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Alternative conformations of human replication protein A are detected by crosslinks with primers carrying a photoreactive group at the 3′-end

O.I. Lavrik[a,*], D.M. Kolpashchikov[a], H.-P. Nasheuer[b], K. Weisshart[b], A. Favre[c]

[a] *Novosibirsk Institute of Bioorganic Chemistry, Siberian Division of the Russian Academy of Sciences, 630090 Novosibirsk, Russia*
[b] *Institute for Molecular Biotechnology, D-07708 Jena, Germany*
[c] *Institut Jacques Monod CNRS, 75351 Paris Cedex 05, France*

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Abstract To analyze the influence of single-stranded template extension of DNA duplex on the conformation of human replication protein A (RPA) bound to DNA we have designed two template-primer systems differing by the size of the single-stranded template tail (9 and 19 nucleotides (nt)). Base-substituted photoreactive dUTP analogs were used as substrates for elongation of radiolabeled template-primer by DNA polymerase β in the absence or in the presence of RPA. Following UV-crosslinking it was demonstrated that the pattern of RPA subunit labeling and consequently RPA arrangement near the 3′-end of the primer is stronlgly dependent upon the length of the template extension.

Key words: Protein-nucleic acid recognition; Human replication protein A; Photoreactive dNTP derivative; Photoaffinity labeling

1. Introduction

The eukaryotic single-stranded DNA (ssDNA) binding protein, the so-called replication protein A (RPA), is a stable heterotrimer consisting of subunits with apparent molecular masses of 70, 32 and 14 kDa, respectively, that have been correspondingly termed p70, p32 and p14 (reviewed in [1]). Interaction of this protein with ssDNA has been extensively studied. It has been demonstrated that human RPA binds single-stranded DNA by at least two different modes, whereby complexes are formed in which RPA occupies 8–10 and 30 nucleotides, respectively [2,3]. Scanning transmission electron microscopy has revealed structural differences of these RPA-DNA complexes that are dependent on both homologous protein interactions and the available length of ssDNA [3]. Sequence comparison suggests a total of four potential ssDNA-binding domains in RPA, two located in p70 and one each in the p32 and p14 subunits, respectively [4]. Indeed, crystallographic studies confirmed that both ssDNA-binding domains of p70 are involved in ssDNA binding [5] and that these domains harbor the major ssDNA-binding activity of RPA which is attributed to the 70-kDa subunit [6]. The p32 subunit can be photocrosslinked to ssDNA, however, only as part of the RPA complex and only with low efficiency [4]. In complex with p14 alone the activity of the ssDNA-binding domain of p32 is only manifested if the subunit is truncated N- and C-terminally [7].

A DNA duplex with an extended template strand represents an appropriate model of the DNA structure operating at the DNA replication fork. However, the study of RPA interaction with this kind of DNA structure is only at its beginning. Photoaffinity labelling techniques have been applied to analyze the interaction of RPA subunits with the primer of a partial duplex DNA [8]. The results clearly demonstrated extensive crosslinking of p32. In contrast, only limited crosslinking of p70 and no crosslinking of p14 were observed. The data corroborate that the p70 subunit binds predominantly to the single-stranded part of a partial duplex DNA whereas the p32 subunit is located near and might even contact the 3′-end of the primer [8].

It has recently been shown that the p32 subunit of RPA monitors the growth state of RNA-DNA primers within replicating SV-40 chromosomes (G. Kaufmann, personal communication). In addition, p32 might be involved in monitoring S phase progression at least in yeast [9]. Therefore it is reasonable to assume that the contact of p32 with the DNA primer end is important for RPA function at the replication fork. Intriguingly, the p32 subunit is phosphorylated in a cell-cycle dependent manner within the replication initiation complex [1,10] and this modification might impose some regulations on RPA's activities [11].

