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Eukaryotic Single-Stranded DNA Binding Proteins: Central Factors in Genome Stability

Sandra Broderick, Kristina Rehmet, Claire Concannon, and Heinz-Peter Nasheuer

Abstract The single-stranded DNA binding proteins (SSBs) are required to maintain the integrity of the genome in all organisms. Replication protein A (RPA) is a nuclear SSB protein found in all eukaryotes and is required for multiple processes in DNA metabolism such as DNA replication, DNA repair, DNA recombination, telomere maintenance and DNA damage signalling. RPA is a heterotrimeric complex, binds ssDNA with high affinity, and interacts specifically with multiple proteins to fulfil its function in eukaryotes. RPA is phosphorylated in a cell cycle and DNA damage-dependent manner with evidence suggesting that phosphorylation has an important function in modulating the cellular DNA damage response. Considering the DNA-binding properties of RPA a mechanism of "molecular counting" to initiate DNA damage-dependent signalling is discussed. Recently a human homologue to the RPA2 subunit, called RPA4, was discovered and RPA4 can substitute for RPA2 in the RPA complex resulting in an "alternative" RPA (aRPA), which can bind to ssDNA with similar affinity as canonical RPA. Additional human SSBs, hSSB1 and hSSB2, were recently identified, with hSSB1 being localized in the nucleus and having implications in DNA repair. Mitochondrial SSBs (mtSSBs) have been found in all eukaryotes studied. mtSSBs are related to prokaryotic SSBs and essential to main the genome stability in eukaryotic mitochondria. Recently human mtSSB was identified as a novel binding partner of p53 and that it is able to stimulate the intrinsic exonuclease activity of p53. These findings and recent results associated with mutations in RPA suggest a link of SSBs to cancer.

Keywords DNA replication · DNA repair · DNA damage response · Single-stranded DNA · Signalling

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Abbreviations

9-1-1 complex	Rad9-Hus1-Rad1 complex (fission yeast and human) equivalent to the Rad17-Mec3-Ddc1 complex in budding yeast
aRPA	alternative replication protein A
A-T	Ataxia telangiectasia
ATM	ataxia telangiectasia-mutated
ATR	ATM-Rad 3-related protein
ATRIP	ATR-interacting protein
BER	base excision repair
BRCA1/2	breast cancer-associated protein 1/2
CDK	cyclin-dependent kinase
DBD	DNA-binding domain
DNA-PK	DNA-dependent protein kinase
DDR	DNA damage response
DSB	double-strand break
dsDNA	double-stranded DNA
E. coli	*Escherichia coli*
HR	homologous recombination
mt	mitochondrial
MBP	maltose binding protein
MMR	mismatch repair
NER	nucleotide excision repair
OB-fold	oligosaccharide/oligonucleotide-binding fold
PCNA	proliferating cell nuclear antigen
PIKK	phosphoinositol-3 kinase-like protein kinase
Pol α	DNA polymerase α–primase
Pol δ	DNA polymerase δ
Pol ε	DNA polymerase ε
Pot1	protector of telomeres 1
RAD	Radiation-induced mutation
RPA	replication protein A
ROS	reactive oxygen species
SSB	single-stranded DNA-binding protein
ssDNA	single-stranded DNA
S. cerevisiae	*Saccheromyces cerevisiae*
SV40	simian virus 40
TopBP1	topoisomerase II-binding protein 1

General Overview

Single-stranded DNA binding proteins (SSBs) are involved in almost every aspect of eukaryotic DNA metabolism including DNA replication, DNA recombination and all major types of DNA repair such as nucleotide excision, base excision,

