Role of the 70-kDa Subunit of Human Replication Protein A (I)

SINGLE-STRANDED DNA BINDING ACTIVITY, BUT NOT POLYMERASE STIMULATORY ACTIVITY, IS REQUIRED FOR DNA REPLICATION*

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Replication protein A (RPA), also known as human single-stranded DNA-binding protein, is a three-subunit protein complex with multiple functions. Here, we investigated the role of the 70-kDa RPA subunit (p70) in DNA replication, by generating a series of deletion mutants. Mutant p70, which lacked 50 amino acids at the C-terminus, failed to interact with the 11-kDa RPA subunit (p11) and, when deleted further at the C terminus, was unable to interact with either the 34-kDa subunit (p34) or with p11, suggesting that p70 directly interacts with both p34 and p11. Studies with purified RPA mutants indicated that deletions at the N-terminal domain of p70 had very little effect on RPA's single-stranded DNA (ssDNA) binding activity, whereas deletion of amino acids 169–246 significantly weakened the DNA binding ability of RPA. By deleting amino acids 296–373 or 374–458, we totally abolished p70's ssDNA binding activity, suggesting that multiple p70 domains are involved in DNA binding. Two p70 domains, the N-terminal domain and the DNA binding domain, were required to stimulate DNA polymerase (pol) α, yet the DNA binding domain alone supported pol δ activity. Interestingly, RPA containing p70 with a zinc-finger domain deletion retained its DNA binding activity, but inhibited pol α and δ activity. RPA that lacked ssDNA binding activity failed to support simian virus 40 (SV40) DNA replication in vitro, whereas mutant RPA that lacked pol α stimulatory activity (including the zinc-finger p70 mutant) functioned normally. We conclude that RPA's DNA binding activity, but not its pol α stimulatory activity, is required for DNA replication.

Human replication protein A (also known as human single-stranded DNA-binding protein) is a three-subunit complex originally identified as an essential factor for the replication of simian virus 40 (SV40) origin-containing DNA in vitro (Wobbe et al., 1987; Fairman and Stillman, 1988; Wold and Kelly, 1988). It consists of 70-, 34-, and 11-kDa subunits (p70, p34, and p11, respectively), which tightly associate with each other in various eukaryotic RPA preparations (Fairman and Stillman, 1988; Brill and Stillman, 1989; Mitis et al., 1993; Brown et al., 1993), suggesting its functional activity is highly conserved. RPA complexes assemble in an ordered process (Stigger et al., 1994; Henricksen et al., 1994), and the cellular localization of the individual subunits varies during the cell cycle (Murti et al., 1996). In interphase, the RPA subunits colocalize to form the complex. During metaphase, however, p70 localizes to the mitotic spindle pole, p34 associates with condensed chromosomes, and p11 is excluded from the chromosomes (Murti et al., 1996), which suggests that RPA assembly is cell cycle-regulated.

In replication, RPA mediates unwinding of SV40 origin-containing DNA in the presence of SV40 T-antigen (T-Ag) and topoisomerase. It interacts with SV40 T-Ag and the DNA polymerase α-prime complex (pol α-prime) (Dorrreter et al., 1992; Lee and Kim, 1995), which is necessary for the initiation of SV40 DNA replication (Collins and Kelly, 1991; Melendy and Stillman, 1993; Lee and Kim, 1995). RPA is also likely to function in elongation because it stimulates pol α, pol δ, and pol ε activity to elongate primed template DNA (Kenny et al., 1989; Lee et al., 1991). RPA also stimulates the 3'–5'–exonuclease activity of pol δ, which suggests a possible role in pol δ's proof-reading activity (Lee, 1993).

In addition, RPA is essential for nucleotide excision repair of UV-damaged DNA. It is involved in an early stage of repair since the addition of UV gene products can replace the RPA requirement in this repair process (Coverly et al., 1992). Recently, RPA was shown to interact with Xeroderma pigmentosum group A complementing protein (XPA) (He et al., 1995; Matsuda et al., 1995; Lee et al., 1995), a protein that recognizes damaged DNA. The RPA-XPA complex appears to have a greater affinity for damaged DNA than does XPA alone (Li et al., 1995), suggesting a possible role for RPA in the formation of a repair initiation complex at the damage site. It is not clear, however, whether RPA is also involved in the later stages of repair.

