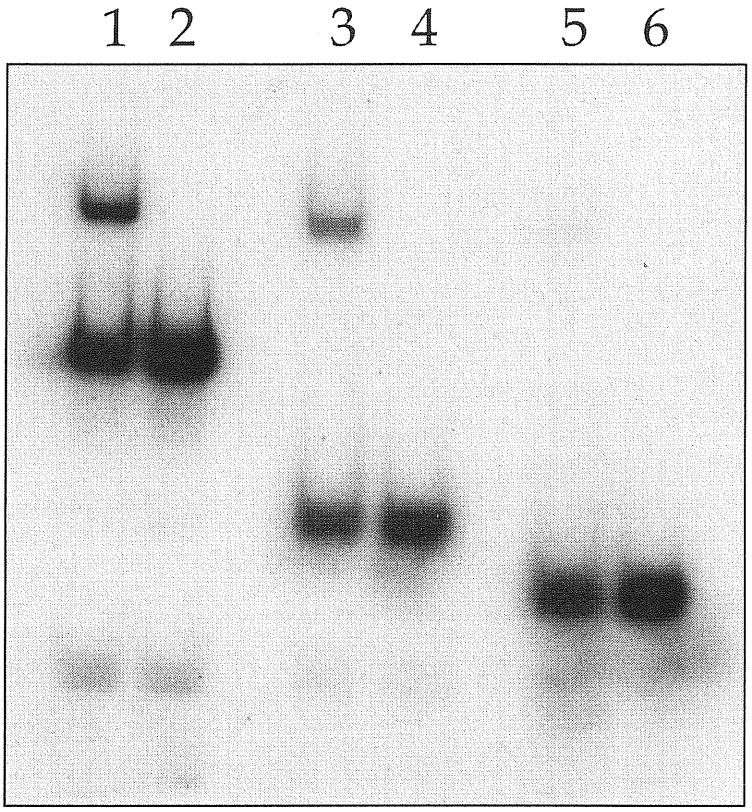

Figure 9. **EMSA assay with tailed substrates**. The EMSA assay was carried out with three different DNA substrates having 3'poly T of 10, 25 and 75 nt. The incubation buffer was the same buffer as used in helicase assay except ATP was not included. The concentration of RECQ1 used for all incubations was 1 nM. Lane 1, 3, 5 were the incubations of RECQ1 with the substrates having 3'tail of 75 T, 25 T and 10 T. Lane 2, 4, 6 were the free DNA controls of substrates having 3'tail of 75 T, 25 T and 10 T.

3.1.4 Stimulation effect of hPPA on the helicase activity of RECQ1

Human replication protein A (hRPA) stimulates specifically the DNA unwinding activity of the WRN and BLM helicases [13, 14]. In our experiments, we saw that also the helicase activity of RECQ1 is specifically stimulated by the addition of hRPA. In fact, RECQ1 (1nM) alone could not unwind a DNA substrate with a duplex region of 33bp, but with the addition of hRPA or *E. coli* single strand binding protein (ESSB) more then 40% of substrate was unwound. On the other hand, kinetics and concentration dependence studies showed that it is necessary to use a concentration of ESSB

(3 μM) ten fold higher than that of hRPA (0.3 μM) to obtain the same stimulation of the helicase activity (Figure 10A and B). To gain more insight into the mechanism of stimulation by these single strand DNA binding proteins, the strand displacement was expressed as function of the ratio (*R*) of the concentration of SSB-units per concentration of DNA-binding site (given by the concentration of the ssDNA substrate in nucleotides divided by the number of oligonucleotides covered by each unit). In this way, the new analysis takes in account that hRPA covers ~30 nt when binding to DNA [81], while ESSB binds ~35 nt [82] (Figure 10C). The plot showed that, at a concentration of hRPA that coats the ssDNA molecules in the helicase reaction (96 nM heterotrimer), 15% of the 33-mer is unwound, while no unwinding is detectable at a coating concentration of ESSB (82 nM). Similarly at an *R* value of 3, 30% of the duplex was released in the presence of hRPA, but no unwinding was observed with ESSB suggesting that even at high *R* values the ESSB stimulates poorly the helicase activity of RECQ1. This observation is further supported by the results obtained with the 109 bp duplex (Figure 11). This long substrate can be unwound only when RPA is present, while ESSB fails to catalyze the unwinding even at a concentration ten fold higher than the one used for RPA and after three hours of reaction.

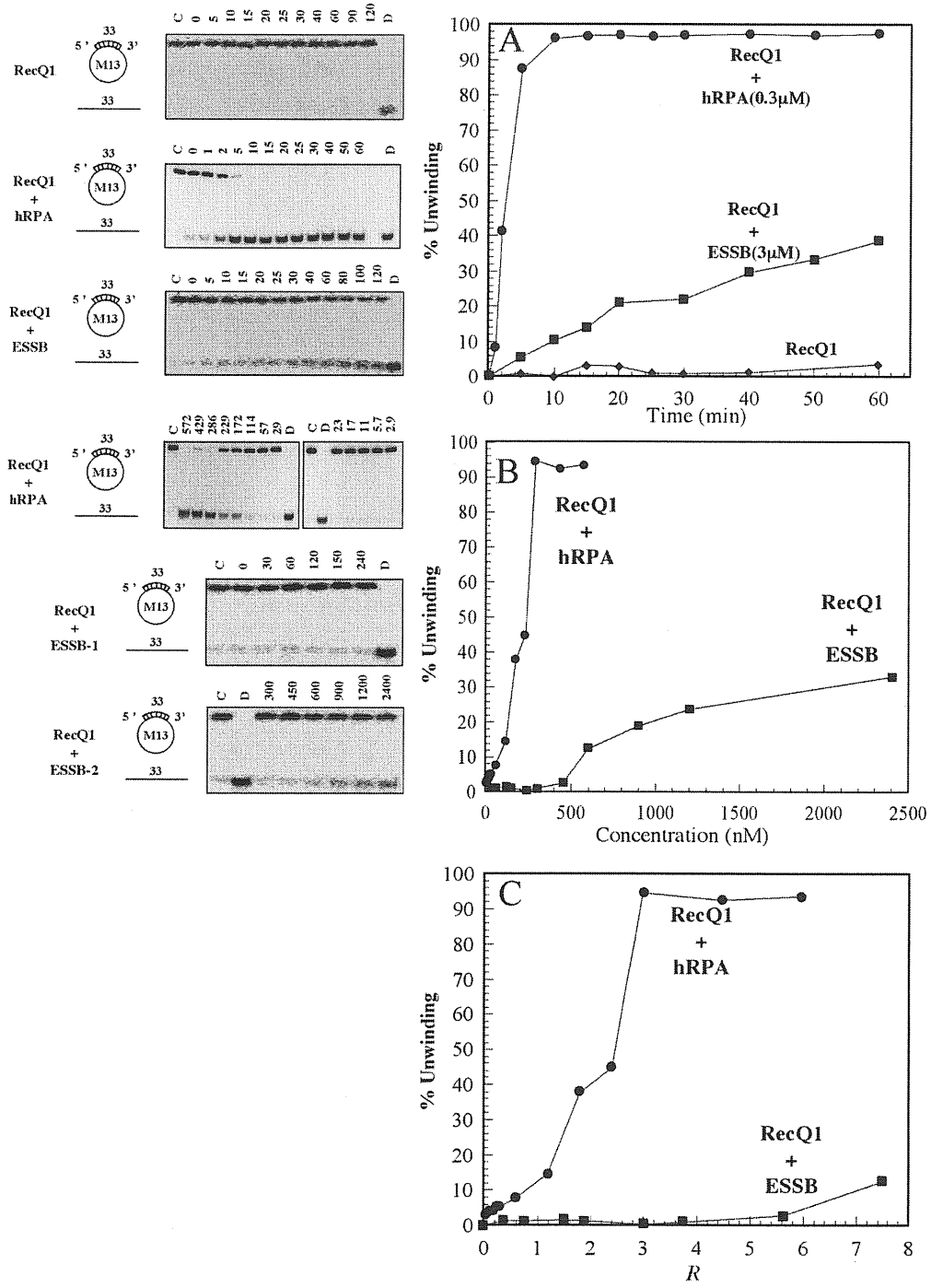

Figure 10. **Stimulation of the helicase activity of RECQ1 by hRPA and ESSB.** The substrate used in all experiments was the 33-bp partial duplex shown in Fig. 7*H*, and the concentration of RECQ1 was always 1 nM. *A*, the kinetics of unwinding were determined using RECQ1 alone (◆) and in the presence of 0.3 μM hRPA (●) or 3 μM ESSB (■). The reaction times (in minutes) are indicated above each lane in the autoradiograms. *B*, concentration dependence studies were carried out at increasing concentrations of hRPA (●) and ESSB (■). The protein concentrations (nanomolar) are indicated above each lane in the autoradiograms. *C*, the percentage of unwinding is expressed as a function of *R*, defined as the ratio of the concentration of single-strand binding protein units to the concentration of DNA-binding units.

