Replication protein A (RPA), the major eukaryotic single-strand DNA (ssDNA)-binding protein, is essential for replication, repair, recombination, and checkpoint activation. Defects in RPA-associated cellular activities lead to genomic instability, a major factor in the pathogenesis of cancer and other diseases. ssDNA binding activity is primarily mediated by two domains in the 70-kDa subunit of the RPA complex. These ssDNA interactions are mediated by a combination of polar residues and four conserved aromatic residues. Mutation of the aromatic residues causes a modest decrease in binding to long (30-nucleotide) ssDNA fragments but results in checkpoint activation and cell cycle arrest in cells. We have used a combination of biochemical analysis and knockdown replacement studies in cells to determine the contribution of these aromatic residues to RPA function. Cells containing the aromatic residue mutants were able to progress normally through S-phase but were defective in DNA repair. Biochemical characterization revealed that mutation of the aromatic residues severely decreased binding to short ssDNA fragments less than 20 nucleotides long. These data indicate that altered binding of RPA to short ssDNA intermediates causes a defect in DNA repair but not in DNA replication. These studies show that cells require different RPA functions in DNA replication and DNA repair.

Significance:

This study elucidates the molecular defects of RPA mutants that cause cell cycle arrest. This study indicates that DNA repair and replication require different RPA-DNA interactions. a role in coordinating DNA metabolism with cell cycle and in the DNA damage response pathway in the event of abnormal cellular functions (6–9). Expression of RPA is up-regulated in tumors including breast and colon cancers (10, 11). Loss of heterozygosity of the RPA locus has been linked to cancer development (12, 13) and abnormal checkpoint signaling (14). Loss of heterozygosity of RPA has also been demonstrated to cause genome instability in mice, suggesting a role for RPA in cancer progression (15, 16).

RPA binds all ssDNA sequences with high affinity (17–20) and interacts with multiple protein partners (3). RPA is essential for all DNA repair pathways including base excision repair, nucleotide excision repair, mismatch repair, and double-stranded break repair (4, 21, 22). In these pathways, RPA is generally one of the first proteins to interact with the DNA and is involved in damage recognition, excision, and resynthesis reactions, specifically interacting with repair proteins including XPA, XPG (Xeroderma pigmentosum group G protein), uracil-DNA-glycosylase, and MRN (MRE11, RAD50, NBS1) (23–27). RPA interactions with Rad51 and recombination mediators are necessary for the formation of the Rad51 filament and recombination (28–30). Known RPA interaction partners involved in checkpoint activation include ATM, ATR-ATRIP, Rad9-Rad1-Hus1 complex, and p53 (4, 31).

The RPA complex is composed of three subunits, RPA1, RPA2, and RPA3, all of which are essential for cell viability and chromosome stability (1). These subunits contain six structurally related DNA-binding domains (DBD) that all can interact with ssDNA (1, 3, 20, 32, 33) The largest subunit, RPA1, is composed of four DBDs and is responsible for high affinity ssDNA binding and many protein-protein interactions (Fig. 1). The N-terminal domain DBD-F contains many protein interaction sites involved in DNA repair, recombination, and checkpoint activation (8, 34–36). The central domains, DBD-A and DBD-B, comprise the ssDNA-binding core of the complex (37, 38). The C-terminal domain DBD-C is involved in trimerization with the two other RPA subunits, interacts with ssDNA, and plays a role in recognizing DNA damage (3, 39). Current models of RPA binding suggest that DBD-A, -B, -C, and DBD-D in RPA2 all contribute to ssDNA binding (see models in Fig. 1) (3). However, the ssDNA-binding core (DBD-A and -B) is the primary determinant for complex binding activity; it is both
necessary and sufficient for high affinity binding of RPA to DNA (37). The high affinity DNA-binding core in the RPA1 subunit contains a series of polar and aromatic residues that contact the ssDNA, including four aromatic residues that are involved in stacking between the bases of the DNA (38, 40, 41). These four aromatic residues, Phe-238 and Phe-269 in domain A and Trp-361 and Phe-386 in domain B, are highly conserved in eukaryotes with other species having either the same or another aromatic residue at these positions (Fig. 1). The aromatic residues are also conserved in the other DBDs in RPA (42). In contrast, the polar residues of the RPA-ssDNA interface are poorly conserved between domains (42, 43). The high conservation of the aromatic residues suggests that they are important for some aspect of RPA function. However, only Phe-238 is required for viability in yeast (42). Mutation of these residues individually causes only modest reduction of the affinity of the RPA complex for ssDNA (37). When both aromatic residues in a DBD are mutated to alanine, the binding of that domain is affected (44), but the overall affinity of the RPA complex is only reduced approximately 1 order of magnitude (37, 40, 44). Mutation of these aromatic residues does, however, cause significant defects in RPA function. For example, when mutant forms of RPA with double aromatic mutations in either DBD-A or DBD-B were analyzed in human cells, it was found that they caused cells to accumulate in G2/M phase, suggesting they cause a cell cycle defect (45). In addition, these mutant forms could not localize to sites of DNA repair (repair foci) after DNA damage, suggesting that they do not function properly in DNA repair. However, these defects do not appear to be simply caused by lowered affinity for ssDNA because other mutant forms of RPA that have a lower affinity for ssDNA were found to be fully functional in vivo (45).

