Dissection of Functional Domains of the Human DNA Replication Protein Complex Replication Protein A*

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Replication protein A (RPA) is a mammalian single-stranded DNA binding factor essential for DNA replication, repair, and recombination. It is composed of three subunits of 70, 34, and 13 kDa (Rpa1, Rpa2, and Rpa3, respectively). Deletion mapping of the Rpa2 subunit identified the domain required for interaction with Rpa1 and Rpa3 which does not include the N-terminal domain that is phosphorylated during S phase. Deletion mapping of Rpa1 defined three domains. The C-terminal third of the Rpa1 polypeptide binds Rpa2 which itself forms a bridge between Rpa1 and Rpa3. The N-terminal third of the Rpa1 polypeptide binds Rpa2 which itself associates with proteins containing acidic transcriptional activator domains such as p53, VP16, and the DNA repair protein p53 requires the N-terminal third of Rpa1 with some contribution from the C-terminal third. The evolutionarily conserved putative zinc finger near the C-terminus of Rpa1 was not required for binding to single-stranded DNA, Rpa2, or p53. However, all three subdomains of Rpa1 and the zinc finger were essential for supporting DNA replication in vitro. These experiments are a first step toward defining peptide components responsible for the many functions of the RPA protein complex.

RPA1 is absolutely required in the in vitro SV40-based DNA replication reaction (1–3) and is also important for many other DNA-mediated processes. It is required for replication in Xenopus egg extracts, for the successful passage of yeast through the large budded stage corresponding to S phase, for excision repair of pyrimidine dimers, and for recombination (4–7). The three-subunit RPA complex binds to single-stranded DNA and modulates the function of DNA polymerases α and δ. It also physically interacts with the SV40 origin-binding protein T antigen and with DNA polymerase α (8–12). In addition RPA associates with proteins containing acidic transcriptional activator domains such as p53, VP16, and the DNA repair protein XP-G. These interactions have been proposed to inhibit DNA binding by RPA and to recruit RPA for replication and repair (13–16). Interaction with p53 is of particular interest because RPA bound to p53 failed to bind single-stranded DNA (15). The 70-kDa subunit (Rpa1) can bind to single-stranded DNA on its own but cannot support DNA replication. The 34-kDa subunit (Rpa2) is phosphorylated in a cell cycle-dependent manner by multiple kinases which include the cdk kinases and the DNA-dependent protein kinase (17–19). The phosphorylation of Rpa2 is induced by γ-irradiation (20) and this form of phosphorylated RPA fails to support DNA replication (20). We have recently discovered a homolog of the middle subunit, Rpa4, which is expressed selectively in some quiescent tissues apparently uncomplexed with Rpa1 and Rpa3 (21). In order to understand how the various subunits of RPA interact with each other, how RPA binds DNA, and how the activity of the protein may be regulated by protein-protein interactions and post-translational modifications, we have mapped the functional domains of the three subunits of RPA using dcdNs coding for the human RPA subunits (22–24). Gomes and Wold (25) have used C-terminal deletions of Rpa1 to map regions required for binding to single-stranded DNA and to Rpa2/Rpa3. This report confirms and extends their results.

Three approaches have been taken for studying the mutant forms of RPA subunits. The first uses the yeast two-hybrid/interaction trap method for studying protein-protein interactions between the subunits. The second uses proteins made by in vitro transcription and translation in co-immunoprecipitation and binding assays to analyze protein-protein or protein-nucleic acid interactions. The third uses recombinant RPA holocomplexes to confirm the findings from the earlier assays and to determine the domains of Rpa1 essential for SV40-based DNA replication. Together, these results provide a functional map of the RPA complex and suggest that, beside the binding of single-stranded DNA and the recruitment of Rpa2 and 3 to the replication apparatus, the Rpa1 subunit executes additional functions essential for DNA replication.

MATERIALS AND METHODS

Plasmid Constructions—The plasmids important for this study are listed in Table I. pEGRPA1 has been described (15). A DNA fragment containing the coding region of RPA3 was made by PCR from phRPA3 and cloned between EcoRI and XhoI sites of pEG202 to make pEGRPA3. pl GRPA3 was made by transferring the EcoRI-XhoI fragment of pEGRPA3 to pLG 4–5. Yep-RPA2 has been described as pl M403 (5). PCR was performed with appropriate primers to synthesize a DNA fragment that contained the coding region of RPA2 (from phRPA2) flanked by BamHI sites. This PCR product was cloned into the BamHI site of pJKS1 such that the RPA2 reading frame was oriented in the same direction as the lacZ gene to make p102. After making p102, the entire RPA2 reading frame was sequenced with multiple primers to ensure that there were no PCR-induced mutations.

