Physical and Functional Mapping of the Replication Protein A Interaction Domain of the Werner and Bloom Syndrome Helicases*

Kevin M. Doherty‡, Joshua A. Sommers‡, Matthew D. Gray‡, Jae Wan Lee‡, Cayetano von Kobbe‡†, Nicolas H. Thomà, Raichal P. Kurekattil‡‡, Mark K. Kenny‡‡, and Robert M. Brosh, Jr.‡‡

From the ‡Laboratory of Molecular Gerontology, NIA, National Institutes of Health, Baltimore, Maryland 21224, the §MDG Associates, Seattle, Washington 98126, the Structural Biology Program, Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York, New York 10021, and the ¶Département of Emergency Medicine, Albert Einstein College of Medicine, Montefiore Medical Center, Bronx, New York 10467

The single-stranded DNA-binding protein replication protein A (RPA) interacts with several human RecQ DNA helicases that have important roles in maintaining genomic stability; however, the mechanism for RPA stimulation of DNA unwinding is not well understood. To map regions of Werner syndrome helicase (WRN) that interact with RPA, yeast two-hybrid studies, WRN affinity pull-down experiments and enzyme-linked immunosorbent assays with purified recombinant WRN protein fragments were performed. The results indicated that WRN has two RPA binding sites, a high affinity N-terminal site, and a lower affinity C-terminal site. Based on results from mapping studies, we sought to determine if the WRN N-terminal region harboring the high affinity RPA interaction site was important for RPA stimulation of WRN helicase activity. To accomplish this, we tested a catalytically active WRN helicase domain fragment (WRN1,2-R) that lacked the N-terminal RPA interaction site for its ability to unwind long DNA duplex substrates, which the wild-type enzyme can efficiently unwind only in the presence of RPA. WRN1,2-R helicase activity was significantly reduced on RPA-dependent partial duplex substrates compared with full-length WRN despite the presence of RPA. These results clearly demonstrate that, although WRN1,2-R had comparable helicase activity to full-length WRN on short duplex substrates, its ability to unwind RPA-dependent WRN helicase substrates was significantly impaired. Similarly, a Bloom syndrome helicase (BLM) domain fragment, BLM422–1290, that lacked its N-terminal RPA interaction site also unwound short DNA duplex substrates similar to wild-type BLM, but was severely compromised in its ability to unwind long DNA substrates that full-length BLM helicase could unwind in the presence of RPA. These results suggest that the physical interaction between RPA and WRN or BLM helicases plays an important role in the mechanism for RPA stimulation of helicase-catalyzed DNA unwinding.

Within the last decade, several genetic disorders with premature aging and/or cancer have been identified in which a gene member of the RecQ helicase family is mutated (1, 2). RecQ helicases share a centrally located domain of ~450 residues that contains the seven conserved helicase motifs (for review, see Ref. 3). The founding member of the RecQ family, Escherichia coli RecQ helicase, has been extensively studied biochemically and has been genetically implicated in DNA recombination. A single yeast RecQ helicase, Sgs1 or Rqh1, is found in the budding yeast Schizosaccharomyces pombe and fission yeast Caenorhabditis elegans, respectively, and these helicases are thought to be important in the cellular response to DNA-damaging agents and maintenance of genome stability. RecQ helicases have also been identified in a number of higher eukaryotes, including Xenopus laevis (focus forming activity 1 (FFA-1)2), Drosophila melanogaster (DmBLM and DmRecQ5), and Caenorhabditis elegans (WRN-1, Ce-RCQ5, HIM-6, and RECQL/Q1). These helicases have proposed functions in DNA replication or repair; however, the precise details of their roles in cellular pathways of DNA metabolism are still under investigation.

There are five human RecQ helicases: 1) WRN, defective in Werner syndrome (WS); 2) BLM, defective in Bloom syndrome (BS); 3) RECQL4, defective in Rothmund-Thomson syndrome and RAPADILINO; 4) RECQ1; and 5) RECQ5. The human RecQ helicase disorders have distinctly different clinical phenotypes; however, WS, BS, and Rothmund-Thomson syndrome are all characterized by genomic instability, an elevated cancer incidence, and/or particular aspects of premature aging. Human diseases have not yet been genetically linked to mutations in RECQ1 or RECQ5. The prominent roles of RecQ helicases in the maintenance of genome stability suggest that RECQ1 and RECQ5 helicases are also likely to be important in vivo.

In addition to the conserved helicase motifs, the majority of RecQ helicases have a second conserved region of ~80 amino acids located C-terminal to the helicase domain designated RecQ-Ct (RQC) (4). Although the conserved helicase domain is responsible for coupling nucleotide hydrolysis to DNA unwinding, recent studies suggest that the RQC motif plays an important role in protein interactions (5), nuclear localization (6), and/or DNA binding (7). The WRN protein is unique among the

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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

‡ Current address: Centro Nacional de Investigaciones Oncologicas (CNIO), Telomeres and Telomerase Group, Madrid 28029, Spain.

¶ To whom correspondence should be addressed: Laboratory of Molecular Gerontology, NIA, National Institutes of Health, 5600 Nathan Shock Drive, Baltimore, MD 21224. Tel.: 410-558-8578; Fax: 410-558-8157; E-mail: BroshR@grc.nia.nih.gov.
Mapping of RPA Interaction Domain on WRN and BLM Helicases