**EXPERIMENTAL PROCEDURES**

**Construction of RPA1 Mutants**—For cell culture studies, an expression plasmid containing an enhanced green fluorescent protein (GFP)-tagged version of RPA1 was modified using QuikChange site-directed mutagenesis to mutate selected aromatic residues to alanine as published previously (45).

**RNAi Knockdown and Replacement of RPA1**—The method for knockdown of endogenous RPA1 and expression of exogenous RPA1 was as described (45). In brief, HeLa or U2OS cells (obtained from the American Type Culture Collection) grown

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**FIGURE 1. Conserved aromatic residues in RPA1 subunit.** Top, schematic of RPA1 subunit with the structural domains and flexible linkers indicated. The positions of the conserved aromatic residues in DBD-A and -B are shown. F238, Phe-238; F269, Phe-269; W361, Trp-361; F386, Phe-386. Middle, alignment of the amino acid sequences of DBD-A and -B from various species. Conserved aromatic residues are in bold. Conservation of all residues is indicated under the sequences: asterisk, identical; colon, strongly similar; period, weakly similar. S. cerevisiae, Saccharomyces cerevisiae. Bottom, schematics of WT or FAB deletion mutant of RPA interacting with dT30 or dT15. DNA-binding domains of RPA are indicated by rectangles with each domain designated with the appropriate letter. DNA is represented by thick black lines, and protein-DNA interactions are represented by vertical lines.
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in DMEM with 10% calf serum at 37 °C with 5% CO₂ were seeded in six-well tissue culture plates at 2 × 10^6 cells per well. Small interfering RNA (siRNA) (200 pmol) was added 24 h after wells were seeded to knock down endogenous RPA1. Transfections were performed with 5 μl of Lipofectamine 2000 (Invitrogen) per well. At 24 h after transfection of siRNA, cells were transfected with 250 ng of plasmid expressing GFP fusion of wild-type RPA1 (WT) or mutant RPA1. The RPA1 siRNA target sequence was 5′-GGAAUUAUGUCGUAAUGCA-3′, corresponding to the 3′-UTR of RPA1.

Flow Cytometry Analysis—Cells were harvested from wells at specified times after transfection of siRNA, washed with PBS, and fixed overnight in 70% methanol. Cells were rehydrated in PBS for 30 min and washed in PBS. 0.1 mg/ml propidium iodide was added to each sample for analysis of DNA content. For analysis of Chk2 activation, cells were incubated in 1:100 phospho-Chk2 primary antibody (Cell Signaling) overnight and then in 1:100 phosphoethrin secondary (Invitrogen) for 2 h. Cells were examined on a FACScan II, and the data were analyzed using FlowJo software (Tree Star). For synchronization studies, cells were treated with 5 μg/ml aphidicolin for 24 h. The cells were released into medium and collected at 0, 8, and 24 h after release.