The BamHI fragment of p102 was cloned into pEG202 to obtain pEGRPA2. The deletions in RPA2 were made as follows. pEGRPA2 was cut with NcoI (sites in RPA2 and in the polylinker of EG202 downstream from RPA2) and ligated, to obtain pEG107. The NcoI fragment

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1 The abbreviations used are: RPA, replication protein A; SV40, Simian virus 40; GST, glutathione S-transferase; PCR, polymerase chain reaction; mAb, monoclonal antibody.
TABLE I

| Plasmid     | Relevant genotype | Rpa1/Rpa2/Rpa3 fragment | Base plasmid |
|-------------|-------------------|-------------------------|--------------|
| pEG202      | His3; LexA under control of an ADH promoter | 1–616, Rpa1 | pEG202 |
| pSH18-34    | URA3; lacZ gene under control of B lacZ operators | 1–616, Rpa1 | pEG202 |
| pJG4–5      | LexA fused to Rpa1 | 1–616, Rpa1 | pEG202 |
| pEGRPA1     | LexA fused to Rpa1 | 1–270, Rpa2 | pEG202 |
| pEGRPA3     | LexA fused to Rpa3 | 1–121, Rpa2 | pEG202 |
| pEG222–411  | LexA fused to Rpa1 | 1–222, 412–616, Rpa1 | pEG202 |
| pEG457–616  | LexA fused to Rpa1 | 457–616, Rpa1 | pEG202 |
| pEG1–492    | LexA fused to Rpa1 | 1–492, Rpa1 | pEG202 |
| pYEP51-RPA2 | Rpa2 | 1–270, Rpa2 | YEP51 |
| pGRPA2      | Activation domain fused to Rpa2 | 1–270, Rpa2 | pG4–5 |
| pG12        | Activation domain fused to Rpa2 | 43–270, Rpa2 | pG4–5 Activation |
| pG109       | Activation domain fused to Rpa2 | 102–270, Rpa2 | pG4–5 Activation |
| pG115       | Activation domain fused to Rpa2 | 134–270, Rpa2 | pG4–5 Activation |
| pG118       | Activation domain fused to Rpa2 | 163–270, Rpa2 | pG4–5 Activation |
| pG117       | Activation domain fused to Rpa2 | 1–163, Rpa2 | pG4–5 Activation |
| pG116       | Activation domain fused to Rpa2 | 1–134, Rpa2 | pG4–5 Activation |
| pG107       | Activation domain fused to Rpa2 | 1–101, Rpa2 | pG4–5 Activation |
| pG124       | Activation domain fused to Rpa2 | 43–163, Rpa2 | pG4–5 Activation |
| phRPA2      | Rpa2 under control of a T7 promoter | 1–270, Rpa2 | pKS |
| phRPA3      | Rpa3 under control of a T7 promoter | 1–120, Rpa3 | pKS |
| phRPA1      | Rpa1 under control of a T7 promoter | 1–616, Rpa1 | pKS |
| prevRPA1    | Rpa1 under control of a T3 promoter | 1–616, Rpa1 | pKS |
| phRPA1Cla   | Rpa1 under control of a Clal site in 3′-untranslated region | 1–616, Rpa1 | pKs |
| p1-221      | Rpa1 under control of a T7 promoter | 1–221, Rpa1 | phRPA1 |
| p1-309      | Rpa1 under control of a T7 promoter | 1–309, Rpa1 | phRPA1 |
| p1-492      | Rpa1 under control of a T7 promoter | 1–492, Rpa1 | phRPA1 |
| p222–411    | Rpa1 under control of a T7 promoter | 1–222, 412–616, Rpa1 | phRPA1Cla |
| p278–616    | Rpa1 under control of a T7 promoter | 278–616, Rpa1 | phRPA1Cla |
| p1-372      | Rpa1 under control of a T3 promoter | 1–372, Rpa1 | prevRPA1 |
| p1-522      | Rpa1 under control of a T3 promoter | 1–522, Rpa1 | prevRPA1 |
| p349–616    | Rpa1 under control of a T3 promoter | 349–616, Rpa1 | prevRPA1 |
| pm1-616     | Rpa1 under control of a T7 promoter | 1–616, Rpa1 | pKS |
| pm1-616     | Rpa1 under control of a T7 promoter | 1–616, Rpa1 | pKS |
| pm1ldRPA    | Rpa1, 2, and 3 under control of a T7 promoter | 1–616, Rpa1 | pET11d |
| pm1ldRPA    | Rpa1, 2, and 3 under control of a T7 promoter | 1–616, Rpa1 | pET11d |
| p1ldRPA     | Rpa1, 2, and 3 under control of a T7 promoter | 1–616, Rpa1 | pET11d |
| p1ldRPA     | Rpa1, 2, and 3 under control of a T7 promoter | 1–616, Rpa1 | pET11d |
| p3atRPA     | Rpa1, 2, and 3 under control of a T7 promoter | 278–616, Rpa1 | pET3a |
| p3atRPA     | Rpa1, 2, and 3 under control of a T7 promoter | 278–616, Rpa1 | pET3a |

