Interaction of the p70 subunit of RPA with a DNA template directs p32 to the 3′-end of nascent DNA

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Abstract Human replication protein A is a heterotrimeric protein involved in various processes of DNA metabolism. To understand the contribution of replication protein A individual subunits to DNA binding, we have expressed them separately as soluble maltose binding protein fusion proteins. Using a DNA construct that had a photoreactive group incorporated at the 3′-end of the primer strand, we show that the p70 subunit on its own is efficiently cross-linked to the primer at physiological concentrations. In contrast, crosslinking of the p32 subunit required two orders of magnitude higher protein concentrations. In no case was the p14 subunit labelled above background. p70 seems to be the predominant subunit to bind single-stranded DNA and this interaction positions the p32 subunit to the 3′-end of the primer.

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

1. Introduction

The eukaryotic replication protein A (RPA) is a stable heterotrimer consisting of three subunits with apparent molecular masses of 70, 32 and 14 kDa, respectively. RPA plays essential roles in the processes of DNA replication, repair and recombination [1]. To fulfil its function, RPA is equipped with a single-stranded DNA (ssDNA) binding activity. The interaction of RPA with ssDNA has been extensively studied. However, to evaluate the role of RPA in the process of DNA replication, a DNA duplex with a protruding template strand is presumably the more appropriate model for the DNA structure operating at the replication fork. For the study of RPA's interaction with this kind of DNA structure, the technique of photoaffinity labelling has been successfully applied to analyze the loading of RPA and the positioning of its subunits [2]. The technique permits even to identify unstable protein-nucleic acid complexes [3]. 5-(*N*-(2-nitro-5-azidobenzoyl)-trans-3-aminopropenyl-1)-2′-deoxyuridine-5′-triphosphate (NAB-4-dUTP), a nucleotide analog with an attached photoreactive group, proved to be useful as a substrate for elongation of a radiolabelled primer-template in the presence or the absence of RPA [2]. Both the p70 and the p32 subunits can be cross-linked to the primer containing the analog at its 3′-end. The p70 subunit appears to bind to the single-stranded part of the DNA duplex, whereas the p32 subunit can locate near the 3′-end of the primer, dependent on the template configuration. Crosslinking of the smallest RPA subunit (p14) was not identified at any conditions. Specific interactions of p32 with nascent SV40 DNA were also revealed by in situ UV-crosslinking [3]. The dynamic characteristics of the cross-linked DNA, its size distribution, fork polarity and RNA primer content indicated that p32 contacts growing RNA-DNA primers, which are the products of DNA polymerase α-primase.

Although it seems clear that p70 binds predominantly to the ssDNA region and p32 contacts, if at all, the primer terminus, it is not known how each of the single subunits contributes per se to DNA binding. Here, we demonstrate that it is mainly the p70 subunit that can contact DNA on its own and that p32 contacts are only available in the complex through positioning by the p70 subunit.

2. Materials and methods

2.1. Materials

Recombinant mammalian DNA polymerase β (pol β) was purified as described [4]. RPA was expressed in *Escherichia coli* and purified as outlined [5,6]. Rainbow-colored protein molecular mass markers were from Amersham, pre-stained markers from New England Biolabs and the 10 kDa ladder from Gibco BRL. T_4 polynucleotide kinase was purchased from New England Biolabs. [γ-^{32}P]ATP was from ICN. Synthetic oligonucleotides were obtained from GENSET. Nensorb −20 columns were from Du Pont. NAB-4-dUTP was synthesized according to Wlassoff et al. [7]. Individual RPA subunits were expressed as maltose binding protein (MBP) fusion proteins and isolated as will be described elsewhere.

2.2. Band shift assays

Band shift assays contained in a 10 μl mix, 20 mM HEPES-KOH pH 7.8, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 200 ng (corresponding to 0.083 pmol) M13mp18 DNA (Pharmacia) and the indicated amounts of RPA or RPA subunit MBP fusion proteins. After 30 min at 37°C, the sample was supplemented with 2 μl load buffer (10 mM HEPES-KOH pH 7.5, 1 mM EDTA, 25% Ficoll 400, 0.1% bromophenol blue) and electrophorezed in a 1% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). The gel was stained with 0.5 μg/ml ethidium bromide and DNA was visualized on an UV-transilluminator.

