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Replication Protein A: Single-stranded DNA's first responder : Dynamic DNA-interactions allow Replication Protein A to direct single-strand DNA intermediates into different pathways for synthesis or repair

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Summary

Replication Protein A (RPA), the major single-stranded DNA-binding protein in eukaryotic cells, is required for processing of single-stranded DNA (ssDNA) intermediates found in replication, repair and recombination. Recent studies have shown that RPA binding to ssDNA is highly dynamic and that more than high-affinity binding is needed for function. Analysis of DNA binding mutants identified forms of RPA with reduced affinity for ssDNA that are fully active, and other mutants with higher affinity that are inactive. Single molecule studies showed that while RPA binds ssDNA with high affinity, the RPA complex can rapidly diffuse along ssDNA and be displaced by other proteins that act on ssDNA. Finally, dynamic DNA binding allows RPA to prevent error-prone repair of double-stranded breaks and promote error-free repair. Together, these findings suggest a new paradigm where RPA acts as a first responder at sites with ssDNA, thereby actively coordinating DNA repair and DNA synthesis.

Keywords

DNA replication; DNA repair; Recombination; single-strand DNA-binding protein; DNA-interactions

Introduction

Single-stranded DNA binding proteins (SSBs) are essential in all organisms. Originally, SSBs were thought to function by coating single-stranded DNA (ssDNA) to prevent formation of secondary structure and protect from degradation by nucleases. Later studies showed that both bacterial and eukaryotic SSBs interact with specific protein partners to promote efficient processing of single-stranded intermediates in DNA replication, repair and recombination [1,2] and have multiple modes of interacting with ssDNA [3].

In eukaryotic cells, the major single-stranded DNA binding protein is Replication Protein A (RPA). Several recent studies have drastically changed our understanding of how RPA interacts with ssDNA and functions in cells. First analysis of DNA binding mutants has

shown that affinity for ssDNA (as measured by binding to oligonucleotides) does not directly correlate with RPA activity [4,5]. In addition, single molecule studies have shown that RPA binding to ssDNA is highly dynamic [6,7] and suggest that this dynamic binding reduces processing by error-prone pathways while promoting recombination repair of double-strand breaks (DSBs) in yeast [8]. This suggests that the RPA-ssDNA complex plays an active role in determining how different ssDNA intermediates are channeled into selected pathways. Finally, it has been shown that deficiencies in RPA lead to elevated mutation rates, genome instability and -- in extreme cases -- catastrophic genome damage [9]. In this article we give an overview of these recent findings and discuss how they suggest a new paradigm for RPA function.

RPA is the first responder in the cells when DNA metabolism is disrupted

RPA is required for cellular replication, repair, and recombination [1,10,11]. RPA also functions in coordination of the cellular response to DNA damage, and is required for activation of cellular checkpoints [12-14]. The common feature of all pathways requiring RPA is that each has ssDNA intermediates; however, different pathways have intermediates that differ in length, adjacent structures (e.g. DNA end vs. duplex DNA) and associated proteins. This means that RPA must be able to recognize and facilitate the selective processing of diverse ssDNA intermediates.

RPA is an abundant protein in cells, and it binds to ssDNA with subnanomolar (nM) affinity [15,16]. Thus, any single-stranded region formed in genomic DNA is immediately bound by RPA. The resulting RPA-ssDNA complex then interacts with protein partners to coordinate the processing of the ssDNA [1,11]. The mechanism by which RPA is able to direct different ssDNA intermediates to different pathways and coordinate replication, recombination and repair is not understood.

RPA has a highly flexible structure

RPA is composed of three subunits of 70-, 32-, and 14-kDa (RPA1, RPA2, and RPA3, respectively) [1,10,17]. Each of the RPA subunits contains one or more DNA-binding domains (DBD) composed of an oligonucleotide/oligosaccharide-binding fold [18]. DBDs are designated with letters A-F (Figure 1A). The three subunits of RPA form a very stable complex with one DBD in each subunit interacting to form the trimerization core (DBD-C, -D, -E) [19]. All the other parts of RPA extend from the trimerization domain on flexible protein linkers. The flexible, often long, unstructured linkers allow the other domains in RPA to rotate independently and to adopt a variety of conformations [20].