During replication, two of RPA's main functions, single-stranded DNA-binding activity and stimulation of DNA pol α activity, are mediated through the p70 subunit (Wold and Kelly, 1988; Kenny et al., 1989; Erdlie et al., 1991; Gomes and Wold, 1995). The p34 subunit interacts with SV40 T-Ag during initiation, which is necessary for efficient DNA replication (Lee and Kim, 1995). It is phosphorylated at the G5/S boundary and dephosphorylated during G2/mitosis (Din et al., 1990; Dutta and Stillman, 1992). Phosphorylation of p34 can also be induced by DNA damage (Liu and Weaver, 1993; Carty et al., 1994), suggesting a role for RPA phosphorylation in DNA metabolism. The function of p11 has not yet been determined.

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1 The abbreviations used are: SV40, simian virus 40; ssDNA, single-stranded DNA; pol, polymerase; T-Ag, T-antigen; RPA, replication protein A; XPA, X. pigmentosum group A complementing protein A; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.
In this study, we focused on the importance of p70 to RPA's function in DNA replication. We found that p70 has two principal functional domains: a ssDNA binding domain and a pol α stimulation domain. By mutating these domains, we found that only the ssDNA binding activity of p70 is required for RPA to function in DNA replication.

**EXPERIMENTAL PROCEDURES**

DNA, Cell Extracts, Proteins, and Antibodies—PSV01EP, SV40 T-Ag, human pol α-prime complex, human pol δ, proliferating cell nuclear antigen (PCNA), and activator 1 (A1; also known as replication factor C, RF-C) were purchased as described previously (Lee et al., 1987). A polyclonal antibody to p70 from rabbit, monoclonal antibodies to p70 (70A, 70B, and 70C), and a monoclonal antibody to p34 were described previously (Stigger et al., 1994).

Recombinant Baculovirus Construction—N-terminal and C-terminal deletion mutants of p70 were generated by polymerase chain reaction (PCR) using the full-length cDNA and the following sets of primers: 5′-CAGT ACC TCA CTA CAA TGCT ACT CCT-3′ (p70CTERM) and 5′-AGG ATC CAT GAA CAC TCT CTC TAA-3′ for p70Δ2-50; p70CTERM and 5′-AGG ATC CAT GGT TTT GAA TGC AGC ACC A-3′ for p70Δ2-100; 5′-AGG ATC CAT GGG CCA GCT GAG GCT GCT-3′ (p70NTERM) and 5′-AAC CAT GGG TGT GCC CCC AGA-3′ for p70ΔN300; 5′-AAC CAT GGG TGT GCC CCC AGA-3′ for p70Δ2-300; and p70NTERM and 5′-AAC CAT GGG TGT GCC CCC AGA-3′ for p70Δ300-500. To generate mutants with internal deletions, we prepared two PCR products for each mutant (one from the N-terminal side and the other from the C-terminal side of the deletion) and ligation them. The following sets of primers were used to synthesize the PCR products required: p70NTERM and 5′-AAC CAT GGT TCT ATC TTT ACA-3′, and 5′-AAC CAT GAC GCC CCA GCT GAG GCT-3′ for p70Δ1-200; 5′-AAC CAT GGG TGT GCC CCC AGA-3′ for p70Δ1-300; and 5′-AAC CAT GGG TGT GCC CCC AGA-3′ for p70Δ1-500. By mutating these domains, we found that only the ssDNA binding activity of p70 is required for RPA to function in DNA replication.

**RESULTS**

**Detection Map and Immunoblotting Analysis**—Previous studies have indicated that several of RPA’s functions are associated with its pol subunit: single-stranded DNA binding activity (Erdie et al., 1991), DNA unwinding (Kenny et al., 1990), and the stimulation of pol α and pol δ (Kenny et al., 1989). To investigate the role of p70 in RPA function, we have constructed a set of p70 mutants that contain deletions at the N-terminal, the C-terminal, or in internal regions (Fig. 1A). These mutants were cloned into baculovirus expression vectors and were successfully expressed in insect cells (S9), as evidenced by immunoblotting analysis (Fig. 1B).