In the present work we have analyzed the crosslinking of RPA subunits to partial duplex DNAs of different primer-template configurations. Single-stranded template extensions of 19 and 9 bases, respectively, were applied for synthesis of photoreactive primers and following crosslinking using both dUTP carrying photoreactive arylazido groups attached by a spacer of 10–12 Å and 4-thiothymidine-5′-triphosphate (sTTP) in which the photoreactive group is part of the nucleotide analog itself. We demonstrate that the contact of p32 to the primer and hence its arrangement within the DNA bound RPA complex is strictly dependent on the length of the template extension.

2. Materials and methods

2.1. Materials

Recombinant mammalian DNA polymerase β (pol β) was purified as described previously [12]. RPA was expressed in *E. coli* and purified as outlined elsewhere [13,14]. Rainbow colored protein molecular mass markers were from Amersham, T_4 polynucleotide kinase from New England Biolabs and [γ-^{32}P]ATP from ICN. Synthetic oligonucleotides were obtained from GENSET. Nensorb-20 columns were purchased from Du Pont. 5-[*N*-(2-nitro-5-azidobenzoyl)-*trans*-3-ami-

*Corresponding author. Fax: (7) (38) 3233 3677.
E-mail: lavrik@niboch.nsc.ru

Abbreviations: RPA, human replication protein A; pol β, mammalian DNA polymerase β; ssDNA, single-stranded DNA; NAB-4-dUTP, 5-[*N*-(2-nitro-5-azidobenzoyl)-*trans*-3-aminopropenyl-1]deoxyuridine-5′-triphosphate; NAB-7-dUTP, 5-[*N*-[*N*-(2-nitro-5-azidobenzoyl)glycyl]-*trans*-3-aminopropenyl-1]deoxyuridine-5′-triphosphate; sTTP, 4-thiothymidine-5′-triphosphate; SDS, sodium dodecylsulfate; PAG(E), polyacrylamide gel electrophoresis; nt, nucleotide

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nopropenyl-1]deoxyuridine-5′-triphosphate (NAB-4-dUTP) [15] and sTTP [16] were synthesized as described. Synthesis of 5-[*N*-[*N*-(2-nitro-5-azidobenzoyl)glycyl]-*trans*-3-aminopropenyl-1]deoxyuridine-5′-triphosphate (NAB-7-dUTP) will be described separately.

2.2. Radioactive labeling of oligonucleotide primers

Dephosphorylated primers were 5′-phosphorylated with T_4 polynucleotide kinase and [γ-^{32}P]ATP as described [17]. Unreacted [γ-^{32}P]ATP was removed by passing the reaction mixture over a Nensorb-20 column according to the manufacturer's instructions.

2.3. Primer-template annealing

The primer and template strands were mixed at a molar ratio of 1:1 and heated at 90°C for 1 min. The mixture was then allowed to cool down slowly to room temperature. The sequences of the primers and templates used were as follows:

System 1 5′-GGTTCGATATCGTAGTTCTAGTGTATAGCCCCTACC-3′
3′-CACATATCGGGGATGG-5′
System 2 5′-CGTAGTTCTAGTGTATAGCCCCTACC-3′
3′-CACATATCGGGGATGG-5′

2.4. Photochemical crosslinking

RPA was labeled with a photoreactive primer that was synthesized in situ in a primer elongation reaction catalyzed by pol β using NAB-4-dUTP or NAB-7-dUTP or sTTP, respectively. Reaction mixtures (10–20 μl) contained 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 50 mM KCl, 1.4 μM pol β, 0.7 μM template-5′ [^{32}P] primer, 10 μM of photoreactive analog and as indicated 0.46 or 0.7 μM RPA. The reaction mixtures were incubated at 25°C for 30 min to allow elongation of the primers. Then the mixtures were spotted on Parafilm that was placed on ice and UV irradiated for 20 min with Baush and Lomb monochromator equipped with an HBO W super pressure mercury lamp. UV light of 320 nm was used for crosslinking NAB-4-dUTP and NAB-7-dUTP, whereas UV light of 335 nm was employed to crosslink sTTP. Reactions were stopped by adding Laemmli buffer and heating. The photochemically crosslinked protein-DNA samples were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [18]. Dried gels were subjected to autoradiography or quantified using a Phosphorimager (Molecular Dynamics).