Band shift assays were also set up using 5′-labelled oligonucleotides. In these cases, the assay contained 2 fmol (10 000 cpm) of the indicated oligonucleotide instead of M13mp18 ssDNA. After incubation,

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Abbreviations: pol β, mammalian DNA polymerase β; NAB-4-dUTP, 5-(*N*-(2-nitro-5-azidobenzoyl)-trans-3-aminopropenyl-1)-2′-deoxyuridine-5′-triphosphate; SDS, sodium dodecyl sulfate; PAG(E), polyacrylamide gel (electrophoresis); MBP, maltose binding protein

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the samples were separated on 10% polyacrylamide gels in TBE (89 mM Tris-borate, 89 mM boric acid) at 160 V until the bromophenol blue dye had migrated half into the gel. The gel was dried and exposed to X-ray films.

2.3. Radioactive labelling of oligonucleotide primers

Dephosphorylated primers were 5′-end-phosphorylated with T_4 polynucleotide kinase as described [8]. Unreacted [γ-^{32}P]ATP was removed by passing the mixture over a Nensorb-20 column using the manufacturer's suggested protocol.

2.4. Primer-template annealing

Primer-templates were annealed at a molar ratio of 1:1, first by heating the mixture at 90°C for 1 min that was then allowed to slowly cool to room temperature. The sequences of the primer and template used were as follows: 5′-GGTTCGATATCGTAGTTCTAGTGTATAGCCCCTACC-3′, 3′-CACATATCGGGGATGG-5′

2.5. Primer elongation in the presence of photoreactive dNTP analogs

Conditions for elongation of oligonucleotides by photoreactive analogs of dNTP were identical to those used for photocrosslinking. 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 products were analyzed by electrophoresis followed by autoradiography.

2.6. Photochemical crosslinking

RPA and its individual subunits were labelled with a photoreactive primer synthesized in situ using NAB-4-dUTP in a reaction of primer elongation catalyzed by pol β. Reaction mixtures (10 μl) contained the following standard components: 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 50 mM KCl, 2 μM pol β, 0.8 μM template-5′-[^{32}P]primer and 10 μM NAB-4-dUTP. The concentrations of RPA or individual RPA subunits were as indicated. The reaction mixtures were incubated at 25°C for 30 min to allow elongation of the primer. Then, the mixtures were spotted on parafilm that was placed on ice and UV-irradiated for 20 min with a Baush and Lomb monochromator equipped with a HBO W super pressure mercury lamp producing UV-light of 315 nm. Reactions were stopped by addition of Laemmli buffer and heating. The photochemically cross-linked protein-DNA samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [9] and analyzed by autoradiography or with a Phosphorimager.

3. Results

In order to unravel the roles of the individual subunits during DNA binding, we aimed to express them separately and to use them in crosslinking experiments taken out of the context of the heterotrimeric complex. It has been reported earlier that attempts to express RPA subunits individually in a soluble form were unsuccessful [10]. To increase the solubility of the subunits, we expressed them as MBP fusion proteins (Fig. 1A). We were able to express all fusion proteins in a soluble form. The fusion parts of these proteins can change their biological properties. Indeed, the fusion RPA subunits cannot associate in heterotrimer structure, but we suggest that they retain DNA binding activity. It should be noted that the molecular mass of each MBP fusion protein increases to 48 kDa when compared with the natural one.

In band shift experiments, we detected DNA binding activity to be associated with the fused p70 subunit, but not with the other two subunits (Fig. 1B). The MBP-RPA70 protein seemed to interact less well with ssDNA of shorter length than the heterotrimeric protein (Fig. 1C). As expected, none of the proteins interacted with an 8-mer oligonucleotide (lanes 2 and 3). Such complexes have been reported in the case of the heterotrimer to be unstable and only detectable in band shift assays after crosslinking [11]. However, whereas RPA was