containing the C-terminal part of RPA2 from the same digestion was cloned into pEG202 (B8) (21) to obtain pEG109. p102 was linearized by partial digestion with BamHI, and then cut in the RPA2 coding region with BglII. After ligation the plasmid containing the C-terminal part of RPA2 from the BglII site was obtained (p113). The EcoRI-BamHI fragment from p113 was cloned into pEG202 to obtain pEG113. p102 was linearized with BglII, the ends filled in with Klenow polymerase, and blunt ends ligated to obtain p102ΔBgl which has a four base pair insertion that disrupts the RPA2 reading frame at the BglII site. The BamHI fragment from p102ΔBgl was cloned into the BamH1 site of pEG202 to obtain pEG116. The N-terminal 560-base, and the C-termi- nal 330-base, BamH1 to BglII fragments of p102 were cloned into the BamH1 site of pEG202 to obtain pEG117 and pEG118 respectively. PCR with appropriate primers was used to make a DNA fragment encoding RPA2 from A43 to the C terminus this fragment was digested with EcoRI and BamH1 or EcoRI and BclI. The 755-base EcoRI-BamHI fragment and the 420-base EcoRI-BclI fragment was cloned between EcoRI and BamH1 sites of pEG202 to obtain pEG121 and pEG124, respectively. To make the pEG derivatives which express the deletions of RPA2 fused to the acidic activation domain, EcoRI-Xhol fragments from each of the pEG plasmids described above were cloned between the EcoRI and Xhol sites of pG4–5 (except for pG121, for which the EcoRI-Sall fragment from pEG121 was transferred to pG4–5). As an additional safeguard against unintentional mutations in the RPA2 coding region, pGRPA2 was also made by a second strategy. A DNA fragment containing the RPA2 coding region was synthesized by PCR and cloned between the BamH1 and EcoRI sites of EG202, and the EcoRI-Xhol fragment from this secondary plasmid transferred to JG4–5 to make the second version of pGRPA2.

The deletion derivatives of Rpa1 were made as follows. p1–616 was the original phRPA1 clone obtained from Dr. T. Kelly where the RPA1 cDNA is cloned into pKS+. Between EcoRI sites such that the RPA1 gene is downstream from the T7 promoter. The EcoRI fragment was recloned into pKs+ in the reverse orientation to obtain prevRPA1. phRPA1 was cut with ClaI (sites in the untranslated region downstream from RPA1) and ligated to obtain prevRPA1Cla. PhRPA1 was cut with HindIII or with Xhol and ligated to obtain p1–219 and p1–309, respectively. phRPA1Cla was cut with HindIII and ligated to obtain p1–222–411. PhRPA1 was cut with BclI and EcoRV (in the polylinker), the ends filled in and ligated to obtain p1–492. prevRPA1 was cut with Xbal or with Spal and ligated to obtain p1–372 and p1–522, respectively. prevRPA1 was cut with Xhol and ligated to obtain p349–616,
and pRPA1ΔCl was cut with PstI and ligated to obtain p278–616. The pEG202-based constructs expressing deletions of RPA1 were made as follows. pEG1–616 is the same as pEGRPA1. NcoI-Xhol fragment of p122–411 and NcoI-Sall fragment of p-129 were cloned between NcoI and Xhol sites of pEG202 (B8) to obtain pEG122–411 and pEG1–492, respectively. A DNA fragment of RPA1 coding for residues 222–411 was synthesized by RT-PCR with the appropriate primers and cloned between EcoRI and BamHI sites of pEG202 to obtain pEG457–616. The plasmids for expressing RPA holocomplex with deletions or mutations in RPA1 were derived from p1IdtRPA which expressed wild-type human RPA in bacteria and which were provided by Dr. Marc Wold (26). The RPA1 coding region of p1IdtRPA was deleted to obtain p11dtRPA (see below, “Site-directed Mutagenesis of RPA1”). The internal HindIII fragment of RPA1 coding region of p11dtRPA was deleted to obtain p11dtRPA222–411. RPA1 amino acid 278–403 flanked by NcoI and XhoI sites of this plasmid was then replaced by the XhoI-BamHI fragment of p1IdtRPA to obtain p3aRPA1 278–616. RPA3-containing EcoRI fragment from p1IdtRPA and RPA2-containing BamHI fragment from p11dtRPA were successively cloned into p3aRPA1 278–616. The RPA1 sequence 1555–1590 with underlined nucleotides changed from TGG to AAATTCGGTGTC is complementary to human Rpa1 (amino acids 500 and 503) are both changed to serine. Primer 1591–1621 was the same as the corresponding sequence of human RPA1. Primer 1591–1621 was 5-phosphorylated, and 15 pmol each of primer were used in PCR on 0.5 pmol p1RPA1 template by Taq polymerase and Taq extender. The PCR cycling parameters are as follows: segment 1, 1 cycle of 94°C 4 min, 50°C 2 min, 72°C 2 min; segment 2, 8 cycles of 94°C 1 min, 56°C 2 min, 72°C 1 min; segment 3, 1 cycle of 72°C 5 min. By keeping the number of cycles low the chances of unintentional PCR-induced mutation are decreased. The PCR reaction is thus used to create a linear DNA fragment corresponding to the whole RPA1 plasmid except the mutations incorporated in the primers. 1 μl of DpnI (which cuts methylated template DNA) and Pfu DNA polymerase were added at 37°C for 30 min to simultaneously select against the parental template DNA and to polish the ends of products, respectively. After heat inactivation of the enzymes (72°C for 30 min) the PCR product was purified by electrophoresis and then used in transformation. The resulting plasmids were screened for the incorporation of the SalI site designed in the mutagenic primer, and candidate plasmids were sequenced to confirm the mutation and rule out adjoining secondary mutations.

Two transformations and β-Galactosidase Assays—Saccharomyces cerevisiae strain EGY40 (MATa trpl1 ura3 his3 leu2::pLexApoLEU2) (27) were transformed with the various plasmids, and transformed yeast was selected by the appropriate nutritional markers according to standard protocols. Briefly, the Jg plasmids contain TRP1, the E. coli plasmids contain H153, and the lacZ-containing plasmids pSH18–34 contains the URA3 marker. β-Galactosidase assays were done on log phase cultures by standard protocols and presented as β-glucuronidase units. lacZ activity was measured in bacterial cell extracts depleted of RPA (1), which have been supplemented with 1% Triton X-100, 1% Trasylol as described in Keshav et al. (21).