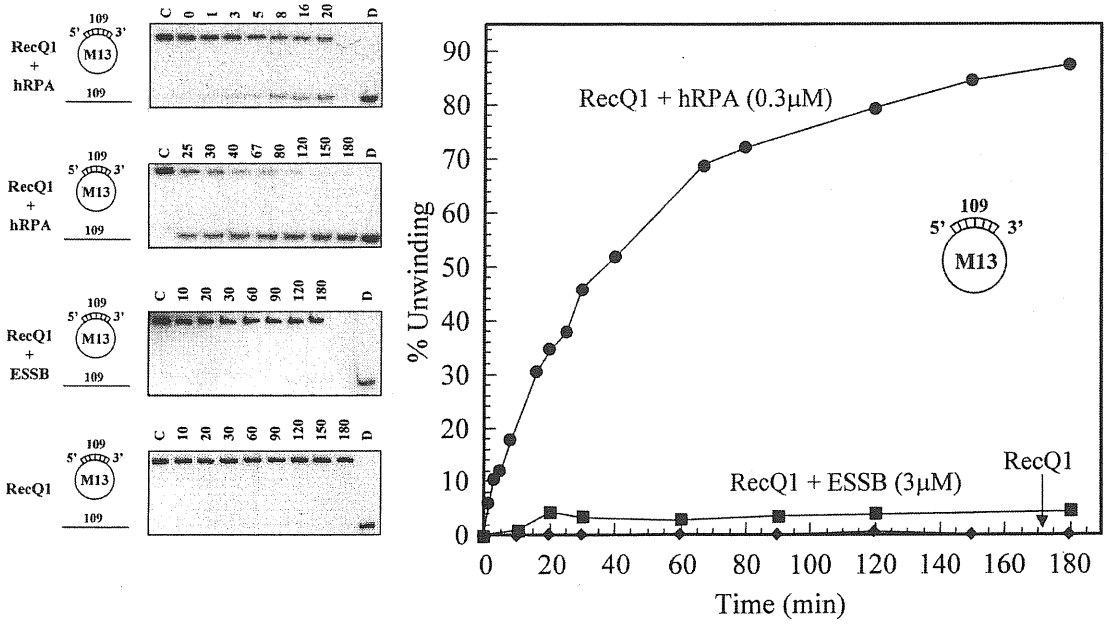

Figure 11. Kinetics of unwinding of a 109-bp partial duplex substrate in the presence of hRPA. The kinetics of unwinding were determined using RECQ1 alone (♦) and in the presence of 0.3 μM hRPA (●) or 3 μM ESSB (■). The concentration of RECQ1 was always 1 nM. The reaction times (in minutes) are indicated above each lane in the autoradiograms.

3.2 Characterization of recombinant RECQ1

3.2.1 Expression of recombinant RECQ1

The purification procedure of RECQ1 from HeLa cells involves several chromatographic steps and allows the production of very limited amounts of protein (< 0.1 mg). Since this amount is insufficient to perform rigorous and quantitative enzymic studies, we decided to develop a new strategy for the expression of recombinant RECQ1.

Two closely related isoforms of RECQ1 were first cloned independently by two separate laboratories, one encoding a protein of 649 aa and the other of 659 aa [12, 66]. The only difference between these two isoforms is found at the C-terminal end. Sequence alignment of the two isoforms indicates that the 659 aa seems to have three additional amino acids substitutions if compared to the shorter variant: A175D, C543S, and T566A. Nonetheless, these substitutions may be attributed to sequencing errors since especially the Cys 543 is highly conserved in the RecQ helicase family. More recently, two other smaller isoforms have been identified yet their enzymic activity has not been determined [67]. We amplified by PCR the cDNA encoding the 649 aa variant of RECQ1 starting from lymphocytes prepared from human peripheral blood and we cloned the amplified gene in the

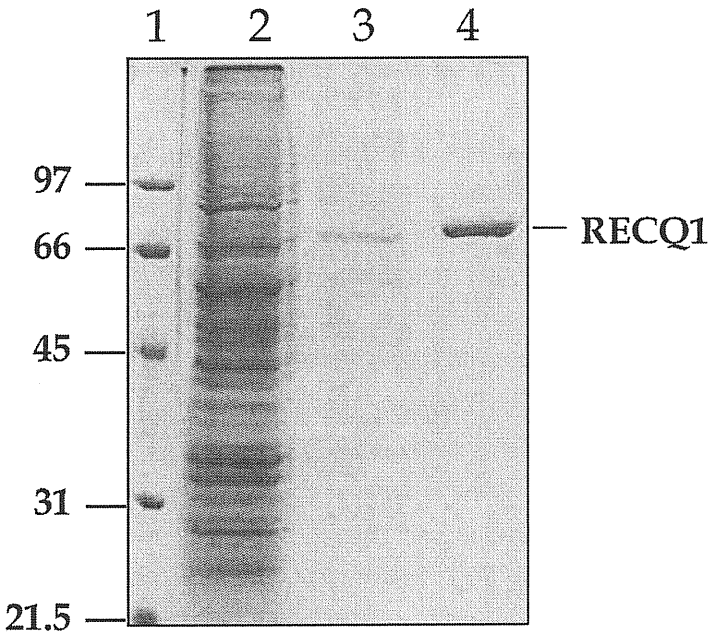

Figure 12. SDS–PAGE analysis of *RECQ1* gene expression in insect sf9 cells. Lane 1, low range molecular mass markers (in kDa); lane 2, lysate of sf9 cells infected with recombinant RECQ1 baculovirus; lane 3, washes of TALON metal affinity resin with 500 mM NaCl and 12. 5 mM imidazole; lane 4, elution from TALON metal affinity resin with 100 mM NaCl and 120 mM imidazole. The gel was 10% SDS–polyacrylamide stained with Coomassie blue R250. Lane 4 contained 3.75 µg of purified recombinant RECQ1.

appropriate expression vector. The recombinant RECQ1 helicase was first expressed in the *E. coli* expression strain BL21 (DE3). The final product displayed the proper molecular weight, but, as already observed by other laboratories, the protein did not show any DNA binding or unwinding activity [67]. Thus, I decided to use the baculovirus expression system already adopted by other laboratories to express proteins part of the RecQ family [83, 84]. In this way, I was able to express active RECQ1 with high yield (~0.75 mg of RECQ1 from 1.5×10^8 infected Sf9 cells). The purity of the purified protein was tested by SDS-PAGE. The silver-stained gel showed only a single band with a molecular mass of ~75 kDa (Figure. 12). The identity of the protein was confirmed by mass spectrometry (Table 2). The activity of recombinant

Table 2. Amino acid sequences of the peptides of RECQ1 identified by mass spectrometry.

Positions	*Sequence*
32-39	QQELIQKK
71-78	EDFPWSGK
118-119	EVFLVMPTGGGK
151-167	LKQLGISATMLNASSSK
172-180	WVHAEMVNK
186-193	LIYVTPEK
237-242	ALGILK
292-306	QKPSNTEDFIEDIVK
394-404	SMENYYQESGR

495-498	DLIK
502-522	QAEELNEKLTPLKLIDSWMGK
529-539	VAGVVAPTLPR
571-599	KIGPKANLLNNEAHAITMQVTKSTQNSFR
619-637	NSGNFQKKAANMLQQSGSK

RECQ1was identical to that of the native RECQ1 purified from the nuclear extract of HeLa cells [15] (data not shown).

