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Different activities of the largest subunit of replication protein A cooperate during SV40 DNA replication

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Abstract Replication protein A (RPA) is a stable heterotrimeric complex consisting of p70, p32 and p14 subunits. The protein plays a crucial role in SV40 minichromosome replication. Peptides of p70 representing interaction sites for the smaller two subunits, DNA as well as the viral initiator protein large T-antigen (Tag) and the cellular DNA polymerase α-primase (Pol) all interfered with the replication process indicating the importance of the different p70 activities in this process. Inhibition by the peptide disrupting protein–protein interactions was observed only during the pre-initiation stage prior to primer synthesis, suggesting the formation of a stable initiation complex between RPA, Tag and Pol at the primer end.
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1. Introduction

Replication protein A (RPA), a hetero-trimeric protein involved in various aspects of DNA metabolism, is highly conserved in its subunit structure, DNA binding activity and posttranslational modifications in all examined eukaryotes [1,2]. The requirement of all three genes coding for the p70, p32 and p14 subunits for cell viability in yeast argues that RPA functions in vital reactions as a complex [3]. The integrity of the human trimer is also needed for Simian virus 40 (SV40) cell-free in vitro DNA replication, since neither the p70 subunit alone nor a sub-complex of p32 and p14 were able to support the reaction and antibodies directed against any one of the subunits were able to inhibit the process [4,5].

Human RPA's tight association to single-stranded DNA (ssDNA) is mediated primarily by p70 [4,6]. The subunit is oriented on the DNA in a polar fashion and positions the p32 subunit in the vicinity of the 3′-end of a primer-template junction [7]. Binding occurs in three modes, whereby the protein occludes 8–10, 12–23 and 28–30 nucleotides, respectively [8–10]. The crystal structure of the ssDNA binding domain of p70 bound to DNA comprises two structurally homologous sub-domains, the so-called oligosaccharide/oligonucleotide binding (OB)-folds, which form the DNA binding domains A and B (DBD-A and DBD-B) [8]. The ssDNA lies in a cleft extending from one domain to the other [11]. OB-folds corresponding to other putative DNA binding domains have been mapped to the N- and C-termini of p70 (DBD-C and DBD-F) and both the p32 (DBD-D) and p14 (DBD-E) subunits [12–16]. α-helices C-terminally located to OB-fold motifs of DBD-C, -D, and -E are arranged in parallel and mediate trimerization [9].

The orchestration of SV40 in vitro DNA replication requires the interaction of RPA with the viral initiator protein T-antigen (Tag) and the cellular DNA polymerase α-primase (Pol) and these interactions were mediated mainly by p70 and p32 [17–20]. Interaction sites of different strengths have been mapped to DBD-A and N-terminal sequences of p70 and to a winged helix–loop–helix motif in the C-terminus of p32 [18,21,22].

To probe the importance of p70's interaction with the other two subunits, DNA and its replicative partner proteins we have expressed specific regions corresponding to formerly defined structural domains and sub-sequences of those [23]. These peptides displayed properties expected from previous deletion analysis. Peptide inhibition studies revealed that the cooperation of all activities of the p70 subunit is essential for the initiation process and that a stable primosome is assembled on a primer, which acts in a processive manner.

2. Materials and methods

2.1. Cloning and expression of RPA subunits

RPA subunit sequences were amplified from a cDNA library obtained with the 5′-RACE kit of GibcoBRL from human 293S cells using primer pairs that allowed for directional cloning in amplification and expression vectors. Subunits were either expressed alone or linked together in various combinations. Likewise sub-regions of p70 were expressed alone or co-transcriptionally with p14 and p32.

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2.2. Expression and purification of proteins

Baculovirus expressed Tag and Pol were immunoaffinity purified. Bacterially expressed RPA, his-tagged RPA (H_6-RPA) as well as topoisomerase I from calf thymus were purified by a combination of affinity, adsorption and ion-exchange chromatography steps. Maltose binding protein (MBP)-fusion proteins were affinity purified on amylose resin according to the manufacturer's instructions (New England Biolabs). All p70 derived fusions were soluble and concentrations of 1 mg/ml and higher were obtained. If required, fusion peptides were concentrated to 5–10 mg/ml using Centricon P-20 centrifugal filter units (Millipore).

2.3. Protein–protein interactions

17.5 pmol RPA (0.116 μg/pmol) were incubated with an equal amount of Tag or Pol, which were coupled to beads via specific antibodies, in the absence or presence of a 100-fold molar excess of peptides. The amounts of co-precipitated RPA were quantified by densitometric analysis of western blots developed with enhanced chemoluminescence (ECL, GE Healthcare). Alternatively, Tag and Pol were passed over amylose columns to which MBP-fusion proteins were coupled at a ratio of 1 mg/ml. Bound proteins were eluted and detected by western blotting using ECL.

2.4. Protein–DNA interactions

100 pmol of RPA (0.116 μg/pmol) or MBP-fusion peptides were incubated with 0.083 pmol circular single-stranded M13mp18 DNA (2.4 μg/pmol). Free DNA was separated from protein–DNA complexes by agarose gel electrophoresis. DNA was visualized by ethidium bromide staining.