Yeast Two-hybrid Reporter Assay—A two-hybrid screen was performed as previously described (9). Briefly, L40 S. cerevisiae reporter strain, pBTM116 (LexA DNA-binding domain) and pVP16 (herpes simplex virus VP16 transcriptional activation domain) fusion vectors were kindly provided by Dr. Stanley Hellenberg. Yeast cells were purchased from BIO 101 (La Jolla, CA), and 3-amino-1,2,4-triazole (3-AT) was from Sigma. LexA-WRN fusion protein constructs were created using the expression vector pBTM116 (21). This plasmid carries the TRP1 gene for selection in yeast and a multiple cloning site inserted after the 202-amino-acid open reading frame of the E. coli lexA gene, which encodes an SOS function regulatory protein and is driven by a yeast ADE2 promoter. In this vector, sites between the C terminus of the LexA protein and specific domains of the human WRN protein were created by insertion of PCR-generated WRN cDNA domains into the multiple cloning sites of pBTM116 as previously described (6). Briefly, the full-length WRN cDNA fragment, construct WRN+, was created by insertion of the unique BamHI-SapI WRN cDNA fragment into the BamHI and Sall (filled in) sites of WRN domain within pBTM116. To create WRN-1/2, a single copy of the acid 27-amino acid repeat was removed from construct WRN+ by deletion of the unique AflII-AflII fragment of the WRN cDNA, which occurs within the repeated sequence. To create the full deletion of the repeated sequence, WRN-1/2, a PCR fragment was generated from WRN cDNA using primers TH1 (5′-GCC AGA TCT TGG AAA CAA CTT CAC-3′) and THAR (5′-CGG GTT AAG CTC AGT AGA TT-3′), which was digested with BamHI and AflII and then substituted for the BamHI-AflII WRN fragment of the pBTM116 vector. To delete the addition of a novel AflII site located just before the acidic repeat sequence, which becomes fused upon ligation to the distal AflII site within the endogenous WRN message. To create the WRN+, WRN-1/2, and WRN+ fusion protein constructs, regions of the wild-type or deleted repeat domains were amplified directly from the corresponding full-length constructs as previously described (9). These PCR products were then inserted into the pBTM116 vector at the chosen restriction sites. VP16-WRN fusion protein constructs were created using the expression vector pVP16 (21). This plasmid carries the LEU2 gene for selection in yeast and a multiple cloning site inserted after the herpes simplex virus VP16 transcriptional activation domain. In-frame fusions between the C terminus of the VP16 protein, and specified domains of the human WRN protein were created by insertion of PCR-generated WRN cDNA domains into the multiple cloning sites of pVP16. Fusion protein constructs were created by PCR amplification of individual full-length VP16 protein subunits (cDNAs were kindly provided by Dr. Marc Wold) using primers containing restriction sites compatible with pBTM116 (for LexA fusions) or pVP16 (for VP16 fusions) multiple cloning sites. For C-terminal truncated versions of the RPA70 subunit, stop codons were introduced within PCR reverse primers directly after the specified amino acids were removed. In the case of the C-terminal domain of WRN, the phenotypic activity of the constructed yeast strain was assessed by transferring the strain to high 3-AT concentration to low 3-AT concentration to low (i.e., from high 3-AT concentration to low) to ensure efficient transfer throughout the series. Relative growth on 3-AT-containing plates was assessed after 10 days of incubation at 30 °C. All reporter assays were performed in at least three different experiments. Reporter strength was essentially invariable within multiple co-transformants (original colonies isolated) of each construct pair in all 3-AT growth experiments.

Proteins—Recombinant hexahistidine-tagged WRN protein was overexpressed using a baculovirus/Sf9 insect cell system and purified as described previously (22). Glutathione S-Transferase (GST) fusion proteins of WRN were subcloned into the bacterial expression plasmid pGEXCS (Amersham Biosciences) as described elsewhere (23). In-frame fusions of GST-2R (WRN residues 406–525) or GST-3R (WRN residues 406–525 with intervening residues 424–475 deleted) were

Biochemical analyses of the purified recombinant WRN protein from a number of laboratories, including ours, have characterized the catalytic activities and protein interactions of WRN (for review, see Refs. 5 and 10). WRN protein has been shown to unwind relatively short DNA duplexes of 20–30 bp in the absence of any auxiliary factors with a 3′ to 5′ polarity (11–13). However, WRN is a relatively poor helicase on longer DNA duplexes such as a 34 bp forked duplex (14) or 69 bp M13 partial duplex DNA substrate (15), suggesting that the enzyme has limited processivity in the unwinding reaction. WRN helicase can catalyze unwinding of longer DNA duplexes up to 851 bp in a reaction dependent on replication protein A (RPA) (15), a single-stranded DNA-binding protein that is implicated in the processes of DNA replication, recombination and repair, and transcription (16). Consistent with the functional interaction between WRN and RPA, the two proteins physically interact (15, 17); however, the mechanism for stimulation of WRN-catalyzed DNA unwinding is not well understood.

To address the mechanism for RPA stimulation of WRN helicase activity, we have begun to evaluate the importance of the physical interaction between WRN and RPA for the stimulation of helicase activity. In this work, we have mapped the RPA binding sites on WRN protein using multiple approaches and identified a high affinity RPA interaction site in the N terminus. Biochemical analyses of a purified recombinant WRN protein fragment, WRNH, that contains the helicase core domain and RQC motif, but lacks the N-terminal region and C-terminal regions of WRN, retains its ability to unwind short (28 bp) M13 partial duplexes nearly as efficiently as full-length WRN helicase; however, WRNH is severely compromised in its ability to unwind longer (50–850 bp) DNA substrates that the full-length WRN protein can efficiently unwind in the presence of RPA. These results demonstrate that WRN helicase binds tightly to RPA via its N-terminal domain and suggest that this physical protein interaction with RPA is likely to be important in the mechanism of RPA-dependent stimulation of WRN helicase activity.

In addition to WRN, human BLM (18), RECQ1 (19), and RECQ5β (20) helicases also physically and functionally interact with RPA, but the details of how RPA stimulates the unwinding activities of these DNA helicases are also largely unknown. Similar to our findings with WRN, we report here that BLM helicase contains a high affinity N-terminal RPA interaction site. A BLM recombinant fragment lacking this RPA interaction site, but containing the RQC and HRD motifs, was shown to be defective in unwinding long DNA duplex substrates despite the presence of RPA. Our studies indicate that both WRN and BLM helicases contain an N-terminal RPA binding domain that is important for the stimulation of helicase activity by RPA. The important cellular roles of RPA in DNA metabolism are likely to involve specific interactions with human RecQ helicases in pathways that maintain genome stability.
created by inserting the PCR products, generated in the same way as for the two-hybrid system, into the BamHI site within the multiple cloning site of the pOECS vector. GST-WRN fragment fusion proteins were expressed and purified as described previously (25). A recombinant WN protein fragment (exact boundaries to be described elsewhere), designated WN<sub>H</sub>, was overexpressed using a baculovirus insect cell system and purified using immobilized glutathione beads. WN<sub>H</sub> contains the entire helicase domain (motifs I–VI) and the conserved RQC region but lacks the N-terminal sequence, including the acidic repeats as well as the C-terminal region after the RQC motif (Fig. 5). The Hexahistidine-tagged recombinant BLM protein, was overexpressed in S. cerevisiae and purified as previously described (24). Expression constructs for maltose-binding protein (MBP) fusion proteins of BLM fragments (25) and the BLM<sub>629-1292</sub> protein fragment were kindly provided by Dr. Ian Hickson (Cancer Research UK Laboratories). MBP-BLM fusion proteins were purified as previously described (25). Briefly, MBP-BLM fusion proteins were expressed in BL21(DE3) cells (New England Biolabs) that had been transformed with the pMAL-C2 expression plasmids containing various portions of the BLM cDNA (MBP-BLM<sub>1-47</sub> and MBP-BLM<sub>696-1415</sub>). BL21(DE3) cells transformed with plasmid encoding MBP were used for control experiments. Overnight transformed bacterial cultures were used to inoculate 1 liter of Luria Bertani (LB) medium, supplemented with 25 μg/ml ampicillin and 100 μg/ml IPTG, which was then incubated at a 1:10 dilution, and the cultures were grown at 37 °C to an A<sub>600</sub> of ~0.5. Isopropyl-1-thio-β-D-galactopyranoside was then added to a final concentration of 0.4 mM, and the cultures were allowed to grow for an additional 3 h before being chilled on ice for 30 min. After centrifugation at 10,000 rpm in a Beckman JA10 rotor, the cell pellet was resuspended in 50 ml of column buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, 1 mM dithiothreitol) supplemented with complete protease inhibitor mixture (Roche Applied Science) at the manufacturer’s recommended concentration. Cells were lysed by sonication, and the lysate was clarified by centrifugation at 35,000 rpm (Ti-60 rotor; Beckman) for 1 h. The beads were subsequently washed three times with 500 μl of lysis buffer, and split into two aliquots, one for binding experiments and one for determination of background signal in Western blot analysis. For binding experiments, protein-bound beads were incubated for 1 h at 4 °C with 1.2 μg of purified recombinant RPA in 250 μl of buffer D (50 mM HEPES, pH 7.1, 100 mM KCl, 10% glycerol). The beads were subsequently washed three times with 500 μl of buffer D and eluted by boiling treatment in 40 μl of Laemmli buffer. Proteins were electrophoresed on 10% polyacrylamide SDS gels and transferred to polyvinylidene difluoride membranes. Control membranes were stained with Amido Black reagent to demonstrate equal loading of protein samples. Membranes were probed with mouse monoclonal anti-RPA antibody (Ab-1, 1:20, Oncogene Research Products), followed by horse anti-mouse IgG-horseradish peroxidase (Vector) and visualized using the ECL detection system (Amersham Pharmacia Biotech). We have utilized both in vivo and in vitro assays to map the domain of the WRN protein important for binding to RPA and to further characterize the physical and functional interactions between the single-stranded DNA-binding protein and the helicase. Our results have been interpreted to provide a better understanding of how the protein interaction between WRN and RPA might be important in the mechanism for RPA stimulation of WRN helicase activity.