3.2.2 Determination of oligomerization state of the recombinant RECQ1

Gel filtration and Dynamic Light Scattering (DLS) experiments were performed in order to investigate further the oligomerization state of recombinant RECQ1 in solution (Figure. 13). Gel filtration experiments showed that RECQ1 has an apparent molecular mass of 158 kDa suggesting that this protein is a dimer in solution (Figure. 13A). This result perfectly agrees with our previous glycerol gradient and gel filtration studies performed with the RECQ1 protein purified from HeLa cells where I had measured a sedimentation coefficient of 7.3 ± 1.7 S and a Stokes radius of 49.5 ± 10.5 Å, corresponding to a native molecular mass of 160 ± 18 kDa [15]. To further support these results, DLS measurements were conducted on RECQ1 in the 2.0 to 3.0 μM concentration range. The data clearly showed that the correlation time of RECQ1 is higher than that measured for BSA (Figure. 13B). This result provides strong experimental evidence that purified recombinant RecQ1 is not a monomer in solution. From the analysis of the data with the

Dynamic v6.0 software, we derived a hydration radius of 5.4 ± 0.6 nm for RECQ1 compared to a hydration radius of 3.5 ± 0.4 nm for the BSA control that has a molecular mass of 66 kDa. The value of the hydrodynamic radius did not change in the presence of a ssDNA fragments of 50 nucleotides,

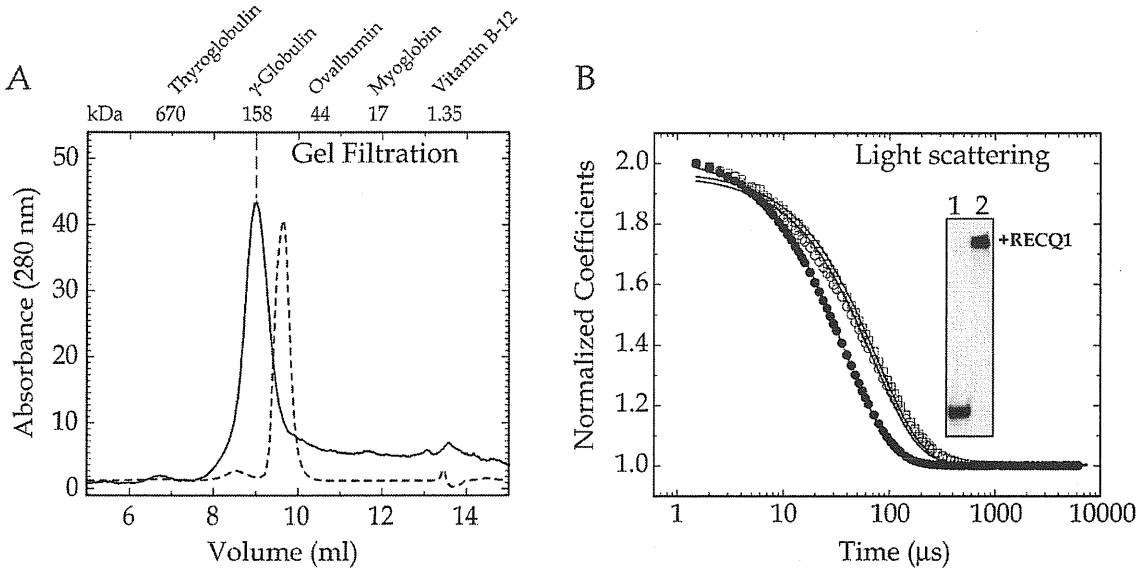

Figure 13. Gel filtration and dynamic light scattering analysis. (**A**) Gel filtration experiments were performed as described in Materials and Methods. The TSK-GEL G3000SW$_{XL}$ column was calibrated with thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (45 kDa), myoglobin (17 kDa) and vitamin B_{12} (1.35 kDa). The amount of RECQ1 loaded was 37.5 μg. The solid curve shows the elution profile of RECQ1 and the dashed curve the elution profile of BSA (66 kDa). (**B**) Normalized auto-correlation function (ACF) for control BSA (solid circles), RECQ1 alone (open circles) and RECQ1 + 50mer ssDNA + ATP + Mg^{2+} (open squares). Measurements were carried out at room temperature (23 ± 0.1°C) with a protein concentration of ~2.5 μM in buffer (50 mM Tris–HCl pH 7.5, 100 mM NaCl, 1 mM DTT). Solid lines are the fit with the built-in regularization method (Dynamics v6.0 from Protein Solutions). Radii were 3.5 ± 0.4 nm for BSA and 5.4 ± 0.6 nm for all the RECQ1 samples. (Insert) Band shift assay with the 50 nt ssDNA probe in the absence (1) and presence (2) of RECQ1. The experiments were carried out with 1 μM RECQ1 and 0.4 nM DNA as described in Materials and Methods.

with or without 5 mM $MgCl_2$ and 5 mM ATP. EMSA experiments confirmed that RECQ1 binds the 50 nucleotides DNA fragment under our reaction conditions (Figure. 13B). Moreover, using fluorescence a dissociation constant (K_d) ≤ 0.1 μM was measured for RECQ1 binding to the 50 nt ssDNA fragment indicating that RECQ1 is fully bound to DNA under the reaction conditions

used for the DLS experiments (date not shown). Thus, our DLS data indicate that only one RECQ1 complex is able to bind a 50 nt fragment and that RECQ1 maintains the same dimeric structure in the free and DNA bound forms.

3.2.3 Unwinding properties of recombinant RECQ1

A series of M13 partial duplex DNA substrates of 17, 25, 50, 110, and 216 bp were prepared in order to characterize the effect of the duplex length on the helicase activity of RECQ1. Unwinding experiments were carried out at increasing concentrations of RECQ1 in a buffer containing 20 mM Tris-HCl (pH 7.5), 8 mM dithiothreitol, 5 mM $MgCl_2$, 5 mM ATP, 10 mM KCl, 4% (w/v) sucrose, 80 μg/ml bovine serum albumin (BSA) (Figure. 14). The figure shows that while a short DNA duplex of 17 bp can be easily unwound using 1 nM RECQ1, longer DNA duplexes require significantly more enzyme to be opened. The 25 bp duplex was completely unwound in 30 min in the presence of 150 nM RECQ1, while using the same enzyme concentration only 50% of the 50 bp duplex was unwound. Further increase in the concentration of RECQ1 did not result in a significant enhancement of the percent of substrate unwound. The reason why this longer duplex could not be completely unwound even at high concentration of RECQ1 may be due to strand reannealing during the unwinding reaction as already observed for other helicases [21, 85]. The 110 bp duplex was the longest DNA substrate among those tested that can be partially unwound by RECQ1. In fact, almost 20%

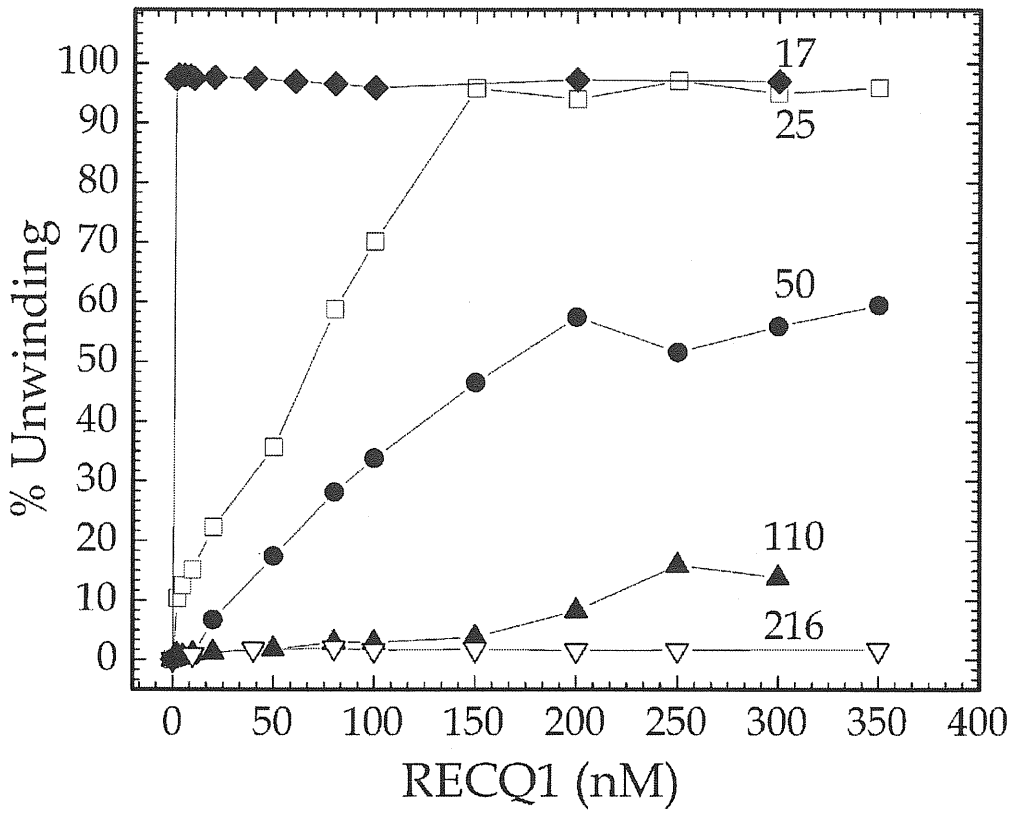

Figure 14. Unwinding studies with DNA substrates of increasing duplex length. Helicase assays were performed as described in Materials and Methods. The indicated amount of RECQ1 was used to unwind the M13mp18 partial duplex substrates of 17 (black diamond), 25 (open square), 50 (black circle), 110 (black triangle) and 216 bp (open triangle). The concentration of the substrate was always 0.4 nM. After incubation at 37°C for 30 min, the reaction mixtures were resolved on a 12% non- denaturing polyacrylamide gel.

of this substrate was unwound in the presence of 250 nM RECQ1 in 30 min, while no unwinding was detected with the 216 bp substrate.