Immunofluorescence Analysis—HeLa cells were seeded on coverslips in six-well tissue culture plates and exposed to RNAi knockdown and replacement of RPA1 as described previously. At 92 h after transfection of siRNA, cells were exposed to DNA-damaging agent, either 20 μm camptothecin for 4 h or 2 mM hydroxyurea (HU) for 3 h. Coverslips were washed twice in cold CSK buffer (10 mmol/liter HEPES (diluted from 1M stock at pH 7.8), 300 mmol/liter sucrose, 100 mmol/liter NaCl, and 3 mmol/liter MgCl₂). Where indicated, non-chromatin-bound RPA was extracted with CSK, 0.5% Triton X-100 for 5 min. Coverslips were fixed with 4% formaldehyde for 20 min and then washed thrice with PBS. Coverslips were incubated in blocking solution (5% calf serum, PBS) for 1 h at room temperature and then in primary antibody at 1:500 overnight at 4 °C. Primary antibodies used were RPA2 (71-9A) (1:300) (46), RPA9 (mabRPA9; B. Stillman, Cold Spring Harbor Laboratory), phosphorylated H2AX (p-H2AX; 1:600) (Cell Signaling), and phosphorylated checkpoint protein 2 (phospho-Chk2; 1:500) (Cell Signaling). Coverslips were washed thrice with PBS and then incubated in anti-rabbit Texas red secondary antibody (Cell Signaling) at 1:800 for 2 h. Coverslips were washed in PBS, incubated in DNA staining solution DAPI (1 μg/μl) for 5 min, washed again in PBS, and mounted to slides. Slides were examined with a Leica immunofluorescence microscope, and images were collected with SPOT software (Diagnostic Instruments, Inc.) Confocal images of replication foci were collected with a Leica immunofluorescence microscope, and images were visualized using a Fujifilm imager and quantified using the Multi Gauge software, and the fraction of free ssDNA was plotted against RPA concentration. Apparent binding constants were calculated by fitting data to the Langmuir binding equation using non-linear least squares fitting (KaleidaGraph-Synergy software).

RESULTS

Conserved Aromatic Residues in ssDNA-binding Core of RPA are Essential for Normal Cell Cycle Progression—A series of double alanine substitutions was analyzed to understand the role of the conserved aromatic residues in DBD-A and -B in RPA function. In addition to AroA and AroB, the first or the second aromatic residue in each domain was mutated to give Aro1 (F238A,W361A) and Aro2 (F269A,F386A), respectively (Table 1). Previous analysis has shown that only Aro1 causes a large decrease in ssDNA binding activity of the RPA complex; Aro1 has an affinity almost 3 orders of magnitude lower than wild-type RPA, whereas the other three mutant forms have an affinity within 1 order of magnitude of RPA (37, 40). To determine the function of these mutants in cells, each was expressed in HeLa cells after knockdown of endogenous RPA1. Treatment of cells with siRNA targeting the 3′-UTR of RPA causes depletion of endogenous RPA1 to less than 5% of normal levels (45). Cells are concurrently transfected with plasmids expressing GFP-tagged RPA1 construct that does not contain the
3′-UTR. The GFP tag allows selection of the GFP-expressing cells by flow cytometry analysis or immunofluorescence. The transfection efficiency of the aromatic residue-expressing plasmids was identical to that of the wild-type RPA1 plasmid (supplemental Fig. 1A). We have previously shown that the level of expression of exogenous RPA1, AroA, and AroB in this system is comparable with the endogenous RPA1 (45). We confirmed that all four aromatic mutants are expressed at similar levels to that of wild-type GFP-RPA1 by quantitating the average GFP fluorescence intensity per cell for each mutant (supplemental Fig. 1B).

Cells lacking RPA exhibit an increase of cells in S-phase due to a replication defect and in G2/M due to a defect in DNA repair (which causes checkpoint activation) (Fig. 2). This phenotype is complemented by expression of exogenous GFP-RPA1, which results in a cell cycle distribution the same as untreated cells (Fig. 2). Expression of RPA with any single aromatic residue mutated to alanine resulted in a normal cell cycle distribution (data not shown). Expression of Aro2 also restored a normal cell cycle distribution. As was observed previously (45), both AroA and AroB were able to partially rescue cell cycle progression but also caused a large increase in the number of cells in the G2/M peak (Fig. 2). In contrast to the other Aro mutants, expression of Aro1 causes a very abnormal cell cycle distribution and significant cell death (Fig. 2 and data not shown). Aro1 binds ssDNA with an affinity ~0.1% of wild-type RPA (1% of the other Aro mutants) and does not support DNA replication in vitro (37). We have observed other RPA1 mutants with severe ssDNA-binding defects that are non-functional (16, 45). We conclude that Aro1 does not support RPA function in cells. These data indicate that the aromatic residue mutants are not functioning normally and that the severity of the defect depends on the combination of aromatic residues mutated.