**Determination of the Domain(s) of p70 That Interact with Other RPA Subunits**—RPA p70 forms a stable complex with p34 and p11, but a much less stable complex with either p34 or p11 alone (Stigger et al., 1994), suggesting that both p34 and p11 must interact with p70 to form a stable RPA complex. We and others have shown previously that the p34 and p11 subunits form stable complexes both in vivo and in vitro (Stigger et al., 1994; Henricksen et al., 1994). Here, we examined the interaction of p70 with wild-type p34 and wild-type p11. Immunoprecipitation with an anti-p70 polyclonal antibody revealed that N-terminal deletion mutants of p70 (p70Δ2-50 and p70Δ2-100) formed a stable complex with p34 and p11. In contrast, a 50-amino acid deletion at the C terminus (p70Δ50C) of p70 abolished its interaction with p11, and significantly reduced its interaction with p34, when immunoprecipitated by the anti-p70 antibody (Fig. 2A). The weak coprecipitation of p34 with all of our p70 mutants, shown in Fig. 2A, was likely due to nonspecific (rather than specific) interaction between p70 and p34, since immunoprecipitation experiments carried out at different times often showed almost...
no coprecipitation of p34 with the p70 mutant that lacks the C-terminal domain (data not shown). When we used an anti-p34 antibody, the p70D50C mutant was coimmunoprecipitated with p34, but this interaction was abolished if further deletions were made at the C terminus of p70 (Fig. 2B).

Coimmunoprecipitation of p11 with p34, shown in Fig. 2B, is due to p11's ability to form a subcomplex with p34 in the absence of p70 (Stigger et al., 1994). Our results indicate that: (i) both p34 and p11 interact with RPA p70, (ii) the 50 amino acids at the C terminus of p70 are necessary for the interaction of p70 with p11, and (iii) amino acids 50–110 at the C terminus of p70 are probably involved in the interaction of p70 with p34. All internal deletion mutants of p70 formed stable complexes with p34 and p11, with no noticeable differences between them (Fig. 2).

Domains of RPA p70 Recognized by Monoclonal Antibodies

70A, 70B, and 70C—Previous studies with monoclonal antibodies to p70 (70A, 70B, and 70C) showed that all three antibodies inhibit DNA replication, but have different behaviors in other assays that require RPA, such as pol α stimulation, pol δ stimulation, and DNA unwinding (Kenny et al., 1990). 70A and 70B neutralize RPA’s ability to stimulate pol α, but have no effect on pol δ stimulation or DNA unwinding, whereas 70C inhibits RPA’s function in pol δ stimulation or DNA unwinding, but has no effect on pol α stimulation. Accordingly, we mapped the regions of p70 recognized by the individual monoclonal antibodies. The polyclonal antibody to p70 and the monoclonal antibody 70C both immunoprecipitated all of the p70 mutants (Fig. 3, A and D); however, monoclonal antibodies 70A and 70B did not immunoprecipitate p70 lacking the N-terminal domains (∆2–50 and ∆2–100) and only weakly immunoprecipitated AJ K (Fig. 3, B and C). Poor immunoprecipitation of ∆TU by 70A and 70B may be due to deletion of the zinc finger domain of p70,

leading to a conformational change in the N-terminal domain (see “Discussion”). Our finding that both 70A and 70B recognized the N-terminal domain of p70 is in keeping with the previous observation that both antibodies had an inhibitory effect on pol α stimulation but no effect on pol δ activity or DNA unwinding. This suggests that the N-terminal domain of p70 is responsible for pol α stimulation.

Purification of Deletion RPA Mutants—To identify the domains of p70 involved in replication, it is essential to prepare an RPA complex containing mutant p70, p34, and p11 since all three subunits are required for RPA to function in replication (Stigger et al., 1994; Lee and Kim, 1995). RPA mutants were

2 E. Stigger and S.-H. Lee, data not shown.
purified from insect cells transfected with recombinant baculoviruses encoding p34, p11, and various RPA mutants (Fig. 4). We should point out that, in this particular gel, less p11 appeared to associate with the ΔLM mutant than with any of the other mutants. We believe this is a staining artifact because immunoblots and other staining experiments depict normal levels of p11 association with this mutant. Because the p70 mutants that lack the C-terminal domain (p70Δ50C, p70Δ110C, p70Δ200C, and p70Δ300C) do not form a complex with p34 and p11, they were not included in these studies.