double-strand break, and DNA mismatch repair (NER, BER, DSBR, and MMR, respectively) (Binz et al., 2004; Fanning et al., 2006; Wold, 1997). However, RPA is also a major player in DNA damage signalling. SSBs are not only essential to maintain the integrity of the genome in the nucleus of eukaryotic cells but they have also been found in eukaryotic mitochondria as being essential for the DNA metabolic activities in this cellular compartment. Recently the long established view, that the heterotrimeric protein complex replication protein A (RPA) is the only nuclear SSB whereas the mitochondrial SSB (mtSSB) is the only other eukaryotic SSB and has exclusively functions in mitochondria, has been challenged: an "alternative" form of heterotrimeric RPA (aRPA) was found and biochemically analysed, in which the second largest canonical RPA subunit RPA2 is substituted by a homologue RPA4 (Keshav et al., 1995; Mason et al., 2009). The heterotrimeric complex aRPA binds to ssDNA but seems not to be associated with any DNA repair pathway tested whereas aRPA rather inhibits the activity of canonical RPA in simian virus 40 (SV40) DNA replication *in vitro* (Keshav et al., 1995; Mason et al., 2009). These differential activities of RPA and aRPA might open new avenues in the regulation of DNA metabolic pathways. Moreover, Richard et al. (2008) reported two additional human genes coding for SSBs, hSSB1 and hSSB2. They are located on chromosomes 12q13.3 and 2q32.3, respectively. One of them, hSSB1, has been characterized in some detail and its domain organization is more closely related to archael SSB than to eukaryotic RPA (Richard et al., 2008). Detailed biochemical and cell biological analyses of hSSB1 suggested that the protein may play a role in DNA repair (Richard et al., 2008). In addition, human mtSSB may have additional functions to its well-known role in the mitochondrial DNA replication since human mtSSB was determined as a protein-binding partner of p53 tumour suppressor and able to stimulate the exonuclease activity of p53 (Mummenbrauer et al., 1996; Wong et al., 2009). This review provides a summary of the present knowledge of the structures, functions and activities of eukaryotic SSBs as well as their impact on human diseases. Taking the properties of RPA-DNA complexes into consideration the review discusses a "molecular counting" mechanism involved in the initiation of DNA damage signalling.

Replication Protein A

RPA is a heterotrimeric complex composed of three subunits with a size of 70, 32 and 14 kDa, which are known as RPA70, RPA32 and RPA14 or alternatively RPA1, RPA2 and RPA3, respectively, with all three subunits being essential in yeast (Binz et al., 2004; Fanning et al., 2006; Wold, 1997). RPA was first identified in human cell extracts as an essential factor in SV40 DNA replication but RPA complexes have been since determined in all eukaryotes with conserved subunit structure and amino acid sequences (Binz et al., 2004; Fanning et al., 2006; Wold, 1997). Prior to the detection of RPA, several proteins binding to ssDNA cellulose were identified in biochemical analyses but RPA was the first found to be directly associated with DNA metabolism (Fairman and Stillman, 1988; Grosse et al., 1986; Jong et al.,

1985; Wobbe et al., 1987; Wold and Kelly, 1988). RPA preferentially interacts with single-stranded DNA (ssDNA) in a high affinity mode whereas it binds with a much lower binding affinity to double-stranded DNA (dsDNA) and RNA (Nasheuer et al., 1992; Wobbe et al., 1987; Wold and Kelly, 1988). RPA is known to be a crucial component in DNA replication, DNA recombination and DNA repair (Braet et al., 2007; Wold, 1997; Iftode et al., 1999).