Expression and Purification of Recombinant RPA—The proteins were expressed and purified as described previously (26). The protein complexes were named according to the Rpa1 mutant present in the complex.

RESULTS

Protein-Protein Interactions between the RPA Subunits—The yeast two-hybrid system (27) was used to analyze interactions among the RPA subunits. In this system a DNA binding protein (lexA) is fused to the protein of interest (Rpa1 in EgRpa1 or Rpa3 in EgRpa3). This protein cannot activate a specially designed promoter upstream from a lacZ gene unless a second protein is present which contains a generic transcriptional activation domain fused to a domain that interacts stably with the first fusion protein.

The S. cerevisiae strain EGY40 carrying the reporter plasmid pSH18–34 (lacZ gene under control of a lexA operator) and pGRPA1 (expresses a fusion between the lexA DNA binding domain and human Rpa1 protein) did not have significant β-galactosidase activity. Plasmid pJ GRPA2 expresses a protein containing a synthetic transcriptional activation domain fused to human Rpa2. When pJ GRPA2 was transformed into the yeast (EGY40::pSH18–34, pEGRPA1), and the expression of the Jg Rpa2 fusion protein induced with galactose, significant β-galactosidase activity was produced (Fig. 1). This suggests that the Jg Rpa2 fusion protein interacted with the EgRpa1 fusion protein at the lexA operator and created a transcription activator at the lacZ promoter. In similar experiments, Jg Rpa2 also interacted with the EgRpa3 fusion protein, but Jg rpa3 did not interact with the EgRpa1 fusion protein. These results support a model where the Rpa2 subunit interacts with both
the Rpa1 and the Rpa3 subunit and thus has the potential to form a bridge between the other subunits.

This model was tested directly by adding the Rpa2 protein in trans to the two hybrids, EgRpa1 and JgRpa3. If Rpa2 forms a bridge between Rpa1 and Rpa3, one would predict that while EgRpa1 and JgRpa3 fail to interact with each other on their own, the expression of Rpa2 in these strains of yeast would result in an EgRpa1-Rpa2-JgRpa3 interaction establishing a functional transcription activator at the GAL10 promoter. Yeast carrying the pSH18–34 plasmid was transformed with the pJGRPA3, pEGRPA1 and either a control vector Yep51, or carrying the pSH18–34 plasmid was transformed with the pEGRPA3 (interaction with RPA3) and the indicated JG plasmids expressing various derivatives of RPA2. Averages of at least two different colonies are presented (in units described in the text) with the standard deviations in parentheses. Significant β-galactosidase activity indicates association between the proteins expressed from the JG and EG plasmids.

The deletions of Rpa2 affected interaction with both Rpa1 and Rpa3. Thus the C-terminal boundary of the Rpa1/Rpa3 interaction is between amino acids 102 and 134. On the C-terminal side, deletion of the last 108 amino acids abolished interaction with both Rpa1 and Rpa3. Thus the C-terminal boundary of the Rpa1/Rpa3 interaction is between amino acids 102 and 134. On the C-terminal side, deletion of the last 108 amino acids abolished interaction with both Rpa1 and Rpa3. The region of Rpa1 from amino acid 222–411 of Rpa1 still retained considerable interaction. Thus the region of Rpa1 from amino acid 412 to the C terminus is necessary for interacting with Rpa2 in this assay.

When human Rpa1 was synthesized in a rabbit reticulocyte lysate, a monoclonal antibody to human Rpa2, p34–20, specifically immunoprecipitated the 70 kDa Rpa1 subunit (Fig. 4A, lane 3). This was not due to cross-reaction of the p34–20 antibody with p70, because (i) the antibody did not recognize p70 on an immunoblot (data not shown), and (ii) boiling of the translation mixtures to disrupt Rpa1-Rpa2 interactions prevented co-immunoprecipitation of Rpa1 by the p34–20 antibody (data not shown).

RPA is routinely found in the cytoplasmic fractions when cells are disrupted. Therefore rabbit Rpa2 could be present in the reticulocyte lysate and p34–20 could co-immunoprecipitate Rpa1 by virtue of its association with the rabbit protein. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected in the absence of Rpa1 they co-sedimented at 3–4.6 S, consistent with a Rpa2/Rpa3 complex of 45–70 kDa. When translated on its own in rabbit reticulocyte lysates Rpa1 still sediments as a broad peak of 5–8 S consistent with the size of RPA holocomplex (120–150 kDa). When Rpa2 and Rpa3 were translated in the absence of Rpa1 they co-sedimented at 3–4.6 S, consistent with a Rpa2/Rpa3 complex of 45–70 kDa. When translated on its own in rabbit reticulocyte lysates Rpa1 still sediments as a broad peak of 5–8 S consistent with the size of Rpa1-2-3 complex. The Rpa1 from all fractions could be precipitated by anti-Rpa2 antibody. Taken together, these results suggest that even when Rpa1 is translated on its own, it forms a complex with unlabeled rabbit Rpa2 – 3 from the reticulocyte lysates.