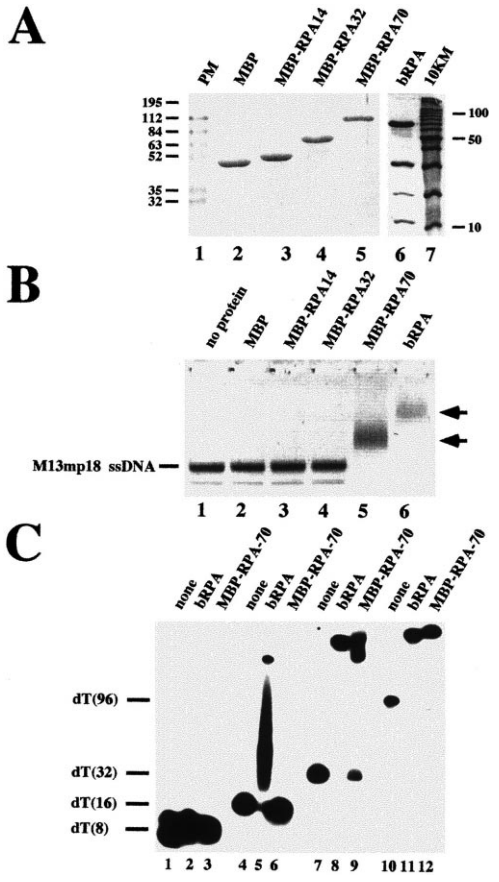

Fig. 1. DNA binding properties of RPA subunits. A shows Coomassie-stained gels of MBP fusion proteins (lanes 2–5: 2 μg each) and of bacterially-expressed RPA (5 μg: lane 6). Note that the band that runs approximately at 20 kDa in lane 6 is a degradation product of the p32 subunit. Molecular masses of pre-stained markers are given on the right margin, whereas those of selected peptides of the 10 kDa ladder are given at the right. B shows band shift experiments in which 10 pmol of the indicated proteins were incubated with 0.1 pmol M13mp18 ssDNA. The position of ssDNA is indicated on the left, whereas shifted complexes are identified by arrows at the right. In C, 5 pmol of the indicated proteins was used in binding assays along with 2 fmol oligo(dT_8) (lanes 1–3), oligo(dT_{16}) (lanes 4–6), oligo(dT_{32}) (lanes 7–9) and oligo(dT_{96}) (lanes 10–12) (each 5000 cpm/fmol), respectively. Complexes were resolved on a 10% native polyacrylamide gel. The gel was dried and autoradiographed. The positions of the different free oligos are shown on the left.

able to bind to a 16-mer fairly stable, the fusion p70 protein was not (compare lanes 5 and 6). The smear below the shifted band in lane 5 must be due to DNA molecules that lost RPA during electrophoresis due to unstable RPA binding. In addition, binding to a 32-mer seemed to be less stable in the case of the p70 fusion protein (compare lanes 8 and 9). Whereas a single shifted band is detected with the heterotrimer, a smear is visible for the fusion protein. It is only with a 96-mer that binding seemed to be equally efficient for both proteins (compare lanes 11 and 12).

After characterization of the DNA binding properties of the MBP fusion proteins, we next used them in crosslinking experiments. A base-substituted arylazido derivative of dUTP, designated NAB-4-dUTP (Fig. 2), has been used in this study. This analog is not only an effective substrate of pol β [2] but moreover allows UV-irradiation to be performed with light at

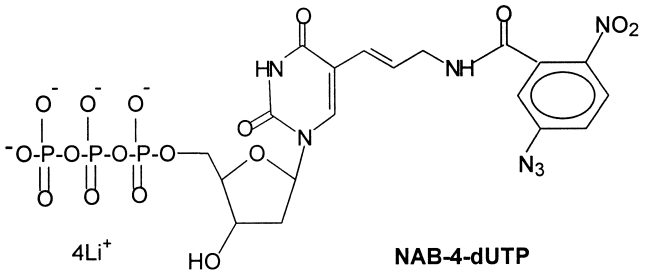

Fig. 2. Structural formula of NAB-4-dUTP.

wavelengths greater than 310 nm, which is far beyond the absorbance maxima of nucleic acids and proteins. We have worked out conditions that enabled us to introduce a single photoreactive dNMP into the 3′-end of a 5′-^{32}P-labelled primer using a primer elongation reaction catalyzed by pol β [12]. Fig. 3 clearly shows that the initial primer (lane 1) is consumed in the reaction and only the final product is obtained in the presence of both TTP (lane 2) and NAB-4-dUTP (lane 3). The decreased mobility of the elongated primer seen with the analog (compare lanes 2 and 3) is a consequence of the bulkiness of the photoreactive group of NAB-4-dUTP [13].