RPA1 contains four DBDs (Figure 1A). DBD-F at the N-terminus of RPA (also known as the N-terminal domain) can interact with DNA but is thought to primarily function as a regulatory, protein-interaction domain [4,13,21]. DBD-A, -B, and -C are primarily required for binding ssDNA but also interact with protein partners. DBD-C is part of the trimerization core [19]. DBD-A has 5-10 fold higher affinity for ssDNA ($K_d=1.7 \mu\text{M}$) than the other DNA binding domains (DBD-B $K_d=16 \mu\text{M}$) [22,23]. In addition the short linker that connects DBD-A and DBD-B allows these two domains to act as a high affinity binding site with an affinity ~ 100 fold higher than isolated domains [23].

RPA2 is composed of two structured domains: a central DNA binding domain (DBD-D) and a C-terminal winged helix domain (wh) (Figure 1A). DBD-D interacts with ssDNA and is part of the trimerization core while the winged helix domain is primarily involved in protein interactions. In addition, the N-terminal domain of RPA2 (called the phosphorylation domain; Pd) is unstructured and becomes multiply phosphorylated after DNA damage [11,24].

RPA3 is composed exclusively of an OB-fold (DBD-E) that interacts weakly with DNA [25] and is part of the trimerization core [26].

Cellular RPA levels, genome stability, and prevention of replication catastrophe

Loss of any of the subunits of RPA is lethal [4,27], and non-lethal mutations in RPA can cause DNA repair defects and genome instability [28-30]. It is also clear, that while under normal circumstances the cellular pool of RPA is sufficient for all required DNA transactions, reduction in the cellular level of RPA is deleterious [4,31]. In mice, haploinsufficiency of RPA causes a high rate of lymphoid tumors and a shortened lifespan [32,33]. In humans, microdeletions or microduplications of the RPA1 gene that lead to moderate changes in protein levels cause defects in the cellular DNA damage response [34,35].

The essential role of RPA in preserving genome stability was highlighted in the recent paper by Toledo and coworkers [9]. Under replication stress, the number of ssDNA regions is drastically increased due to fork stalling. RPA is recruited to these sites where it helps stabilize the stalled forks and signal for ATR activation to stop the cell cycle and ongoing replication [36]. Under normal conditions, RPA binding to stalled replication forks allows eventual reestablishment of replication [37-39]. Toledo and his colleagues used high throughput microscopy to monitor RPA binding and chromosome breakage in cells under replication stress [9]. They showed that if the level of ssDNA becomes high enough that there is insufficient RPA (called “RPA exhaustion”) there is rapid conversion of the single-stranded DNA to double-strand breaks, which causes replication catastrophe and cell death. RPA exhaustion depends on the relative levels of RPA and single-stranded DNA. Cells with reduced levels of RPA experience catastrophe at lower levels of ssDNA/replication stress, and over expression of RPA allows cells to tolerate higher levels of stress. Furthermore, the ATR DNA damage check point plays a role preventing RPA exhaustion: RPA-bound ssDNA tracks activate ATR, which inhibits processes that generate ssDNA, such as new origin activation.

RPA flexibility and DNA binding modes of RPA

RPA binds single-stranded DNA with high affinity; however, the flexibility of the RPA complex has made it difficult to study RPA binding to DNA structurally. It was only in 2012 that the structure of RPA from *Ustilago maydis* stably bound to single-stranded DNA was determined (Figure 1B; [40]). This structure and other biochemical analysis show that four

DBDs in RPA (A-D) form a stable complex with ~30 nt of ssDNA. RPA binds ssDNA directionally with DBD-A at the 5' end and DBD-D at the 3' end of the complex [40-42].

