



Implication of RPA32 phosphorylation in S-phase checkpoint signalling at replication forks stalled with aphidicolin in *Xenopus* egg extracts

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ABSTRACT

Activation of the replication checkpoint relies upon uncoupling of DNA polymerases and helicase activities at replication forks, which in multicellular organism results in production of long stretches of single-stranded DNA bound by the trimeric, single stranded DNA binding protein, the RPA complex. Binding of RPA to this substrate promotes synthesis of replication intermediates that contributes to checkpoint activation by allowing binding of the 9–1–1 checkpoint clamp. The RPA32 kDa subunit is also phosphorylated during this process but its role in checkpoint signalling is unclear. Here we have investigated the requirement for RPA32 phosphorylation in checkpoint activation in *Xenopus* egg extracts. We show that phospho-deficient mutants of RPA32 stimulate checkpoint signalling at replication forks arrested with aphidicolin at both the initiation and the elongation step of DNA replication, without affecting DNA synthesis. In contrast, we show that phospho-mimetic RPA32 mutants do not stimulate checkpoint activation at unwound forks. These results indicate that the hypophosphorylated, replication fork-associated form of RPA32 functions in S-phase-dependent checkpoint signalling at unwound forks in *Xenopus* egg extracts while RPA32 phosphorylation may be implicated in other pathways such as repair or restart of arrested replication forks.

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1. Introduction

Checkpoints are feedback mechanisms that coordinate cell cycle phases thus ensuring that each phase of the cell cycle will be faithfully completed before the onset of the next one. DNA damage triggers activation of specific checkpoints that delay the cell cycle to facilitate DNA repair. Detection and signalling of DNA damage during S-phase is crucial in maintenance of genomic stability. This checkpoint, also known as replication checkpoint, depends upon the ATR kinase, a member of the PIKK family ([1] for review). Inhibitors of replicative polymerases, such as aphidicolin, or inhibitors of nucleotide synthesis (hydroxyurea), inhibit the enzymatic activity of DNA polymerases, however the helicase activity is not affected. The result of this enzymatic uncoupling is the production of single-stranded DNA (ssDNA) generated by DNA unwinding by the helicase [2–5]. This DNA substrate is bound by the major eukaryotic ssDNA binding protein, the trimeric RPA complex, that stimulates replicative DNA polymerases to synthesise small repli-

cation intermediates that are binding sites for the essential checkpoint clamp, the 9–1–1 complex [1,4,6–9]. The interaction of 9–1–1 with ATR bound to its partner, the ATRIP protein, and the TopBP1 protein promotes ATR activation. Some types of DNA damage, such as interstrand crosslinks, natural replication forks barriers or specialized chromatin structures, also arrest the progression of the helicase, so that no replication fork uncoupling is produced [10]. In this situation checkpoint activation occurs in a single stranded-independent way since replication forks are not unwound. Both events lead to a more or less pronounced arrest of the replication fork, also known as replication stress.

RPA is a complex made of three unrelated subunits, of 70, 32 and 11 kDa that plays an essential role in initiation and elongation step of DNA synthesis [2,11,12]. In addition RPA also participate in DNA repair, recombination and checkpoint activation [13]. We have very recently shown that RPA nucleation at stalled forks is dispensable for checkpoint activation [7] leaving the open question about the function of extensive RPA nucleation at arrested forks. The RPA32 subunit is phosphorylated at the N-terminus at forks arrested with hydroxyurea [14], and upon DNA damage [15–18] but the role of this phosphorylation in activation of the replication checkpoint is unclear. A previous work in mammalian cells has demonstrated that phospho-deficient RPA32 mutants associates with replication foci and forks arrested with hydroxyurea, while

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phospho-mimetic mutants of RPA32 do not, instead they appear to associate with proteins implicated in DNA repair and/or replication forks restart [19].

Using cell-free extracts derived from activated *Xenopus* eggs that faithfully reproduce S-phase in a very synchronous fashion *in vitro*, we have explored the role of RPA32 subunit phosphorylation on two CDK and one ATM/ATR consensus sites on checkpoint activation and DNA synthesis. Our results show that these RPA32 phosphorylation mutants do not interfere with extensive recruitment of RPA at arrested forks. Moreover we show that phosphorylation-deficient, replication fork-associated RPA2, and not constitutive phosphorylated RPA32 stimulate checkpoint activation when replication forks are stalled with aphidicolin. Finally we also show that these mutants do not affect the progression of DNA synthesis in the absence of replication stress, suggesting that phosphorylation of RPA32 at forks arrested with aphidicolin is not implicated in activation of replication checkpoint.

2. Materials and methods

2.1. *Xenopus* egg extracts

Cytoplasmic extracts were prepared as previously described [20], snap frozen in liquid nitrogen and stored at -80°C . Upon thawing, extracts were supplemented with cycloheximide (250 $\mu\text{g}/\text{ml}$) and an energy regeneration system (1 mM ATP, 2 mM MgCl_2 , 10 mM creatine kinase, 10 mM creatine phosphate). For RPA depletion experiments, extracts were incubated with specific antibodies at 4°C for 40 min twice. Depleted extracts were reconstituted with energy regeneration system, and sperm nuclei (2000 nuclei/ μl of extract). When required egg extracts were supplemented with 50 $\mu\text{g}/\text{ml}$ of aphidicolin (Sigma).