**Aromatic mutant properties**

| Name | Mutations | Relative binding (dT) | Normal cell cycle | Support DNA replication | Effective DNA repair |
|------|-----------|----------------------|------------------|------------------------|----------------------|
| Aro2 | F269A,F386A | 13%                  | Yes              | Yes                    | No                   |
| AroA | F238A,F269A | 23%                  | No               | Yes                    | No                   |
| AroB | W361A,F386A | 15%                  | No               | Yes                    | No                   |
| Aro1 | F238A,W361A | 0.2%                 | No               | No                     | No                   |

We next determined whether cells expressing aromatic residue mutants retained the ability to activate the DNA damage checkpoint. Reconstituted cells were treated with camptothecin and collected, and phosphorylation of checkpoint kinase 2 was monitored by flow cytometry (phospho-Chk2, a marker of cellular checkpoint activation (50)). The mock-treated sample without DNA damage treatment shows the background intensity of staining in the absence of induced DNA damage (Fig. 3B). When mock-treated cells were exposed to DNA damage, the level of phospho-Chk2 increased as demonstrated by the rightward shift of the peak (Fig. 3B). We then analyzed GFP-positive cells expressing different forms of RPA1. Similar increases in phospho-Chk2 were observed after DNA damage in cells expressing wild-type RPA1, AroA, AroB, or Aro2 (Fig. 3B). These data demonstrate that the RPA mutants do not interfere with cellular checkpoint activation after exogenous DNA damage.

We next determined the proportion of checkpoint activation in cells expressing different forms of RPA1 in the absence of exogenous damage. HeLa cells expressing different forms of RPA1 were grown on coverslips, fixed, and stained with an antibody to phospho-Chk2. The percentage of cells with phospho-Chk2 staining was quantitated. (Only cells expressing GFP-RPA were considered in reconstitution experiments.) The mock-treated cells had a very low number of cells showing staining for phosphorylated Chk2, whereas the cells lacking RPA1 had a dramatic increase in the proportion of cells with phosphorylated Chk2 (Fig. 3C). Reconstitution with wild-type RPA lowered the number of cells with phospho-Chk2 to background levels, similar to the mock-treated cells (Fig. 3C). Cells expressing only AroA or AroB had a high percentage of cells with phospho-Chk2 staining (Fig. 3C). The level of phospho-Chk2 observed with AroA and AroB was similar to that observed in cells depleted for RPA1. In contrast, cells expressing Aro2 had levels of phospho-Chk2 similar to wild-type RPA1-expressing cells. Similar activation was observed by assessing phosphorylation of H2AX. The activation of Chk2 was the result of high spontaneous levels of DNA damage because cells expressing AroA and AroB also had high levels of γ-H2AX in the absence of exogenous DNA damage (supplemental Fig. 2A). Taken together, these data indicate that spon-
taneous DNA damage accumulates in cells expressing AroA or AroB but not in cells expressing Aro2. This endogenous DNA damage causes checkpoint activation in the absence of exogenous DNA damage.

Aro2-expressing Cells Are Not Able to Recover after DNA Damage—To investigate whether cells expressing aromatic mutants are able to recover after DNA damage, a damage recovery assay was carried out. HU is an inhibitor of ribonucleotide reductase and causes the arrest of cells in early S-phase. This arrest is normally rapidly eliminated after removal of the HU. Cells were treated with 2 mM HU for 3 h followed by a recovery period of 24 h. Time points were taken immediately following DNA damage and after 24 h of recovery. Cells were fixed and stained for phosphorylated Chk2, and the percentage of phospho-Chk2 cells was quantified. Less than 5% of the mock-treated non-damaged cells showed phospho-Chk2 staining (Fig. 4). Immediately following DNA damage treatment, 34% of the mock-treated cells showed detectable phospho-Chk2 staining. This percentage is similar to the proportion of cells in S-phase in an unsynchronized population and is consistent with HU causing a DNA damage response during S-phase. After the 24-h recovery period, the number of cells showing Chk2 activation had returned to the baseline levels, suggesting complete recovery from exogenous DNA damage and alleviation of the cellular checkpoint after 24 h. In contrast, more than half of the RPA1 knockdown cells exhibited phospho-Chk2 staining prior to HU treatment. Phospho-Chk2 staining increased modestly after HU treatment and remained unchanged after the 24-h recovery period, suggesting that cells cannot recover from DNA damage when lacking RPA function. Cells reconstituted with wild-type RPA1 had low phospho-Chk2 staining (Fig. 4). HU treatment caused the level of phos-
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Phospho-Chk2 staining increased to ~45% treatment, and following the recovery period, the population had returned to baseline levels of phospho-Chk2. We observed similar results monitoring phosphorylation of H2AX (supplemental Fig. 2B). We conclude that cells reconstituted with wild-type RPA1 can repair HU damage and recover after DNA damage similar to untreated cells. Cells expressing AroA or AroB have high levels of phospho-Chk2 and \( \gamma \)-H2AX in the absence of DNA damage treatment (Fig. 4 and supplemental Fig. 2B). The percentage of cells with phospho-Chk2 staining was quantified visually. At least 50 cells were counted for each sample set. A representative experiment is shown.