**RESULTS**

We have utilized both in vivo and in vitro assays to map the domain of the WRN protein important for binding to RPA and to further characterize the physical and functional interactions between the single-stranded DNA-binding protein and the helicase. Our results have been interpreted to provide a better understanding of how the protein interaction between WRN and RPA might be important in the mechanism for RPA stimulation of WRN helicase activity.
of RPA (RPA70, RPA32, and RPA14) were fused to the herpes simplex virus transcriptional activation protein VP16, in the plasmid pVP16. In addition, two N-terminal fragments of RPA70, designated RPA701–168 and RPA701–308, and a C-terminal fragment of RPA70, designated RPA70308–616, were fused to VP16. The yeast strain L40 was then used to screen for interaction sites between the expressed WRN and RPA fusion proteins in vivo. Every combination of constructs was examined by individual co-transfections (crosses) of construct pairs, and RPA32 was used in both orientations as a control for intra-RPA subunit interactions. Co-transformed yeast cells were first plated on a media lacking tryptophan and leucine to select for colonies containing both LexA and VP16 fusion protein constructs. Colonies were then restreaked and replica-plated to a series of plates containing media lacking tryptophan, leucine, and histidine. 3-AT, an inhibitor of histidine biosynthesis, was used at various concentrations to titrate the strength of the histidine reporter (data not shown).

The results of the initial crosses between the WRN and RPA fusion protein construct pairs revealed that there were no significant interactions observed in crosses between any of the RPA subunits and the WRN exonuclease, helicase, or C-terminal domains examined (data not shown). The RPA32 subunit showed strong interaction with the smaller RPA14 subunit and modest, but significant, interaction with both constructs containing the C-terminal region of RPA70. The intra-RPA subunit interactions we observed were exactly as previously reported (17, 29). These results indicate a requirement of the WRN-RPA interaction is optimized when both of the repeated 27 amino acids were present specifically the acidic repeat of WRN, is associated with the RPA70 acidic region (Table I). Those WRN proteins containing the full acidic repeated sequence (VP-WRN1/2, VP-WR1/2), revealed the greatest ability to transactivate the reporter gene when co-expressed with the RPA70308 fusion protein. The reporter strength observed was decreased by ~33% when full-length WRN containing one-half of the repeated sequence was expressed with RPA701–308. Similarly, the reporter strength observed was reduced by ~29% when the WRN fragment containing only one-half of the repeated sequence (VP-WR1/2) was co-expressed with RPA701–308. Because all these fragments contain short sequences that flank the acidic direct repeat (residues 406–423 and 478–525), the flanking sequence (VP-WR1/2) was also tested for interaction with the individual RPA subunits and the fragments of the RPA70 subunit. No significant interactions were observed with this small (65 residues) fragment for any of the protein pairs examined (Table I). These results demonstrate that WRN, and more specifically the acidic repeat of WRN, is associated with the RPA70 subunit in vivo and suggest that the WRN-RPA interaction is optimized when both of the repeated 27 amino acids were present in either the full-length WRN protein or the short WRN fragment harboring the repeat itself and the flanking sequences.

Two-hybrid screens using two additional regions of RPA70 (RPA701–168 and RPA70308–616) did not reveal an interaction with any of the WRN regions tested; however, the C-terminal half of RPA70 (RPA70308–616) did interact with RPA32 (Table I), as also observed in the opposite orientation in this study (data not shown), and as expected from previous reports (17, 29). These results indicate a requirement of the region between residues 168–308 in the RPA 70-kDa subunit for the interaction with WRN.

The full-length RPA70 subunit did not interact with any of the WRN protein fragments. This may be due to the poor solubility or improper folding of the RPA70 subunit expressed in the absence of RPA32 and RPA14 (30–32). It was demon-
strated that expression of full-length RPA70 or RPA701–507 resulted in poor solubility of either the full-length or truncated RPA70 subunit, whereas most of the expressed RPA701–442 was present in the soluble fraction (32). The authors suggested that in the absence of RPA32 and RPA14, full-length RPA70 has a different conformation than the more soluble RPA70 C-terminal deletion mutants, including RPA701–442. These results suggest an explanation for why RPA701–308, but not full-length RPA70, interacted strongly with full-length WRN as demonstrated by our yeast two-hybrid studies. Recently, the WRN interaction domain was mapped to residues 100–300 of RPA70 (17), a result consistent with our findings. In the work from the Loeb laboratory, a direct interaction between WRN and the purified soluble recombinant RPA701–442 (lacking the C-terminal 174 amino acids) was reported, enabling the authors to conclude that RPA stimulates WRN helicase activity by its interaction with RPA70 (17). In this study, we have focused our efforts on determining the RPA interaction site on WRN protein.

