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Coordinated Regulation of Replication Protein A Activities by Its Subunits p14 and p32*

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Klaus Weisshart‡§, Pavel Pestryakov¶, Richard W. P. Smith‡‖, Hella Hartmann‡, Elisabeth Kremmer, Olga Lavrik¶, and Heinz-Peter Nasheuer‡ ‡‡§§**

*From the ‡Institute of Molecular Biotechnology, Beutenbergstrasse 11, Jena 07745, Germany, the ¶Institute of Chemical Biology and Fundamental Medicine, Siberian Division of the Russian Academy of Science, Prospect Lavrentieva 8, Novosibirsk 630090, Russia, the **GSF, Institut für Immunologie, Marchioninistrasse 25, München 81377, Germany, and the ‡‡National University of Ireland, Galway, Ireland*

The heterotrimeric replication protein A (RPA) has multiple essential activities in eukaryotic DNA metabolism and in signaling pathways. Despite extensive analyses, the functions of the smallest RPA subunit p14 are still unknown. To solve this issue we produced and characterized a dimeric RPA complex lacking p14, RPAΔp14, consisting of p70 and p32. RPAΔp14 was able to bind single-stranded DNA, but its binding mode and affinity differed from those of the heterotrimeric complex. Moreover, in the RPAΔp14 complex p32 only minimally recognized the 3′-end of a primer in a primer-template junction. Partial proteolytic digests revealed that p14 and p32 together stabilize the C terminus of p70 against degradation. Although RPAΔp14 efficiently supported bidirectional unwinding of double-stranded DNA and interacted with both the simian virus 40 (SV40) large T antigen and cellular DNA polymerase α-primase, it did not support cell-free SV40 DNA replication. This inability manifested itself in a failure to support both the primer synthesis and primer elongation reactions. These data reveal that efficient binding and correct positioning of the RPA complex on single-stranded DNA requires all three subunits to support DNA replication.

The multifunctional replication protein A (RPA)[1] is required for eukaryotic DNA replication, repair, and recombination as well as for signaling pathways after DNA damage (1–6). The protein complex consists of three subunits, p70, p32, and p14, named according to their molecular masses of 70, 32, and 14 kDa, respectively. RPA interacts preferentially with single-stranded DNA but also recognizes double-stranded DNA (ssDNA and dsDNA, respectively) via the four DNA binding domains DBD-A, B, and C, located on p70, and DBD-D, on p32 (7, 8). In addition to these four well established DBDs, the RPA complex contains two related, structurally defined oligonucleotide oligosaccharide binding fold domains, which had been named DBD-E in p14 and DBD-F of the N terminus of p70 (9). RPA has additional functions as a communication interface because it interacts with most key players of the aforementioned processes as well as with various tumor suppressors, suggesting a direct function of RPA in cancer development (2, 5, 10–12).

Recent studies have shown that RPA plays a central role in DNA damage signaling in human and yeast (1, 2, 5, 6). Human RPA interacts with ATR via ATRIP (Ataxia Telangiectasia mutated and Rad3-related and ATR Interacting Protein, respectively) to signal the existence of stretches of ssDNA in the genome after DNA damage (1, 6). In addition, RPA interacts with the tumor suppressor protein p53, which is mutated in more than 50% of all tumors (13). In yeast the p70 subunit is involved in the S phase DNA damage checkpoint, and mutations in the gene encoding the 32-kDa subunit result in defective S phase progression (14). Moreover, p32 is specifically phosphorylated during the cell cycle and after DNA damage, suggesting that the RPA activities are regulated (2, 5). Recently, the negative charge of these phosphorylated residues was mimicked by the introduction of aspartates, which interfered with the DNA unwinding activity of the RPA complex and the loading of mutant RPA into the chromatin (9, 15). These findings support the notion that the cooperation of the RPA subunits is necessary for its full functions. During the initiation of DNA repair processes RPA binds to ssDNA of the partially unwound DNA in a polar fashion (16). The RPA subunit p32 is positioned by p70 on partial duplex DNA such that it contacts the 3′ terminus of the primer (17, 18). This polarity allows RPA together with XPA to direct the two endonucleases XPF and XPG for their precise DNA cleavage 3′ and 5′ of a lesion on the damaged strand (16). In addition to these repair processes, RPA also participates in DNA recombination reactions (19–21).

In the eukaryotic cell cycle, RPA is necessary for activation of the prereplication complex to form the initiation complex and for the ordered loading of essential initiator functions, *e.g.* the DNA polymerase α-primase (pol·prim) complex, to origins of replication (3, 22). To study the DNA functions of RPA at replication forks, the cell-free SV40 DNA replication system

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§ Present address: Carl Zeiss Jena GmbH, Carl-Zeiss-Promenade 10, Jena 07745, Germany.

‖ Present address: University of Glasgow, Institute of Virology, Church St., Glasgow G11 5JR, Scotland, United Kingdom.

§§ To whom correspondence should be addressed: Dept. of Biochemistry, National University of Ireland, Galway, the Cell Cycle Control Laboratory, University Rd., Galway, Ireland. Tel.: 00353-91-512-409; Fax: 00353-91-512-504; E-mail: h.nasheuer@nuigalway.ie.

[1] The abbreviations used are: RPA, replication protein A; BSA, bovine serum albumin; DBD, DNA binding domain; dsDNA, double-stranded DNA; DTT, dithiothreitol; MBP, maltose-binding protein; pol·prim, DNA polymerase α-primase; ssDNA, single-stranded DNA; SV40, simian virus 40; TAg, T antigen.

has been used extensively (2, 5). The replication of SV40 DNA requires the coordination and cooperation of multiple factors. To synthesize primers and to initiate DNA replication RPA, pol·prim, and SV40 T antigen (TAg) work in concert at the SV40 origin of DNA replication. Then proliferating cell nuclear antigen is loaded onto the DNA by replication factor C (3, 22). To exchange the initiator DNA polymerase, pol·prim, for the processive elongation enzyme, DNA polymerase δ, multiple protein contacts between these proteins and RPA must take place (3, 22). In this regard, the polarity of the RPA·DNA complex enables the p32 subunit to monitor the length of the RNA·DNA primers during lagging strand DNA synthesis in mammalian cells (23).

Genetic data have revealed that all three subunits are essential in budding yeast (24). Although detailed structural data of all RPA subunits have been published, the biochemical function of p14 is still unknown (8, 25). To investigate the role of p14 in eukaryotic DNA metabolism, we produced a protein complex of p70 with p32 (RPAΔp14, RPA lacking p14) and compared it with the heterotrimeric RPA. The biochemical analyses show that the RPAΔp14 complex retains its abilities to interact with DNA, TAg, and pol·prim. In addition, these recombinant proteins support the bidirectional unwinding step catalyzed by TAg. However, the RPAΔp14 complex bound less efficiently to short ssDNA stretches, which have lengths of 32 nucleotides or fewer. Moreover, in RPAΔp14 p32 no longer interacts efficiently with dsDNA to ssDNA transition structures, which arise during the unwinding of dsDNA during DNA replication and repair. Therefore, p14 most likely coordinates the association of the two larger RPA subunits with these DNA structures. This interaction seems to be essential for the function of RPA because investigation in the cell-free SV40 DNA replication system revealed that a complex containing all three RPA subunits is necessary to support both origin-dependent primer synthesis and the elongation stage of DNA synthesis. These findings show that p14 is required for RPA binding of DNA structures, which occurs during DNA replication and DNA repair processes.

MATERIALS AND METHODS

Expression of p70 and p70-containing Protein Complexes—Soluble p70 was expressed as a C-terminal fusion to the maltose binding protein (MBP) yielding MBP-p70 as described earlier (17). Likewise, p14 and p32 were expressed as MBP fusion proteins to yield MBP-p14 and MBP-p32, respectively. MBP-p70 was also expressed by a polycystronic RNA along with both or either of the two smaller subunits yielding the complexes MBP·RPA (consisting of MBP-p70, p32, and p14), RPAΔp14 (consisting of MBP-p70 and p32, but lacking p14), and RPAΔp32 (consisting of MBP-p70 and p14, but lacking p32) (26). Human RPA was expressed additionally in bacteria as a heterotrimeric complex with (bH_6-RPA) or without (bRPA) an oligohistidine fused to p70 and purified as described in the presence of 10 μM $ZnCl_2$ (26–28).