During chromosomal DNA replication RPA is associated with the initiation and elongation process (Wold, 1997; Iftode et al., 1999). In the eukaryotic cell cycle RPA is necessary for activation of the pre-replication to form the initiation complex and for the ordered loading of essential initiator functions, e.g. the DNA polymerase α–primase (Pol α) complex, to origins of replication (Nasheuer et al., 2002, 2007; Oehlmann et al., 2007). Studies of the SV40 DNA replication system revealed that SV40 TAg, RPA, topoisomerase I and Pol α closely interact to allow initiation of the leading strand synthesis at the viral origin of replication (Hurwitz et al., 1990; Khopde et al., 2008; Murakami et al., 1992; Nesper et al., 1997; Oehlmann et al., 2007; Ott et al., 2002; Smith et al., 2002; Taneja et al., 2007a, 2007b, Trowbridge et al., 1999; Voitenleitner et al., 1997; Weisshart et al., 2000, 2004a). First, RPA is required very early during the unwinding of the origin DNA in cooperation with the replicative helicase SV40 TAg and, secondly, RPA supports Pol α to synthesize the first Okazaki fragment (Oehlmann et al., 2007; Schub et al., 2001; Smith and Nasheuer, 2002; Taneja et al., 2007a, 2007b). In addition, RPA serves as "fidelity clamp" for Pol α (Maga et al., 2001). The latter does not have an intrinsic proof-reading exonuclease but can interact with the tumour suppressor protein p53 with p53 having 3′ to 5′ exonuclease activity able to remove misincorporated nucleotides and damaged DNA (Melle and Nasheuer, 2002; Mummenbrauer et al., 1996, Wong et al., 2009). At the transition from the initiation to the elongation reaction in eukaryotic DNA replication RPA is involved in the DNA polymerase switch from Pol α, which has a DNA polymerase with intermediate processivity, to DNA polymerase δ (Pol δ), which in association with proliferating cell nuclear antigen (PCNA) has high processivity (Hübscher et al., 2000, 2002; Nasheuer et al., 2007; Yuzhakov et al., 1999). Multiple RPA-DNA and RPA-protein interactions are necessary for the elongation reaction of DNA replication including those to the clamp loader replication factor C (RF-C) and PCNA, the actual replication clamp that is the processivity factor of Pol δ. Here, the polarity of the RPA-DNA complex enables the RPA2 subunit to monitor the length of the RNA-DNA primers during lagging strand DNA synthesis in mammalian cells (Mass et al., 1998). Interestingly the early initiation steps have been found to be host-specific in various polyomaviral DNA replication systems (Brückner et al., 1995; Mahon et al., 2009; Schneider et al., 1994; Smith et al., 2002; Stadlbauer et al., 1996).

In cellular DNA replication with linear chromosomes as the template, RPA is also involved in the control of telomerase activity to replicate chromosomal ends, the telomeres. Human HeLa cells deficient in RPA show a decrease in the ability of telomerase to elongate DNA primer (Rubtsova et al., 2009). Addition of small amounts of purified RPA to RPA-depleted cell extracts restores the telomerase activity but adding an excess of RPA to these extracts inhibits the enzyme

activity. In contrast, prokaryotic SSB does not stimulate telomerase activity but actually inhibits the enzyme (Rubtsova et al., 2009). Moreover, RPA interacts with Pot1 and Werner helicase to prevent instability of telomeres (Ahn et al., 2009). However, RPA together with Blooms or Werner helicase may also be involved in the fusion of chromosome ends after telomere loss (Wang and Baumann, 2008).

During the initiation of DNA repair processes RPA binds to ssDNA of the partially unwound DNA in a polar fashion (de Laat et al., 1998). RPA2 is positioned by RPA1 on partial duplex DNA and contacts the 3′-terminus of the primer (Kolpashchikov et al., 1999). This polarity allows RPA together with XPA to direct the two endonucleases XPF and XPG for their precise DNA cleavage 3′ and 5′ of a lesion on the damaged strand (de Laat et al., 1998). The gap filling process will then occur by additional interaction between RF-C, PCNA and Pol δ or ε. In addition to these NER processes, RPA also participates in various steps of homologous recombination (HR) (Daboussi et al., 2002; Song and Sung, 2000; West, 2003). Recent studies revealed that the protein CtIP performs the resection of DSB and the production of ssDNA region in S and G2 phase to allow the activation of ATR-dependent DNA damage signalling and the binding of RPA to these DSBs (Sartori et al., 2007). In budding yeast, the coordinated interactions of RPA, Rad51 and Rad52 modulate the formation of Rad51 nucleoprotein filaments, which mediate DNA strand exchange, a key step in HR (Song and Sung, 2000; Sugiyama and Kowalczykowski, 2002). In mammals, Rad51 colocalizes with the tumour suppressor proteins BRCA1 and BRCA2 in DNA damage-induced nuclear foci and forms a tight complex with BRCA2, which in turn influences the activities of their partner recombination factors and the efficiency of recombination (Pellegrini et al., 2002). Moreover, BRCA1, the BRCA2-Rad51 complex, and Rad54 cooperate with RPA during the strand invasion reaction of HR (Shiloh, 2003; van Komen et al., 2002). Recent findings suggest that RPA controls DNA recombination and genome stability by associating with BRCA2, which might be involved in tumour suppression (Wong et al., 2003). RPA is also involved in additional flavors of DNA repair. RPA has been implicated in BER due to its interaction with human uracil DNA glycosylase and XRCC1 stressing its central role in eukaryotic DNA metabolism (Levy et al., 2009; Mer et al., 2000).