No interactions were detected whatsoever between any RPA subunit and full-length WRN without the direct repeat (WRN \(^{-}\)) or WRN fragments harboring the helicase domain and/or the entire C-terminal sequence (WRN\(_{522-1432}\) and WRN\(_{861-1432}\)) or a portion of the WRN C-terminal sequence (1162–1432, WRN\(_{1162-1432}\), data not shown). Neither the RPA14 nor RPA32 subunits interacted with any region of WRN protein, but they did show a very strong interaction with each other (Table I), as expected from previous reports (28, 29).
The yeast two-hybrid data suggest that the acidic direct repeat domain of WRN may mediate the physical interaction between WRN and the RPA 70-kDa subunit. To verify the yeast two-hybrid data and further map the physical interaction site, we performed WRN affinity pull-down experiments. Various WRN-GST fusion proteins were expressed and bound to glutathione beads and were subsequently used in pull-down assays with purified RPA heterotrimer, shown by Amido Black staining of the RPA70, RPA32, and RPA14 subunits immobilized on the membrane (Fig. 3). The GST-2R fusion contains the region of WRN406–525, which contains the entire acidic direct repeat and the flanking sequences present. The GST-0R is a fusion of GST to the region of WRN406–525 flanking the direct repeat sequences but without the direct repeat in the middle (residues 424–475). After a binding incubation in the presence of DNase I (2 μg/ml) to eliminate the potential for indirect interactions mediated through co-binding to DNA, the protein samples were resolved by SDS-PAGE and transferred to membranes for Amido Black staining to verify equal protein loading followed by Western blot analyses to probe for bound RPA. As shown in Fig. 3, approximately equal amounts of GST-0R and GST-2R were used in the affinity pull-down experiments. RPA, as detected with an antibody against the RPA70 subunit, was identified in the eluted samples from the GST-2R pull-down experiment (Fig. 3, lane 5). Little to no RPA was detected when the pull-down was performed using the GST-0R-bound glutathione beads (Fig. 4, lane 3) or when RPA was omitted from the binding incubation (Fig. 3, lane 4). Similar results were obtained in the presence of ethidium bromide (10 μg/ml), indicating that the interaction between RPA and GST-2R was not DNA mediated (data not shown).

The results from the yeast two-hybrid studies and the WRN affinity pull-down experiments suggest that the direct repeat elements acting alone can mediate an interaction with RPA70; however, we wanted to ascertain whether the interaction would be retained by a WRN protein fragment that contained the direct repeat but in the context of a significantly greater length of flanking sequence. To address this, we performed RPA binding experiments using glutathione beads with GST-WRN239–499 (Fig. 4A) bound as the affinity reagent. As shown in Fig. 4B, ~25% of the RPA input was co-precipitated with GST-WRN239–499 (lane 4) by comparison with the control (lanes 1 or 8). GST was unable to co-precipitate RPA (lane 2). The results from the WRN affinity pull-down experiments support the findings from the yeast two-hybrid studies that the WRN direct repeat elements mediate a physical interaction with the RPA70 subunit.

Although it was evident that the N-terminal region of WRN, residues 239–499, mediated an interaction with RPA70, it was conceivable that other regions of WRN might also be associated with RPA70. To address this possibility, we examined the ability of other recombinant GST-WRN fragments bound to glutathione beads to pull-down RPA. Whereas glutathione beads coated with GST-WRN949–1092 (Fig. 4B, lane 3) or GST-WRN1072–1432 (Fig. 4B, lane 7) bound RPA weakly, a significant fraction of the RPA70 input was bound not only by GST-WRN239–499 but also by GST-WRN949–1432 (lane 5) and GST-WRN949–1092 (lane 6), suggesting that the RQC region, which overlaps with WRN949–1092 (Fig. 4A) and mediates other WRN protein interactions (2, 5), might also bind RPA70. In control experiments, pull-downs were performed in the presence of DNase I (2 μg/ml) to confirm that the interaction between WRN and RPA70 was not DNA mediated (data not shown).
Mapping of RPA Interaction Domain on WRN and BLM Helicases

G斯特-WRN_{239–499}, GST-WRN_{949–1092} or GST-WRN_{949–1432} and RPA was not mediated by DNA (Supplemental Fig. S1). Similar results were obtained in the presence of ethidium bromide (10 μg/ml), also indicating that the interaction between RPA and the WRN protein fragments was direct and not DNA-mediated (data not shown).

Physical Analysis of the WRN-RPA Protein Interaction—Results from the affinity pull-down experiments and two-hybrid studies suggested that RPA physically interacts with certain discrete domains of the WRN protein. ELISA studies were performed to explore the specificity and affinity of the WRN-RPA interaction. Increasing amounts of RPA (0–96 nM, heterotrimer) were incubated in the presence of 3% BSA with WRN or the specified WRN protein fragment (18 nM) that had been previously immobilized on microtiter wells. Bound RPA protein was detected by a mouse monoclonal antibody against RPA70. As shown in Fig. 5, the colorimetric signal was both dose-dependent and saturable with full-length WRN protein. The specificity of this interaction was demonstrated by the very low absorbance values (0.062 A_{490}) for wells that had been pre-coated with BSA compared with the intense signal obtained with WRN. A purified recombinant GST-WRN_{239–499} fragment (Fig. 4A) that contained the N-terminal acidic repeated element also displayed a dose-dependent and saturable signal for interaction with the bound RPA. Although the WRN_{239–499} RPA signal was ~2-fold less than that obtained using full-length WRN, the plateau was approached at the same concentration of WRN_{239–499} and full-length WRN, 22 nM (Fig. 5). The colorimetric signal obtained from either WRN-RPA or WRN_{239–499}-RPA interaction was resistant to the presence of ethidium bromide (10 μg/ml, data not shown), indicating that a contaminating DNA bridge was not responsible for the positive signal. In comparison to WRN or WRN_{239–499}, only a very weak signal was detected when WRN_{1072–1432} was incubated with the RPA-bound wells (Fig. 5). A detectable signal was observed for the RPA interaction with a recombinant WRN protein fragment, WRN_{14–1443}, that contains the entire helicase domain (motifs I–VI) and the conserved RQC region but lacks the N-terminal sequence, including the acidic repeats as well as the C-terminal region after the RQC motif; however, the A_{490} values were significantly lower than that obtained for either WRN_{239–499} or full-length WRN and failed to reach a plateau at the highest RPA concentration tested, 96 nM (Fig. 5), suggesting that the WRN_{N-R}-RPA interaction was of considerably lower affinity than that of WRN_{239–499}-RPA or WRN-RPA.

Specific binding of RPA to WRN- or WRN_{239–499}-coated wells was analyzed according to Scatchard binding theory. The data were analyzed by Hill plots as described under “Materials and Methods.” The transformed data were linear, indicating a single site on RPA for binding to either WRN or WRN_{239–499}. The apparent dissociation constants (K_{d}) for WRN-RPA and WRN_{239–499}-RPA were 7.2 nM and 11.7 nM, respectively (Table I). These results, together with yeast two-hybrid and affinity pull-down studies, demonstrate a direct physical interaction between WRN and RPA mediated by the N-terminal domain of WRN harboring the direct repeat elements. WRN_{239–499} binds to RPA with similar affinity to that observed with the WRN-RPA interaction.