The ssDNA Binding Domain of p70—First, we examined whether mutating p70 affected RPA’s ssDNA binding activity. As shown previously, wild-type RPA formed stable complexes with oligo(dT)$_{50}$, which appeared as bands in the polyacrylamide gel. Deletions of the N-terminal domain [Δ2–100 and Δ101–169 (ΔJK)] and the zinc-finger domain (Δ459–507; ΔTU) had very little effect on RPA’s ssDNA binding activity, although these deletions did significantly change the gel mobility pattern of RPA (Fig. 5). On the other hand, deletion of amino acids 169–246 (ΔLM) significantly reduced RPA’s ssDNA binding activity, while deletion of amino acids 296–373 (ΔPO) or 374–458 (ΔRS) abolished ssDNA binding activity (Fig. 5). Deletion of amino acids 247–296 (ΔNO) also abolished RPA’s ssDNA binding activity (data not shown). Taken together, our results indicate that RPA’s ssDNA binding activity requires multiple regions of p70.

Stimulation of pol α and pol δ Activity—RPA was thought to be involved in the elongation step of replication because it stimulates pol α and δ to elongate a primed DNA template (Kenny et al., 1989). In fact, RPA’s stimulation of pol α activity could be due to a specific interaction with pol α during elongation. We, therefore, examined RPA mutants for their ability to stimulate pol α and pol δ activity. Wild-type RPA stimulated pol α activity about 2-fold, whereas RPA mutants lacking DNA binding activity (mutants ΔLM, ΔPO, and ΔRS) had virtually no effect on pol α (Fig. 6A). On the other hand, RPA containing either N-terminal deletion p70 mutants (Δ2–100 and ΔJK) or p70 with a deletion at the zinc finger region (ΔTU), strongly inhibited pol α activity. Mutant RPA:p70Δ2–50 also failed to stimulate pol α stimulation (data not shown). This result is consistent with the antibody experiment (Fig. 3), in which the N-terminal domain of p70 was shown to be responsible for pol α stimulation. Our results also suggest that the DNA binding domain of p70 is distinct from its pol α stimulation domain, and that both domains are required for pol α stimulation. Interestingly, the zinc-finger mutant ΔTU contains both the DNA bind-

**Fig. 4.** SDS-PAGE of purified RPA containing either wild-type or mutant p70. RPA was purified as described under “Experimental Procedures.” Proteins were electrophoretically separated on a 12% SDS-polyacrylamide gel, and visualized by Coomassie Blue staining.

**Fig. 5.** The ssDNA binding activity of RPA mutants. Two concentrations (75 and 150 ng) of either wild-type or mutant RPA, as indicated at the top of the figure, were incubated with 100 fmol of 5'-32P-labeled (dT)$_{50}$ for 5 min at room temperature. The RPA-DNA complexes were separated from free DNA by 5% polyacrylamide gel electrophoresis (acrylamide:bisacrylamide = 79:1).

**Fig. 6.** The effect of wild-type and mutant RPA on pol α and pol δ activity. A, RPA and pol α activity. Reaction mixtures (30 μl) contained 0.05 unit of pol α-primease, 0.1 μg of poly(dT)$_{50}$oligo$_{174}$ and the indicated amount of wild-type (C), RPA:p70Δ2–100 (G), RPA:p70Δ110C (J), RPA:p70ΔLM (B), RPA:p70ΔPO (△), RPA:p70ΔRS (A), and RPA:p70ΔTU (+). B, RPA and pol δ activity. Reaction mixtures (30 μl) contained 0.05 unit of pol δ (0.05 μg), 0.2 μg of PCNA, 0.4 μg of A1, 0.1 μg of singly primed DNA, and the indicated amount of wild-type (C), RPA:p70Δ2–100 (G), RPA:p70Δ110C (J), RPA:p70ΔLM (B), RPA:p70ΔPO (△), RPA:p70ΔRS (A), and RPA:p70ΔTU (+).
ing and the pol α stimulation domains, yet it still inhibited pol α activity. We also examined the effect of RPA mutants on the stimulation of pol δ activity (Fig. 6B). In contrast to pol α stimulation, RPA mutants lacking the N-terminal domain of p70 (Δ2–100 and Δ1 K) stimulated pol δ activity in the presence of PCNA and A1 (RF-C), whereas p70 mutants that lacked DNA binding activity (ΔLM, ΔPQ, and ΔRS) had no effect on pol δ activity. This result indicates that the DNA binding domain of p70, but not the pol α stimulation domain, is required for pol δ stimulation. The zinc-finger p70 mutant ΔTU also inhibited pol δ activity.