Purification of Proteins—SV40 TAg and the human pol·prim complex were expressed in High Five cells (Invitrogen) infected with recombinant baculoviruses and purified as described previously to (29–31). Topoisomerase I was purified from calf thymus according to Strausfeld and Richter (32). MBP- and His-tagged fusion proteins were purified on amylose (New England Biolabs) or TALON (Clontech) resin, respectively, according to the manufacturer's instructions.

If proteins were used for protein-protein interaction studies, SV40 TAg and pol·prim were treated coupled to the immunoaffinity matrix with 0.05 unit/μl benzonase (Merck) at room temperature for 30 min in a buffer containing 30 mM Hepes-KOH, pH 7.8, 5 mM $MgCl_2$, 1 mM DTT prior to elution (26). Before loading a Mono Q on a Smart system (Amersham Biosciences) column (30), RPA was dialyzed against the same buffer and treated with benzonase. After elution from the Mono Q column, peak fractions were pooled ands dialyzed against storage buffer (20 mM Hepes-KOH, pH 7.8, 1 mM DTT, 0.1 mM EDTA, 50 mM NaCl, 10% glycerol).

Immunoprecipitation—10 μg of specific monoclonal antibodies (PAb101 for TAg and SJK237-71 for pol·prim) were coupled to 20 μl of 50% (v/v) protein G-agarose beads in 100 μl of binding buffer (50 mM Hepes-KOH, pH 7.9, 100 mM KCl, 7 mM $MgCl_2$, 0.25% inositol, 0.25 mM EDTA, 0.05% Nonidet P-40) for 1 h at 4 °C. The beads were then washed four times with 1 ml of wash buffer (30 mM Hepes-KOH, pH 7.9, 100 mM KCl, 7 mM $MgCl_2$). 2 μg of a protein, for which the antibodies were specific, was than loaded on the beads in a 100-μl reaction in binding buffer. After 1 h at 4 °C, the beads were again washed four times with 1 ml of wash buffer. The beads were than incubated with 2 μg of the protein of interest in 100 μl of reaction buffer containing 2% BSA for 2 h at 4 °C. After washing four times as before, the beads were boiled in 20 μl of sample buffer. Eluted proteins were run on a 10% SDS-gel and detected after electrotransfer (33) with the ECL system (Amersham Biosciences).

Band Shift Assays—The oligo$(dT)_{32}$ was radioactively labeled according to Sambrook *et al.* (34). Band shift assays contained in a 10-μl mixture 20 mM Hepes-KOH, pH 7.8, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 1 pmol of oligo$(dT)_{32}$, and the indicated amounts of RPA or RPA subcomplex. After 30 min at room temperature, the sample was supplemented with 2 μl of loading buffer (10 mM Hepes-KOH, pH 7.5, 1 mM EDTA, 25% Ficoll 400, 0.1% bromphenol blue) and separated in a native 6% polyacrylamide gel in 0.5× TBE buffer (89 mM Tris borate, 89 mM boric acid). The gel was dried, and DNA was visualized by autoradiography using a PhosphorImager (Amersham Biosciences).

Primer Labeling and Elongation in the Presence of Photoreactive dNTP Analogs—The PT32 primers (5′-$(T)_{32}$AGTGTTATAGCCCCTAC-C-3′and 3′-ACAATATCGGGGATGG-5′) were annealed to the template at a molar ratio of 1:1 in 10 mM Tris-HCl, pH 7.5, 10 mM KCl, by heating the mixture at 95 °C for 5 min and then slowly cooling it down to room temperature (43). Primers were 3′-end labeled at 37 °C in a 300-μl reaction mixture containing 50 mM Hepes-KOH, pH 7.8, 50 mM KCl, 10 mM $MgCl_2$, 0.32 μM DNA polymerase β, 0.54 μM partial duplex DNA PT32, 1 μCi of [α-^{32}P]dCTP, and 2.5 μM unlabeled dCTP. After 5 min of incubation, photoreactive NAB-4-dUTP or 4-S-TTP was added at a final concentration of 5 μM. Then the mixture was incubated further for 15 min at 37 °C. Reactions were stopped by heating for 5 min at 95 °C and cooling rapidly on ice for 5 min. After this treatment, denatured DNA polymerase β was precipitated and removed by centrifugation for 15 min at 15,000 × *g*. Resulting mixtures were heated at 95 °C for 5 min and cooled down slowly to room temperature to allow the radioactively labeled photoreactive primer to reanneal to the template strand.

Photochemical Cross-linking of Protein-DNA Complexes—RPA was labeled with a photoreactive primer in a 20-μl reaction mixture containing 50 mM Hepes-KOH, pH 7.8, 50 mM KCl, 10 mM $MgCl_2$, 0.5 μM radioactively labeled photoreactive partial duplex DNA, and 0.5 μM RPA or RPA subcomplexes (43). Reaction mixtures were incubated for 5 min at room temperature, placed in a micro-Eppendorf tube on ice, and irradiated through the open top for 10 min using the Stratalinker UV cross-linker (Stratagene) equipped with lamps producing UV light of 312 nm. The reaction mixtures were quenched with SDS loading buffer and boiled for 15 min. Photochemically cross-linked proteins were separated by 13.5% SDS-PAGE. Gels were dried and exposed to x-ray films (Kodak Biomax, Sigma).

SV40 in Vitro Replication—*In vitro* replication reactions were done essentially as described previously (35) with slight modifications. Crude human extracts were depleted of cellular RPA by passing the extract over two consecutive 70B antibody columns (36, 59). The 60-μl reaction was performed in the presence of 30 mM Hepes-KOH, pH 7.8, 7 mM magnesium acetate, 1 mM EGTA, 0.5 mM DTT, 4 mM ATP, 0.2 mM each CTP, GTP, and UTP, 0.1 mM each dGTP and dTTP, 0.05 mM each dCTP and dTTP, 40 mM creatine phosphate, 80 μg of creatine kinase, 5 μCi each [α-^{32}P]dCTP and [α-^{32}P]TTP, 6.5 pmol of SV40 TAg, 200 ng of pUC-HS DNA, containing the complete SV40 origin sequences (36), and 190 μg of S100 extract (depleted or undepleted) prepared from human 239S cells. To reconstitute RPA-depleted cell extracts, up to 5 pmol of RPA or RPA subcomplexes was added as indicated.

After a 90-min incubation at 37 °C, 5 μl of the reaction was spotted on DE81 paper to measure the incorporated nucleotides (34). To the remaining reaction, EDTA, SDS, and proteinase K were added to final concentrations of 20 mM, 0.65%, and 1.7 mg/ml, respectively, and incubation continued for another 30 min. The sample was extracted once with phenol/chloroform, and DNA was passed over a G-50 spin column (Roche Applied Science) into TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA) to remove unincorporated nucleotides. Then DNA was ethanol precipitated and dissolved in 20 μl of TE buffer. 5 μl of dissolved DNA was digested with EcoRI and EcoRI/DpnI, respectively, and products were separated in a 0.8% agarose gel in TAE buffer in TAE buffer (40 mM Tris acetate, 1 mM EDTA). The gel was dried and exposed to x-ray films.

Origin DNA Unwinding Assays—Unwinding reactions (total volume of 10 μl) were carried out with 200 ng of supercoiled closed circular pUC-HS DNA, containing the complete SV40 origin sequences (36), 30 mM Hepes-KOH, pH 7.9, 1 mM DTT, 7 mM magnesium acetate, 4 mM ATP, 40 mM creatine phosphate, 0.1 mg/ml creatine kinase, 0.25 mg/ml BSA, 1.5 pmol of topoisomerase I, and 5 pmol of RPA or RPA subcomplex as indicated. The reaction was started by adding 1 pmol of TAg. After 1 h at 37 °C, the reaction was stopped by adding proteinase K, EDTA, and SDS to final concentrations of 0.3 mg/ml, 6 mM, and 0.3%, respectively. After deproteination at 37 °C for 30 min, the DNA was ethanol precipitated, redissolved in loading buffer (10 mM EDTA, 2% Ficoll, 2% sucrose, 1 μg/ml ethidium bromide, 0.01% bromphenol blue, 0.1% SDS), and separated in 1.5% agarose gels. The gels were then photographed. Unwound DNA form U was determined by densitometric scanning of the ethidium bromide-stained DNA. Quantification was performed by comparing the intensities of the reaction products with those of DNAs of a known concentration range.