Physical Interactions of RPA with DNA

The RPA binds to ssDNA with high specificity and affinity but it also interacts with template primer systems. Especially RPA2 interacts with the 3′- and 5′-ends of a primer that is hybridized to a template whereas RPA binds with significantly lower affinity to dsDNA (Kolpashchikov et al., 1999; Nasheuer et al., 1992; Wold and Kelly, 1988). The heterotrimeric RPA complex associates with DNA in two different confirmations; in an elongated conformation to long stretches larger than 30 nucleotides of ssDNA whereas it is also known to bind ssDNA fragments of 8–10 residues in a globular conformation (Wold, 1997; Iftode et al., 1999).

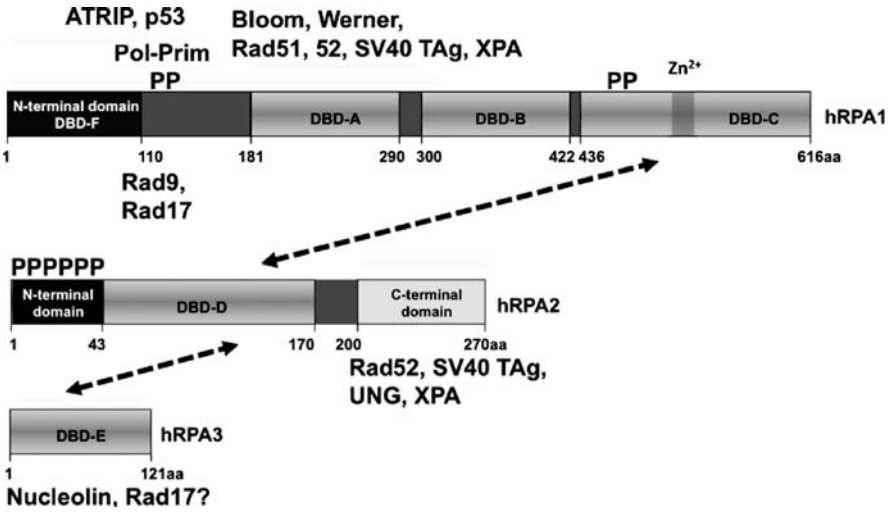

Fig. 1 Physical interactions of RPA. A schematic diagram of the structural and functional domains of the RPA subunits presents the DNA-binding domains (DBDs). Regions of RPA interacting with other proteins and the Zn finger domain are illustrated. Phosphorylation sites in RPA1 and the N-terminus of RPA2 are marked with P. The *arrows* highlight the physical interactions within the RPA complex itself

The RPA complex contains six OB-folds (Oligosaccharide/oligonucleotide-binding fold) with at least one found in the core domain of each subunit (Fig. 1). The OB-fold consists of a five-stranded beta-sheet coiled to form a barrel-helix and an alpha-helix that connects the third and fourth strands (Murzin, 1993). The high affinity ssDNA activities are located in the largest subunit RPA1 (Kenny et al., 1990; Nasheuer et al., 1992; Wold and Kelly, 1988). RPA1 contains four OB-folds, referred to as DNA-binding domains (DBDs) F, A, B and C following their arrangement from the N- to the C-terminus whereas the RPA2 subunit contains DBD-D and RPA3 has DBD-E (Fig. 1). RPA1 has not only DNA-binding activity (Kenny et al., 1990) but is also involved in protein-protein interactions (Dornreiter et al., 1992; He et al., 1995; Weisshart et al., 2000, 2004b). The C-terminus of RPA1 is required for stable interactions with the smaller two RPA subunits (Gomes et al., 1996) whereas the N-terminus is involved in multiple physical interactions with other DNA metabolic factors (reviewed by (Fanning et al., 2006; Wold, 1997) and summarized in Fig. 1). The redox status of RPA seems to significantly affect initial interactions of RPA with ssDNA but has no effect after RPA formed a stable complex with DNA suggesting that redox regulation of the zinc-finger may be involved in mediating the initial RPA-ssDNA interaction to form a stable RPA-ssDNA complex (Bochkareva et al., 2000; You et al., 2000).