SV40 DNA Replication Activity-To determine whether RPA’s ssDNA binding activity and its ability to stimulate pol α and pol δ, are required for replication, we examined the effect of RPA mutants on SV40 DNA replication. RPA mutants lacking the N-terminal domain of p70 (Δ2–100) and amino acids 101–169 (ΔJK) supported SV40 replication less well than did wild-type RPA, whereas mutants defective in DNA binding (ΔLM, ΔPQ, and ΔRS) did not support SV40 replication at all (Fig. 7). Surprisingly, the zinc-finger mutant ΔTU, a strong inhibitor of both pol α and pol δ, efficiently supported DNA replication. To further investigate this, we examined these mutants during the elongation of SV40 DNA (Fig. 8). The reaction was preincubated in the absence of deoxyribonucleoside triphosphates (dNTPs) and terminated at different time points after dNTP addition to assess elongation of the nascent DNA. Mutant RPA (Δ2–100N and ΔTU) caused about a 2-fold decrease in the synthesis of nascent DNA chains, and in the rate of DNA elongation. This inhibition is significantly less than that observed in the polymerase stimulation reactions. These data strongly suggest that RPA’s DNA binding activity is essential for replication, but that its ability to stimulate pol α and pol δ activity is not.

DISCUSSION

RPA is a critical component of many DNA metabolic processes, including replication and repair. In this report, we characterized the role of one of the RPA subunits, p70, in DNA replication. Previously, we have shown that p34 and p11 form a complex, to which p70 is added, thus forming the RPA complex (Stigter et al., 1994). From the immunoprecipitation experiment shown in Fig. 2, we have determined that the p70 subunit interacts with both p34 and p11. Consistent with Gomes and Wold (1995), the C-terminal 50 amino acids of p70 are responsible for its interaction with p11 and the adjacent C-terminal domain is required for p70’s interaction with p34. Considering that the deletions we made are relatively large (more than 50 amino acids), it is possible that the domains of

p70 that interacts with p34 and p11 overlap. We now know that each RPA subunit interacts with both of the other two subunits, which may explain why the RPA complex is so stable.

Previous studies with anti-p70 monoclonal antibodies showed that both 70A and 70B neutralized pol α activity, while 70C inhibited DNA unwinding and pol δ activity. These data are consistent with our immunoprecipitation study, which indicated that both 70A and 70B recognized the N-terminal domain of p70, and that loss of this domain resulted in the loss of pol α stimulation activity. Together, these results strongly indicate that the N-terminal domain of p70 is responsible for pol α stimulation, and probably interacts directly with pol α. Antibody 70C, however, showed no specific affinity for any p70 domain; it immunoprecipitated all of our p70 deletion mutants. It may be that the epitope recognized by this antibody is repeated within the p70 molecule, or that it lies at a junction between two mutants, such that the antibodies recognize both mutants. It is interesting to speculate that if the epitope for 70C lies within the DNA binding domain of p70, and this domain is indeed repeated within the p70 molecule, that this could support our ssDNA binding data, with different p70 deletion mutants, that suggest p70 contains several DNA binding domains. It is interesting to point out that 70C significantly inhibited phosphorylation of RPA p34 by DNA-dependent protein kinase in the presence of ssDNA,3 further supporting the possibility that the epitope for 70C is within the DNA binding domain.