For a better comparison of the results obtained with the different substrates, it is helpful to express the unwinding data as an apparent rate of base pairs unwound per min per RECQ1 helicase unit (bp/min/RECQ1) [21]. The apparent unwinding rates for the 25 and 50 bp partial duplex were almost identical. In fact, at a RECQ1 concentration of 50 nM, the rates were 0.00238 and 0.00240 bp/min/RECQ1 respectively for the 25 bp and the 50 bp

duplex. Likewise, at a RECQ1 concentration of 100 nM, the unwinding rate was 0.00234 bp/min/RECQ1 for the 25 bp duplex and 0.00232 for the 50 bp duplex. Thus, the concentration of RECQ1 that is required for unwinding is proportional to the length of the duplex to be unwound. On the other hand, the unwinding activity of RECQ1 was dramatically reduced with longer DNA duplexes. In fact, only a small fraction of the 110 bp duplex could be unwound by RECQ1 and no unwinding was detected for the 216 bp duplex even at the highest RECQ1 concentration tested. Therefore, RECQ1 is able to efficiently unwind *in vitro* only short DNA substrates.

3.2.4 Stimulation of recombinant RECQ1 helicase activity by hRPA

The unwinding assays with RECQ1 purified from the nuclear extract of HeLa cells showed that the helicase activity was specifically stimulated by hRPA [15]. On the other hand, the small amount of HeLa RECQ1 available did not allow us to perform studies with DNA duplexes longer than 110 bp. The baculovirus expression systems allowed the purification of milligram quantities of active RECQ1. Thus, the recombinant protein was incubated with the 216 bp duplex substrate in the presence of increasing concentrations of hRPA (Figure. 15A). In control reactions, we verified that hRPA alone (300 nM) did not denature the 216 bp DNA duplex. Similarly, RECQ1 alone (300 nM) was unable to unwind this substrate, even though more than 16% of the substrate was unwound when hRPA was added at a concentration of 100 nM and 70% unwinding was reached by increasing the hRPA concentration to 300

nM. Moreover, kinetic studies performed in the presence of 300 nM RECQ1 and 300 nM hRPA showed that the 216 bp substrate could be completely unwound if the incubation time was longer than 80 min (Figure 15B). On the other hand, no unwinding was detected when *E. coli* single-strand binding protein (ESSB) was used instead of hRPA even at a concentration of 3 μM. Strand displacement can be expressed as function of the ratio (*R*) of the concentration of SSB-units per concentration of DNA-binding site as already described before (see paragraph 3.1.4). As such, the analysis takes into account that hRPA covers ~30 nts when binding to DNA [81], while ESSB binds ~35 nts [82]. The plot shows that 16% of the substrate was unwound at a concentration of hRPA that coated the ssDNA molecule in the helicase reaction (96 nM) and that, at an *R* value of 3, 70% of the substrate was unwound in 45 min. On the other hand, ESSB failed to catalyze unwinding even at a concentration 10-fold higher than that used for hRPA and at an *R* value of 36.

In order to determine the maximum number of base pairs that can be unwound by RECQ1 in the presence of hRPA, we performed a systematic study using DNA substrates with DNA duplex regions of 301, 416, 501, 603, and 807 bp (Figure 16). The results showed that, under our experimental conditions, RECQ1 is able to unwind up to 501 bp since no unwinding was detectable for the 603 and 807 bp duplexes.

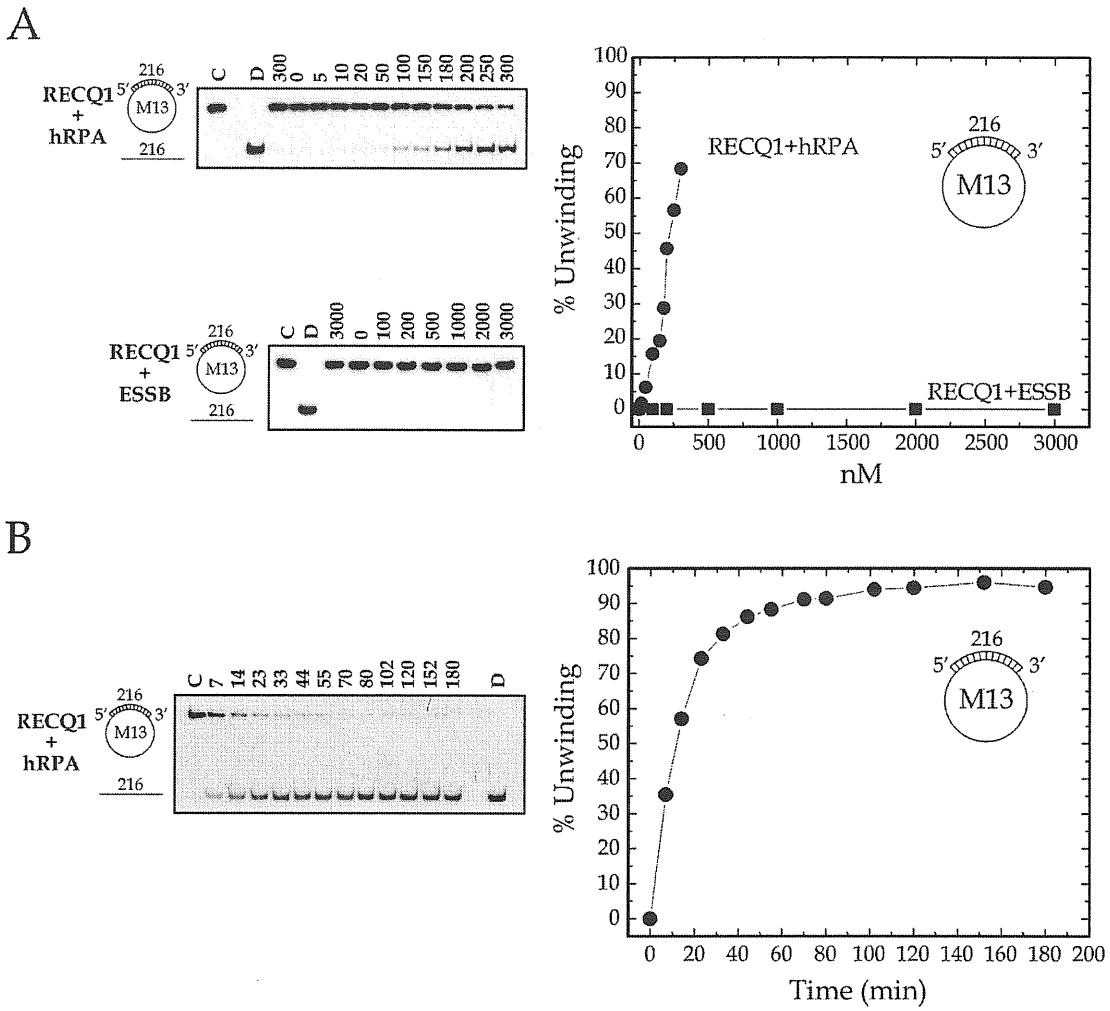

Figure 15. Stimulation of RECQ1 helicase activity on a 216 bp duplex substrate by hRPA and ESSB. (A) The indicated amount of hRPA (filled circle) or ESSB (filled square) was preincubated with 216 bp duplex DNA substrate (0.4 nM). The reactions were initiated by adding 300 nM RECQ1 and carried on at 37°C for 45 min. The reaction mixtures were resolved on a 6% non-denaturing polyacrylamide gel. The concentrations (nM) of hRPA and ESSB are indicated above each lane in the autoradiogram. Lanes C and D are control assays without enzyme and with heat-denatured substrate. The graph shows the percentage of unwinding versus the concentration of single-stranded binding protein. (B) Kinetics of unwinding of the 216 bp duplex DNA substrate by RECQ1 (300 nM) in the presence of hRPA (300 nM). The experiment was performed using the same reaction conditions as described above. At the indicated times (min), 20 µl of the reaction mixture were removed and quenched with 50 mM EDTA. The mixtures were resolved on a 6% non-denaturing polyacrylamide gel.