Analysis of DNA Unwinding Reactions Catalyzed by WRN and a WRN Helicase Domain Fragment—The results from yeast two-hybrid studies, WRN affinity pull-down experiments, and ELISA measurements together suggest that the N-terminal region of WRN harboring the acidic repeat is important for the physical interaction with the RPA 70-kDa subunit. To better understand the importance of the WRN-RPA physical interaction for their functional interaction, we set out to test a WRN protein that lacked the acidic direct repeats in functional helicase assays on long DNA duplex substrates in the presence of RPA. A baculovirus construct encoding full-length WRN protein lacking one or both of the direct repeats was constructed; however, the engineered recombinant WRN proteins were insoluble under a number of viral infection and/or cell lysis conditions, preventing us from being able to purify them. We next sought to test a catalytically active WRN helicase domain fragment, WRNH-R, which lacks the N-terminal region containing the acidic direct repeat sequence (Fig. 4A). Although WRN_{N-R} retains the RQC domain, the recombinant fragment lacks a C-terminal region containing the HRD motif.

![Image]

**Table I**

| RecQ protein/fragment | K_{d} with RPA |
|------------------------|---------------|
| WRN                    | 7.20          |
| WRN_{239–499}          | 8.05          |
| WRN_{14 R}             | ND            |
| WRN_{1072–1432}        | ND            |
| BLM                    | 6.51          |
| BLM_{1–477}            | 5.80          |
| BLM_{966–1470}         | ND            |

* ND, K_{d} was not determined.
We first investigated the DNA unwinding activity of WRNH/R on a 19-bp forked duplex substrate. The forked duplex was previously demonstrated to be a preferred helicase substrate of full-length WRN (27). The substrate was efficiently unwound by full-length WRN (20 and 40 fmol) in the 15-min reaction (Supplemental Fig. S2). At lower protein levels (2.5 and 5 fmol), WRN and WRNH/R unwound similar percentages of the DNA substrate. Slightly more substrate was unwound by WRN compared with WRNH/R at the 10-fmol protein amount. At 20-fmol protein, full-length WRN protein unwound a 1.53-fold more of the substrate than WRNH/R. At 40 fmol of protein, ~88% of the forked duplex was unwound by WRN, whereas 74% of the substrate was unwound by WRNH/R. Overall, the results suggest that WRNH/R is only modestly compromised in its helicase activity on a 19-bp forked duplex compared with the full-length recombinant WRN protein.

We next wanted to compare the helicase activity of WRNH/R with full-length WRN on a series of increasing length M13 partial duplex substrates, because we have previously found that WRN requires the presence of RPA to efficiently unwind the longer duplex tracts. Because WRNH/R was slightly less active than full-length WRN in its ability to unwind the 19-bp forked duplex, a series of WRN or WRNH/R protein concentrations were tested on a 28-bp M13 partial duplex substrate to determine protein concentrations that would yield comparable levels of unwinding for full-length WRN and the WRNH/R helicase domain fragment. In a 15-min reaction, it was determined that a similar percentage of the 28-bp M13 partial duplex substrate was unwound by 20 nM WRNH/R compared with 15 nM WRN (Supplemental Fig. S3A). We then examined the unwinding kinetics for the M13 28-bp partial duplex using these protein levels over a 32-min time course (Supplemental Fig. S3B). Increasing levels of DNA unwinding by WRNH/R were observed throughout the early time points; helicase activity achieved a plateau of ~90% substrate unwound at 15 min. A quantitative comparison demonstrated similar kinetics of unwinding by WRN (15 nM) and WRNH/R (20 nM) throughout the 15-min time course on the 28-bp partial duplex substrate.

Having normalized the helicase activity of WRN and WRNH/R on a short 28-bp M13 partial duplex substrate, we next compared the helicase activities of full-length WRN and the WRNH/R fragment on longer M13 duplex substrates of 50, 70, and 100 bp. As expected from our previous characterization of WRN helicase activity on longer duplex DNA substrates (14, 15), WRN alone, in the absence of RPA, did not displace the annealed labeled strand complementary to the M13 sequence for these longer M13 partial duplex substrates (data not shown). RPA alone also did not denature any of these partial duplex substrates (data not shown). In the presence of RPA (87 nM heterotrimer), WRN (15 nM) efficiently unwound a 50-bp M13 partial duplex substrate, resulting in 94% of the substrate unwound in 2 min (Fig. 6A). In contrast, WRNH/R unwound only 7% of the substrate by 2 min. Although a linear increase in unwinding of the 50-bp partial duplex substrate by WRNH/R was observed up to 8 min, only approximately one-third of the substrate was unwound compared with the amount of substrate unwound by wild-type WRN within 2 min. We next compared the helicase activities of WRN and WRNH/R on a longer 70-bp M13 partial duplex (Fig. 6B). Full-length WRN had unwound 88% of this substrate by 2 min, compared with only 3.7% of the substrate being unwound by WRNH/R during the same time period. By the end of the 32-min time course, WRNH/R had unwound 48.9% of the 70-bp substrate, which was significantly less (~2-fold) than the helicase activity catalyzed by full-length WRN after only 2 min. On a 100-bp partial duplex, WRN unwound ~30% of the substrate in 2 min; in contrast, hardly any detectable unwinding of the 100-bp partial duplex was observed by WRNH/R in the same 2-min period (Fig. 6C). A kinetic analysis of the helicase activity data over the first 4 min of the reaction demonstrated that WRN unwound 8.9% of the 100-bp partial duplex per minute. In contrast, WRNH/R poorly unwound the 100-bp substrate over the entire time course, resulting in only 9.7% of the duplex DNA unwound after 32 min (Fig. 6D). Thus, WRNH/R unwound 0.6% of the 100-bp substrate per min, a 14.3-fold lower rate compared with the reaction catalyzed by full-length WRN.

In the presence of RPA, WRN can unwind very long DNA duplexes, including an 849-bp M13 partial duplex substrate (15). As previously published (15), we found that neither WRN nor RPA alone can denature this duplex DNA (data not shown). However, in the presence of RPA, WRN was able to unwind a significant fraction of the 849-bp partial duplex substrate by the end of the 32-min incubation (Supplemental Fig. S4A). In contrast, WRNH/R failed to unwind the 849-bp DNA duplex in either the presence or absence of RPA (Supplemental Fig. S4B). From these results, we conclude that, although WRNH/R can efficiently unwind a short 28-bp M13 partial duplex substrate, this WRN helicase domain fragment is severely compromised in its ability to unwind longer DNA duplex substrates in which the unwinding activity of the full-length WRN helicase is dependent upon the presence of RPA.