2.5. Primer elongation in the presence of photoreactive dNTP analogs

Reaction conditions for the elongation of oligonucleotides by photoreactive analogs of dNTP were identical to those of the photocrosslinking experiments. DNA synthesis was initiated by adding polymerase and carried out for 30 min at 25°C. The reaction was terminated by adding 10 μl of 90% formamide, 50 mM EDTA and 0.1% bromophenol blue. The mixture was heated for 3 min at 80°C and the products were analyzed by electrophoresis [17] followed by autoradiography.

3. Results

Base-substituted arylazido derivatives of dUTP and sTTP have been employed in this study, since these analogs are effective substrates of pol β [8,16]. The structure of the analogs is shown in Fig. 1. In addition, the photoreactive properties of dUTP analogs and sTTP allow for UV irradiation by light at a wavelength greater than 310 nm, which is far beyond the absorbance maxima of nucleic acids and proteins. Note that the reactive moiety is separated from dUTP by a 10- and 12-Å spacer in NAB-4-dUTP and NAB-7-dUTP, respectively, whereas in sTTP the reactive group is part of the nucleotide analog.

We have worked out conditions that allowed us to introduce single photoreactive moiety into the 3′-end of a ^{32}P-labeled primer using a primer elongation reaction catalyzed by pol β (see Fig. 2, lanes 3, 4 for primer-template system 1). Fig.

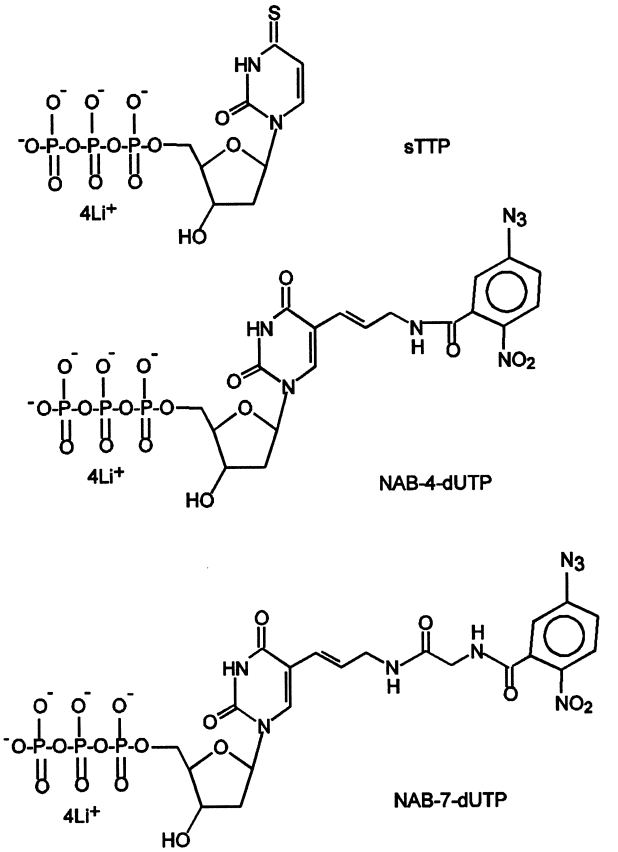

Fig. 1. Structural formulae of photoreactive analogs of dUTP.

2 lane 2 demonstrates primer elongation with TTP. This held also true if RPA was present in the assay or for the primer-template system 2 (data not shown). We have consequently used these conditions for the synthesis of photoreactive primers before UV crosslinking in the presence or in the absence of RPA (see below).