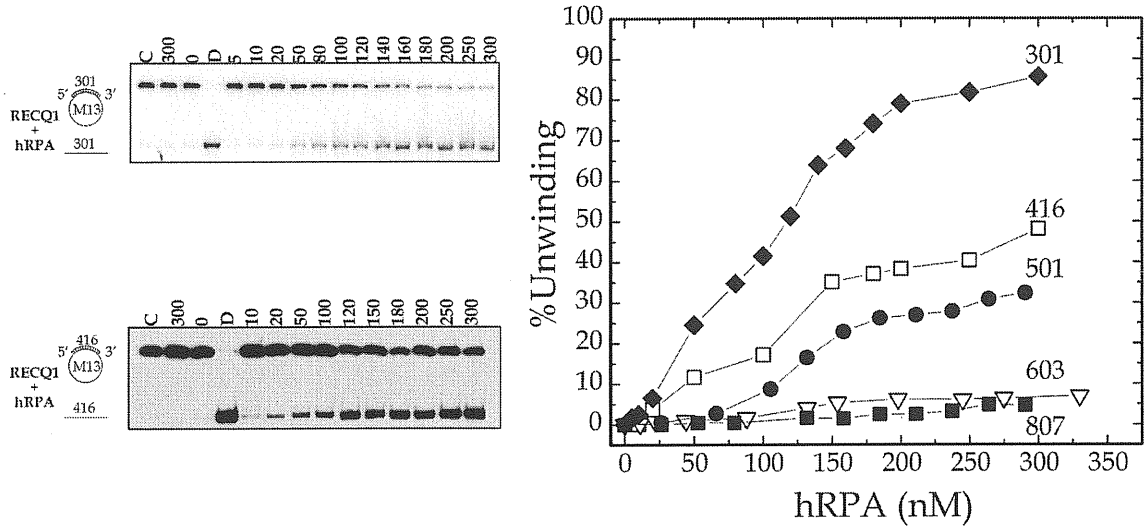

Figure 16. Unwinding studies with DNA substrates of increasing duplex length in the presence of hRPA. The indicated amount of hRPA was preincubated with different DNA substrates containing partial duplexes of 301 (filled diamond), 416 (open square), 501 (filled circle), 603 (open triangle) and 807 bp (filled square). The concentration of the substrate was always 0.4 nM. The reactions were initiated by adding 300 nM RECQ1. The reaction was incubated at 37°C for 120 min. The reaction mixtures were resolved on a 6% non-denaturing polyacrylamide gel. The percentage of unwinding is expressed as a function of hRPA concentration. The hRPA concentrations (nM) are indicated above each lane in the autoradiogram. Lanes C and D are control assays without enzyme and with heat-denatured substrate.

3.2.5 Effect of hRPA on the rate of ATP hydrolysis

The ATPase activity of RECQ1 was measured in the absence and presence of DNA cofactors of varying length. Successively, the consequences of the addition of hRPA on the rates of ATP hydrolysis were analyzed (Figure 17). Little or no ATPase activity was detected in the absence of DNA in agreement with previous studies performed with other DNA helicases [21, 86]. In the presence of ssDNA cofactors, the reaction of ATP hydrolysis followed a classical Michaelis-Menten kinetics over an ATP concentration range of 1 to 800 μM. The kinetic constant (k_{cat}) for ATP hydrolysis with a short ssDNA fragment of 17 nt was 31 ± 2 min^{-1}. The k_{cat} value increased by

less than fold 2 in the presence of a DNA cofactor of 25 nt and by a factor of 4 in the presence of the long M13 ssDNA circle (Table 3). The same experiments were repeated at two different ssDNA concentrations and no change in the initial rates for ATP hydrolysis was observed indicating that the reaction was saturated with respect to ssDNA (data not shown). The addition of different concentrations of hRPA (from 50 to 300 nM) did not induce an enhancement in k_{cat} (Table 3). On the contrary, at the highest hRPA concentration (300 nM) we observed a 2-fold inhibition on the rate of ATP hydrolysis (data not shown). These result are similar to that obtained by Brosh and co-workers studying the effect of hRPA on the ATPase activity of BLM and cannot be easily explained since it is only observed with M13 and not with the shorter ssDNA effectors [21]. Our results indicate that hRPA does not increase the ATPase activity of RECQ1 and that the stimulation of the helicase activity of RECQ1 by hRPA must be due to other factors.

Table 3. Comparison of the k_{cat} (min^{-1}) for ATP hydrolysis of RECQ1, BLM, and WRN. The k_{cat} values for RECQ1 have been measured in the presence of various DNA effectors with and without hRPA.

RECQ Helicase	*DNA Effector*	k_{cat}	*References*
		min^{-1}	
hsRECQ1	17mer	31.14±2.29	
	25mer	56.95±1.51	

	M13mp18	126.29±3.44	
hsRECQ1+hRPA	25mer	58 ± 1.48	
	M13mp18	133.18 ± 3.44	
HsBLM	M13mp18	1163±358	[21]
hsBLM$^{642-1290}$	M13mp18	1328.4	[56]
hsWRN	M13mp18	58.4±17	[20]
DmRECQ5	M13mp18	900±120	[17]

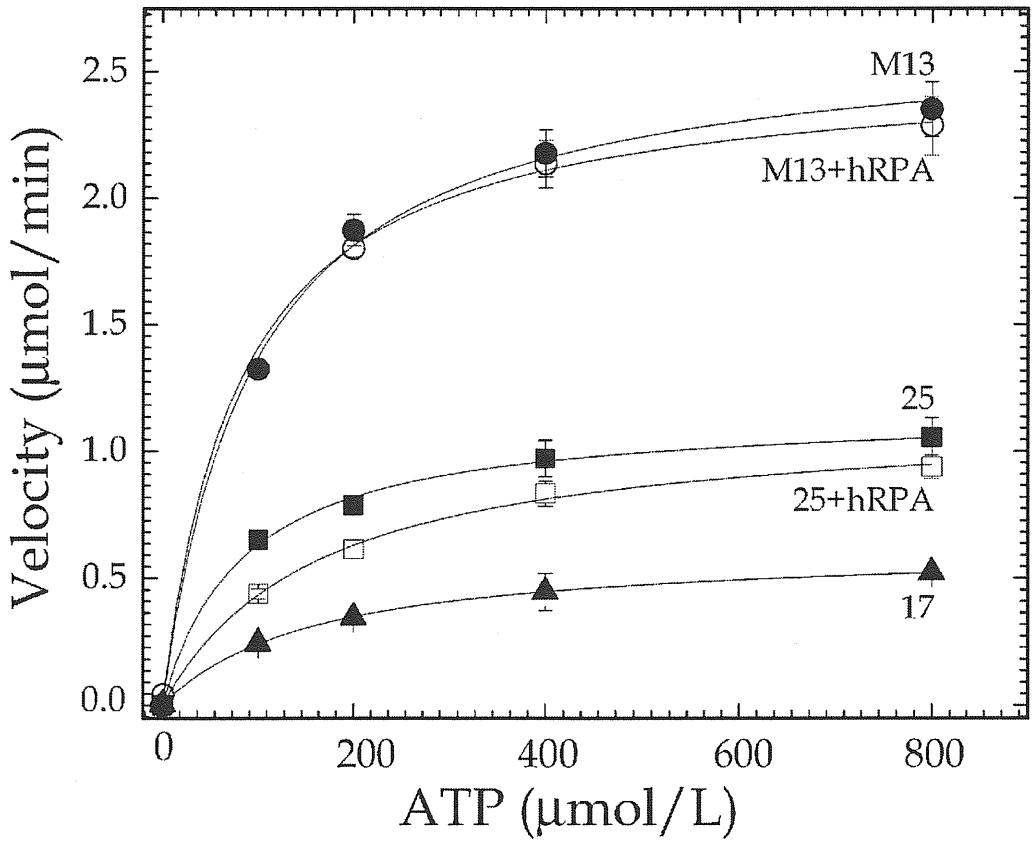

Figure 17. Analysis of the ATPase activity of the recombinant RECQ1 and effect of hRPA on rate of ATP hydrolysis. Filled circles, reactions with M13mp18; open circles, reactions with M13mp18 and hRPA (50 nM); filled squares, reactions with 25 nt ssDNA; open squares,

reactions with 25 bp ssDNA and hRPA (50 nM); filled triangles, reactions with 17 bp ssDNA. The initial velocities for ATP hydrolysis were expressed as a function of the ATP concentration. The experimental points were fitted to the Michaelis–Menten equation: $V_0 = V_{max}X/(K_m + X)$, where V_0 is the initial velocity and X is the substrate concentration (ATP). Each value represents the mean of at least five independent measurements.