Primer Synthesis Assays—Standard primer synthesis reactions (initiation reactions) were done essentially as described (36, 37). However, to avoid differences in the efficiency of the unwinding reaction unwound DNA instead of supercoiled DNA was used as a substrate for the initiation. To uncouple the unwinding from the primer synthesis reaction a two-step procedure was applied. In the first step (unwinding reaction) a 40-μl assay was set up with bRPA, keeping the buffer conditions as before, but omitting CTP, GTP, UTP, and dNTPs. Moreover, all proteins and DNA were used at 5× higher concentrations in this first step of the assay. After 30 min at 37 °C, the reaction was stopped by adjusting the reaction to 10 mM EDTA. The sample was extracted once with phenol/chloroform, and DNA was passed over a G-50 spin column into TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA) to remove unincorporated nucleotides.

The primer synthesis reaction contained in a 40-μl reaction 30 mM Hepes-KOH, pH 7.8, 7 mM magnesium acetate, 1 mM EGTA, 0.5 mM DTT, 4 mM ATP, 0.2 mM each GTP and UTP, 2 μM CTP, 40 mM creatine phosphate, 40 μg/μl creatine kinase, 10 μCi of [α-^{32}P]CTP, 5 pmol of RPA or RPA subcomplex, 6.5 pmol of SV40 TAg, 10 units of topoisomerase I, 1 pmol of pol·prim, and about 200 ng of unwound pUC-HS. After a 60-min incubation at 37 °C 5 μl was spotted on DE81 paper to quantify incorporated nucleotides (34). Reaction products were precipitated in the presence of 0.8 M LiCl, 10 mM $MgCl_2$, and of 10 μg of carrier tRNA. The precipitate was dissolved in 35% formamide, 8 mM EDTA, 0.1% bromphenol blue, and 0.1% xylene cyanol FF for 30 min at 65 °C, heated for 3 min at 95 °C, and separated in 20% denaturing polyacrylamide gels at 600 V until the bromphenol marker had nearly migrated to the bottom of the gel. The wet gels were exposed to an x-ray film.

Primer Elongation Assays—The elongation system (monopolymerase system) was set up essentially as described previously (37) with slight changes. Primed SV40 DNA was prepared as described above but performing the initiation reaction in the presence of bRPA and without uncoupling the unwinding reaction. In addition, the CTP concentration was increased to 0.2 mM, radioactive nucleotides were absent, and 5× higher amounts of all proteins and DNA were used. After 60 min the reaction was stopped by adjusting it to 10 mM EDTA, and the sample was extracted once with phenol/chloroform. Then DNA was passed over a G-50 spin column into TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA) to remove unincorporated nucleotides.

For the elongation step, the standard reaction contained in a final volume of 40 μl 30 mM Hepes-KOH, pH 7.8, 7 mM magnesium acetate, 1 mM EGTA, 0.5 mM DTT, 4 mM ATP, 0.2 mM each CTP, GTP, and UTP, 0.1 mM each dATP, dGTP, and dTTP, 2 μM dCTP, 40 mM creatine phosphate, 40 μg/μl creatine kinase, 10 μCi of [α-^{32}P]dCTP, 5 pmol of RPA or RPA subcomplex, 6.5 pmol of SV40 TAg, 10 units of topoisomerase I, 1 pmol of pol·prim, and about 200 ng of pUC-HS DNA. After a 60-min incubation at 37 °C 5 μl was spotted on DE81 paper for quantification of incorporated nucleotides (34). EDTA, SDS, and proteinase K were added to final concentrations of 20 mM, 0.65%, and 1.7 mg/ml, respectively, and incubation continued for another 30 min. The sample was extracted once with phenol/chloroform, and DNA was passed over a G-50 spin column into TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA) to remove unincorporated nucleotides. DNA was ethanol precipitated in the presence of 10 μg of carrier tRNA. Then the nucleic acids were dissolved in 20 μl of alkaline loading buffer (50 mM NaOH, 1 mM EDTA, 5% Ficoll 400, 0.025% bromcresol green) and separated in alkaline 1.5% agarose gels in 50 mM NaOH, 1 mM EDTA for 10 h at 150 mA with circulating buffer in the cold. The gel was fixed in 10% trichloroacetic acid, dried, and exposed to x-ray films.

Limited Proteolysis of RPA and Identification of Proteolytic Fragments—2 μg of the indicated protein complexes were treated at room temperature with the indicated amount of trypsin in a 10-μl reaction mixture containing 30 mM Hepes-KOH, pH 7.8, 1 mM EDTA, 70 mM $MgCl_2$, and 10 mM DTT. After a 30-min incubation reactions were terminated by the addition of SDS loading buffer and heating to 95 °C for 5 min. The proteolyzed products were separated by 16% SDS-PAGE and analyzed by Coomassie Brilliant Blue G-250 staining and immunoblotting.

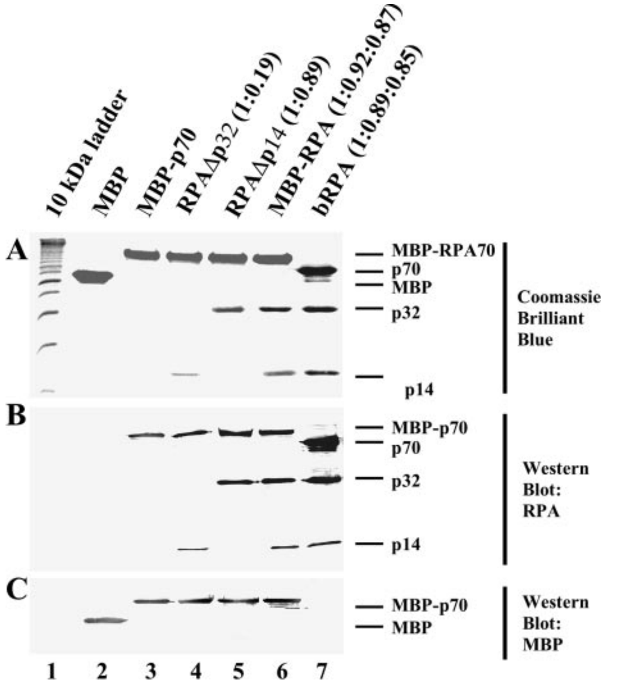

FIG. 1. **Expression of various RPA subunit configurations.** *A*, Coomassie Brilliant Blue-stained gel of the indicated proteins (MBP, 42.5 kDa, *lane 2*; MBP-p70, *lane 3*), dimeric (RPAΔp32, *lane 4*; RPAΔp14, *lane 5*), and heterotrimeric protein complexes (MBP·RPA, *lane 6*; bRPA, *lane 7*), 4 μg each. The ratio of RPA subunits (in the order 70:32:14) as determined by densitometric scanning is indicated for the dimeric and heterotrimeric complexes. *B* and *C* show Western blots of 1-μg proteins that have been probed with polyclonal RPA and MBP antisera, respectively. The 50-kDa protein of the 10-kDa ladder (*lane 1*) has a higher intensity than the other marker proteins; the positions of the MBP fusion proteins and the single subunits are indicated on the *right*. MBP has a molecular mass of 42.5 kDa.

RESULTS

Expression of Various RPA Subunit Combinations—To investigate the importance of each RPA subunit in eukaryotic replication, we expressed different RPA subcomplexes and tested their activities during the primer synthesis and primer elongation steps. Although earlier reports showed that the production of the soluble p70 is dependent on p14 and p32, several groups have described the expression of soluble MBP fusion proteins of p70 (17, 38–40). These studies provoked us to study the ability of p70 to form subcomplexes with the smaller two subunits.