In a previous report we have demonstrated that photoreactive primers synthesized in situ by DNA polymerases with NAB-4-dUTP as a substrate of primer elongation can be used for the subsequent analysis of the interaction of RPA with DNA by UV crosslinking [8]. To determine the influence of the template length on the mode of RPA interaction with the junction of the primer-template, i.e. near the 3′-end of the primer, we have compared RPA crosslinking products obtained with two different DNA duplexes. The first duplex had a single-stranded DNA extension of 19 nucleotides (nt) (system 1) whereas the second one was designed to have a 9-nt overhang (system 2) which presumably is long enough for the binding of one RPA molecule according to published results [3]. The latter complex has been reported to be rather unstable and could only be identified by using chemical crosslinking with glutaraldehyde [3]. However, we hypothesized that photochemical crosslinking is able to fix unstable RPA complexes with DNA. Fig. 3 presents the results obtained with the two DNA primer-template systems using NAB-4-dUTP and NAB-7-dUTP. As can easily be seen, pol β was readily labeled and hence crosslinked to both DNA substrates in the absence of RPA (Fig. 3, lanes 3, 5, 7 and 10). If the primer-template system 1 with the 19-nt extension was used a reduction in pol β crosslinking in the presence of RPA was observed,

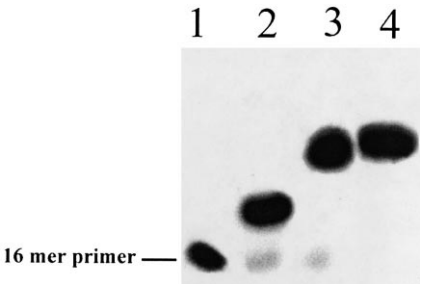

Fig. 2. Substrate properties of NAB-4-dUTP and NAB-7-dUTP in the reaction of primer elongation catalyzed with pol β. All reaction mixtures contain 1.4 μM pol β, and 0.7 μM template-^{32}P-labeled primer (system 1) (for other conditions see Section 2). Lane 1 contained pol β and template-primer; lane 2 contained pol β, template-primer and 10 μM TTP; lane 3 contained pol β, template-primer and 10 μM NAB-4-dUTP; lane 4 contained pol β, template-primer and 10 μM NAB-7-dUTP. Reaction mixtures were incubated for 30 min at 25°C. The reactions were terminated and products were analyzed by electrophoresis followed by autoradiography (see Section 2).

and labeling of the p70 and p32 subunits, respectively, took place (Fig. 3, lanes 8 and 9). Phosphorimaging analysis showed that the efficiency of p32 labeling in that case was approximately 15 times higher in comparison with p70. In the case of the primer-template system 2 with the 9-nt extension only weak labeling of p32 was detected by using NAB-4-dUTP or NAB-7-dUTP for primer elongation (Fig. 3, lanes 4