**N-terminal Region of Bloom Syndrome Helicase Binds RPA and Is Important for RPA Stimulation of BLM Helicase Activity**—The compromised ability of WRNH/R to unwind long RPA-dependent DNA substrates suggested that the N-terminal domain of WRN, which mediates the high affinity physical interaction with RPA, is likely to be important for the functional interaction between RPA and WRN. Although the N-terminal sequence of BLM protein does not display as great an extent of sequence homology to WRN as found in the helicase and RQC domains, BLM, like WRN, contains acidic regions located before the conserved helicase domain that may be important in mediating a similar physical interaction with RPA. To address this possibility, we tested a purified recombinant MBP-BLM protein fragment that contains three highly acidic regions of the BLM protein, BLM<sub>1–447</sub> (Fig. 7A), for direct binding to RPA using the ELISA assay. As observed for full-length BLM protein, the MBP-BLM<sub>1–447</sub> fragment exhibited a dose-dependent and saturable signal for its interaction with the immobilized RPA. Although the BLM<sub>1–447</sub>-RPA signal was ~2-fold less than that obtained using full-length BLM, the plateau was approached at the same concentration of BLM<sub>1–447</sub> and BLM, 45 nM (Fig. 7B). The colorimetric signal obtained from either BLM-RPA or BLM<sub>1–447</sub>-RPA interaction was resistant to the presence of ethidium bromide (10 μg/ml, data not shown), indicating that a contaminating DNA bridge was not responsible for the positive signal. In comparison to BLM or BLM<sub>1–447</sub>, a considerably weaker signal was detected when a C-terminal BLM fragment harboring the RQC and HRD domains was incubated with the RPA-bound wells (Fig. 7B), suggesting that the BLM<sub>966–1470</sub>-RPA interaction was of considerably lower affinity than that of BLM<sub>1–447</sub>-RPA or BLM-RPA. Specific binding of RPA to BLM- or BLM<sub>1–447</sub>-coated wells was analyzed according to Scatchard binding theory. The transformed data were linear, indicating a single site on RPA for binding to either BLM or BLM<sub>1–447</sub>. The apparent dissociation constants (Kd) for BLM-RPA and BLM<sub>1–447</sub>-RPA were 6.51 nM and 5.80 nM, respectively (Table II).

Previously, we determined that RPA can stimulate BLM helicase to efficiently unwind M13 partial duplex substrates as long as 259 bp, and to a lesser extent a long 849-bp partial
In the current study, under slightly modified reaction conditions, we observed that BLM helicase was able to efficiently unwind a 849-bp M13 partial duplex in the presence of RPA, resulting in 93% of the substrate unwound, whereas no detectable BLM unwinding was observed in the absence of RPA (Fig. 8). To address the potential functional importance of the N-terminal region of BLM protein, which physically interacts with RPA, for BLM helicase activity on a long DNA duplex substrate, we tested a catalytically active MBP-BLM recombinant protein, BLM642–1290, for unwinding of the 849-bp partial duplex substrate. This particular recombinant BLM helicase domain fragment retains DNA helicase activity on short linear DNA duplexes (33) and an M13 69-bp partial duplex substrate similar to that of full-length BLM. As shown in Fig. 8, very little (<1%) unwinding of the 849-bp partial duplex substrate by BLM642–1290 was detected in the presence of RPA. These results suggest that the N-terminal domain of the BLM protein, which physically binds to RPA, is important for unwinding of long DNA duplexes in which the BLM helicase reaction is dependent upon the presence of RPA.

FIG. 6. Stimulation of WRN or WRN_{H-R} helicase activities by RPA on increasing lengths of M13 partial duplex DNA substrate. WRN protein (15 nM) or WRN_{H-R} (20 nM) was incubated with RPA (87 nM) and the 50-bp (A), 69-bp (B), or the 100-bp (C) M13 partial duplex substrates (0.125 nM) under the standard helicase reaction conditions as described under “Materials and Methods.” Reactions were quenched at the indicated time points. Helicase reaction products were resolved on native 10% polyacrylamide gels. Phosphorimaging images of representative gels are shown. A heat-denatured DNA substrate control is indicated by the filled triangle. D, percent displacement of the radiolabeled DNA strand from the M13 partial duplex substrate was plotted versus time for helicase reactions containing WRN (left) and WRN_{H-R} (right) and the 50-bp (filled circle), 69-bp (open circle), or 100-bp (filled square) M13 partial duplex substrates. Helicase data represent the mean from at least three independent experiments with S.D. indicated by error bars.
DISCUSSION

RPA is an essential protein in the cellular processes of DNA replication, repair and recombination, and RNA transcription (16). In addition to its ability to bind ssDNA with high affinity and a defined polarity, the heterotrimeric protein physically interacts with a number of nuclear DNA-metabolizing proteins and modulates their functional or catalytic activities in a specific manner (for review, see Ref. 16). We have been interested in how the DNA-unwinding reactions catalyzed by human RecQ helicases are stimulated by RPA, because heterologous ssDNA-binding proteins either poorly or completely fail to stimulate DNA unwinding by human WRN (15), BLM (18), RECQ1 (19), or RECQ5β (20) depending on the duplex length or helicase in question. Of the human RecQ DNA helicases examined, WRN, BLM, and RECQ1 all physically interact with RPA, further supporting the notion of a specific functional interaction between the helicase and the single-stranded DNA-binding protein. In this work, we sought to define the physical interaction site(s) between RPA and WRN and to investigate whether the WRN-RPA physical interaction is important for RPA-dependent stimulation of WRN-catalyzed DNA unwinding.

The results from our mapping studies reveal that WRN has a high affinity RPA interaction site located within its N terminus, and a second site of lower affinity in its C terminus. In addition to its high affinity, the N-terminal RPA interaction site is of particular interest, because it harbors a perfectly and directly repeated acidic sequence element that was shown to give a strong signal for interaction with RPA70 in vivo by the yeast two-hybrid assay. Up to this point, although a number of the conserved domains or motifs of the WRN protein have been extensively characterized, the importance of the N-terminal region harboring the acidic direct repeats for WRN catalytic function or protein interactions had not been characterized.

Interestingly, the Xenopus homolog of WRN, FFA-1, is unique among the eukaryotic RecQ homologs in that it contains one-half of the identical direct repeat found in WRN (8). Molecular and cellular studies in the Xenopus system have provided some insight into the biological importance of the interaction between RPA and the eukaryotic RecQ helicase FFA-1. The assembly of
RPA-containing replication foci in *X. laevis* egg extracts requires FFA-1 (34). FFA-1 binds to nuclear chromatin at an early stage of formation of functional replication origin complexes and enables RPA to subsequently bind to the foci. RPA was shown to physically interact with FFA-1 and stimulate its helicase activity in a specific manner, because the T4 gene 32 ssDNA-binding protein did not stimulate FFA-1 helicase activity (35). The region of FFA-1 required for the RPA interaction was found to reside within the N-terminal 291 amino acids, a region that contains the single acidic repeat element of FFA-1 (35). The notion that RPA stimulates FFA-1 helicase activity through a direct protein interaction was further supported by the observation that addition of a GST-FFA-1 fragment, that mediates the physical interaction with RPA to a reaction mixture containing RPA and FFA-1, inhibited FFA-1-catalyzed unwinding of a DNA duplex substrate in a dose-dependent manner but did not affect binding of RPA to ssDNA (35). Presumably, the inhibitory GST-FFA-1 fusion protein can prevent RPA stimulation of FFA-1 helicase activity by competing against the full-length FFA-1 for interaction with RPA. Consistent with this idea, the FFA-1 fragment harboring the RPA interaction site can also exert a dominant negative effect on DNA replication in reconstituted *Xenopus* nuclei, suggesting a biological importance for the FFA-1-RPA interaction (35).