3.2.6 Analysis of physical interaction between hRPA and RECQ1

Far western assays

I performed Far Western experiments to study the possible physical interaction of RECQ1 with hRPA. For these experiments, hRPA was immobilized on a Hybond-P PVDF membrane that was successively incubated with recombinant RECQ1. After washing the unbound proteins, conventional Western analysis was performed to detect the RECQ1 eventually bound to hRPA (Figure. 18A). Bovine serum albumin (BSA) was added as negative control. In addition, a membrane containing hRPA was incubated with buffer alone to verify that there was no cross-reactivity of the anti-histidine antibody with hRPA. The Far Western showed a single band at the position of the 70 kDa subunit of hRPA, while no binding was detected for BSA and for the 32 and 14 kDa subunits of hRPA. This result indicates that RECQ1 specifically interacts with the 70 kDa subunit of hRPA. The same experiment carried out with the Ku heterodimer, a protein that plays a central role in non-homologous end-joining (NHEJ). The result shows that RECQ1 does not interact with Ku.

ELISA assays

To further confirm a direct association between RECQ1 and hRPA, ELISA experiments with the recombinant purified proteins were performed in the laboratory of Dr. Robert M. Brosh at the Laboratory of Molecular Gerontology, National Institute on Aging, Baltimore, Maryland (USA). Increasing amounts of hRPA were incubated in wells that had been previously coated with RECQ1 (18 nM) (Figure 18B). After washing, the RECQ1-hRPA complex was detected with a mouse monoclonal antibody raised against the 70 kDa subunit of hRPA and a colorimetric assay was used to build binding curves that reached saturation at hRPA concentrations ≥40 nM. The specificity of this interaction was demonstrated by the absence of color in wells that had been precoated with BSA rather than RECQ1. Data analysis yielded an apparent dissociation constant (K_d) of 6.2 nM that is similar to that previously described for the interaction of BLM with hRPA [14]. Moreover, the same experiments performed in the presence of ethidium bromide (50μg/ml) yielded almost identical binding curves demonstrating that the RECQ1-hRPA interaction is not mediated by DNA (data not shown).

Co-immunoprecipitation experiments

In collaboration with the laboratory of Dr. Brosh, we performed co-immunoprecipitation experiments from HeLa nuclear extracts using a polyclonal antibody directed against RECQ1 to make sure that a physical interaction between RECQ1 and hRPA exist also in cell nuclei. The anti-RECQ1 antibody precipitated both RECQ1 (data not shown) and hRPA proteins from normal human nuclear extract (Figure 19 lane 4). Approximately 5% of the hRPA from the HeLa nuclear extract input

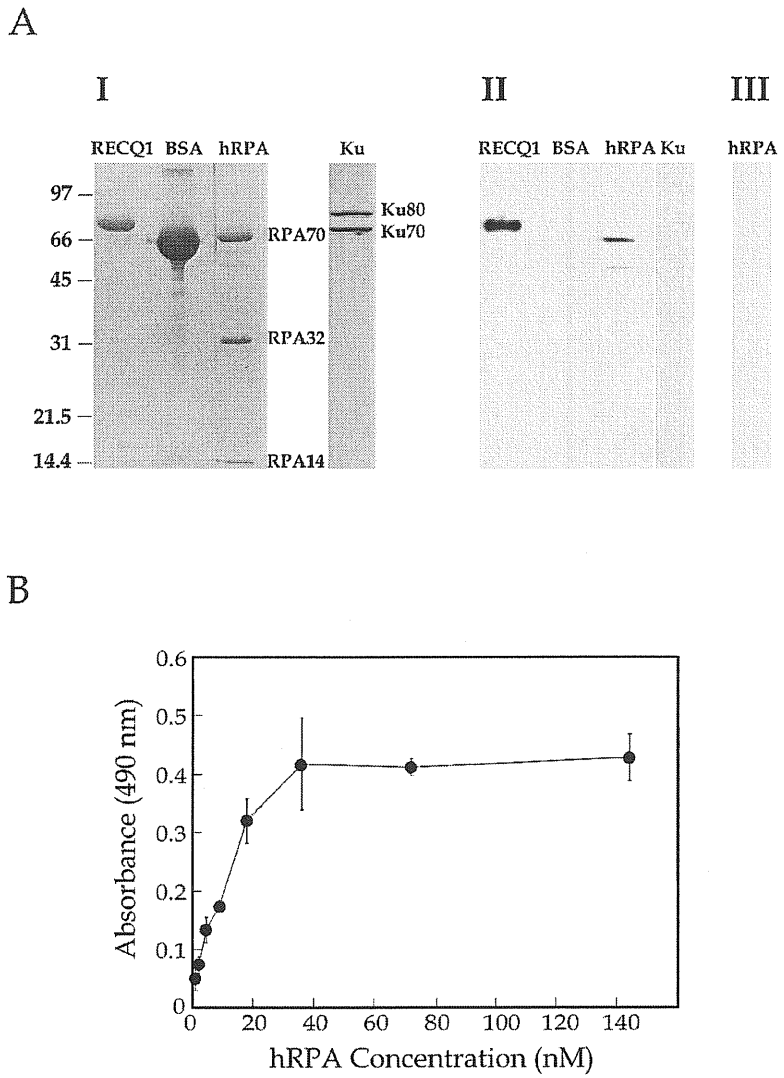

Figure 18. RECQ1 physically interacts with the 70 kDa subunit of hRPA. (A) Detection of the RECQ1–hRPA complex by far western analysis. Purified recombinant RECQ1, BSA, hRPA, and Ku were subjected to SDS–PAGE. I, Coomassie blue staining of the gel; II, proteins were transferred to a Hybond-P membrane and then incubated with recombinant RECQ1, western blotting using anti-6His monoclonal antibody then being used to detect the presence of RECQ1 on the membrane; III, in control experiments Hybond-P membrane containing hRPA was incubated with buffer alone to verify that there was no cross-reactivity between the anti-6His monoclonal antibody and hRPA. (B) Detection of RECQ1–hRPA complex by ELISA. RECQ1-coated wells (18 nM application) were incubated with increasing amounts of hRPA protein for 1 h at 30°C. Wells were aspirated and washed three times and bound hRPA protein was detected by ELISA using a mouse monoclonal antibody against hRPA (70 kDa subunit) protein. Absorbance readings at each point were corrected by subtracting a background A_{490} reading generated with BSA-coated wells.

(Figure. 19 lane 2) was co-immunoprecipitated with RECQ1 using the anti-RECQ1 antibody. Control experiments using normal rabbit IgG incubated in

the HeLa nuclear extracts confirmed the specificity of the anti-RECQ1 antibody (Figure 19. lane 5). Analogous experiments carried out in the presence of ethidium bromide demonstrated that this DNA intercalating drug does not affect the co-immunoprecipitation of hRPA with RECQ1 (Figure 19 lane 3). This result indicates that the interaction between RECQ1 and hRPA occurs also in the cell nuclei and is not mediated by nucleic acids as already observed with the EMSA assay.