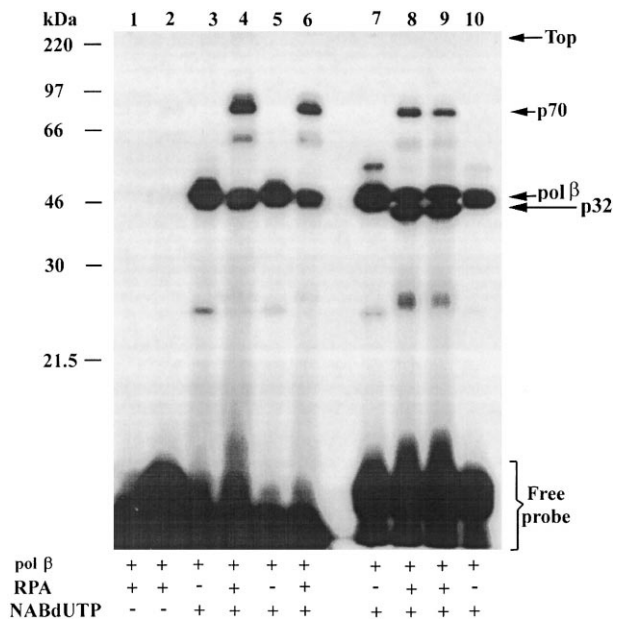

Fig. 3. Photoaffinity labeling of RPA by photoreactive primers by using 'short' and 'long' template systems (system 1 and system 2). Reaction mixtures were composed of 1.4 μM pol β, 0.7 μM template-^{32}P-labeled primer, 10 μM NAB-4-dUTP (lanes 3, 4, 7, 8) or 10 μM NAB-7-dUTP (lanes 5, 6, 9, 10), 0.46 μM RPA (lanes 1, 2, 4, 6, 8, 9) (for other conditions see Section 2). Control reaction mixtures (lanes 1, 2) contained pol β, template-primer and RPA. Lanes 1, 3, 4, 5, 6 contained system 2 as a template-primer, lanes 2, 7, 8, 9, 10 contained system 1. Reaction mixtures were incubated for 30 min at 25°C and then UV irradiated for 20 min (λ 320 nm). The UV-crosslinked protein-DNA complexes were separated by SDS-PAGE and visualized by autoradiography. The positions of the free probe (excess of ^{32}P-labeled oligonucleotide) and protein markers are indicated.

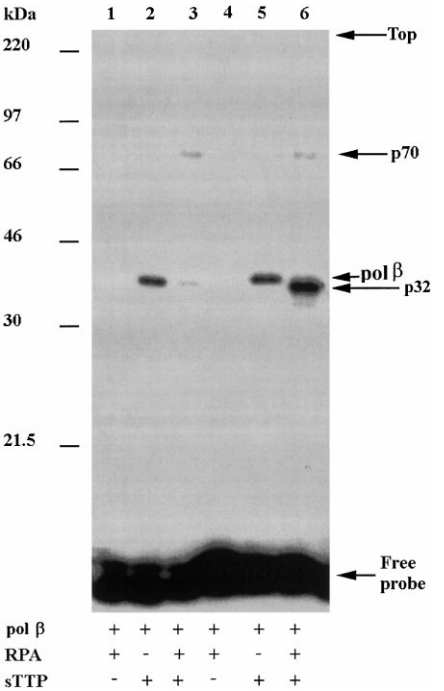

Fig. 4. Photocrosslinking of photoreactive primers synthesized by using sTTP and 'long' and 'short' template-primer duplex. All reaction mixtures contained 1.4 μM pol β, 10 μM sTTP, 0.46 μM RPA and 0.7 μM template-^{32}P-labeled primer: system 1 (lanes 4, 5, 6) and system 2 (lanes 1, 2, 3) (for other conditions see Section 2). Control reaction mixtures (lanes 1, 4) contained pol β, RPA and template primer: system 2 and system 1, respectively. Reaction mixtures were incubated for 30 min at 25°C and then UV irradiated by monochromatic UV light (λ 335 nm). UV-crosslinked protein-DNA complexes were separated by SDS-PAGE and visualized by autoradiography. The positions of the free probe and protein markers are indicated.

and 6). Again the presence of RPA led to a concomitant decrease of pol β labeling (compare lanes 3 and 4, lanes 5 and 6). In contrast labeling of p70 and its proteolytic 54-kDa fragment was readily demonstrated. The level of p70 labeling was higher using both of the analogs in the case of the 9-nt template extension system in comparison with the 19-nt extension template system (Fig. 3, compare lanes 4 and 6 to lanes 8 and 9). The conclusion was proved by Phosphorimager estimation. Results were identical if RPA was added to the reaction mixture just prior to primer-synthesis or before UV irradiation after the primer had been synthesized (data not shown).

Similar results were obtained with sTTP as the substrate of primer elongation (Fig. 4), though crosslinking efficiencies in that case were lower than with NAB-4-dUTP or NAB-7-dUTP. Again, pol β is efficiently crosslinked to either DNA substrate in the absence of RPA (Fig. 4, lanes 2 and 5) and the extent of crosslinking is reduced by RPA (Fig. 4, lanes 3 and 6). Labeling of p32 occurs only in the case of system 1 with the more extended template tail (Fig. 4, lane 6). When the template strand has an extension of only 9 nt (system 2), p32 crosslinking was not observed (Fig. 4, lane 3). The level of p70 labeling was higher in the case of the DNA substrate with the 9-nt extension in comparison to the one with the 19-nt extension (Fig. 4, compare lanes 3 and 6). It was proved by Phosphorimaging analysis.