One would then predict that the domain of Rpa1 shown in the two-hybrid interaction assay to be important for association with Rpa2 will also be important for co-immunoprecipitation of in vitro translated Rpa1 by p34–20 antibody. Several deletion derivatives of Rpa1 were synthesized in the rabbit reticulocyte lysate, and immunoprecipitated by p34–20 examined (Fig. 4B and summarized in Fig. 8). As expected from the results of the yeast interaction trap, co-precipitation by p34–20 antibody was seen with the Δ222–411 derivative of Rpa1 (lane 7) but not with the Rpa1-492 derivative (lane 10). The 1–522 derivative was weakly associated with rabbit Rpa2, although the significance of this association is unclear. The absence of any association of the 1–372 derivative, and of strong association of the 349–616 protein, again emphasizes that the C-terminal part of Rpa1 is necessary for interaction with Rpa2.
important for association with Rpa2. The minimal domain is confirmed to be between amino acids 412 and 616.

Region of Rpa1 Involved in Binding Single-stranded DNA—
RPA can bind to single-stranded DNA at salt concentrations in excess of 0.5 M NaCl, and we were interested in determining which portions of Rpa1 were necessary for this high avidity binding of DNA. Full-length Rpa1 (1–616) was synthesized in vitro in rabbit reticulocyte lysates (containing rabbit Rpa2 and 3), bound to single-stranded DNA cellulose matrix and washed with 0.5 M NaCl. The binding of Rpa1 to single-stranded DNA was resistant to elution by 0.5 M NaCl (Fig. 5A, lane 2, and summarized in Fig. 8). Both 1–522 and 1–492, which bound Rpa2 very poorly or not at all, could still bind single-stranded DNA at 0.5 M NaCl (Fig. 5B, lanes 6 and 10). 1–372 was poor in single-stranded DNA binding activity (Fig. 5B, lane 4) putting the C-terminal limit of the DNA binding region between 372 and 492; 278–616 could, while 349–616 could not, bind single-stranded DNA at 0.5 M NaCl (Fig. 5B, lanes 6 and 8), although they were both capable of associating with Rpa2. We conclude that for binding single-stranded DNA at 0.5 M NaCl, Rpa1 requires a minimal domain between amino acids 278 and 492.

If the NaCl concentration of the DNA binding reaction and of the washes is reduced to 0.1 M NaCl, the N-terminal part of Rpa1 (1–309 or 1–219) now binds to single-stranded DNA, although this association is sensitive to 0.5 M NaCl (Fig. 5C). Therefore, Rpa1 has a second single-stranded DNA binding domain (amino acids 1–219), but binding of DNA through this domain is disrupted by high salt concentrations.

The absence of background “binding” of Rpa1 to the negative control CL6B beads argues against the nonspecific aggregation of the in vitro translated proteins on single-stranded DNA cellulose beads. To rule out the possibility that the binding to DNA was secondary to the association of Rpa1 with an unknown DNA binding protein from rabbit reticulocyte lysates, selected recombinant RPA complexes containing deletion derivatives of Rpa1 were purified to homogeneity and tested in a nitrocellulose filter binding assay (Fig. 7B). The results corroborate those obtained in Fig. 5.

Region of Rpa1 Required for Binding p53—We have reported that RPA bound to p53 fails to bind single-stranded DNA (15). One explanation could be that the overlapping regions of Rpa1 are required to bind the two ligands, so that the ligands are mutually exclusive. To determine if this was the case, we used the deletion derivatives of Rpa1 to map the region required to bind p53. Rpa1 and deletion derivatives were synthesized in vitro and bound to glutathione agarose beads coated with either GST or GST-p53 (Fig. 6A, summarized in Fig. 8). A small C-terminal deletion increased binding to p53 (1–616 versus 1–522, lanes 3 and 21). This result was obtained consistently and could indicate that the C-terminal 94 amino acids of Rpa1 somehow interfere with association with p53. Comparison of 1–492 to 1–372 (lanes 6 and 15) suggests that the C-terminal limit of the p53 binding region lies between 372 and 492. 1–278 is important for the binding of p53 because 278–616 is unable to bind p53. The requirement for this region is emphasized by the comparison of the p53 binding activity of 1–522 Rpa1 versus 278–522 Rpa1 (lanes 12 and 21). However, the 1–278 region alone did not bind p53 (e.g., 1–309, lane 18).

We confirmed and extended the results using purified RPA holocomplexes with selected deletion derivatives of Rpa1 (Fig. 7A, described below) and analyzed for binding to p53 (Fig. 6B).
RPA with Δ222–411 Rpa1 bound to p53 indicating that the middle third of Rpa1 was not required for this activity (Fig. 6B, lane 9). The failure of RPA with 278–616 Rpa1 to bind p53 (Fig. 6B, lane 12) is consistent with the results in Fig. 6A (lane 9) and confirms that the N-terminal 1–278 amino acids of Rpa1 are essential for the interaction. Taken together, we conclude that the N-terminal 221 amino acids of Rpa1 together with residues in the 411–492 region are sufficient for binding p53.