The role of RPA1 in human cancer and maintaining cell survival and chromosomal stability in mammalian cells has been highlighted since a mutation changing the amino acid L230P in DBD-A (Wang et al., 2005). Heterozygous mice having the mutation in only one of the coding genes were viable but develop lymphoid tumours

whereas mice, which are homozygous for this mutation (carrying the mutation in both genes coding for RPA1), died during embryonic development. The primary mouse embryonic fibroblasts from heterozygous mice had defects in DSB repair showing chromosomal breaks and aneuploidy (Wang et al., 2005). These findings suggests that role of RPA1 in metabolism is vital for chromosome stability and tumour suppression (Wang et al., 2005).

In contrast to RPA1, the specific role of the two smaller subunits especially RPA3 is still poorly understood. In RPA2, DBD-D has low binding affinity for ssDNA (Dickson et al., 2009). DBD-E in RPA3 provides a structural role and is required for the stable heterotrimeric formation of RPA (Wold, 1997; Iftode et al., 1999). RPA undergoes a conformational change upon binding to ssDNA, which can be analysed by partial proteolytical digestion. RPA3 appears to have a protease resistant structure since even after complete digestion of the two larger subunits RPA3 was shown to be resistant to proteases. RPA2 has a partial resistance to protease digestion resulting in a rapid removal of the N-terminus (Gomes et al., 1996). RPA2 consists of three structural domains; an N-terminal domain, which functions in RPA phosphorylation (Fig. 1), a central DNA binding domain (DBD-D) responsible for subunit interactions and a C-terminal domain with protein-protein interaction activity (Arunkumar et al., 2005; Mer et al., 2000; Nuss et al., 2005; Oakley et al., 2001; Ott et al., 2002 reviewed in Fanning et al., 2006). Upon binding to ssDNA RPA1 becomes more resistant to proteolytic cleavage whereas RPA2 becomes more sensitive (Gomes et al., 1996; Pestryakov et al., 2003). In contrast, in prokaryotic *E. coli* SSB protease sensitivity only increases if the ssDNA oligonucleotide is long enough to allow cooperative binding (Gomes et al., 1996). In yeast it was recently shown that the DNA-binding activity of RPA2 is not essential whereas protein interaction activities of DBD-D of RPA2 are required for viability (Dickson et al., 2009).

Photocrosslinking using nucleotide analogues has been shown to be a valuable tool to analyse structure and functions of DNA binding proteins in DNA metabolic pathways (Hartmann et al., 1988; Lavrik et al., 1998 and "Nucleotide Excision Repair in Higher Eukaryotes: Mechanism of Primary Damage Recognition in Global Genome Repair" by Rechkunova and Lavrik, this book). Studies with RPA and a variety of nucleotide analogues that can be specifically activated by UV revealed that RPA binds in an ordered fashion to ssDNA and that in partial double-stranded DNA the DBD-C and DBD-D are involved in the specific interactions of RPA with 3′-end of a primer annealed to a longer oligonucleotide (Pestryakov et al., 2003, 2004). Recently photocrosslinking techniques revealed that RPA3, which contains an OB-fold, also interacts with DNA (Salas et al., 2009).