To this end, p70 was expressed as an MBP fusion protein in bacteria and affinity purified in the absence of any other RPA subunit (MBP-p70; Fig. 1*A*, *lane 3*). In addition, MBP-p70 was coexpressed with p14 but without p32 (RPAΔp32, *lane 4*), with p32 but lacking p14 (RPAΔp14, *lane 5*), and with both small subunits (MBP·RPA, *lane 6*). Compared with the MBP (*lane 1*), the MBP-p70 fusion protein showed the expected increase in the molecular mass, suggesting that p70 is expressed as a full-length protein. As a control, the heterotrimeric complex was also expressed in bacteria from a polycistronic mRNA as described earlier (bRPA, *lane 7*) (27). The Coomassie Brilliant Blue-stained gel and the immunoblots unambiguously identified the presence of each subunit (Fig. 1*B*) and MBP (Fig. 1*C*)

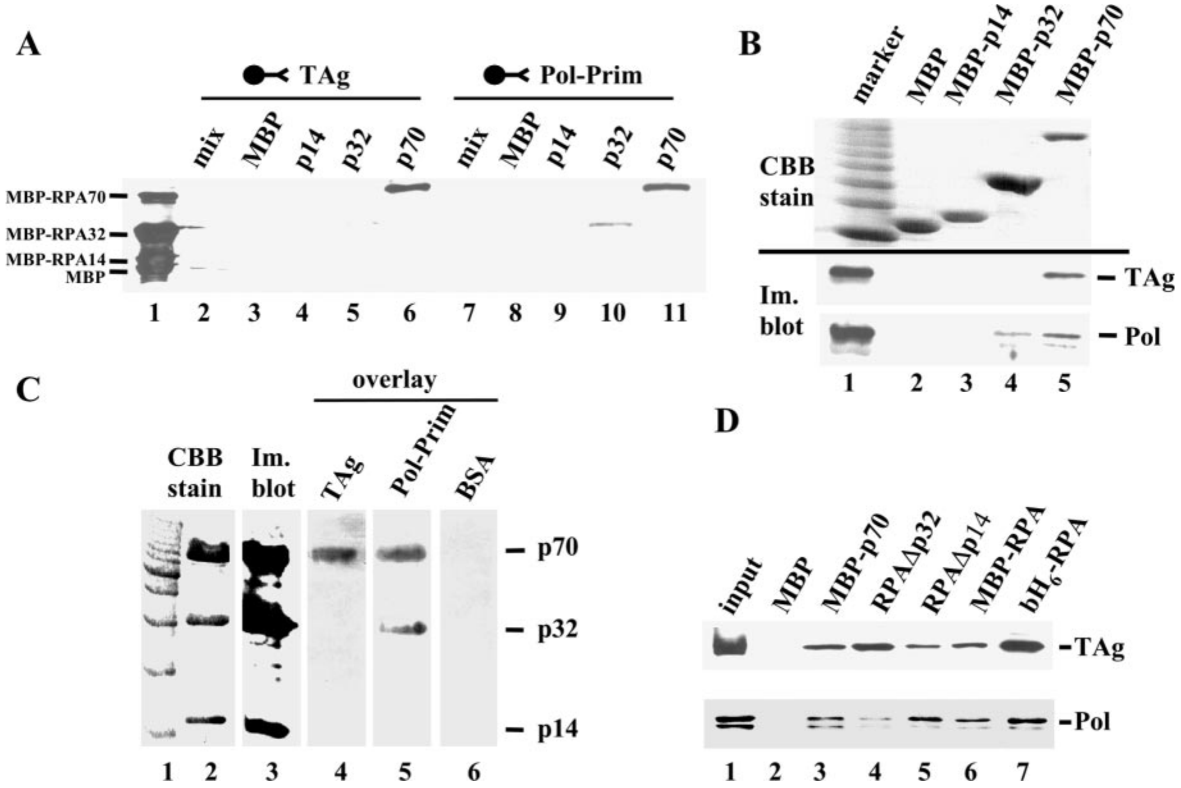

FIG. 2. **Interaction of RPA and subcomplexes with TAg and pol·prim.** In *A*, associations of MBP fusion proteins with TAg or pol·prim were tested in affinity pull-down assays. TAg (*lanes 2–6*) and pol·prim (*lanes 7–11*) were coupled via monoclonal antibodies PAb101 and SJK 237-71, respectively, to protein G-Sepharose resin, which was loaded into columns. Subsequently, MBP (42.5 kDa, *lanes 3* and *8*), MBP-p14 (*lanes 4* and *9*), MBP-p32 (*lanes 5* and *10*), and MBP-p70 (*lanes 6* and *11*) were loaded onto the antibody columns. A mixture of all MBP fusion proteins was also incubated with antibody resins to which no protein had been bound (*mix*, *lanes 2* and *7*). Bound proteins were analyzed by Western blotting using an anti-MBP serum. *Lane 1* represents 1/10 of input material of all MBP fusion proteins. Their positions are indicated at the *left*. *B*, affinity pull-down assays using beads that were loaded with MBP (*lane 2*), MBP-p14 (*lane 3*), MBP-p32 (*lane 4*), and MBP-p70 (*lane 5*). The Coomassie Brilliant Blue-stained proteins (*CBB stain*) in the *upper image* show the quantities of each MBP fusion protein coupled to ~10 μl of resins. The 10-kDa ladder, of which the 50-kDa protein has a higher intensity than the other marker proteins, is presented in *lane 1*. The *lower image* shows a Western blot analysis (*Im. blot*) of eluted fractions from the columns, over which TAg (*upper part*) or pol·prim (*lower part*) has been passed (*lanes 2–5*). *Lane 1* represents 1/10 of the input material of TAg or pol·prim as shown at the *right*. The positions of TAg and the p180 subunit of pol·prim (*Pol*) are indicated at the *right*. *C*, protein-stained SDS gel (*lanes 1* and *2*) and various protein blots developed in parallel (*lanes 4–6*). The purity of RPA was determined by Coomassie Brilliant Blue-stained SDS gel (*lane 2*) along with the 10-kDa ladder (*lane 1*). One blot was stained with a mixture of monoclonal antibodies recognizing subunits of RPA (*lane 3*). The other blots show protein overlay assays (far Western blots), in which 20 μg of RPA was separated by SDS-PAGE, renatured in the gel, and transferred to nitrocellulose filters. These immobilized RPA subunits then served as the solid phase (*lanes 4–6*). The filters were incubated with TAg (*lane 4*), pol·prim (*lane 5*), or BSA (*lane 6*). Bound proteins were detected by polyclonal antisera against TAg (*lane 4*) or pol·prim (*lane 5*). To exclude cross-reactivity of the antibodies with RPA subunits, the BSA-incubated filter was treated with a mixture of both antisera (*lane 6*). The positions of p70, p32, and p14 subunits are indicated on the *right*. *D*, 24 pmol of TAg and pol·prim, respectively, were passed over affinity resins, to which the indicated proteins had been coupled. After elution of the column, the presence of TAg and pol·prim was analyzed by immunoblotting. *Lane 1* corresponds to 1/20 of the input material.

in the affinity-purified complexes, proving their integrity. MBP-p70 was equally well expressed in the absence or presence of different combinations of p14 and p32 (Fig. 1*A*, *lanes 3–7*). Densitometric scanning of the Coomassie Brilliant Blue-stained gel revealed that all subunits were present in nearly equimolar amounts (*lanes 5–7*) with the exception of RPAΔp32 (*lane 4*). In this case the p14 polypeptide was reproducibly about 5-fold underrepresented compared with p70. The results show that both subunits can bind *per se* to the soluble p70 polypeptide, but p14 does so less efficiently than p32. The lack of an exact 1:1:1 ratio of p70:p32:p14 in RPA might be caused by staining variations of the different subunits. This variation was not the result of a variation of the expression levels because all three subunits were expressed to nearly the same levels (data not shown).