Each of the DNA-binding domains interacts with approximately 4-6 nucleotides of single-stranded DNA [43]. Early reports suggested that there were multiple modes of RPA binding, including an unstable 8 nt binding mode [44] and a stable 30 nt binding mode [15,44]. The 8-10 nt binding mode has only been observed after cross linking [44] or when RPA interacts with very short oligonucleotides [45]. In contrast, detailed thermodynamic analysis of RPA binding identified only 18-20 and 28-30 nt binding modes [7,16]. It seems likely that these two stable modes of binding represent 3 and 4 DBDs interacting with DNA (Figure 1A). So the current paradigm is that RPA binds single-stranded DNA by sequentially engaging DNA-binding domains, hence creating more stable complexes as more DNA was bound. This simple model accounts for many of the properties of RPA binding: the affinity of RPA decreases as DNA length decreases [15], and RPA adopts different conformations depending on the length of DNA bound[45]. However, the recent studies of RPA, discussed below, suggest that the interaction of individual DNA-binding domains is not sequential, and that RPA-binding is better described by a dynamic model.

RPA binding to ssDNA is dynamic

Recently, two papers have shown that RPA interactions with DNA are highly dynamic and suggest that microscopic interactions of individual DNA-binding domains may contribute significantly to RPA function. Gibb and coworkers used single molecule imaging of yeast RPA on single-strand DNA curtains to examine RPA binding at a molecular level [6]. When RPA binds ssDNA, it forms a stable complex that very rarely dissociates. However, if there is free RPA or other ssDNA-binding proteins present, bound RPA was found to rapidly exchange. This suggests that in the presence of other ssDNA-binding proteins, RPA can be rapidly removed from the DNA. The second study, by Nguyen and coworkers, analyzed individual molecules of RPA bound to single-stranded DNA [7]. This analysis showed that human RPA rapidly diffuses along single-stranded DNA without dissociating. The rate of diffusion of RPA ($\sim 5000 \text{ nt}^2 \text{ sec}^{-1}$ at 37°C) is ten times faster than that of *E. coli* SSB [7]. This is consistent with the two proteins having different mechanisms of diffusion and RPA binding being more dynamic. Furthermore, these studies showed that this rapid diffusion was productive, and promoted the destabilization of adjacent DNA hairpins [7]. Both studies suggest that the DNA binding properties of RPA are the result of having multiple DBDs linked in a flexible structure interacting with ssDNA. The microscopic affinity of each DBD is modest, but together they give the complex very high affinity (a subnanomolar macroscopic dissociation constant.)

Together, these binding studies suggest a dynamic model of RPA binding in which RPA binds stably to ssDNA tracks but is able to rearrange its conformation as individual DBDs microscopically dissociate and rebind (Figure 2). In this model, microscopic rearrangement allows RPA (i) to adopt different conformations on ssDNA, (ii) to rapidly diffuse along ssDNA, (iii) to destabilize secondary structure and short duplex regions, and (iv) to provide small single-stranded regions that can act as nucleation sites for the binding of other proteins (leading to RPA displacement) (Figure 2). It seems likely that this dynamic binding allows

RPA to bind to different ssDNA intermediates, and it could contribute to RPA directing intermediates into different pathways for DNA synthesis or repair.

Not all RPA binding to ssDNA is equivalent

This dynamic model for RPA binding to ssDNA may help explain a persistent mystery regarding RPA activity. This mystery is that RPA affinity for single-stranded oligonucleotides does not directly correlate with RPA function. Mutational analysis of the DNA-binding sites in RPA has generated forms with reduced ssDNA-binding activity. In this class, some mutations that reduce the affinity of the complex by two orders of magnitude are fully functional in cells, while other mutations that have a higher affinity for oligonucleotides are partially or completely inactive [4]. In particular, mutation of conserved aromatic residues in the DNA binding sites of DBD-A or -B cause a separation-of-function phenotype: the mutants support DNA replication but are defective in DNA repair [5]. These results suggest that high affinity for ssDNA is not sufficient for the full function of RPA, and that replication and repair require different RPA-DNA interactions. It now seems likely that the observed loss in activity arises because the mutants are affecting the kinetics or microscopic interactions of individual DBDs in the dynamic RPA-ssDNA complex. These data suggest that altering the dynamics in the RPA-ssDNA complex affects activity without a comparable effect on the macroscopic affinity constant.