The RPA Complex and Its Binding to Proteins

The RPA Complex and DNA Replication

RPA forms a heterotrimeric complex in eukaryotic cells which can also be produced by co-expression of RPA subunits in *E. coli* (Henricksen et al., 1994; Weisshart

et al., 2004b). However, the assembly of the RPA complex is only partially understood. Recent studies using the expression of RPA subunits in *E.coli* suggest the formation of the RPA complex requires a specific assembly of its subunits with the RPA2 and RPA3 interact to form a stable subcomplex, (Henricksen et al., 1994). The subunit RPA1 is difficult to express in *E. coli* but the RPA1 fusion protein with maltose binding protein (MBP) is soluble and functionally active (Weisshart et al., 2004b). MBP-RPA1 forms a soluble and stable complex with RPA2 whereas MBP-RPA1 only weakly binds the smallest subunit RPA3. However, all these purified RPA sub-complexes do not support DNA replication whereas when all three subunits were co-expressed a stable, heterotrimeric RPA complex is formed, which has similar properties to human RPA and is indistinguishable in DNA replication assays from RPA purified from human cells (Henricksen et al., 1994; Weisshart et al., 2004b).

RPA physically interacts with various proteins required for DNA replication and DNA repair and DNA damage signalling (summarized in Fig. 1 and Fanning et al., 2006; Wold, 1997). Recent investigations revealed that protein interaction functions of DBD-D (domain of RPA2) are essential in yeast whereas DNA-binding activities of RPA2 are dispensable for viability of yeast cells (Dickson et al., 2009) underlining the importance of physical RPA-protein interactions for cell functions. RPA physically binds to replication factors such as Pol α and Cdc45 (Bauerschmidt et al., 2007; Braun et al., 1997; Weisshart et al., 2000, 2004b). Polyomaviral DNA replication systems such as SV40, mouse polyomavirus, and the human polyomaviruses BKV and JCV have served as model systems to study eukaryotic DNA replication with the SV40 DNA replication being the best understood. Only a single viral protein the large TAg is necessary for polyomaviral DNA replication and RPA is specifically required for TAg-mediated unwinding of DNA templates containing the viral origin of replication (Dodson et al., 1987; Wold et al., 1988). Studies of the polyomavirus DNA replication systems revealed that SV40 TAg, RPA, topoisomerase I and Pol α closely interact to allow initiation of the leading strand synthesis (Oehlmann et al., 2007). SV40 TAg binds to two regions of RPA one in the N-terminus of RPA1 and the other in the C-terminus of RPA2 (Ott et al., 2002; Fanning et al., 2006). In addition, these interactions may enhance the maturation of Okazaki fragments during DNA replication (Bartos et al., 2006). In addition to the association with TAg, RPA physically interacts with Pol α to support its initiation functions, to serve as "fidelity clamp" for Pol α and to support the polymerase switch from Pol α to Pol δ (Maga et al., 2001). These physical interactions of RPA and Pol α have been localized to the N-terminus of RPA1 and to RPA2 whereas p180 and both primase subunits of are physically involved on the Pol α side (Braun et al., 1997; Weisshart et al., 2000, 2004b).

As mentioned above, RPA interacts with proteins involved in DNA repair such as the nucleotide excision repair proteins XPA and XPG, the tumour suppressor protein p53, and transcriptional activators such as VP16 (He et al., 1993). In addition, the association with p53, which is stimulated by DNA damage, inhibits functions of RPA in DNA replication (Binz et al., 2004; Dutta et al., 1993). RPA physically interacts with Blooms and Werner helicase (Doherty et al., 2005). RPA physically binds

to human uracil DNA glycosylase and XRCC1 linking RPA to BER and stressing its central role in multiple pathways of eukaryotic DNA metabolism (Levy et al., 2009; Mer et al., 2000). The binding to Pot1 and Werner helicase may prevent instability of telomeres whereas together with Blooms or Werner helicase RPA supports the fusion of chromosome ends but only after telomere loss (Wang and Baumann, 2008; Ahn et al., 2009). In summary, the physical RPA-protein interactions serve various important functions to prevent genome instability and cancer in eukaryotic organisms.