We have shown previously that the p70 and p32 subunits of RPA can be cross-linked to photoreactive oligonucleotides synthesized in situ by DNA polymerases with NAB-4-dUTP as a substrate. When partial DNA duplexes with extended template tails were applied, a more intensive labelling of p32 occurred over p70, whereas the opposite was true if the template tail was short [2,12]. In contrast, under no conditions, a remarkable crosslinking of the p14 subunit was observed. It was hypothesized that the p70 subunit interacts with the single-stranded tail of the DNA template and positions the p32 subunit near or away from the 3′-end of the primer dependent on the single-stranded extension size used. The p14 subunit is located relatively far from the 3′-end of the primer no matter what kind of template is employed [2].

For crosslinking experiments, we chose physiological relevant concentrations (0.4 μM) of the individual RPA subunits and the RPA heterotrimer [1] (Fig. 4). The p70 subunit fusion protein was cross-linked to the photoreactive 5′-^{32}P-labelled primer (lane 2). Increasing the protein concentration yielded higher amounts of cross-linked product (compare lane 2 with lane 7). In contrast, adding either the same amount of the p32 fusion, the p14 fusion or MBP alone did not result in their crosslinking (lanes 3, 4 and 5). Note that each protein covalently bound to the primer runs with a molecular mass that is 6–8 kDa greater than that for the unmodified protein during SDS-PAGE and each fusion MBP-RPA subunits has an apparent molecular mass of 48 kDa greater than that of the native one. When the polypeptides p70, p32 and p14 were added together, it was again only the p70 fusion protein that was cross-linked. The crosslinking efficiency was to the same extent as the one seen with this subunit alone (compare lane 6 to 2). We want to stress that the subunits do not associate to each other if combined individually (data not shown). Indeed, it was found earlier that the subunits have to be co-expressed in order to built the heterotrimeric complex [10,14].

If we increased protein concentrations for the p32 subunits in the range of two orders of magnitudes, i.e. to 50 μM, we observed specific crosslinking of this subunit to an extent that clearly exceeded the crosslinking of MBP that served as a negative control (compare lanes 8 and 10). In contrast, no labelling above the background of the p14 fusion protein was detected (compare lanes 9 and 10). If all three subunits were present at high concentrations, weak labelling with both the p70 and the p32 fusion proteins took place. Again, no crosslinking to the p14 fusion protein was seen (lane 11). Note that both subunits cross-linked less well than is seen with the individual subunits (compare lanes 7 and 8 to lane 11) or in the case of p70 if all three fusion proteins were present in lower amounts (compare lanes 5 and 11). This is probably due to a competition between p70 and p32 for the same or overlapping binding sites.

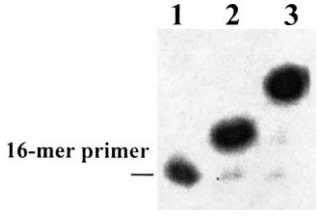

Fig. 3. Characterization of NAB-4-dUTP as a substrate. Reactions of primer elongation by pol β were initiated in the presence of either TTP (lane 2) or NAB-4-dUTP (lane 3). All reaction mixtures contained in addition the standard components as described in Section 2.