To gain some insight into the WRN-RPA functional interaction, we examined the helicase activity of a recombinant helicase core domain fragment, WRNH$_{42}$, that contains the conserved RecQ helicase motifs and RQC region but lacks the high affinity RPA interaction site in the N terminus as well as a C-terminal portion, on a series of partial duplex DNA substrates that varied in their number of base pairs. These studies led to the conclusion that, although this WRN helicase domain fragment is only mildly reduced in its helicase activity on short DNA duplex substrates, its ability to unwind DNA duplex substrates of 50 bp or greater is seriously impaired despite the presence of the WRN auxiliary factor RPA in the reaction mixtures. Although it is tempting to conclude that the absence of the high affinity RPA interaction site in the WRN helicase core domain fragment is solely responsible for the poor unwinding of the longer duplex substrates, other factors may contribute. The slightly reduced helicase activity of WRNH$_{42}$ on short duplex substrates compared with the wild-type enzyme suggests that regions outside the WRN helicase domain may affect the intrinsic catalytic activity of the helicase, particularly for longer duplexes. For example, in addition to the high affinity RPA interaction site, WRNH$_{42}$ also lacks the HRD motif in the C-terminal region of the full-length WRN protein. Recent evidence demonstrates that a C-terminal WRN fragment harboring this motif has the ability to bind various oligonucleotide-based duplex DNA substrates (7). It is conceivable that the HRD motif may contribute to the interaction of full-length WRN with the DNA substrate by conferring processivity to the enzyme when RPA is present in the helicase reaction. An argument that can be made against this explanation for the defective helicase activity of WRNH$_{42}$ is that the BLM helicase domain fragment BLM$_{42}$, which retains the HRD motif but lacks the high affinity RPA interaction site, is also defective in unwinding RPA-dependent DNA duplex substrates that the full-length BLM enzyme can unwind efficiently in the presence of RPA. However, further molecular studies will be necessary to address the functional importance of the HRD motif in the catalytic activities of WRN or BLM proteins.

It was recently reported that the RPA70 subunit stimulates WRN helicase activity to the same extent as the RPA heterotrimer (17). Further mapping demonstrated that the N-terminal half of RPA70 (residues 1–441) was sufficient for stimulation of WRN helicase activity. A mutant heterotrimer lacking residues 1–168 of RPA70 stimulated WRN helicase activity as efficiently as wild-type RPA heterotrimer, suggesting that the WRN-RPA functional interaction is mediated by residues 169–441 (17). By ELISA assays, it was determined that the WRN binding domain for RPA70 resides within residues 100–300 and overlaps with the ssDNA binding domain, residues 150–450 (17). Thus the WRN interacting domain and ssDNA binding domain of RPA overlap with each other, suggesting that the ssDNA and WRN-protein binding activities of RPA70 are functionally intertwined.

Structural studies of RPA have revealed that the N-terminal region of the human RPA70 subunit folds into a five-stranded anti-parallel β-barrel (36). Two loops on the side of the structure (residues 31–42 and 87–92) form a large basic cleft containing five arginines and a lysine. This cleft has been thought to be a region that may bind acidic motifs on interacting proteins. Although the minimal physical interaction site for WRN was mapped to residues 100–300 of RPA70, it was also shown that mutant RPA heterotrimers containing N-terminal truncations of RPA70, including deletion of the first 112 amino acids, impaired the physical interaction between RPA and WRN (17). In summary, the results of the Loeb study (17) are consistent with ours in that the N-terminal half of RPA70 mediates WRN binding. We have extended these observations by mapping the RPA interaction sites on the WRN protein, showing that an N-terminal region of WRN mediates a high affinity interaction with RPA.

We had previously characterized a physical and functional interaction between BLM helicase and RPA (18). BLM and RPA were shown to interact via the RPA70 subunit, similar to WRN. Here we report that the domain for RPA70 binding resides within the N-terminal region of BLM and that a helicase domain fragment of BLM lacking the RPA70 interaction site is unable to unwind long DNA duplexes that the full-length BLM helicase can efficiently unwind if RPA is present. Our studies suggest that human RecQ helicases, which physically and functionally interact with RPA, may do so via domains of the helicase that are functionally conserved but do not display extensive sequence homology. The fact that helicase domains of both BLM and WRN that lack the RPA interaction domain can efficiently unwind short duplex tracts suggests that the RPA interaction domain is folded independently of the catalytic helicase domain. Although BLM does not have the same acidic repeat elements that WRN does, the RPA70 interaction site of BLM contains three highly acidic regions that may be important in the RPA interaction. Importantly, the results from the BLM/WRN helicase and RPA interaction studies suggest that the physical interaction with RPA is an important component of the mechanism for the stimulation of DNA helicase activity.

The biological significance of the WRN-RPA or BLM-RPA interaction may be highly relevant to our understanding of the DNA metabolic defects in WS and BS. WS cells have a prolonged S phase (37), asymmetry of DNA replication fork progression (38), slower rate of repair associated with DNA damage induced in S-phase, reduced induction of RAD51 foci, and a higher level of strand breaks (39). The *in vivo* evidence implicating WRN in the recovery of DNA synthesis after replication arrest poses the question of whether WRN functions together with RPA in a critical step to resolve a key replication or recombinational intermediate that arises from fork stalling or collapse. RPA has been found to co-localize with WRN upon replication arrest (40) and DNA damage (41), suggesting that the two proteins may indeed collaborate to perform certain cellular functions(s).
The most characteristic features of BS cells are an elevated frequency of chromosome breaks and exchanges (42, 43) as well as an increase in the level of reciprocal exchanges between sister chromatids (44). Like WS cells, BS cells display aberrant replication and abnormalities in DNA replication that include an extended S phase and the accumulation of abnormal replication intermediates compared with normal cells (45, 46). It is conceivable that BLM helicase and RPA may also act together to facilitate efficient DNA replication and/or recombination.

*S. cerevisiae* has a sole RecQ homologue, Sgs1. sgs1 loss of function mutations result in the development of age-associated phenotypes, including a 60% reduction in life span and nucleolar fragmentation (47). An sgs1 deletion mutant exhibits genomic instability characterized by elevated homologous and homeologous recombination throughout the genome, particularly within the ribosomal DNA array (47, 48). It was reported that expression of either human BLM or WRN can suppress the hyper recombination phenotype of the sgs1 mutant (49), suggesting that the human RecQ helicases have a role in maintaining genomic stability by preventing aberrant recombination.

DNA polymerase stabilization at stalled replication forks requires the ATM-related kinase Mec1 and Sgs1 (50). A model was proposed whereby Sgs1 helicase resolves aberrantly paired DNA structures at stalled forks to maintain single-stranded DNA that allows RPA and Mec1 to promote DNA polymerase association. The observation that Sgs1 is present at replication forks and binds RPA is suggested to be important for polymerase assembly/stabilization. In future studies, insight into the biological importance of the WRN-RPA or BLM-RPA interactions at stalled forks may be gained by genetic complementation studies using WRN or BLM mutants that are specifically defective in their interaction with RPA.