Interaction of the Various RPA Complexes with TAg and Pol·Prim—First we investigated the abilities of the single subunits to bind to both TAg and pol·prim. It is not yet clear which subunits of RPA interact with TAg and pol·prim, and different assays have shown different outcomes (10, 39–41). To reconcile the different results we employed various biochemical test systems including pull-down assays (Fig. 2, *A* and *B*) and far Western blots (Fig. 2*C*). TAg and pol·prim were coupled via antibodies to a matrix, and soluble MBP fusion proteins were passed over a column of the respective resins (Fig. 2*A*). The MBP-p70 was retained by both the TAg- and pol·prim-specific columns (*lanes 6* and *11*), whereas MBP-p32 was only bound by the latter (compare *lanes 5* and *10*). MBP and MBP-p14 did not associate with either TAg or pol·prim (*lanes 3* and *4*, and *8* and *9*). Binding was not the result of a nonspecific affinity of MBP-p70 or MBP-p32 to the resins because the MBP fusion proteins did not interact with the antibody-coupled resins (*lanes 2* and *7*). In an opposite setup, the MBP fusion proteins were coupled to amylose resin, and TAg or pol·prim was passed over the respective columns (Fig. 2*B*). Although the MBP and MBP-p14 columns were not able to retain either TAg or pol·prim (*lanes 2* and *3*), both proteins bound to the MBP-p70 column (*lane 5*). On the other hand, only pol·prim, not TAg, was associated with MBP-p32 (*lane 4*). We also tested the ability of TAg and pol·prim to bind to the different subunits in far Western blots, in which the separated RPA subunits served as the immobilized phase (Fig. 2*C*, *lane 3*). Whereas TAg reacted exclusively

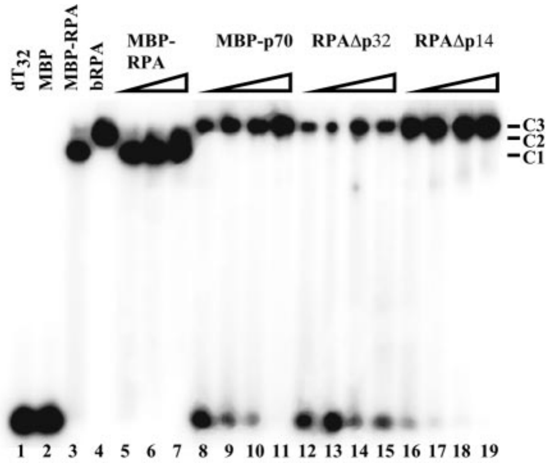

FIG. 3. **Binding of RPA and RPA subcomplexes to oligo(dT)$_{32}$.** Increasing amounts (0.62, 1.25, 2.5, and 5 pmol) of heterotrimeric MBP·RPA (*lanes 3* and *5–7*, respectively), MBP-p70, RPAΔp32, and RPAΔp14 (*lanes 8–11*, *12–15*, and *16–19*, respectively) were incubated with 1 pmol of radioactively labeled oligo(dT)$_{32}$. The protein-DNA complexes were separated by nondenaturing PAGE (10% acrylamide). Bound and free DNA were determined by autoradiography using a Storm PhosphorImager. For comparison, the gel shift of oligo(dT)$_{32}$ was also analyzed by nondenaturing PAGE and autoradiography in the presence of only assay buffer (*lane 1*), MBP, and bRPA (5 pmol of protein, *lanes 2* and *4*, respectively).

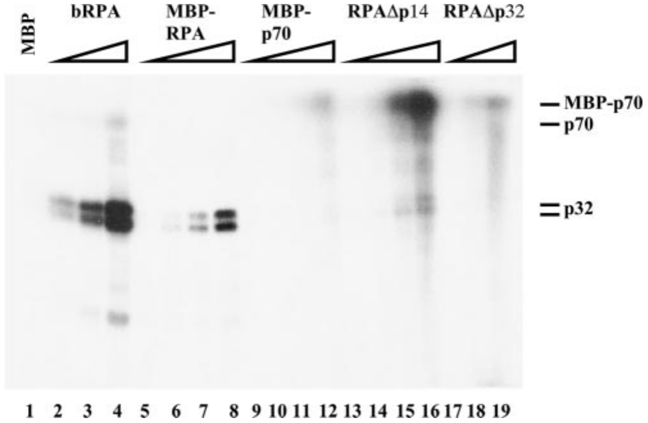

FIG. 4. **RPA cross-linked to a primer 3′-end of a partial DNA-duplex.** RPA was UV-induced cross-linked to the photoreactive radioactive primer-template probe (42). Photochemically cross-linked protein-DNA samples were separated by 13.5% SDS-PAGE. The dried gel was subjected to autoradiography. Designations of the cross-linked polypeptides are shown on the *right*. *Lane 1* represents the photoreactive radioactive primer-template irradiated with UV in the presence of 2 μg of MBP as a negative control. Increasing amounts (0.62, 1.25, 2.5, and 5 pmol) of indicated, recombinant purified proteins were incubated with the primer-template construct and then irradiated with UV. RPA subunits cross-linked to the 3′-end of the primer using a partial DNA duplex with a 5′-protruding tail of 32 nucleotides are indicated.

with the p70 subunit (*lane 4*), pol·prim bound to both the p70 and the p32 subunits (*lane 5*). With BSA as the first overlay, there was no staining observed for any of the subunits, demonstrating that the antibodies used to detect bound TAg and pol·prim did not cross-react with any of the RPA subunits (*lane 6*).

Next we assayed the ability of different subcomplexes to bind to TAg and pol·prim. To this end, the different RPA complexes were immobilized on amylose resins, and the interaction partners were passed over the respective columns (Fig. 2*D*). Any one of the various RPA assemblies containing MBP-p70 was able to retain TAg as well as pol·prim (*lanes 3–6*) albeit to a somewhat lesser extent than bH$_6$-RPA (bacterially expressed human RPA fused to oligohistidine), which was coupled via oligohistidine-specific antibodies to protein G-Sepharose (*lane 7*). The MBP column did not show any binding (*lane 2*). It should be stated that all of the complexes tested contain the p70 polypeptide, and it has been established earlier that p70 possesses binding sites for both TAg and pol·prim. It should also be noted that the fractions of proteins bound to the RPA subcomplexes and p70 were in the same order of magnitude, which we have measured for the interaction of pol·prim and TAg.

Interaction of Various RPA Complexes with DNA—RPA associates with ssDNA mainly via its p70 subunit (2). As expected, all p70-containing complexes and p70 itself interacted with circular ssM13mp18 DNA (data not shown). To evaluate further the binding mode of each of the complexes, linear 5′-labeled oligo(dT)$_{32}$ was used (Fig. 3). The p70-containing protein complexes and p70 itself formed stable complexes with oligo(dT)$_{32}$. Three protein-DNA complexes C1, C2, and C3, which had different mobilities, could be separated by nondenaturing gel electrophoresis (Fig. 3). At low concentrations (0.62 and 1.25 pmol), MBP·RPA forms the complex C1 with oligo(dT)$_{32}$, which has the highest mobility of the three (Fig. 3, *lanes 3* and *5*, respectively). Upon increasing the protein concentrations of MBP·RPA a second protein-DNA complex C2 was detected. Both protein-DNA complexes C1 and C2 were also found with bRPA (Fig. 3, *lane 4*; data not shown). However, the disappearance of the C1 complex and the appearance of the C2 complex require one concentration step in the presence of bRPA, whereas MBP·RPA reproducibly showed the presence of both complexes over a wider concentration range (data not shown). By comparing the amount of shifted protein-DNA complexes, MBP·RPA seems to bind to oligo(dT)$_{32}$ with slightly lower affinity than bRPA (Fig. 3, compare *lanes 7* and *4*; data not shown). In contrast to heterotrimeric MBP·RPA, the polypeptide MBP-p70 and the two heterodimeric RPA subcomplexes, RPAΔp14 and RPAΔp32, showed a reduced affinity to ssDNA and formed a protein-DNA complex C3 with a lower mobility compared with the C1 and C2 complexes. Increasing or decreasing their protein concentration did not yield a mobility shift comparable with C1 and C2. Reproducibly, RPAΔp14 formed a protein-DNA complex that had higher affinity to oligo(dT)$_{32}$ than MBP-p70 alone. The DNA binding activity of p32 might have caused this increase in DNA binding activity. In contrast, RPAΔp32 had the same or a slightly reduced DNA binding activity compared with MBP-p70 (Fig. 3, compare *lanes 8–11* with *12–15*). These results support the notion that p32 but not p14 has DNA binding activities. The slower mobility of the protein-DNA complexes containing MBP-p70, RPAΔp14, or RPAΔp32 than those of bRPA and MBP·RPA can be explained by the binding of multimers of the earlier to DNA or the altered shapes of the protein-DNA complexes.