Following DNA damage, RPA localizes to sites of DNA damage, termed DNA repair foci, along with other DNA damage recognition and repair proteins (45, 51). These foci can be visualized after detergent extraction to remove non-chromatin-bound RPA (45, 51). HeLa cells were treated with camptothecin for 4 h to induce DNA damage, detergent-extracted, and fixed. Exogenous RPA1 (GFP fluorescence) and RPA2 (monoclonal antibody) localization to foci were monitored by confocal fluorescence microscopy. As expected, untransfected control cells showed no GFP staining but strong RPA2 foci (from endogenous RPA) after DNA damage (Fig. 5A). RPA2 levels are not reduced after RPA1 knockdown (45); however, RPA2 and RPA3 do not associate with DNA in the absence of RPA1, and no RPA2 foci were observed after RPA1 knockdown (Fig. 5A). In contrast, knockdown cells reconstituted with exogenous wild-type RPA1 have intense foci containing both RPA1 and RPA2 (Fig. 5A). As shown previously, neither AroA nor AroB was able to localize to sites of DNA damage as demonstrated by the lack of GFP and RPA2 staining (Fig. 5B) (45). Aro2 also did not localize to foci (Fig. 5B). The finding that these mutant forms of RPA1 do not localize to sites of DNA damage, cause spontaneous checkpoint activation, and/or cannot recover after DNA damage indicates that they are defective in one or more DNA repair processes.

Activity in DNA Replication—While analyzing the localization of different forms of RPA after DNA damage, we made an additional observation. A majority of the cells expressing the aromatic mutations had no RPA2 foci, but ~30% of the cells showed a distribution of lower intensity foci (Fig. 5C; supplemental Fig. 3A). These foci were much less intense than those observed with wild-type endogenous or exogenous RPA (Fig. 5). In contrast to the RPA2 foci, GFP foci were not reproducibly observed in any of the damaged cells expressing the aromatic RPA1 mutants. RPA1, RPA2, and RPA3 form an obligate complex, and GFP-tagged proteins have a lower level of fluorescence than fluorescently labeled antibodies (compare wild-type RPA 1-GFP with RPA2-antibodies in Fig. 5A). Thus, it is most likely that in extracted cells, the amount of RPA1 localizing to these faint foci is below the level of detection of GFP. RPA is known to localize to sites of DNA replication and DNA repair (51), and ~30% of the cells in these cultures are in S-phase (e.g. Fig. 2). This suggests that the faint foci are RPA localizing to sites of DNA replication (replication foci). To test this hypothesis, we examined foci formation in undamaged cells expressing different forms of RPA.

In control experiments, RPA2 was visualized in cells expressing only endogenous RPA (mock-treated), and 28% of the cells contained faint foci of RPA2 (Fig. 6A and supplemental Fig. 3A). In parallel experiments, we showed that 34% of unperturbed, extracted cells had proliferating cell nuclear antigen foci. These percentages are the same as the number of cells in S-phase determined by flow cytometry (Fig. 2). In contrast, a majority of the cells in cultures exposed to camptothecin contained RPA2 and proliferating cell nuclear antigen foci (supplemental Fig. 3A), and the foci were much more intense after DNA damage (Fig. 5 and supplemental Fig. 3B). We also observed this difference in foci intensity when unperturbed cells were stained with monoclonal antibody to RPA1 (supplemental Fig. 3B). We conclude that these foci are sites of replication.

HeLa cells were then grown on coverslips and subjected to knockdown/reconstitution with different forms of RPA1. When cells were treated with siRNA to RPA1, only background RPA2 staining was observed (Fig. 6A). No RPA2 foci were observed in cells treated with RPA1 siRNA. After reconstitution, ~30% of cells expressing AroA, AroB, or Aro2 contained replication foci (Fig. 6A). This suggests that AroA, AroB, and Aro2 are able to support cellular DNA replication.