The RPA Complex in DNA Repair Processes – Molecular Counting Capabilities

After binding to ssDNA either during DNA replication or in response to DNA damage, RPA is phosphorylated, and this is thought to be an important event in DNA damage response (DDR) (Binz et al., 2004). Recent observations have indicated an involvement of ATR (ATM and Rad3-related) in the RPA2 phosphorylation in response to stalled replication fork in S-phase generated by genotoxic agents such as UV (Olson et al., 2006). A regulatory network has emerged, in which the collaboration of the proteins ATRIP, TopBP1, and the 9-1-1 complex (ATR Interacting Protein, Topoisomerase II-Binding Protein 1, and Rad9-Hus1-Rad1, respectively) together with RPA and putatively other factors activates the phosphoinositol-3 kinase-like protein kinase (PIKK) ATR after DNA damage (Mordes et al., 2008; Xu et al., 2008; see also "Function of TopBP1 in Genome Stability" by Miiko et al., this book). The regulation seems to require multiple protein complexes or "keys". During checkpoint signalling, RPA binds to a region of ssDNA established after DNA damage. To activate ATR RPA must recruit the kinase via ATRIP to the DNA. However, to initiate a DNA damage-dependent signal transduction, at least, TopBP1 must then associated with the RPA-ATRIP-ATR complex. This occurs via an interaction of RPA with the 9-1-1 complex and here especially Rad9. The latter recruits TopBP1, which in turn will activate ATR (Mordes et al., 2008; Xu et al., 2008). Note that 9-1-1 binds to the same N-terminal region of RPA1 and one RPA complex can only bind either to 9-1-1 or ATRIP (Mordes et al., 2008; Xu et al., 2008):

These findings reveal a multi-component network, which is summarized here as a "multi-key" principle, to activate ATR (similar to the requirement of multiple passwords or "keys" to perform a bank transfer on the internet requires). After DNA damage, the initiation of a DDR signal transduction cascade starts with RPA binding to extended stretches of ssDNA and probably short primers synthesized by Pol α followed by the activation of ATR by protein interactions (Byun et al., 2005; Michael et al., 2000). As described before, RPA has high specificity to transitions from ssDNA to dsDNA, to both to 5′ and 3′ ends (Lavrik et al., 1998; Pestryakov et al., 2004). That way multiple RPA molecules would bind to long DNA stretches, which might also have associated scattered RNA-DNA primers, to fully activate ATR. The coordinated activation mechanism would require a minimum of two RPA molecules, but most likely more, to activate ATR (Mordes et al., 2008; Xu et al.,

2008). Depending on the RPA-ssDNA interaction mode, such as with the extended ssDNA-binding mode of RPA being about 50 nucleotides (Blackwell et al., 1996; Lavrik et al., 1998; Pestryakov et al., 2004), the ssDNA region would be at least 100 or multiples of 50 nucleotides to initiate a sustainable DNA damage signal transduction cascade. This multi-factor interaction requirement for initiation of DNA damage-based signalling pathway and checkpoint activation could be seen as a sign of a "molecular counting mechanism". The hypothesis of a multi-key concept or a molecular counting mechanism is supported by the findings that the initial binding of ATR-ATRIP-RPA complex is followed by the activation of the ATR kinase by TopBP1, which is recruited by the 9-1-1 complex binding to RPA (Mordes et al., 2008; Xu et al., 2008; Zou and Elledge, 2003). However, complete activation of ATR may require additional not yet identified protein complexes, which may also associate with RPA. Hypothesizing that all these activation steps of ATR are based on physical interactions with the RPA-ssDNA complex, the activation of a checkpoint by ATR necessarily requires a threshold level of protein-RPA-ssDNA complexes associating with the damaged DNA and its vicinity – "multiple keys" – and introduce a counting reminiscent to primase with accurate counting capabilities with less fidelity in RNA primer synthesis (Arezi and Kuchta, 2000). In this "molecular counting" hypothesis "one unit" would be about 50 nucleotides of ssDNA. The ability of RPA to perform, or more precisely, to initiate and being the base for a molecular counting process would explain the difference between relatively short ssDNA sequences occurring during DNA replication and initiating no checkpoint contra extended ssDNA stretches being induced after DNA damage such as stalled replication forks, which would initiate a DDR-dependent checkpoint. The former might still activate a basal ATR activity involved in coordinating basic cell functions such as feedback process and explain the essential function of ATR during the cell cycle. If RAD9-RPA interaction would be disrupted the ATR signalling to CHK1 would be impaired and the cell would become hypersensitive to replication stress as well as DNA damage (Cimprich and Cortez, 2008; Cortez et al., 2001).