2.5. Subunit complex formation

Various p70 MBP-fusion proteins were co-transcriptionally expressed with p14, p32 or both. Extracts were passed over amylose resin to capture the MBP fusion and co-eluted subunits were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting. To test for soluble proteins, crude extracts were analyzed in parallel.

2.6. SV40 in vitro DNA replication

SV40 in vitro DNA replication was carried out with S100 extracts prepared from human 239S cells using 0.1 pmol of pUC-HS DNA (2 μg/pmol), which contains the complete SV40 origin. The assay was supplemented with ATP to drive the unwinding reaction catalyzed by Tag, as well as other ribonucleotides (rNTPs) and (radioactive labeled) deoxyribonucleotides (dNTPs) to support the primer synthesis and primer extension reactions catalyzed by Pol. The mix was assembled on ice and the reaction was started by the addition of 6.25 pmol Tag (0.096 μg/pmol). Incubation lasted for 120 min at 37 °C. Five μl of the reaction were spotted on DE81 paper to quantify incorporated nucleotides. After removal of proteins from the rest of the sample by phenol/chloroform extraction DNA was ethanol precipitated and double restricted with EcoRI to linearize the DNA (form II) and DpnI to remove any un-replicated template DNA. Products were separated by agarose gel electrophoresis and analyzed by autoradiography. Peptides were added at different time points to the reaction at a 5–100-fold molar excess over Tag. Alternatively, the replication reaction was stalled at the unwinding, primer synthesis and primer extension steps by omitting ATP, other rNTPs and dNTPs, respectively. Peptides added to these reactions were pre-incubated with the mix for 30 min prior to the addition of the missing nucleotides to relieve the replication block.

2.7. DNA unwinding

For bidirectional unwinding reactions 6.25 pmol Tag (0.096 μg/pmol) were incubated with 0.1 pmol supercoiled SV40-origin containing pUC-HS DNA in the presence of 1.2 pmol toposiomerase I (0.1 μg/pmol) and 4 pmol RPA (0.116 μg/pmol) Where indicated, RPA was replaced by the same amounts of MBP-fusion peptides. Alternatively peptides were added in addition to RPA. Under-wound DNA (form U) was detected by agarose gel electrophoresis and ethidium bromide staining and quantified by densitometric scanning.

Detailed protocols can be obtained in the methods section of the Supplementary data.

3. Results

3.1. Activities of p70 peptides

To probe the importance of different parts of the p70 subunit of replication protein A (RPA) for the replication process we have expressed different domains and sub-regions thereof as soluble maltose binding protein (MBP) fusion proteins. Amino acids 174–330 were sufficient for DNA binding, 1–173 for binding DNA polymerase α-primase (Pol), 174–250 for binding both SV 40 large T-antigen (Tag) and Pol, and 421–525 as well as 525–616 for complex formation with the other two smaller subunits (for a detailed characterization see results section and Figs. 1 and 2 of the Supplementary data).

All fragments containing amino acids 174–250 reduced the amounts of RPA co-precipitated with Pol and Tag, respectively (Fig. 1). Peptide 1–173 reduced the co-precipitated RPA only to a minor extent if tested with Pol, but showed no effect on RPA's interaction with Tag. C-terminal derived peptides did not reduce the amounts of co-precipitated RPA.

Peptide 174–250 was chosen to probe the importance of protein–protein interactions between replicative factors during cell

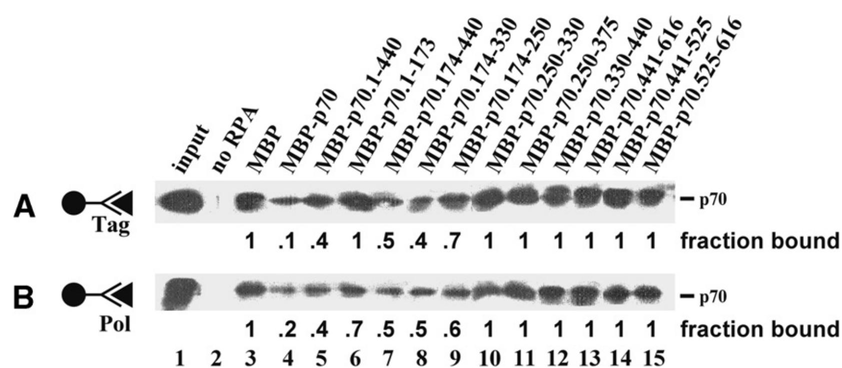

Fig. 1. Interference of p70 peptides with protein interactions. Co-precipitations of 17.5 pmol soluble H_6-RPA were performed with the same amounts of immobilized Tag (Panel A) and Pol (Panel B) in the presence of a 100-fold molar excess of the indicated p70 competitor polypeptides. Bound H_6-RPA was detected with his-tag specific antibodies by western blotting and ECL detection. The numbers below the lanes refer to the fractions of H_6-RPA bound in relation to the amount obtained in the presence of MBP. The position of the p70 subunit bearing the histidin-tag is indicated. Lane 1 represents a loading control of 1/10th of the input material, whereas in lane 2 no RPA was added.

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