To directly determine whether these mutants were able to support progression through S-phase, we studied their function in synchronized cells. After knockdown and reconstitution, HeLa cells were synchronized in late G1 phase with a 24-h treatment with aphidicolin, an inhibitor of DNA polymerases. Cells...
were released into inhibitor-free medium, and the cell cycle distribution was analyzed after 0, 8, and 24 h. Immediately following release from cell cycle inhibition, a majority of cells in all samples were in G1 phase (Fig. 6B). After 8 h, the mock-treated cells have synchronously entered S-phase, as indicated by a thickening and shift of the G1 peak to the right (Fig. 6B). After 24 h, the majority of the cells were clearly G2 phase, demonstrating progression through S-phase and into G2 and mitosis (Fig. 6B). In contrast, a majority of cells lacking RPA1 remain in the G1 peak even at the 24-h time point (Fig. 6B, Knockdown). Progression through S-phase was rescued by introduction of exogenous wild-type RPA1 (Fig. 6B). Cells expressing Aro1 remained in G1. We conclude that Aro1 is not able to support cell cycle progression. (A subset of cells expressing Aro1 appear to begin to progress into S-phase at the 24-h time point. This population is exaggerated due to increasing cell death later in the experiment and is probably caused by cells with incomplete knockdown of RPA1. A similar population is observed in the RPA1-depleted sample.) These data support the conclusion that Aro1 is not functioning in DNA replication and our conclusion that Aro1 is a non-functional form of RPA1. In contrast, cells reconstituted with AroA, AroB, or Aro2 were able to progress through S and into G2 phase (Fig. 6B). This indicates that cells expressing AroA, AroB, or Aro2 are capable of supporting cellular replication.

Molecular Defects of Aromatic Residue Mutants—To determine whether disruption of the aromatic residue mutations alter RPA-protein interactions, ELISA assays were done with three proteins that are known to interact directly with the DBD-A and DBD-B (3). These proteins, the nucleotide excision repair protein, XPA, and the recombination proteins, Rad51 and Rad52, are essential for initiation of nucleotide excision repair and recombination, respectively (21, 52). XPA, Rad51, Rad52, or BSA (as a control for nonspecific binding) was absorbed onto wells of a microtiter plate. Then the wells were incubated with increasing amounts of RPA complexes containing different forms of RPA1. Binding was assessed by the addition of a primary antibody to RPA2. Wild-type RPA was able to interact with XPA, Rad51, and Rad52 as expected (Fig. 7A). Similar interactions were observed with both AroA and AroB over the concentration range assayed, indicating that these protein interactions were not affected by mutation of the aromatic residues in vitro. RPA interacts with a large number of protein partners; however, most RPA-protein interactions are mediated by several protein-interacting domains on RPA (3). These studies suggest that the aromatic residue mutants do not totally disrupt the protein interaction domains in the DNA-binding core of RPA1.

We next examined binding of the mutants to DNA in more detail. The occluded binding site of the full-length RPA complex is ~30 nt (53), and AroA, AroB, and Aro2 all have modestly reduced binding to DNA 30 nt in length (37, 40). However, DBD-A and -B only directly interact with 8–12 nt of DNA (54), and other DBDs (especially DBD-C in RPA1 and DBD-D in
RPA2) are needed for the formation of the stable 30-nt complex (3, 40, 55, 56). With shorter oligonucleotides, fewer DBDs are able to interact with the DNA, and the affinity of the RPA complex is reduced (Fig. 1) (3, 40, 56, 57). This predicts that RPA binding to short oligonucleotides primarily depends on DBD-A and -B and suggests that the aromatic residue mutants may

FIGURE 6. Aro mutants support function in DNA replication. A, RPA at replication foci. HeLa cells were grown on coverslips, and the knockdown (Kdn) and reconstitution method was applied to express WT, AroA, AroB, or Aro2. Non-chromatin-bound RPA was extracted before fixing the slide. Row one, DAPI staining; row two, RPA2 antibody staining. B, HeLa cells were treated and analyzed as described in the legend for Fig. 2. At 72 h after mock or siRNA transfection, cells were synchronized with 5 μg/ml aphidicolin (APH) for 24 h and then released into medium. Flow cytometry was used to analyze DNA content at 0, 8, and 24 h after release. A representative experiment is shown.