Recent findings suggested that RPA interacts tightly with DNA consisting of duplex DNA and protruding 3′- or 5′-ssDNA (16, 42, 43). Therefore, we investigated the binding to the 3′-end of a primer hybridized to a template primer with an oligo(dT)$_{32}$ extension. Wild type RPA and MBP·RPA bound efficiently to the DNA, and as described earlier p32 was exclusively cross-linked to a photoreactive 3′-end of the primer (Fig. 4, *lanes 2–4* and *6–8*, respectively). Qualitatively there was no difference between the two RPA complexes, but the MBP·RPA bound to the 3′-end about 3-fold less efficiently than bRPA (Fig. 4, compare *lanes 7* and *8* with *lanes 2* and *3*). It is important to note that the cross-linked products of p32 run as a doublet in these experiments (Fig. 4, *lanes 2–4* and *6–8*). Depending on the residue of p32, which is cross-linked to the DNA, the mobility of the covalent p32·DNA complex changes, which was determined previously (44).

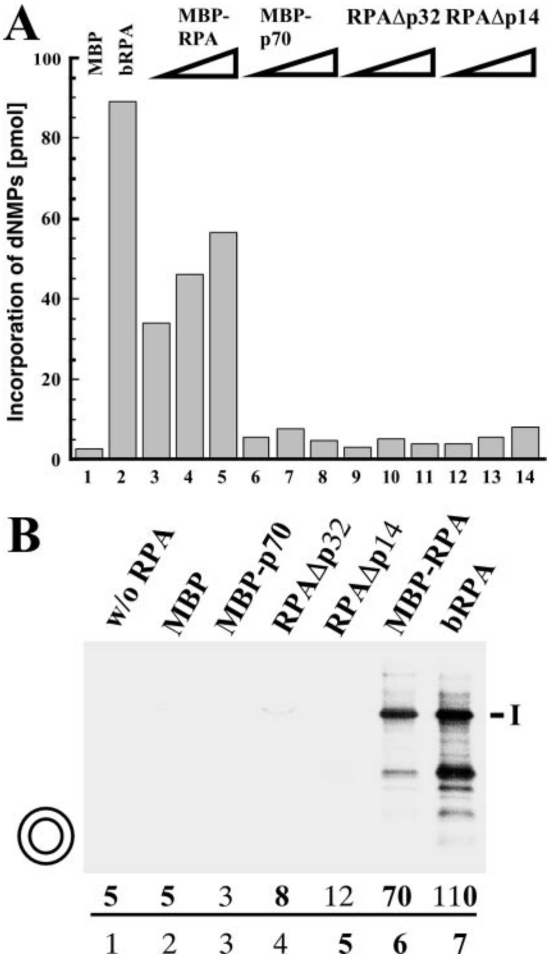

FIG. 5. **Cell-free SV40 DNA replication by the different RPA complexes.** The RPA complexes were tested in a cell-free SV40 DNA replication system using RPA-depleted human extracts (*A* and *B*). In *A* and *B*, the radioactive incorporated DNA replication products, which were synthesized in RPA-depleted crude human extracts supplemented with the indicated proteins (1.25, 2.5, and 5 pmol in *A*; 5 pmol in *B*) were analyzed by acid precipitation and scintillation counting (*A*). After EcoRI plus DpnI digestion, the reaction products were separated by agarose gel electrophoresis and detected by autoradiography (*B*). The position of linear dsDNA (*I*) is indicated on the *right*. The template configuration is shown on the *left* and represents supercoiled plasmid DNA as a substrate (*B*). *Lane 1* shows an assay mix that contained crude extracts without RPA.

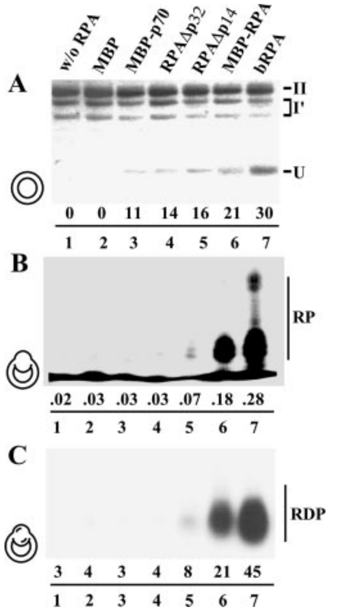

FIG. 6. **Activities associated with recombinant protein complexes.** The RPA complexes were tested in an unwinding assay of origin-containing dsDNA (*A*) using an SV40 origin-containing plasmid DNA and purified proteins. In the unwinding assay, *A*, an origin-containing supercoiled plasmid, sketched as a *double circle* as a substrate on the *left*, was incubated with TAg and topoisomerase I in the absence of MBP, RPA, or RPA subcomplex (*lane 1*, *w/o RPA*), and in the presence of MBP, the indicated MBP fusion protein complexes, or bRPA (*lanes 2–7*). After incubation, DNAs were loaded on an agarose gel with an ethidium bromide-containing loading buffer (1 μg/ml ethidium bromide) and analyzed by gel electrophoresis. The positions of the underwound DNA (*U*), the topoisomers of the plasmid (*I′*), and the relaxed DNA (*II*) are indicated on the *right*. The concentration of each DNA was determined by densitometric scanning of gel with and comparing the intensities of each band with that of a dilution series of plasmid DNA. The yields of unwound DNA are indicated in pmol directly below each *lane*. The activities of the recombinant proteins were characterized further in the SV40 initiation reaction (*B*, synthesis of oligoribonucleotides, *RP*, in the origin of SV40 DNA replication) or in the SV40 monopolymerase elongation reaction (*C*, elongation reaction of the purified unlabeled initiation RNA, which yields RNA·DNA products, *RDP*) using purified proteins. The RNA primers (*RP*) and RNA·DNA primers (*RDP*) are marked on the *right* in *B* and *C*, respectively. The template configuration is shown on the *left* and represents partially unwound DNA (*B*) and partially unwound DNA associated with initiation RNA (*C*) as assay substrates. The initiation activity of the proteins was determined by incorporation of radioactive labeled NTPs, binding of radioactive oligoribonucleotides to DE81, and scintillation counting. In parallel, they were analyzed by denaturing PAGE and autoradiography. The *numbers* directly below the radioactive DNA bands refer to pmol of incorporated NTPs (*B*). *Lane 1* shows the reaction in the absence of RPA. In *C*, the educts of the reaction are products of a preparative initiation reaction as performed in *B* but in the absence of any radioactive NTPs. The incorporation of radioactive dNTPs was then measured by acid precipitation and scintillation counting and, in parallel, by denaturing PAGE and autoradiography (*C*). The *numbers* directly below the radioactive DNA bands refer to pmol of incorporated dNTPs (*C*). *Lane 1* shows the reaction in the absence of RPA.

Omitting p14 resulted in a qualitative change of the cross-linking products. The RPA subunit p32 was no longer able to bind efficiently the 3′-end of the primer and was hardly cross-linked (Fig. 4, *lanes 13–16*). Nevertheless the presence of p32 stimulated the binding and cross-linking efficiency of p70 (Fig. 4, compare *lanes 13–16* with *lanes 9–12*), whereas p14 alone did not influence p70 in these reactions (Fig. 4, *lanes 17–19*). These findings reveal that p14 has a role in the interaction of RPA with these DNA structures, which are intermediates in all DNA metabolic processes.

Activities Associated with RPA Subcomplexes—RPA plays a central role in eukaryotic DNA replication. To analyze the subunit requirements in the DNA replication process, we assayed the activities of the heterotrimeric complexes, of the various dimeric subcomplexes, and of p70 in the *in vitro* SV40 DNA replication system using RPA-depleted crude human cell extracts (Fig. 5, *A* and *B*). None of the individual subcomplexes was able to support cell-free SV40 DNA replication (Fig. 5*A*, *lanes 6–14*, and 5*B*, *lanes 3–5*). In contrast, SV40 DNA replication was supported by bRPA and to a slightly lesser extent by MBP-p70 in complex with the other two subunits (Fig. 5*A*, *lanes 2–4*; 5*B*, *lanes 7* and 6, respectively). The DNA synthesis products were at least in part resistant to DpnI digestion, revealing that DNA replication occurred in the presence of RPA and MBP·RPA (*lanes 6* and *7 highlighted* linear products). However, the DNA replication was in part incomplete yielding some products that could be cleaved by DpnI. These partially DpnI-sensitive DNA synthesis products are common in viral cell-free DNA replication systems (35, 45).