2.2. DNA replication assay

Egg extracts were supplemented with α -[^{32}P] dCTP (3000 Ci/mmol, Perkin Elmer). At the indicated time points samples were neutralised in 10 mM EDTA, 0.5% SDS, 200 $\mu\text{g}/\text{ml}$ Proteinase K (Sigma) and incubated at 37°C over night. Incorporation of radioactive label was determined by TCA-precipitation on GF/C glass fibre filters (Wathmann) following by scintillation counting.

2.3. Antibodies

Anti-RPA32. and RPA70 antibodies were previously described [7,11]. Human RPA32 and RPA70 antibodies were from AbCam. Histone H3 antibody was from Abcam. Anti- $\text{P-S}^{345}\text{Chk1}$ antibody was from Cell Signalling. ORC2 and Cdc45 antibodies were a kind gift of Marcel Méchali (Institute of Human Genetics). Proteins transferred to nitrocellulose membranes were probed with specific antibodies using the SNAPi.d.[®] Western blot system (Millipore). Detection of Western blot signals was obtained by enhanced chemio-luminescence (ECL) using the Luminata[®] reagent (Millipore).

2.4. Immunological methods

RPA antibodies were covalently coupled to recombinant Protein A beads. Complete depletion of RPA was achieved by incubating one volume of egg extract with 60% of antibodies (v/v). For reconstitution experiments, RPA completely-depleted extracts were supplemented with 100 and 200 $\mu\text{g}/\text{ml}$ of recombinant human RPA complex.

2.5. Recombinant proteins

Recombinant *Xenopus* RPA32 wild-type and mutant proteins were previously described [21]. The recombinant human RPA complex was a kind gift of U. Ubscher (University of Zurich, Switzerland).

2.6. Subcellular fractionation procedures

Egg extracts supplemented with demembrated sperm nuclei were diluted 10-fold with ice-cold Xb buffer (10 mM Hepes pH 7.7; 100 mM KCl; 50 mM sucrose; 2 mM MgCl_2 , 5 mM leupeptine, apro-tinin and pepstatin) and centrifuged at 1500g in a Sorvall centrifuge at 4°C for 5 min to sediment nuclei. Nuclei were washed once in ice-cold Xb and detergent-extracted with 0.1% NP-40 for 5 min on ice. Chromatin (pellet) and nucleosolic (supernatant) fractions were obtained by centrifugation at 6000g for 5 min at 4°C . Phosphorylation of Chk1 was detected in nucleosolic fraction.

3. Results and discussion

3.1. Phosphorylation of RPA32 at CDK and ATR consensus sites is not required for RPA nucleation at forks arrested with aphidicolin

In mammalian cells phospho-deficient RPA2 mutants activate normally the ATR-dependent checkpoint [22]. The role of phospho-mimetic mutants was not previously investigated, although it has been reported that phosphorylated RPA32 interacts

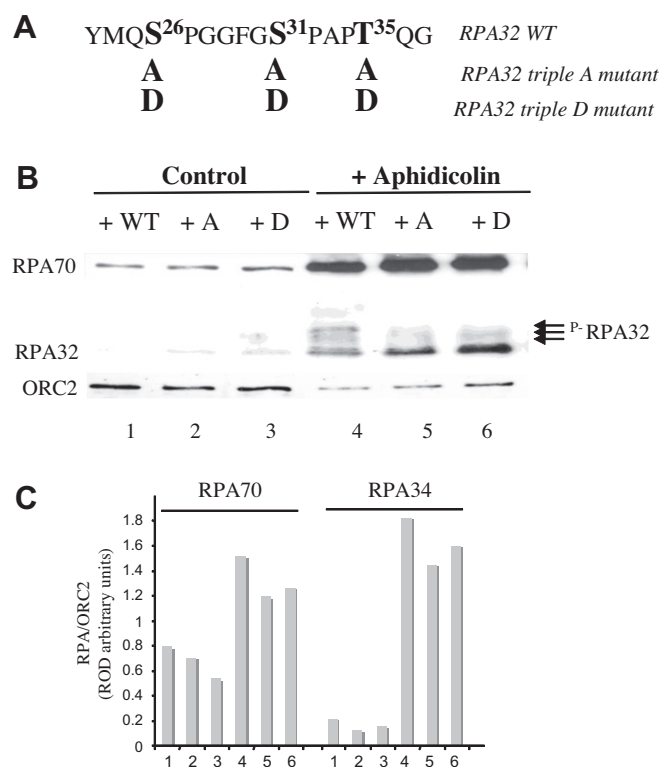


Fig. 1. RPA phosphorylation mutants do not affect the nucleation of RPA at arrested forks. (A) Schematic representation of the *Xenopus* RPA32 N-terminus wild-type, or triple phospho-deficient ("A" mutant) or phospho-mimetic ("D" mutant) mutants in the indicated residues. (B) Western blot of chromat fractions obtained as described in materials and methods upon incubation of sperm chromatin with (+) or without (control) aphidicolin in the presence of different RPA32 recombinant proteins. (C) Quantification of RPA32 or RPA70 accumulation onto chromatin observed in the experiment of panel B normalised to ORC2 loading control. Numbers correspond to lanes of panel B.

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