FIGURE 7. Mutation of aromatic residues does not affect interaction with selected repair proteins, but does affect DNA binding to short ssDNA. A, RPA interactions with repair proteins. Protein-protein interactions between WT (solid line), AroA (dotted line), or AroB (dashed line) and repair proteins were assessed using an ELISA. 1 μg of XPA, Rad51, Rad52, or BSA was coated in wells of a microtiter plate, and then the amount of the indicated form of RPA was added. Non-bound RPA was washed away, and an antibody to RPA2 was used to detect bound protein followed by a secondary antibody linked to HRP. HRP substrate was added, and absorbance was measured at 450 nm. BSA values were subtracted to correct for nonspecific binding. Error bars indicate S.D. 8, RPA binding to different lengths of ssDNA. Binding affinity was measured with gel mobility shift assays to the indicated oligonucleotides. Association constants were determined, and the average from at least three separate experiments with S.D. (error bars) is shown. Stoichiometric binding (S) is indicated; no complex was detected for AroA or AroB with dT15.
have a greater effect on binding to short oligonucleotides. To test these hypotheses, we analyzed binding of the aromatic residue mutants to DNA of different lengths. We initially analyzed both wild-type RPA and a truncated form consisting of the three N-terminal domains of RPA1 (DBD-F, -A, and -B, called FAB) as controls. DBD-F has a low affinity for ssDNA, so binding of FAB is primarily dependent on DBD-A and -B (33). FAB has been previously shown to have a binding affinity similar to the aromatic residue mutants, approximately 1 order of magnitude less than wild-type RPA (58).

Wild-type RPA and FAB bound to long oligonucleotides \((dT30\) and \(dT25\)) with high affinity. Binding of wild-type RPA was stoichiometric and represents the lower limit of the affinity (Fig. 7B). The apparent association constant for FAB was approximately 1 order of magnitude reduced from wild-type RPA with both of these oligonucleotides. The affinities of wild-type RPA and FAB were both reduced proportionally, approximately 1 order of magnitude with \(dT20\) (Fig. 7B; see also the summary of all binding constants in supplemental Table 1). When the DNA was 15 nt in length, the affinities of wild-type RPA and FAB were the same and several orders of magnitude decreased from that observed with longer oligonucleotides. This is consistent with the binding to short oligonucleotides being dependent only on DBD-A and -B.

AroA, AroB, and Aro2 show longer length dependence in DNA binding. All three mutant forms had an affinity for \(dT30\) that was similar to that of FAB (22, 10, and 44% of FAB, respectively). With \(dT25\), Aro2 binding was not significantly different from FAB, whereas AroA and AroB showed decreased binding (16 and 2% of FAB, respectively). A greater decrease was observed with \(dT20\); the association constants for Aro2, AroA, and AroB were reduced to 4, 1, and 1% of FAB, respectively. Any interaction of AroA or AroB with \(dT15\) was below the limit of this assay, but binding of Aro2 to \(dT15\) was detected (Fig. 7B and supplemental Table 1). This indicates that under conditions in which only DBD-A and -B interact with DNA, mutation of both conserved aromatic residues in either domain disrupts the formation of a stable RPA-DNA complex. In contrast, mutation of Phe-269 and Phe-386 in Aro2 has a more modest effect on the interactions of the core DNA-binding domain. This is consistent with Aro2 having a more moderate phenotype than the other aromatic residue mutants. We conclude that mutation of the conserved aromatic residues has only a modest effect on binding to ssDNA greater than 25 nt in length and has a significantly larger disruption of the interaction with ssDNA oligonucleotides 20 nt and shorter. These results indicate that the aromatic residues are important for forming stable interactions with short ssDNA intermediates.

**DISCUSSION**

DBD-A and -B of the RPA1 subunit interact with ssDNA through both polar and non-polar residues. Four aromatic residues in these domains, Phe-238, Phe-269, Trp-361, and Phe-386, are responsible for the non-polar, base-stacking interactions. These aromatic residues are important for RPA function; mutation of these conserved residues causes a loss of viability in yeast (42) and cell cycle arrest in human cells (45) (Fig. 2). However, the defect that causes this loss of function has been poorly understood. The aromatic residues were initially thought to be essential for ssDNA binding; however, single mutations and most combinations of double aromatic residue mutations have only modest effects on binding to 30 nt (Fig. 7B) (37, 40, 44). In these studies, we show that these mutations have a large effect on interactions with short oligonucleotides (<20 nt). These results confirm that aromatic residue base stacking is important for RPA-DNA interactions and indicate an essential role for DBD-A and -B in binding to short DNA fragments. Disruption of base-stacking interactions in either DBD-A or DBD-B prevents RPA from stably interacting with ssDNA 15 nt or shorter. These results are consistent with previous studies that showed that isolated DBD-A or DBD-B interacts with ssDNA very weakly and that both DBD-A and DBD-B were needed for stable binding to DNA (32, 40, 41).