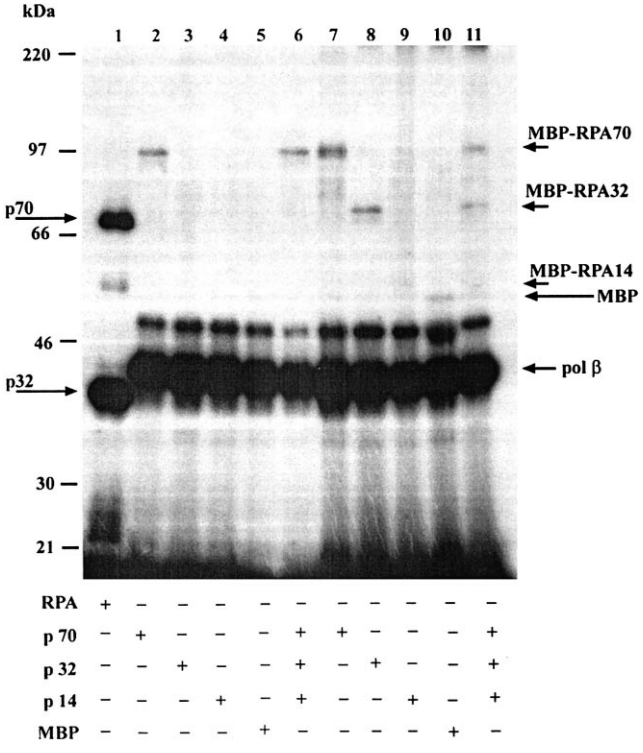

Fig. 4. Photoaffinity labelling of RPA and its individual subunits. Reactions were set up that contained either the heterotrimeric RPA (lane 1), MBP-RPA70 (lanes 2, 6 and 7), MBP-RPA32 (lanes 3 and 8), MBP-RPA14 (lanes 4 and 9), MBP (lanes 5 and 11) or a mixture of MBP fusion proteins (lanes 7 and 11). Protein concentrations used were 0.4 μM (lanes 1–6), 1 μM (lane 7) and 50 μM (lanes 8–11), respectively. Molecular weight marker positions are indicated at the left, the position of cross-linked proteins on the right.

A total different crosslinking pattern was revealed if the heterotrimeric complex is used in the crosslinking studies (lane 1). Here, both the p70 and the p32 subunits were cross-linked efficiently, however, with a clear preference for the latter subunit. Crosslinking efficiencies clearly exceeded those seen with the individual subunits. This is also revealed by a more efficient competition of pol β by the heterotrimer compared to those observed with the single subunits (compare lane 1 with lanes 7 and 8).

4. Discussion

The interaction of RPA with ssDNA has been intensively studied using various techniques including X-ray crystallography [1]. It was shown that RPA could adopt different configurations, dependent on the available length of the ssDNA. However, one of the important questions concerning the function of RPA still remains to be investigated, i.e. how it interacts with the DNA structure operating at the replication fork. In this respect, it is important to unravel the mode of RPA binding with DNA during the synthesis of the nascent DNA and to determine the interrelationship of RPA and DNA polymerases around the 3′-end of the growing primer.

A step forward in this direction was the use of crosslinking techniques [2,3]. A model of the mode of RPA binding to a DNA duplex with a template strand extended over the 3′-end has been suggested by us earlier on the basis of crosslinking data [2]. In this model, the p70 subunit is responsible for binding to the single-stranded region and for positioning of the p32 subunit in relation to the primer end. The orientation of the p32 subunit towards the 3′-end of the primer is strongly dependent on the RPA conformation that in turn is modulated by the length of the template extension [12]. In this report, we wanted to investigate the DNA binding properties of the single subunits in order to test the hypothesis that it is the p70 subunit which makes the crucial contacts to DNA and that through its way of binding positions the other subunits. Indeed, experimental data obtained earlier speak in favor of the p70 subunit being primarily responsible for binding to ssDNA [1].

In using soluble MBP fusion proteins of individual RPA subunits, we show here that it is predominantly the p70 subunit that interacts with ssDNA on its own, albeit that the affinity of the p70 fusion protein for shorter ssDNAs seemed to be less compared to the trimeric protein (Fig. 1). It could be the influence of the fusion part of MBP-RPA70. In addition, we demonstrate that p70 alone or uncomplexed with the two other RPA subunits can interact with an extended template tail of a DNA duplex at physiological relevant protein concentrations (0.1–1 μM) [15] as revealed by p70 crosslinking to the photoreactive dNMP moiety introduced into the 3′-end of the primer (Fig. 4).

Crosslinking of the p32 subunit to the photoreactive primer was detected only at very high protein concentrations (50 μM) that could be the consequence of p32 DNA binding by its DNA binding domain [16,17]. We cannot rule out conclusively that the p32 crosslinking observed reflects merely nonspecific interactions with DNA. But we think it is more unlikely than a specific binding. First, labelling was above background levels and second, p32 could compete with p70 if used at higher concentrations. Earlier reports showed that this DNA binding activity is revealed only if p32 is in a complex with the other two subunits or if it is truncated [18,19]. Thus, it seems likely that the DNA binding activity of the p32 subunit is hardly accessible in the single subunit and becomes available in the trimeric protein only after p70 is bound to DNA.

We did not obtain any crosslinking above background levels for the p14 fusion protein even when we used elevated concentrations of the protein. We conclude that the p14 subunit is not involved in a direct interaction with DNA and speculate that its role consists in the assembly of the RPA oligomer.

Our data suggest that the largest RPA subunit is mainly responsible for binding ssDNA and that this event results in the proper orientation of the other RPA polypeptides in respect to the DNA template-primer junction. If p32 contacts only the primer or in addition the ssDNA remains to be determined.

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