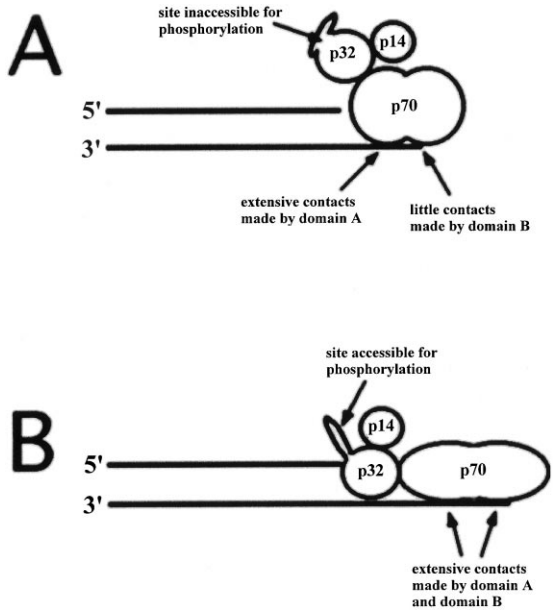

Fig. 5. Model of RPA-DNA interaction. The figure depicts schematically the primer-template strands with the 9-nt (panel A) and 19-nt (panel B) extension as solid bars. The orientation of the upper primer and lower template strands are identified through their 5′- and 3′-ends, respectively. In the 8-nt-binding mode (panel A), the p70 subunit of RPA binds DNA extensively through domain A, wheras domain B makes only few contacts, positioning the p32 and p14 subunits away from the primer end. The N-terminus depicted as a protrusion is not accessible for kinases. The conformation of the complexed RPA adopts an overall globular shape. In the 30-nt-binding mode (panel B) both DNA-binding domains of p70 contact the ssDNA extensively, positioning the p32 subunit in close proximity to the primer end and giving the complex its elongated shape. p14 still points away from the 3′-end and p70 cannot come into close contact to it due to p32 binding. The N-terminus of p32 in this configuration becomes free to be phosphorylated by kinases.

4. Discussion

The interaction of RPA with single-stranded DNA has been intensively studied by using various techniques including X-ray crystallography (for review see [1]). Under physiological conditions RPA binds tightly to ssDNA thereby covering a length of 30 nt [1]. RPA binding to stretches of 8–10 nt has also been observed, however, these complexes are less stable and probably represent intermediate states for binding in the 30-nt mode [2,3]. These data are in agreement with estimations of the dissociation constants for RPA complexes with long and short ssDNA [1].