These results raised the question of which step(s) are not supported by MBP-p70 and the dimeric complexes. To compare their activities each subreaction of the SV40 replication system was tested in the presence of purified proteins. The recombinant proteins were analyzed in (i) the unwinding assays using

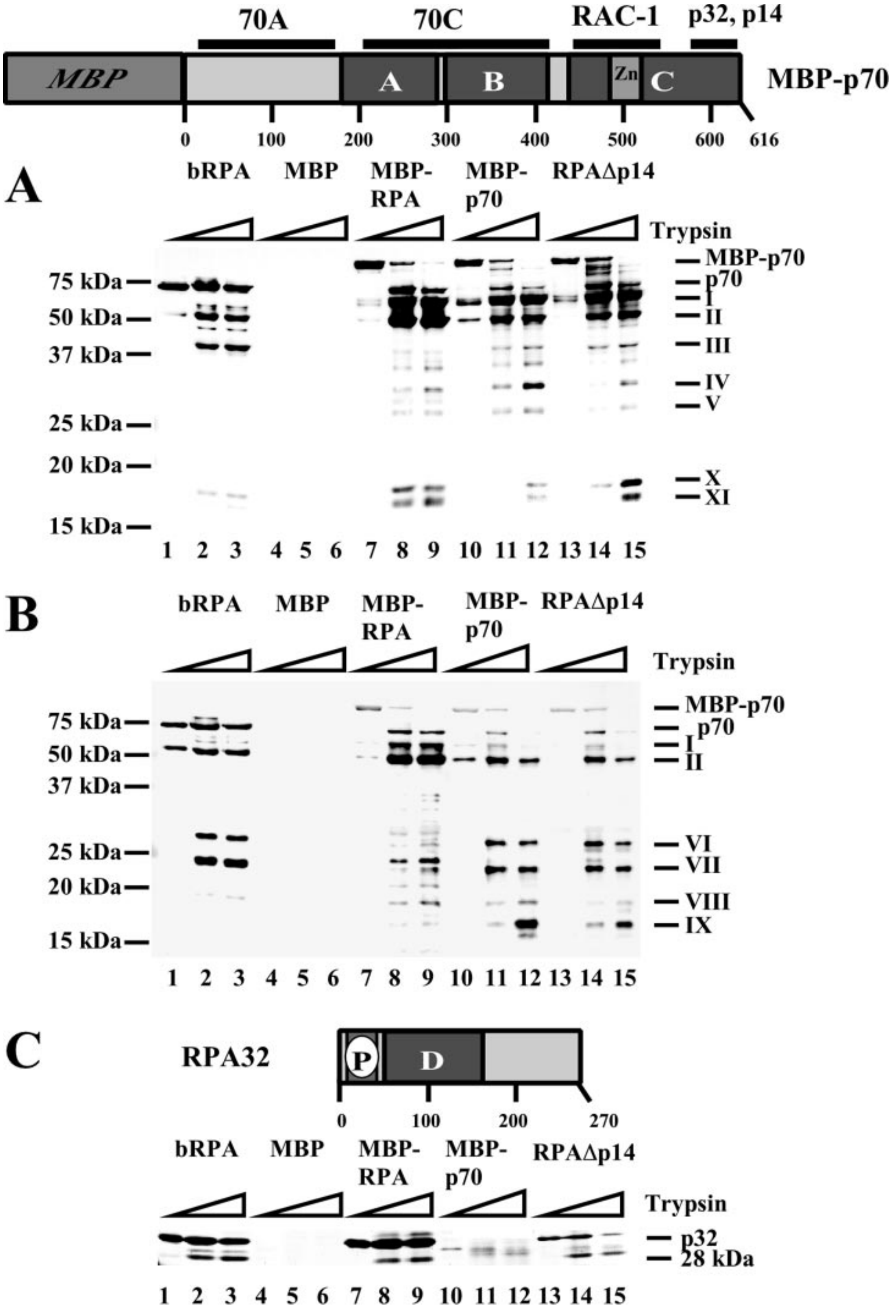

FIG. 7. **Proteolytic stabilities of different RPA subunit complexes.** 2 μg of the indicated protein complexes were incubated with buffer (*lanes 1*, *4*, *7*, *10*, and *13*), a ratio of 1/2000 (*lanes 2*, *5*, *8*, *11*, and *14*), or 1/1000 (*lanes 3*, *6*, *9*, *12*, and *15*) of trypsin for 30 min at room temperature. The reaction was stopped by the addition of SDS loading buffer and heating to 95 °C for 5 min. Each reaction was loaded on a 16% acrylamide SDS gel, and then the proteins were transferred to a polyvinylidene difluoride membrane. The membranes were incubated with either a mixture of mouse monoclonal antibodies 70A and 70C (59) recognizing the N-terminal and the central p70 domain of RPA (*A*), or RAC-1 (43) recognizing the C terminus of p70 (*B*) as indicated. The immunoblot was developed with the appropriate alkaline phosphatase-coupled secondary antibodies and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate staining. The polypeptides produced by trypsin were numbered according to their size (*A* and *B*). In parallel, 6 μg of the indicated protein complexes were treated the same way, but after PAGE the gel was stained with Coomassie Brilliant Blue. In *C*, the regions corresponding to the p32 subunit of RPA and its major proteolysis product with an apparent molecular mass of 28 kDa are shown.

a closed circular, origin-containing dsDNA template (Fig. 6*A*); (ii) the primer synthesis reactions using an unwound template (Fig. 6*B*); and (iii) the primer elongation reactions using an unwound, primed template (Fig. 6*C*). All RPA subunit combinations supported the TAg-mediated unwinding of an origin-containing plasmid (Fig. 6*A*, *lanes 3–7*). All MBP-containing protein complexes were, however, less active in supporting the unwinding reaction than was recombinant bRPA (compare *lanes 3–6* with *lane 7* and the quantification underneath). It is worth mentioning here that ssDNA binding is the only prerequisite to support the unwinding process. It has been shown previously that several ssDNA-binding proteins and p70 alone can fulfill this task, which is consistent with the activity of MBP-p70 in *lane 3* (46–48).

The picture changed substantially when the activities of MBP-p70 and the dimeric complexes were assayed in the origin-dependent primer synthesis and elongation reactions. In contrast to MBP·RPA and bRPA (Fig. 6, *B* and *C*, *lanes 6* and *7*), neither of the RPA subcomplexes nor MBP-p70 supported any of the more complex reactions (*lanes 3–5*). Therefore, p14 together with p32 is necessary for both the initiation of DNA replication at an origin and later during the elongation reactions. These data reveal that p14 is essential to support the initiation and elongation reaction of DNA replication.

p14 and p32 Together Are Necessary to Protect the C Terminus of p70 against Tryptic Digest—Partial digests of RPA with trypsin have allowed the determination of various functional domains of p70 and p32, whereas p14 forms a single proteolytically stable domain (7, 8, 25, 43). We carried out partial tryptic digests to analyze the assembly of RPA. These tryptic digests showed that the recombinant proteins were folded in an appropriate way because the tryptic pattern and stability of p70 and p32 were similar in all digests (Fig. 7). The Western blot developed with the p70-specific monoclonal antibodies 70A and 70C (59) did not reveal any differences in the tryptic patterns of p70 (Fig. 7, compare *A*, *lanes 1–3* and *7–15*). In addition, the Coomassie Brilliant Blue staining of p32 and its proteolysis product of 28 kDa were the same in all complexes (Fig. 7, compare *C*, *lanes 1–3*, *7–9*, and *13–15*). The polypeptides X and XI are the N-terminal fragments, which have been well characterized in earlier publications, are underrepresented in this tryptic digest of RPA but were more pronounced in other experiments (Ref. 43 and data not shown).