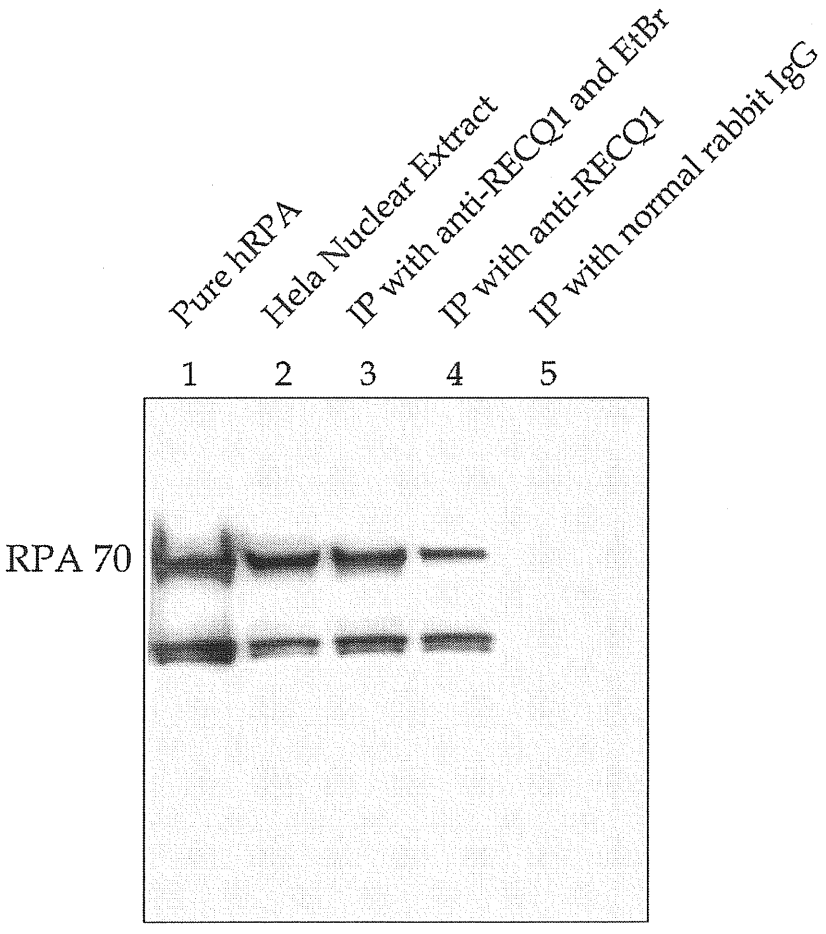

Figure 19. Co-immunoprecipitation of RECQ1 and hRPA from human nuclear extracts with and without ethidium bromide. RECQ1 antibody co-precipitates RECQ1 and hRPA from HeLa nuclear extracts both in the presence and absence of ethidium bromide. The blot was probed with mouse anti-RPA antibody. Lane 1, purified hRPA (250 ng); lane 2, HeLa nuclear extract (54 µg); lane 3, immunoprecipitate from HeLa nuclear extract (1.36 mg) with ethidium bromide (50 µg/ml) using rabbit anti-RECQ1 antibody; lane 4, immunoprecipitate from HeLa nuclear extract (1.36 mg) using rabbit anti-RECQ1 antibody; lane 5, control precipitate from HeLa nuclear extract (1.36 mg) using normal rabbit IgG.

4. Discussion

RECQ1 was first RecQ helicase to be discovered in human cells. Nevertheless, RECQ1 is still one of the least characterized human RecQ helicases in terms of its enzymic activity and biological function. Conversely, three other members of human RecQ helicase family, WRN, BLM and RECQ4, have been widely studied, in the past ten years. The main reason is that these three helicases are responsible for three genetic disorders associated with inherent genomic instability, whereas there are no evidences yet of disorders coupled to mutations in the RECQ1 gene. Even though there are evidences that RecQ helicases play a key role in maintaining genomic stability, their precise biological functions are still uncertain or under debate. In this regard, a detailed characterization of the substrate specificity and unwinding activity of the five human RecQ helicases as well as the discovery of protein partners that affect their enzymatic activity is essential for the understanding of the function of these increasingly studied class of enzymes. In the present work, the RECQ1 helicase was isolated from the nuclear extract of HeLa cells and s its enzymatic activity was characterized in details. Since the amount of native RECQ1 obtained from HeLa cells was insufficient for a quantitative biochemical and enzymic characterization, the production of RECQ1 was successively scaled up by expressing the recombinant protein. Previous studies have reported that RECQ1 expressed either using bacteria or yeast expression system doesn't possess any helicase activity [67, 87]. For this reason, we decided to express the protein using the baculovirus expression

system that has been already successfully used for the expression of WRN. After optimization of the expression condition, approximately 0.75 mg of purified recombinant RECQ1 were obtained from 1.5×10^8 infected Sf9 cells. The identity of the recombinant RECQ1 was verified both by mass spectrometry and western-blot with the specific antibodies. The helicase activity of our recombinant RECQ1 is comparable to that of RECQ1 purified from HeLa nuclear extracts.

Several helicases from different organisms have been identified and characterized to date. Some helicases exist and function as monomers, others as dimers or hexamers [5, 88]. Different mechanisms for DNA unwinding have been proposed for monomeric, dimeric and hexameric helicases, respectively [89]. Therefore, the knowledge of the oligomeric state of the helicase is crucial to understand the mechanism that the enzyme adopts to translocate along the DNA molecule and to dissociate the base pairs during the unwinding process. The recently obtained crystal structure of the core domain of *E. coli* RecQ showed that this protein is a monomer in the absence of DNA [49]. On the other hand, there are contradictory evidences regarding the oligomerization state of the full-length *E.coli* RecQ. A recent biophysical study on the *E. coli* RecQ suggests that this enzyme functions as a monomer when unwinding DNA [59] in contrast with previous studies indicating that *E. coli* RecQ can form a multimer of at least three subunits in solution [52]. The only information available so far on the oligomerization state of human RecQ

helicases has been obtained for BLM and WRN. Electron microscopy and size-exclusion chromatography experiments demonstrated that the full-length BLM forms hexamers [55], while recent studies with the fragment 642-1290 of BLM revealed that $BLM^{642-1290}$ runs as a monomer on a gel filtration column both in solution and in its ssDNA bound form [56]. Similar gel filtration experiments suggested that the 333-amino acid N-terminal fragment of WRN as well as the full-length recombinant protein elute as a trimer [57]. Successively, analysis by atomic force microscopy of the 171-amino acid fragment of WRN, responsible for the exonuclease activity of this enzyme, revealed that this fragment is in a trimer-hexamer equilibrium in the absence of DNA and that this equilibrium is significantly shifted toward the hexamer in the presence of DNA [58]. Our results from glycerol gradient and gel filtration studies on RECQ1 purified from HeLa cells indicate that the native RECQ1 forms dimers in solution. In addition, the sigmoidal shape of the titration curve done at increasing concentration of RECQ1 is indicative of a cooperative behavior suggesting that more than one unit of RECQ1 dimer could be involved in DNA unwinding as seen in the case of other helicases. The size-exclusion chromatography studies with recombinant RECQ1 confirmed that this protein exists as a dimer in solution. To further support this conclusion and in order to see if the oligomerization state of RECQ1 may change in the presence of ssDNA, Dynamic Light Scattering (DLS) experiments were performed both in the presence and absence of a 50 nt ssDNA fragment. The value of the hydration radius for RECQ1 is of 5.4 ± 0.6

nm compared to a hydration radius of 3.5 ± 0.4 nm for the BSA control that has a molecular mass of 66 kDa. The higher value of the hydrodynamic radius confirms that RECQ1 does not exist as a monomer in solution. On the other hand, the value of the radius is higher then that expected for a simple RECQ1 dimer under a spherical shape assumption. This result could be consistent with a highly hydrated complex, a very elongated shape or an oligomeric complex formed by more than two subunits, although this last hypothesis is not supported by our gel filtration results. The value of the hydrodynamic radius does not change when a ssDNA fragment of 50 nt is added to the sample in the presence of 5 mM $MgCl_2$ and 5 mM ATP. Hence, the oligomerization state of RECQ1 does not change when the enzyme is bound to the 50 nt ssDNA probe. The fact that RECQ1 seems to form dimers rather than hexamers indicates that the five human helicases of the RecQ family may form different oligomeric structures and may adopt different mechanisms to unwind DNA.