Immunoprecipitation of RPA mutants (Fig. 2) predicted that the domains for DNA binding activity and pol α stimulation would be distinct and separable. Amino acids 296–373 and 374–458 (mutants ΔPQ and ΔRS, respectively) are essential for ssDNA binding activity, while deletion of amino acids 169–246 (ΔLM) significantly reduced the affinity of RPA to bind DNA (Fig. 3). In addition, deletion of amino acids 101–169 (ΔJK) eliminated the formation of the larger DNA-RPA complex (Fig. 5), suggesting that this domain may be required for RPA’s

3 S.-H. Lee, unpublished observation.
cooperative DNA binding activity. Human RPA, compared to other single-stranded DNA-binding proteins (Escherichia coli SSB, T4 gene 32, or yeast RPA), binds to single-stranded DNA with low cooperativity (Kim et al., 1992; Kim and Wold, 1995). More experiments are needed to clarify the role of this domain (ΔJK) in the DNA binding activity of RPA. Interestingly, the RPA complex containing the zinc-finger p70 mutant (ΔTU) supported efficient ssDNA binding activity. This is consistent with results of similar experiments done with an E. coli-expressed p70 mutant (Gomes and Wold, 1995).

Deletions at either the N-terminal domain (Δ2–100 and ΔJK) or the DNA binding domain (ΔLM, ΔPQ, and ΔRS) of p70 abolished RPA’s function in pol stimulation, suggesting that both domains are necessary for this function. N-terminal deletion mutants (Δ2–100 and ΔJK) not only lost their ability to stimulate pol activity, but actually inhibited pol activity in an assay measuring elongation of a primed DNA template. These N-terminal mutants are, in a sense, analogous to E. coli SSB, T4 gene 32, and adenovirus DNA-binding protein since they all contain DNA binding domains, but lack the pol stimulation domain (presumably pol interaction domain). The absence of this domain results in the inhibition of pol activity. The zinc-finger p70 mutant also inhibited pol activity, leading us to suggest that the zinc-finger domain affects the conformation of p70 such that RPA cannot stimulate pol activity. In contrast to pol stimulation, and with the exception of ΔTU, the RPA mutants (Δ2–100 and ΔJK) that had ssDNA binding activity could stimulate pol δ activity in the presence of PCNA and RF-C, whereas mutants that lacked DNA binding activity could not. Hence, DNA binding activity is required to stimulate pol δ activity. RPA mutants Δ2–100 and ΔJK, however, were less effective than wild-type RPA in stimulating pol δ, and the ΔTU mutant actually inhibited pol δ activity. These results suggest that RPA’s ssDNA binding activity is required, but not sufficient, to stimulate pol δ. RPA p70 has at least two distinct domains, a N-terminal pol stimulation domain, and a broader DNA binding domain (Fig. 9). The DNA binding domain is essential for RPA’s function in replication, but the polymerase stimulation domain is not. Why then, did antibodies 70A and 70B inhibit both pol α activity and SV40 DNA replication (Kenny et al., 1990)? It may be that steric hindrance from the antibodies is more responsible for the inhibitory effect on RPA than that caused by the deletion mutant. Interestingly, both pol α activity and pol δ activity were inhibited by RPA: p70ΔTU. It is possible that the deletion of this domain caused a conformational change at the N terminus of p70, which then affected the polymerase stimulatory activity of the complex (Fig. 6) as well as monoclonal antibody recognition (Fig. 3). The monoclonal antibodies 70A and 70B recognize the native form but not the denatured form of p70 (data not shown). The zinc-finger mutant (RPA:p70ΔTU) supported efficient SV40 DNA replication in vitro, while RPA mutants (Δ2–100 and ΔTU) were much less inhibitory to the elongation of SV40 DNA than they were to the elongation of a primed template DNA (Fig. 8). This result indicates that stimulation of pol activity during elongation is not essential for DNA replication. It is possible that polymerases functioning at the replication fork may be different from those required for elongation of primed template DNA and could be much less affected by the presence of RPA.

However, we cannot rule out the possibility that stimulation of pol activity is required for chromosomal DNA replication or some other DNA metabolic process, such as DNA repair. It is interesting to point out that the zinc-finger mutant did not function in nucleotide excision repair in vitro,4 suggesting that the subunits of RPA have different functions in replication than they do in repair. A detailed analysis of these RPA mutants in nucleotide excision repair would provide further insight into the role of RPA in DNA metabolism.

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