Although DBD-A and -B constitute the high affinity binding core in RPA, the formation of a stable RPA-DNA complex also involves interactions of other DNA-binding domains (40, 44, 55, 56). These interactions cause RPA to have multiple modes of DNA binding (44, 59) that differ in the amount of DNA occluded by the complex and in the number of domains interacting with DNA. In the stable 30-nt DNA binding mode, it is believed that at least four DBDs (A, B, C, and D) interact with DNA (see models in Fig. 1, bottom). Our results indicate that having multiple, independent DNA-binding domains allows RPA binding to DNA to be resistant to mutations that partially disrupt a single domain. AroA, AroB, and Aro2 all bind \(dT30\) with high affinity, suggesting that interactions of the other DBDs are able to partially compensate for reduced binding of the mutated domain. Of these three mutants, AroB shows the greatest decrease in binding with intermediate length oligonucleotides (e.g. \(dT25\), Fig. 7B). This is presumably because disruption of interactions of DBD-B (in the middle of the linear array of binding domains; Fig. 1) makes it more difficult for the domains on either side to simultaneously interact to an intermediate length oligonucleotide. With \(dT20\), the contribution of the domains outside the high affinity binding region is decreased, resulting in the larger observed length dependent binding for the Aro mutants. In contrast, interaction of \(dT15\) is mediated almost entirely via the high affinity binding domains A and B and thus results in the greatest difference in binding affinity of the Aro mutants. The \(dT15\) binding data indicate that disruption of both aromatic residues in the same domain (AroA and AroB) has a larger effect on stability than disruption of Phe-269 in DBD-A and Phe-386 in DBD-B (Aro2).

These studies also show that the four aromatic residues are not functionally equivalent. The four mutant forms analyzed have different pairs of residues mutated. We found that the mutants ranged from being non-functional (Aro1) to severely defective (AroA and AroB) to mildly defective (Aro2). This indicates that Phe-238 and Trp-361 are more important for function than Phe-269 and Phe-386 but that all contribute to DNA interactions and RPA function. These studies also confirm that mutation of both aromatic residues in a single DBD dramatically affects the interactions of that domain with DNA (43, 44).

The aromatic residues are in the interior of the DNA-binding cleft of DBD-A and DBD-B and directly stack with ssDNA. The
different combinations of mutations affect RPA-ssDNA interactions, whereas they interact normally with several proteins that directly interact with these domains. This suggests that the RPA-DNA interface but not the protein interaction sites on DBD-A and -B are disrupted by the aromatic residue mutations. Thus, our data suggest that the separation of function phenotype observed with the aromatic residue mutants is the result of altered RPA-DNA interactions. It has been thought that RPA-DNA interactions were similar across different cellular pathways; however, these findings suggest that different RPA-DNA interactions are needed in repair and replication.

During DNA replication, there is extensive DNA unwinding, long-lived multiprotein complexes form at replication forks, and long ssDNA intermediates are exposed. RPA binds to the ssDNA intermediates and interacts with multiple proteins at the fork. In contrast, during nucleotide excision repair, the ssDNA intermediates are short (less than 30 nt), and RPA is required for assembly of the initial repair complex. This difference in ssDNA intermediate size coupled with the reduced affinity of the aromatic residue mutants suggests that the mutants may be unable to form stable complexes with repair intermediates but can form stable complexes with long replication intermediates. Supporting this model, preliminary kinetic analysis suggests that the aromatic residue mutants dissociate more rapidly from ssDNA than wild-type RPA.

Other pathway differences may contribute to the aromatic residue mutant phenotype. For example, in replication, RPA binds after the replication fork complexes form, and RPA-protein interactions would be expected to stabilize RPA binding to these intermediates. In contrast, during nucleotide excision repair, RPA binding is needed for the formation of the repair complex. Thus, mutations that reduce the stability of RPA-DNA complexes on short ssDNA stretches would be expected to prevent initiation of DNA repair but have minimal effects on DNA replication. Finally, RPA interacts with a large number of protein partners, so we cannot rule out the mutations disrupting specific RPA-protein interactions. However, these studies are more consistent with the repair defect being caused by altered stability or kinetics of binding to short ssDNA intermediates.

Our data indicate that binding to short ssDNA is not required for the function of RPA in DNA replication but is essential for at least some processes in DNA repair. We predict that the aromatic mutations will have different impacts on different repair processes and possibly the repair of different types of DNA lesions. Specifically, we predict that the aromatic mutations will have minimal effect on repair processes that have long ssDNA intermediates (e.g., recombinational repair) but will disrupt nucleotide excision repair and possibly other repair processes. Future studies will be needed to determine the repair pathways affected by these mutations and to finish defining repair-specific functions of RPA.

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