One of the important and still open issues to understand the function of RPA concerns its interaction with the DNA structure operating at the replication fork. It was therefore the intention of this study to take a step forward in this direction and to elucidate the DNA binding mode of RPA during the synthesis of the nascent strand and the interrelationship of RPA and DNA polymerases at the 3′-end of the growing primer. To this end we have used different photoreactive dNTP derivatives to assess the difference in the mode of RPA subunit labeling for DNA duplexes with short (9 nt in length) and extended (19 nt in length) template tails. It was suggested earlier that crosslinking of RPA to photoreactive primers synthesized in situ by DNA polymerases should be sensitive to the alteration of subunit arrangements near the 3′-end of the nascent DNA [8]. As a matter of fact our data clearly show that the orientation of RPA subunits near the 3′-end of the primer is different for the various DNA substrates employed and dependent on the length of the template extension (Figs. 3 and 4). It is only when RPA is able to bind to an extended template tail that its p32 subunit is positioned in close proximity to the 3′-end of the primer, whereas binding to the short extension does not allow p32 to come into close proximity of the primer end. Our results are corroborated by the visualization of both the 8-nt and 30-nt complexes of DNA and RPA in vitro by scanning transmission electron microscopy [3]. The former complex was found to have a globular shape and would represent the binding of RPA to the 9-nt extended template whereas the latter generally had an elongated shape and presumably represents the structure of RPA complexed to the template with the 19-nt single-stranded protrusion, although this single-stranded extension is less than 30 nt long. With both DNA primer-templates no labeling of p14 was observed arguing that this subunit is located relatively far from the 3′-end in either case. The intensity of p70 labeling in the case of the 19-nt extended template was more than one order of magnitude lower in comparison to p32, indicating that this subunit is also positioned away from the primer. Interestingly the level of p70 labeling increased approximately two-fold if the 9-nt extended template was used with which no crosslinking to the p32 subunit took place. This suggests that in this case p70 is able to approach the primer end more closely. We want to suggest here that the interaction of p70 with the single-stranded template tail provides orientation of the other RPA subunits in relation to the 3′-end of the nascent DNA and have incorporated this into the model represented by Fig. 5. In the case of the 9-nt template extension p70 binds DNA closer to the 3′-end and p32 is positioned away from the primer end, whereas in the case of the 19-nt template extension p70 binds the ssDNA further away from the 3′-end and positions p32 close to it. The fact that sTTP in which the photoreactive group is not separated by a spacer from the nucleotide is efficiently crosslinked to RPA (Fig. 4) demonstrates the very close proximity of p32 to the primer end while p14 in any case would be positioned away from the primer end. The scheme of interaction between the three subunits as depicted in Fig. 5, i.e. p32 bridges the other two subunits that do not make direct contact with each other, is based on published results [19], although we are aware of different discussed models of subunit interactions where it is proposed that p70 contacts p32 and p14 separately [20].

What then determines the difference in p70 binding? p70 possesses two DNA binding domains each of which make contact to the single-stranded DNA [5]. However, the predominant contacts are made by the domain located more N-terminally, which is referred to as domain A [19] in the crystal structure where the DNA binding region of p70 was complexed to an octanucleotide. Domain B made only minor contributions to the binding to the nucleotide. It is therefore very attractive to speculate that, if p70 is able to contact 8–10 nt only, it will do so by strongly binding with domain A and with additional weak interactions contributed by domain B, whereas binding in the 30-nt mode allows domain B to make more extensive contact as well. Thus differences in the extent of DNA binding by p70 may bring about different conformational changes and subunit positioning within RPA, allowing

the complex to take a more globular or extended shape in the 8-nt or 30-nt mode, respectively. Indeed structural changes of p70 have been observed upon DNA binding by proteolytic mapping studies [21]. In addition the accessibility of p32 for phosphorylation by DNA dependent protein kinase is dramatically enhanced if RPA is complexed in the 30-nt mode [3] indicating p32 rearrangement upon binding of RPA to DNA. The special arrangement and the accessibility of the p32 subunit could be significant for the interaction of RPA with other factors of the DNA replication machinery [1] and for the regulation of its activity during the cell cycle by phosphorylation [10,11].

It will be extremely interesting to examine whether the globular and elongated conformations represent the only stable states of the complex or extremes of a continuum of conformations. The systems used here are oversimplified as compared to the in vivo processes of replication and gap repair where pol β appears directly involved. It is tempting to suggest that the low affinity globular state is indeed the one in which RPA directly binds (or leaves) ssDNA and then rearranges to a stable elongated complex if space is available on the single-stranded DNA tail. This process could be regulated by the interaction of RPA with DNA polymerases and other factors of the DNA replication machinery.

RPA competes with pol β for the interaction with the 3′-end of the primer regardless of the complexed RPA form (globular or elongated; Figs. 3 and 4). Competition is not dependent on active primer synthesis, since RPA that was added after completion of the primer reduced pol β binding as efficiently. Such competition might facilitate the switch from primer synthesis to the primer elongation step. Indeed, the p32 subunit does interact with short RNA-DNA primers but not with more advanced products (G. Kaufmann, personal communication). Thus the contacts made by p32 to the primer end might serve predominantly the monitoring of the growing primer length during the initiation phase of replication.

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