We previously reported that the N-terminal 1–308 amino acids of Rpa1 is sufficient for p53 binding (15) in contradiction to the data shown in Fig. 6A. The discrepancy between the two results may be due to differences in the way Rpa1 was produced. In the previous study, lexA-Rpa1(1–308) fusion protein expressed in yeast was used for binding to GST-p53, while in the current experiments we used Rpa1 produced in rabbit reticulocyte lysates without any N-terminal fusion for its association with GST-p53. In the previous study the lexA portion of lexA-Rpa1(1–308) may stabilize a weak interaction by artificially increasing the protein-protein contact area between the two proteins, but such a concern does not arise in the current study. Alternatively, in the current study the rabbit reticulocyte lysate may contain proteins which competitively inhibit a weak interaction of 1–308 Rpa1 with GST-p53. Indeed, Rpa1D222–411 produced in rabbit reticulocyte lysates was poor at interacting with GST-p53 (data not shown), although bacterially produced recombinant RPA Δ222–411 containing the same derivative of Rpa1 associated strongly with GST-p53 (Fig. 6B). Therefore, while we can conclude that the N-terminal
activities. Rpa1 were tested for their DNA binding and DNA replication with a buffer containing 50 mM NaCl. The studied in an SV40-based binding assay. Washes were done at 0.5M NaCl.

...recombinant RPA to single-stranded DNA in a nitrocellulose filter containing Rpa2, Rpa3, and indicated versions of Rpa1. 

...B [32P]dAMP incorporated into polynucleotide (in a theoretical 50-

...A putative C4-type zinc finger motif was noted at position 481–503 of human Rpa1, which is evolutionarily conserved in yeast Rpa1 (5, 6, 23). The ability of 1–492 (which deletes 2 of the 4 cysteines) to bind single-stranded DNA suggests that the zinc finger is not required for binding to single-stranded DNA. Since 1–492 could bind p53, the zinc finger is also dispensable for binding p53.

...To determine whether the zinc finger was important for binding Rpa2, point mutations were made in the Rpa1 CDNA which changed the 2 C-terminal cysteines of the putative zinc finger to serines (m1–616). This point-mutated form of Rpa1 was synthesized in vitro, and its ability to bind Rpa2 was measured by co-immunoprecipitation with monodonal antibody p34–20 (Fig. 4A, lane 7). The mutated form of Rpa1 associated with Rpa2 as effectively as wild-type Rpa1. Also, as expected from the deletion derivatives, binding to single-stranded DNA and p53 was unaffected by the loss of the putative zinc finger (Fig. 5A, lane 5; Fig. 6B, lane 6). Therefore, the putative zinc finger is not required for binding any of the three ligands tested, Rpa2, single-stranded DNA, and p53.

...the high affinity DNA binding site). All four forms of Rpa1 which still form a holocomplex with Rpa2 and Rpa3, and indicated versions of Rpa1 were tested for their DNA binding and DNA replication conditions and washes in a buffer containing 0.05 M NaCl in the top). m1–616 RPA and 278–616 RPA had 70 and 40% of the activity of wild-type RPA, respectively at 48 ng/ml. At high salt concentration of binding and washing (0.5 mM NaCl), both wild-type and mutant RPA complexes bound less DNA compared to low salt conditions (Fig. 7B, bottom). m1–616 RPA and 278–616 RPA had 70 and 40% of the activity of wild-type RPA (respectively; 48 ng/ml), while Δ222–411 RPA did not bind any DNA, consistent with the results obtained in Fig. 5B.

...The RPA holocomplexes were tested for their ability to support SV40-based DNA replication in an extract depleted of 278 amino acids of Rpa1 are required for interacting with GST-p53, we cannot yet conclude whether it is sufficient or insufficient for the interaction.

...The Putative Zinc Finger of Rpa1 Is Dispensable for Binding Single-stranded DNA, p53, or Rpa2—A putative C4-type zinc finger motif was noted at position 481–503 of human Rpa1, which is evolutionarily conserved in yeast Rpa1 (5, 6, 23). The ability of 1–492 (which deletes 2 of the 4 cysteines) to bind single-stranded DNA suggests that the zinc finger is not required for binding to single-stranded DNA. Since 1–492 could bind p53, the zinc finger is also dispensable for binding p53. On the other hand, binding to Rpa2 was fairly weak with 1–522, and nonexistent when the C-terminal deletion reaches the zinc finger (1–492). To determine whether the zinc finger was important for binding Rpa2, point mutations were made in the Rpa1 CDNA which changed the 2 C-terminal cysteines of the putative zinc finger to serines (m1–616). This point-mutated form of Rpa1 was synthesized in vitro, and its ability to bind Rpa2 was measured by co-immunoprecipitation with monodonal antibody p34–20 (Fig. 4A, lane 7). The mutated form of Rpa1 associated with Rpa2 as effectively as wild-type Rpa1. Also, as expected from the deletion derivatives, binding to single-stranded DNA and p53 was unaffected by the loss of the putative zinc finger (Fig. 5A, lane 5; Fig. 6B, lane 6). Therefore, the putative zinc finger is not required for binding any of the three ligands tested, Rpa2, single-stranded DNA, and p53.

...DNA binding by the mutant RPA holocomplexes in the DNA cellulose pull-down assays was as predicted from the domain mapping experiments (data not shown). RPA with wild-type, zinc finger- mutated, and 278–616 Rpa1 were all able to bind to DNA in high and low salt concentrations. RPA with Δ222–411 Rpa1 could bind to single-stranded DNA in low but not in high salt concentration.