Dynamic DNA binding and RPA function

Recent functional analysis indicates that the dynamic interactions of RPA with ssDNA help regulate repair pathway selection during the repair of double-strand breaks (DSBs). Homology-mediated recombination (HR) is the major, error-free repair pathway for repairing DSBs in budding yeast. In HR, the 5' strand of the double strand break is resected to produce a 3' overhang [46,47]. RPA participates in resection and then coats the resulting 3' overhang (Figure 3). Subsequent protein interactions with recombination mediators result in the loading of Rad51 that, in turn, catalyzes recombination. HR repair is error-free with no loss of genetic material [48].

After resection of a DSB, a second (alternative) pathway, microhomology-mediated end joining (MMEJ), competes with HR to fix the break. In MMEJ, short homologous single-stranded regions (between eight and twenty nucleotides in length) on the 3' overhangs anneal, the DNA flaps are excised, gaps filled in and the DNA ligated [49]. This pathway is error-prone; any DNA sequences between the regions of microhomology are lost during repair.

A recent paper by Deng and co-workers shows that RPA binding influences the choice of the pathway used in DSB repair: inhibiting MMEJ and stimulating HR [8]. Deng used a novel genetic system that generated two specific double-strand breaks flanked by repeat sequences in an *ADE2* marker gene, and then monitored the phenotype of the repaired junctions to determine the frequency of repair by different pathways. In normal cells, a majority of the repair was by the error-free HR pathway, with very little MMEJ repair occurring. Cells expressing hypomorphic mutants of RPA were also examined. Expression of a point mutant in DBD-F (*rfa1-t11*) -- which functions in replication but is deficient in

recombination and DNA repair [50] -- did not increase the frequency of MMEJ. In contrast, expression of mutant forms with point mutations in the DNA-interaction domains had a much high frequency of MMEJ. (Cells expressing some mutant forms of RPA had more than 300-fold increase in MMEJ.) The mutant forms of RPA that correlated with high MMEJ frequency were also shown to dissociate more rapidly from DNA, and were partly defective in destabilizing (unwinding) duplex DNA. These results suggest that RPA binding inhibits MMEJ by preventing the annealing microhomology regions, and that the dynamic binding of RPA is needed for destabilizing the microhomology regions (Figure 3).

Regulation of RPA binding and remaining questions

It now appears that dynamic RPA interactions are critical for correct processing of different single-stranded DNA intermediates in the cell. However, it is still not clear how the different domains in RPA contribute to these properties. In addition, the mechanism by which mutations that affect the binding of one DBD specifically disrupt DNA repair without affecting DNA replication is not known. More detailed analysis of RPA-DNA interactions, particularly the dynamics of the RPA-DNA complex, is needed in order to understand the contributions of individual DBDs to RPA binding.

RPA is required for telomere maintenance [51-53]. RPA has been shown to bind specifically to telomere sequences and to destabilize G-quadruplex structures [54,55]. It is likely that the dynamic binding of RPA is needed for these activities.

RPA interacts with a number of protein partners in DNA replication, repair, recombination and checkpoint pathways. These interactions are essential for RPA function in these pathways. Most RPA-protein interactions involve parts of the protein outside the DNA-binding sites in DBD-A-D. This suggests a model in which RPA-protein interactions may be directly modulating DNA-binding by regulating individual DBDs or altering the conformation of RPA. It has been previously suggested that protein interactions with DBD-F (the N-terminal domain of RPA1) or with the winged helix domain of RPA2 modulate checkpoint activation and replication, respectively [1,13,14,56,57].