The antibody RAC-1, which recognizes amino acids 441–525 in the C terminus of p70 (43), detects similar tryptic polypeptide named I–VIII in the heterotrimeric RPA complexes (Fig. 7*B*). However, RAC-1 detects one additional tryptic polypeptide IX in MBP-p70 (Fig. 7*B*, *lane 12*) and the heterodimeric complex RPAΔp14 (Fig. 7*B*, *lanes 14* and *15*), which is not present in the tryptic digests of the heterotrimeric complexes, bRPA and MBP·RPA (Fig. 7*B*, *lanes 2*, *3*, *8*, and *9*). Because trimerization of subunits is mediated by a specific α-helix located in the C-terminal region of p70 (8) these findings suggest two possible explanations: p14 binds directly to p70 and hinders the access of trypsin to the C terminus of p70 in the presence of p32. Alternatively the interaction of p14 with p32 changes the binding of p32 to the C terminus of p70, and in this modified complex, p70 is resistant to tryptic digestion. The structural analysis of the RPA trimerization core leaves both alternative mechanisms possible.

DISCUSSION

Eukaryotic DNA replication and DNA repair processes are tightly regulated by multiple protein-DNA and protein-protein interactions. One of the best studied examples of such a process is the association of DNA helicases, RPA, and pol·prim during the initiation of eukaryotic DNA replication. This protein assembly plays a pivotal part in the DNA replication events, which culminate in the unwinding of the origin DNA and the synthesis of the first primer (3, 49–51). The importance of RPA is underlined by genetic studies in yeast which demonstrated that all three RPA subunits are required for viability (24, 52). However, it is unclear whether this is because of the essential functions of RPA in DNA replication, other processes such as DNA repair or recombination, or some other activities in eukaryotic DNA metabolism (2, 5, 6, 53).

Biochemical functions of p70 and p32 have been reported, but the activity of the third subunit p14 is still obscure (2, 5, 10–12). To gain insight into the roles of p14 we produced recombinant protein complexes RPAΔp14 lacking p14 consisting of p70 and p32, RPAΔp32 containing p70 and p14, as well as a heterotrimeric complex MBP·RPA and analyzed their biochemical activities. MBP-p70 associated with p32 equally efficiently in the absence and presence of p14. This result is consistent with the recent finding that a stable RPAΔp14 complex can be produced by partially denaturing the heterotrimeric RPA (54). On the other hand, p14 interacted with p70 to a lesser extent and was underrepresented in the purified MBP-p70 fraction in the absence of p32. These findings suggest that there is probably a weak physical interaction between p14 and p70, which may have escaped detection in the yeast two-hybrid system (13) and that efficient physical binding requires the presence of p32. Proteolysis data (Fig. 7) support the idea that both p14 and p32 bind independently to p70 and abolish tryptic digestion at the C terminus of p70. An alternative and more complex explanation, however, would be that p14 induces conformational changes of p32 which then in turn prevent access of trypsin to the C terminus of p70. The x-ray structure of the RPA trimerization core complex suggests that p14 is positioned in close proximity to both p70 and p32. However, only a comparison of the structure of the RPA trimerization core complex with that of p32 alone in binding to the C terminus of p70 will solve the question of whether the addition of p14 induces structural changes either in p70 or p32. Our result that the C terminus of p70 is sensitive to proteases is consistent with earlier findings that zinc and binding to DNA modulate its tryptic digestion pattern (55).

The recombinant protein complexes were then biochemically investigated to determine the role of p14 in the heterotrimeric RPA complex. The functional integrity of the recombinant proteins as well as the dispensability of p14 for some basic RPA activities were demonstrated because the recombinant proteins interacted with both pol·prim and TAg (Fig. 2), bound ssDNA (Fig. 3), and supported TAg-mediated unwinding of dsDNA containing the SV40 origin (Fig. 6*A*). Based on our data, however, p70 and dimeric complexes were clearly distinguishable from the two heterotrimeric complexes, bRPA and MBP·RPA, in their DNA binding activities. The two heterotrimeric complexes have a higher affinity toward ssDNA and form complexes with mobilities, which differ from those complexes missing one RPA subunit. For further analysis of the DNA binding activities of RPA we turned to more complex naturally occurring DNA structures, which are intermediates of DNA replication and repair (16, 43). Although RPA inefficiently binds to dsDNA, the presence of a short double-stranded region including the 3′-OH group of the hybridized primer adjacent to a stretch of ssDNA increases the affinity of RPA to it compared with ssDNA of the same length (16, 43). The RPAΔp14 has a higher affinity toward ssDNA substrates than MBP-p70 and RPAΔp32, but p32 requires p70 and p14 to interact efficiently with the 3′-end of a primer in such a primer-template system, and, importantly, p32 was unable to bind the 3′-OH group, which is in line with earlier reports that p32 but not p14 contains a DBD (25, 38). Interestingly, the interactions of DBD-C and DBD-D with the 3′-OH group of a primer do not require the presence of DBD-A and DBD-B, whereas DBD-D depends on the presence of DBD-C to bind efficiently to the 3′-end of a primer (44). Consistent with our findings, this active DBD-C·DBD-D complex also contains p14.

The importance of p14 for the function of RPA was underlined by the inability of RPAΔp14 to support cell-free SV40 DNA replication in RPA-depleted extracts. The studies presented here revealed that p14 is required for both the initiation and the elongation reactions. These findings show that the three DBDs located in p70 together with DBD-D of p32, as well as their protein interaction activities, are not sufficient to support all of the DNA replication functions of RPA. Although there are no experimental data that would suggest that p14 interacts directly with DNA (2, 5, 10–12) our findings imply further that the RPA subcomplexes associate with short and long ssDNA substrates in a manner qualitatively different from heterotrimeric RPA and that they therefore possibly do not support cell-free DNA replication. An aberrant association of RPA with other replication proteins is most likely not responsible for the failure of RPAΔp14 to support cell-free DNA replication because RPAΔp14 interacts efficiently with pol·prim and SV40 TAg. In addition, these are the only protein-protein interactions of RPA which are necessary for the initiation and the monopolymerase elongation of SV40 DNA replication with purified proteins (Fig. 6, *B* and *C*; for an overview, see Ref. 3).

Although the structure of p14 is known at the atomic level and it contains an oligonucleotide oligosaccharide binding fold, which can be involved in DNA binding or in protein-protein interactions (25), its activity in cellular DNA metabolism has still been obscure. The qualitative and quantitative differences between heterotrimeric RPA and RPAΔp14 in these biochemical assays suggest for the first time a function for p14, the smallest RPA subunit. During the unwinding of double-stranded origin DNA or during DNA repair, short ssDNA stretches arise at ssDNA to dsDNA transitions (3, 16). Our data show that p14 modulates DNA binding of RPA to these DNA structures most likely by coordinating the association of the two larger subunits with the DNA. This hypothesis is supported by the recent findings that the DBD-D alone recognizes the transition of dsDNA to ssDNA by at least 2 orders of magnitudes less efficiently than in the heterotrimeric wild type RPA complex, whereas complexes containing p14, DBD-C, and DBD-D interact with these DNA structures in a way similar to RPA (16, 43).

The large RPA subunit p70 and RPAΔp14 can perform the multiple protein-DNA and protein-protein interactions of RPA, but nevertheless p14 is necessary for the cell-free initiation of DNA replication in an origin and independently for the elongation reactions of DNA replication *in vitro*. Therefore, it seems likely that p14 acts to position the DNA binding and protein interacting domains of p70 and p32 such as the N terminus of p70, DBD-A, DBD-B, DBD-C, and DBD-D during DNA synthesis by acting as a bridging factor as proposed by Bochkareva *et al.* (8). In addition, p14 is probably involved in the DNA polymerase switch mechanism that is necessary to elongate the RNA·DNA primers of the initiation reaction in a processive manner (56–58). These findings in turn suggest that the interaction of p32 with the 3′-OH group of an ssDNA to dsDNA transition plays an important role in the initiation and elongation reaction and that this association of p32 requires p14. This recently detected RPA activity toward the 3′-OH group of an ssDNA to dsDNA transition is probably also involved in DNA repair because it is necessary for RPA to associate with short ssDNA stretches (16, 43, 44). Thus, p14 plays multiple roles and is essential for the duplication of DNA strands in DNA replication and DNA repair processes.

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Klaus Weisshart, Pavel Pestryakov, Richard W. P. Smith, Hella Hartmann, Elisabeth Kremmer, Olga Lavrik and Heinz-Peter Nasheuer

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