Acknowledgments—We thank Dr. I. Hickson (Cancer Research UK Laboratories) for kindly providing recombinant BLM protein, MBL462-1290, and expression constructs for MBP fusion proteins of BLM. We thank Drs. S. Sharma, R. Gupta, and V. Bohr (Laboratory of Molecular Gerontology, NIA, National Institutes of Health) and Dr. I. Hickson (Cancer Research UK Laboratories) for critical reading of the manuscript.

**REFERENCES**

1. Bachrati, C. Z., and Hickson, I. D. (2008) Biochem. J. 374, 577–606
2. Harrigan, J. A., and Bohr, V. A. (2003) Biochimie (Paris) 85, 1185–1193
3. Hickson, I. D. (2003) Nat. Rev. Cancer 3, 169–178
4. Morezov, V., Mushegan, A. R., Koonin, E. V., and Bork, P. (1997) Trends. Biochem. Sci. 22, 417–418
5. Brosh, R. M., Jr., and Bohr, V. A. (2002) Exp. Gerontol. 37, 491–506
6. von Kobbe, C., and Bohr, V. A. (2002) J. Cell Sci. 115, 3901–3907
7. von Kobbe, C., Thoma, N. H., Czajaowski, B. K., Pavletich, N. P., and Bohr, V. A. (2003) J. Biol. Chem. 278, 52997–53006
8. Yu, C. E., Oshima, J., Fu, Y. H., Wijeman, M. E., Hisama, F., Alisch, R., Matthews, S., Nakura, J., Miki, T., Ouaai, S., Martin, G. M., Mulligan, J., and Schellenberg, G. D. (1996) Science 272, 258–262
9. Balaje, A. S., Machwe, A., May, A., Gray, M. D., Oshima, J., Martin, G. M., Nehlin, J. O., Brosh, R. M., Jr., Orren, D. K., and Bohr, V. A. (1999) Mol. Biol. Cell 10, 2655–2668
10. Opresko, P. L., Cheng, W. H., von Kobbe, C., Harrigan, J. A., and Bohr, V. A. (2003) Carcinogenesis 24, 791–802
11. Gray, M. D., Shen, J. C., Kamath-Loeb, A. S., Blank, A., Sopher, B. L., Martin, G. M., Oshima, J., and Loeb, L. A. (1997) Nat. Genet. 17, 100–103
12. Shen, J. C., Gray, M. D., Oshima, J., and Loeb, L. A. (1998) Nucleic Acids Res. 26, 2879–2885
13. Suzuki, N., Shimamoto, A., Imamura, O., Kurimoto, J., Kitao, S., Goto, M., and Furutchi, Y. (1997) Nucleic Acids Res. 25, 2975–2978
14. Opresko, P. L., Laine, J. P., Brosh, R. M., Jr., Seidman, M. M., and Bohr, V. A. (2001) J. Biol. Chem. 276, 44677–44687
15. Brosh, R. M., Jr., Orren, D. K., Nehlin, J. O., Ruvn, P. H., Kelly, M. K., Machwe, A., and Bohr, V. A. (1999) J. Biol. Chem. 274, 18341–18350
16. Wold, M. S. (1997) Annu. Rev. Biochem. 66, 61–92
17. Shen, J. C., Luo, Y., Kamath-Loeb, A., Wold, M. S., and Loeb, L. A. (2003) Mecl. Ageing Dev. 124, 921–930
18. Brosh, R. M., Jr., Li, J. L., Kelly, M. K., Karow, J. K., Cooper, M. P., Kurekattili, R. P., Hickson, I. D., and Bohr, V. A. (2000) J. Biol. Chem. 275, 23509–23508
19. Cui, S., Arosio, D., Doherty, K. M., Brach, R. M., Jr., Falaschi, A., and Vindigni, J. (2004) Nucleic Acids Res. 32, 2158–2170
20. Garcia, P. L., Liu, Y., Jirin, J., West, S. C., and Janscak, P. (2004) EMBO J. 23, 2882–2891
21. Vojtek, A. B., Holenberg, S. M., and Cooper, J. A. (1993) Cell 74, 265–274
22. Sharma, S., Otterlei, M., Sommers, J. A., Driscoll, H. C., Dianov, G. L., Kao, H. I., Bambara, R. A., and Brosh, R. M., Jr. (2004) Mol. Biol. Cell 15, 734–750
23. Brosh, R. M., Jr., von Kobbe, C., Sommers, J. A., Karna, M., Opresko, P. L., Potrowski, J., Dianova, I., Dianov, G. L., and Bohr, V. A. (2001) EMBO J. 20, 5791–5801
24. Karow, J. K., Newman, R. H., Freemont, P. S., and Hickson, I. D. (1999) Curr. Biol. 9, 797–800
25. Wu, L., Davis, S. L., North, P. S., G ourac, H., Rieu, J. F., Turley, H., Gatter, K. C., and Hickson, I. D. (2000) J. Biol. Chem. 275, 9636–9644
26. Kote, M. K., Schlegel, U., Farnzeaux, H., and Hurwitz, J. (1999) J. Biol. Chem. 265, 7693–7700
27. Brosh, R. M., Jr., Waheed, J., and Sommers, J. A. (2002) J. Biol. Chem. 277, 23286–23245
28. Lin, Y. L., Chen, C., Keshav, K. F., Winchester, E., and Dutta, A. (1996) J. Biol. Chem. 271, 17190–17198
29. Bockkareva, E., Kories, S., Lees-Miller, S. P., and Bockkareva, A. (2002) EMBO J. 21, 1835–1836
30. Erdile, L. F., Heyer, W. D., Koller, and Kelly, T. J. (1991) J. Biol. Chem. 266, 12090–12098
31. Gomes, X. V., and Wold, M. S. (1995) J. Biol. Chem. 270, 4534–4543
32. Henriksson, L. A., Umbricht, C. B., and Wold, M. S. (1994) J. Biol. Chem. 269, 11211–11332
33. Jinan, P., Garcia, P. L., Hamburger, F., Makuta, Y., Shiraishi, K., Imai, Y., Ikeda, H., and Bickel, T. A. (2003) J. Mol. Biol. 330, 29–42
34. Han, Y., Chen, C. Y., Kobayashi, R., and Newport, J. (1998) Nat. Genet. 19, 375–378
35. Chen, C. Y., Graham, J., and Yan, H. (2001) J. Cell Biol. 152, 985–996
36. Jacobs, D. M., Lipton, A. S., Isern, N. G., Daughdrill, G. W., Lowry, D. F., Gomes, A., and Wold, M. S. (1999) J. Biol. Chem. 274, 1321–1331
37. Po, P., Hoehn, H., Runger, T. M., and Martin, G. M. (1992) Exp. Cell Res. 202, 287–287
38. Rodriguez-Lopez, A. M., Jackson, D. A., Iborra, F., and Cox, L. S. (2002) Aging Cell 1, 30–39
39. Pichetti, F., Franchito, A., Mossei, P., and Palitti, P. (2001) Mol. Cell. Biol. 21, 2412–2421