...DNA binding by these holocomplexes was quantitated by a nitrocellulose DNA binding assay (Fig. 7B; binding was in replication conditions and washes in a buffer of 0.05 mM NaCl in the top). m1–616 RPA and 278–616 RPA retained considerable DNA binding activity (60–70% of wild-type RPA activity respectively at 48 ng/ml), while Δ222–411 RPA was weaker in its DNA binding capacity (40% of wild-type activity at 48 ng/ml). At high salt concentration of binding and washing (0.5 mM NaCl), both wild-type and mutant RPA complexes bound less DNA compared to low salt conditions (Fig. 7B, bottom). m1–616 RPA and 278–616 RPA had 70 and 40% of the activity of wild-type RPA (respectively; 48 ng/ml), while Δ222–411 RPA did not bind any DNA, consistent with the results obtained in Fig. 5B.

...The RPA holocomplexes were tested for their ability to support SV40-based DNA replication in an extract depleted of

![Fig. 7. RPA holocomplexes prepared with selected mutants of Rpa1 were tested for their DNA binding and DNA replication activities.](image)
endogenous RPA. Despite the fact that the three mutant RPA holocomplexes bound significant amounts of single-stranded DNA in the salt and RPA concentrations used in the replication reaction, none of them supported DNA replication (Fig. 7C). Both m1–616 RPA and 278–616 RPA bound single-stranded DNA to an extent comparable with that of wild-type RPA but did not support any DNA replication. This result suggests that DNA replication requires additional activities from Rpa1 beside binding to single-stranded DNA and formation of the RPA holocomplex.

**DISCUSSION**

In order to understand how the trimeric RPA protein complex participates in DNA replication, we have determined some of the functional regions of the Rpa1 and Rpa2 proteins. The C-terminal part of Rpa1 binds to Rpa2 and the middle part binds to single-stranded DNA in a salt resistant manner (Fig. 8). Surprisingly, the N-terminal part of Rpa1 also binds to single-stranded DNA, but only at low salt concentrations. p53 uses amino acids from both the N- and C-terminal portion of Rpa1 to bind to the protein. The C-terminal two-thirds of Rpa2 forms a bridge between Rpa1 and Rpa3, although additional contacts between Rpa1 and 3 cannot be ruled out.

Attempts to reconstitute the RPA holocomplex by expression of recombinant polypeptides have suggested that although Rpa1 and Rpa2 can interact to form an easily dissociable complex, Rpa2 and Rpa3 form a stable complex which will associate with Rpa1 to form the Rpa1-2-3 complex (26, 28). We have modified the two-hybrid system to demonstrate that Rpa2 is capable of forming a bridge between Rpa1 and Rpa3. This report is the first demonstration that bridging interaction in a three subunit complex can be studied by a modification of the two-hybrid system. Deletion mapping of Rpa2, however, did not separate the parts of the protein that interact with Rpa1 or Rpa3. Finer mutagenesis may be required to achieve this goal, and since we now have a genetic system for assaying the interaction, it should be possible to mutagenize randomly the cDNA coding for the Rpa2 protein and screen for mutations that selectively affect interaction with Rpa1 or Rpa3. The N-terminal 43 amino acids of Rpa2 containing its sites of phosphorylation by cdk kinases (17) appears to be independent from the region required to associate with Rpa1 and 3.

Rpa1 was readily divisible into regions important for one function but not another. The deletions could potentially disrupt the tertiary structure of Rpa1 or produce insoluble proteins. However, since various deletion derivatives of Rpa1 carried out some functions and not others, we believe that the deletions do not result in a global denaturation of the protein. Also the bead-binding assays were confirmed by two-hybrid assays in yeast and by solution binding assays with soluble recombinant proteins to eliminate artifacts due to insoluble deletion derivatives. The regions indicated in Fig. 8 are required for binding the indicated ligands. The Rpa1-Rpa2 interaction requires the C-terminal one-third of Rpa1, a result consistent with that obtained by Gomes and Wold (25). This region also appears sufficient for association with Rpa2. Although this domain assignment utilized the association of human Rpa1 with rabbit Rpa2 in the reticulocyte lysate the results are the same for the association of human Rpa1 and Rpa2 when the deletions were tested (a) in the yeast two-hybrid assay and (b) for the ability to form the RPA holocomplex when co-expressed with Rpa2 and 3 in bacteria.

Approximately the middle third of Rpa1 (278–492) is important for binding of single-stranded DNA in 0.5 M NaCl, a conclusion reached from the properties of 278–616 (+), Δ222–411 (−), 1–492 (+) and 1–372 (−). Definition of the minimal regions of Rpa1 required to bind Rpa2 and single-stranded DNA is also supported by the binding properties of the smaller products obtained during in vitro transcription-translation of Rpa1 (Fig. 4A, lane 1). Assuming that these products are by initiation from internal methionines, polypeptides of 57, 36, and 29 kDa probably correspond to products initiating at methionines 97, 278, and 349. Since Rpa2 is expected to bind to the C-terminal region of these products, immunoprecipitation with anti-Rpa2 antibody should precipitate all the smaller products, consistent with what is observed (Fig. 4A, lane 3). Single-stranded DNA cellulose however, only binds to products containing 278–492, and therefore should bind only to the internally initiated products of 57 and 36 kDa but not of 29 kDa. This is in fact what is observed (Fig. 5A, lane 2). The DNA binding region of Rpa1 does not contain the zinc finger motif or residues 109–145, the reported region of sequence similarity to E. coli single-stranded DNA binding protein (ssb) (23).