Other key features that characterize DNA helicases are their substrate specificity. The 3′ to 5′ polarity of unwinding was the only information available on the helicase activity of RECQ1 and has been also confirmed by our results [12]. The substrate specificity of RECQ1 was then investigated with a series of DNA probes of different structures and length. As it was previously observed for the BLM and WRN helicases [54], RECQ1 cannot unwind blunt-end DNA substrates. In contrast, the *E. coli* RecQ helicase, is

able to initiate duplex unwinding from a blunt-end terminus [53]. Nevertheless, RECQ1 is able to unwind certain kind of blunt-end duplexes, since it can unwind a blunt-end DNA substrate with a 25 bp "bubble" in the centre, again in analogy to what has been previously observed for WRN and BLM, [54] (Figure 7). From the short size of its N- and C-terminal domains, RECQ1 is the human RecQ helicase, among the three (BLM, WRN and RECQ1), that resembles the most the *E.coli* variant. For this reason, it could have been predicted that RECQ1 could have more similarities to the *E. coli* RecQ than the other two helicases. On the other hand, our findings suggest that the residues responsible for the substrate specificity of these enzymes must reside in central conserved domain rather than in the N- and C-terminal domains and indicate that RecQ helicases from different organisms have different substrate specificities. The effect of the length of the 3′ single strand DNA tails on the unwinding activity of RECQ1 was also investigated (Figure 8). Tail length studies have not been performed before on other human RecQ helicases, but it was shown that Sgs1p from *Saccaromyces cerevisiae* is able to unwind substrates with 3′ tails of only 3 nts. Our results indicate that RECQ1 differs from Sgs1p, since a DNA probe with a 3′ tail of 10 nt is poorly unwound and only when the tail length is increased to 25 nt the more than 70% of the substrate is unwound (Figure 8). A possible explanation is that RECQ1 needs a single strand DNA tail longer than 10 nts to efficiently bind the substrate and start the unwinding. In fact, our EMSA results clearly show that RECQ1 has a very low affinity for the DNA substrate with 10 nt tail,

whereas it binds very efficiently the DNA substrates with 25 nt 50 nt tails. In collaboration with the laboratory of Robert Brosh, we also saw that the unwinding activity of RECQ1 is greatly inhibited on 5′-flap DNA substrate. This substrates resembles a key DNA replication intermediate formed during the synthesis of lagging strand and is easely unwound by WRN even when the 5′ flap ssDNA is very short, 5-10 nt. Other substrates with more complicated structures will be made in the future to complete the characterization of the substrate specificity of RECQ1. For example, it would be also very interesting to study to activity of RECQ1 on Holliday junction, a signature structure of homologous recombination (HR), since these structures are efficiently resolved by WRN and BLM. In addition, BLM and WRN unwind G-quadruplex and triplex structures with a 3' ssDNA tail more efficiently than a duplex with a 3' ssDNA tail. G-quadruplex and triplex DNA structures are considered as "road blocks" of DNA metabolism and are generated during the processes of DNA recombination and replication. The abilities to resolve those structures are certainly the important tasks for the maintenance of genomic stability. Thus, similar studies with RECQ1 will provide valuable information on its function and on the differences existing among the five human RecQ helicases.

The length of the duplex region that can be unwound varies dramatically among helicases. For example, the *E. coli* RecBCD is a highly processive helicase capable of unwinding > 20 kb at a rate of 350 bp s^{-1} [90]

and UvrD, another *E.coli* helicase, can unwind blunt-ended duplex substrates of 2700 bp [91]. On the contrary, a common feature of the human RecQ helicases is that they are not able to unwind long DNA duplexes. The human BLM and WRN helicases can only unwind dsDNA fragments ≤ 53 bp and ≤ 91 bp, respectively [84, 86]. Similarly to BLM and WRN, our results show that RECQ1 can easily unwind dsDNA substrates shorter than 50 bp and less than 20% of a 110 bp duplex is unwound in the presence of 250 nM RECQ1.

On the other hand, considerably longer duplexes can be displaced by RECQ1 if hRPA is added to the reaction mixture. The stimulation effects were observed both using native and recombinant RECQ1. RECQ1 is able to unwind duplexes of 501 bp when hRPA is present. A possible explanation for the hRPA stimulation could be that it coats the single strands generated during the opening of the duplex. Nevertheless, our data with the *E. coli* single-strand DNA binding protein (ESSB) show that only hRPA is able to stimulate the helicase activity of RECQ1 indicating that hRPA performs an additional role in the unwinding reaction rather than simply inhibiting the reannealing of the displaced strands and suggesting that specific intra-molecular interactions may exist between RECQ1 and hRPA. The same specific effect of hRPA has been already observed for BLM and WRN that, in the presence of hRPA, can unwind DNA duplexes of 259 bp and 849 bp, respectively [20, 21]. The difference in number of base pairs that can be displaced by BLM, WRN, and RECQ1 with the assistance of hRPA may be

due to the different experimental conditions used. However, the observation that the unwinding activity of all these three helicases is specifically stimulated by hRPA suggests that they might all be involved in the physiological processes that require hRPA.

In order to shed light on the mechanism of hRPA stimulation, the effect of hRPA on the ATPase activity of RECQ1 was tested. The k_{cat} value for the rate of ATP hydrolysis is 31 ± 2 min^{-1} in the presence of a DNA fragment of 17 bp and increases to 126 ± 3 min^{-1} in the presence of M13 ssDNA as effector (Table 3). These k_{cat} values are very similar to that measured for WRN, but about 10-fold smaller than those determined for BLM and DmRECQ5 [17, 20, 21, 56]. In agreement with previous studies done with BLM and WRN [20, 21], the presence of hRPA does not enhance the ATPase activity of RECQ1 suggesting that the molecular basis for the specific hRPA stimulation of the unwinding activity of these helicases must be different. Brosh and co-workers speculated that hRPA might help the recruitment of the helicase to the ssDNA-dsDNA junction on the ongoing helicase reaction [21]. However, further studies with DNA substrates of different structures and with mutant proteins will be needed to reach a better understanding of the stimulation mechanism.

Moreover, Far Western analysis indicated that RECQ1 physically interacts with the 70 kDa subunit of hRPA. The direct interaction between

RECQ1 and hRPA is substantiated by the results of ELISA and co-immunoprecipitation experiments. ELISA assay with the purified proteins allowed the estimation of an apparent dissociation constant K_d = 6.2 nM for the RECQ1-hRPA complex close to that previously determined for the interaction of BLM with hRPA [14]. The same experiment repeated using the DNA intercalating drug, ethidium bromide, gives analogous results indicating that DNA is not mediating this interaction. Co-immunoprecipitation experiments with HeLa nuclear extracts proved that the RECQ1-hRPA complex is present in the cell nuclei and, also in this case, the addition of ethidium bromide does not disrupt the complex confirming that nucleic acids are not required for the binding of RECQ1 to hRPA. The BLM and WRN helicases also interact with hRPA, although the precise region of the helicases involved in the binding of hRPA and the functional role of this interaction remains an open question. Several proteins involved in different aspects of DNA metabolism have been shown to interact with WRN and BLM [92]. Nevertheless, the precise location of the interaction domain within the respective RecQ helicase has been identified only for some of them [65]. For example, the N-terminus of WRN contains a 3′- 5′ exonuclease domain and mediates the interaction of WRN with Ku70 subunit [23, 93] and PCNA [22], while the C-terminus is responsible for the interaction with p53 [94, 95], Ku80 subunit [93] and FEN1 [78]. The extended N- and C-terminal domains of the BLM helicase mediate its interaction with topoisomerase III [96], MLH1 [64, 97] and RAD51 [98]. RECQ1 lacks the extended N- and C-terminal tails of

WRN and BLM, but contains the so-called RQC motif in its C-terminal tail. The shorter N- and C-terminal domains may explain why RECQ1 is unable to interact with the two subunits of the Ku heterodimer. On the other hand, the fact that RECQ1, BLM and WRN are all able to interact with hRPA suggests that the binding is mediated by a domain conserved among these three helicases. The RQC domain has been shown to be involved in the interaction between BLM and FEN1 [78] and to be required for the helicase activity of WRN [56]. The binding of these helicases to hRPA may also involve the RQC domain. Alternatively, the motifs required for the helicase-hRPA interaction may be contained in the central helicase domain of 450 amino acids conserved among all RecQ helicases.

Human replication protein A is a ssDNA binding protein required for multiple processes in eukaryotic DNA metabolism such as DNA replication, DNA repair, and recombination [81]. Therefore BLM, WRN, and RECQ1 may also be involved in one or several of these processes. In particular, there is an increasing body of evidence suggesting that RecQ helicases are involved in the re-initiation of DNA replication at stalled replication forks since it has been shown that they can remove potential "roadblocks" such as G-quadruplex structures [99-101]. The shared interaction with hRPA indicates that these RecQ helicases might be involved in common physiological processes and work in a complementary fashion such that the absence of any of them may be compensated by the presence of another member of the

family. On the other hand, only BLM is able to interact with RAD51 [98] and MLH1 [64, 97], whereas WRN has the unique capacity of binding Ku [93] and PCNA [22], indicating that these helicases are also likely to be responsible for specific physiological functions. However, more studies will need to be done to reach a better understanding of the function of RECQ1 and of the other members of this fascinating class of enzymes.

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