RPA is post-translationally modified in cells with DNA damage. RPA is hyper-phosphorylated in response to DNA damage [11]. There are multiple phosphorylation sites at the N-terminus of RPA2 and several other less characterized phosphorylation sites on the other subunits [11,58]. RPA phosphorylation is important for recovery from DNA damage and replication stress in S phase [36,39,59], and genotoxic stress in mitosis [39,60]. RPA phosphorylation has also been suggested to regulate homologous recombination after replication arrest [39,61,62]. It is known that phosphorylation modulates both protein and DNA interactions [63,64] but how these changes regulate the cellular DNA damage response remain poorly understood.

RPA is also SUMOylated at lysine residues in the C-terminal domain of RPA1 in response to DNA damage [65]. Mutation of the sites of SUMOylation (K449 and K577) causes cells to be more sensitive to DNA damage [65]. In addition, SUMOylation of RPA appears to stimulate loading of RAD51 at sites of DNA damage [65]. Again, the mechanism of regulation of RPA by SUMOylation is not known.

It seems likely that RPA-protein interactions and post-translational modifications of RPA could be regulating function by affecting the conformations or dynamics of RPA-ssDNA complexes. It is intriguing to speculate that RPA is dynamically integrating DNA interactions, interactions with protein partners, and cell signals (through post-translational modifications) to correctly triage ssDNA into the appropriate pathway for DNA synthesis or repair.

Conclusions

The binding of RPA to single-stranded DNA is essential for genome duplication and stability. RPA binds to single-stranded DNA with high affinity and forms a stable complex with ~30 nucleotides. Formation of the stable RPA-ssDNA complex requires the direct interaction of up to four domains of RPA with single-stranded DNA. The DNA-binding domains in RPA are linked by flexible polypeptides and the RPA-DNA complex has recently been shown to be highly dynamic. It appears that dynamic interactions between individual DBDs and DNA allow RPA to change conformation, rapidly diffuse along ssDNA and efficiently destabilize partly duplex structures. These properties also allow efficient displacement of RPA by other DNA binding proteins. We suggest that dynamic RPA binding actively helps channel different ssDNA intermediates into separate pathways in the cell.

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Abbreviations

RPA	Replication Protein A
ssDNA	single-stranded DNA
DBD-DNA	binding domain
ATR	Ataxia telangiectasia and Rad3 related
DSB	double-strand break
HR	homology-mediated recombination
MMEJ	micro homology-mediated end joining

Figure 1. Structure and ssDNA-binding of RPA

(A) Schematic representation of RPA showing proposed 20 and 30 nucleotide binding modes. RPA domains include DNA binding domains (DBD-A-F), the phosphorylation domain (Pd), winged-helix domain (wh) and linker regions (lines). Stable binding of RPA is thought to involve three or four DBDs interacting with DNA. (B) The structure of *Ustilago maydis* RPA in complex with ssDNA, with the position of specific DBDs in the larger structure indicated [40]. RPA1 is colored in green, RPA2 in blue, RPA3 in red. Single-stranded DNA is represented by a black line.

Figure 2. Dynamic model of RPA binding to ssDNA

In this model, stable macroscopic binding of RPA to ssDNA includes constant, microscopic dissociation and rebinding of individual and subsets of the DBDs (top set of schematics). This rapid binding allows the RPA complex to rearrange and diffuse along ssDNA without dissociation. It also allows RPA to promote helix destabilization at adjacent duplex or hairpin structures (bottom left and right). Microscopic dissociation also generates transient ssDNA regions that provide nucleation sites for other ssDNA-binding proteins that cause displacement of RPA (bottom middle).

Figure 3. RPA regulation of double-strand break repair

Dynamic binding of RPA prevents spontaneous annealing of microhomologous sequences during HR-mediated repair of DSBs (after [8]). Upon formation of a double-strand break, the ends are resected to generate 3' overhangs that are bound by RPA (ovals). DNA sequences with microhomology (orange lines) are exposed as the resection proceeds. RPA binding stimulates error-free HR by interacting with Rad51 and other recombination mediators (not shown) to promote homologous strand invasion and stabilize the displaced strands during recombination. Dynamic binding is predicted to promote displacement of RPA by Rad51. In contrast, dynamic binding of RPA prevents annealing of microhomologous sequences and, thus, inhibits MMEJ.