DNA binding by Rpa1 at 0.15 M NaCl in a Southwestern assay showed that small N-terminal fragments of Rpa1 (e.g. 1–249, 1–326, and so forth) were capable of binding DNA (25). It is likely that the DNA binding these authors observe in the Southwestern assay is the equivalent of the DNA binding we see at 0.1 M NaCl, and can be executed by a salt-sensitive DNA binding site near the N terminus of Rpa1. Gomes and Wold (25) reported a significant drop in affinity when the deletions removed amino acids N-terminal to residue 441 (1–441 had high and 1–326 had low association constants for DNA). The close agreement on the C-terminal limit of the DNA binding region when assaying binding in high salt versus binding with high association constant (residue 492 versus 441) suggests that the salt resistant DNA binding is due to the high affinity binding site. Since 278–616 Rpa1 binds to DNA at high salt, we suggest that the salt-resistant (high affinity?) DNA binding site in the middle of Rpa1 is separate from a salt-sensitive DNA binding site contained in the 1–219 region of the protein.

The existence of separate salt-sensitive and -resistant DNA binding sites on Rpa1 was unsuspected. RPA has been reported to bind to single-stranded DNA in two different modes: one with each RPA molecule covering 8 bases, and the other with each RPA molecule covering 30 bases (29, 30). Different parts of
the Rpa1 molecule with different DNA binding sites could be involved in the two modes of DNA binding. The deletion mapping also shows that the Rpa2 binding region of Rpa1 is dispensable for binding single-stranded DNA with high affinity, implying that Rpa2–3 are not necessary to give Rpa1 a specific structure essential for high affinity association with DNA.

In vitro DNA replication reactions are performed at low salt concentrations (less than 50 mM KCl), conditions under which the low affinity DNA binding site is functional. However, the results reported in Fig. 7 suggest that the salt-sensitive DNA binding site (absent in 278–616, but present in 222–411 Rpa1) is required but not sufficient to support DNA replication. Of course, the salt-sensitive DNA binding site in the N-terminal third of Rpa1 could actually be used in replication to bind a negatively charged protein and not DNA.

Scrutiny of the Rpa1 deletions shows that association with p53 did not correlate with binding to single-stranded DNA or to Rpa2. There was a derivative which bound p53 well but not single-stranded DNA (Δ222–411), others which bound both well (1–522), and a third which bound DNA well but not p53 (278–616). Likewise there were Rpa1 derivatives which bound p53 well but not Rpa2 (1–522 and 1–492), and a derivative which did the reverse (278–616). This confirms our previous observation that the Rpa1–p53 interaction required neither Rpa2 nor single-stranded DNA from the complex (15). The importance of the N-terminal 278 amino acids of Rpa1 for p53 binding is confirmed by a darker exposure of the autoradiogram in Fig. 6A: internally initiated polypeptides in the 1–616, 1–522, and 1–492 input lanes corresponding in size to those expected from initiation at methionine 278 were not bound by GST-p53.

Although p53 binds Rpa1 without displacing Rpa2, it excludes single-stranded DNA from the complex (15). The domain mapping results suggest that the regions of Rpa1 necessary for binding p53 include the N-terminal 221 amino acids and possibly additional residues in the 411–492 region. Therefore p53 could potentially exclude DNA from the N-terminal low affinity DNA binding site (residues 1–219 of Rpa1). Exclusion of DNA from the high affinity DNA binding site (278–492 of Rpa1) could be due to a selective overlap of the DNA and p53 binding sites in the 411–492 region or due to conformation changes induced in Rpa1 by p53. The absence of significant overlap between the p53 and Rpa2 binding sites of Rpa1 explains why p53 does not exclude Rpa2–3 from the p53-Rpa1 complex. Recombinant peptides derived from simple direct repeats of 10–12 amino acid sequences containing bulky hydrophobic residues interspersed with negatively charged residues bind Rpa1 well. Such sequences are commonly noted in “acidic activation domains” of transcription trans-activators like p53 and VP16. Mutations in bulky hydrophobic residues of p53 abolish interaction with RPA. Therefore, the domain of RPA which binds to p53 is likely responsible for more generalized interactions of RPA with other proteins containing “acidic activation domains” such as VP16, yeast Gal4, and the DNA repair protein XP-G.

The ability to divide the Rpa1 subunit into subdomains required for essential activities (holocomplex formation and DNA binding) opened the way toward determining whether other subdomains of Rpa1 are essential for DNA replication. Δ222–411 Rpa1 did not support DNA replication indicating that the evolutionary conserved salt-resistant DNA binding activity present in all single-stranded DNA binding replication proteins is essential for replication. The 278–616 and m1–616 form of Rpa1, however, carried out the core activities of the protein: binding to DNA in high salt concentrations and forming a holocomplex with Rpa2 and Rpa3. Yet, neither of these derivatives supported DNA replication suggesting that other activities of Rpa1 are essential for replication. At present we cannot determine whether these mutations selectively remove a functional domain of Rpa1 required only for DNA replication, or selectively misfold the RPA complex such that DNA binding is allowed but not DNA replication. In either case, determination of the step in the DNA replication reaction blocked with these mutants will shed light on what additional activities or properties are required from RPA to support DNA replication.

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