In contrast, various researchers observed that both ATR recruitment to sites of IR-induced DNA damage and its activation require components of the MRN complex as well as ATM (Adams et al., 2006; Falck et al., 2005; Myers and Cortez, 2006). In HR, interactions of RPA, Rad51 and Rad52 modulate the formation of Rad51 nucleoprotein filament and DNA strand exchange (Sugiyama and Kowalczykowski, 2002). These protein-protein interactions are required for genome stability including meiotic recombination, mating-type switching, and survival after DNA damage (Kantake et al., 2003). In mammals, members of the *RAD52* group interact with the tumour suppressor proteins BRCA1 and BRCA2, which in turn influence the activities of recombination factors and the efficiency of HR (Shiloh, 2003). Moreover, recent findings also suggest that RPA controls HR and genome stability by associating with BRCA2, which might be involved in tumour suppression (Wong et al., 2003). BRAC1, the BRAC2-Rad51 complex and Rad54 cooperate with RPA during the strand invasion reaction of HR (Shiloh, 2003; van Komen et al., 2002).

RPA Phosphorylation

RPA is phosphorylated in a cell cycle-dependent manner in S phase and in M phase of a normal cell cycle and dephosphorylated at the end of M phase (Din et al., 1990; Dutta and Stillman, 1992). Cyclin-dependent kinases (CDKs) phosphorylate human RPA2 at one site S23 in S phase and at two sites S23 and S29, which are both canonical CDK recognition sites, in M phase (Stephan et al., 2009). The phosphorylation of RPA influences the binding to dsDNA and to replication factors since in biochemical characterisations the purified mitotic form of human RPA has a weaker binding to dsDNA, DNA-dependent kinase (DNA-PK) and Pol α (Oakley et al., 2003). Recent studies revealed that the N-termini of RPA1 and RPA2 interact, which is diminished in phosphorylated RPA forms (Binz and Wold, 2008).

In eukaryotic cells RPA is also phosphorylated in response to DNA damaging agents suggesting that DDR pathways regulate RPA (Carty et al., 1994; Pan et al., 1995; Zernik-Kobak et al., 1997; Zhou and Elledge, 2000; Zou and Elledge, 2003). Recently a model for the regulation of RPA function by phosphorylation started to emerge: After DNA damage, RPA associates with the site of damage through direct binding with DNA and repair/recombination factors (Binz et al., 2004). In complex with damaged DNA RPA is involved in the initiation of cellular DDRs. In addition, as a response of the signal transduction pathway RPA is then a target of DNA damage-dependent PIKKs, and ATM, ATR and DNA-PK phosphorylated RPA with the N-terminus of RPA2 being a well known substrate and a marker for DNA damage (Binz et al., 2004; Kaufmann, 2007). These kinases and RPA co-localize at the DNA damage site and also physically interact with RPA. Phosphorylation of the RPA2 subunit in response to UV or ionizing radiation causing a conformational change in the RPA complex, which in turn promotes decreased interactions of RPA with protein involved in DNA replication and PIKKs whereas interactions with the p53 tumour suppressor are increased. Associations with proteins involved in DNA repair remain unchanged (Binz et al., 2004). Importantly it is to remember that the UV damage only induces RPA phosphorylation at DNA damage-dependent sites in S phase whereas ionizing radiation or other DSB-causing agents also lead to RPA phosphorylation at these sites independently of cell cycle phases (Anantha et al., 2008; Rodrigo et al., 2000; Stephan et al., 2009).

After phosphorylation, the mobility of RPA2 during gel electrophoresis is significantly reduced, which suggests a conformational change in the subunit in both human and yeast (*S. cerevisiae*) RPA (Din et al., 1990; Dutta and Stillman, 1992). Up to nine potential phosphorylation sites have been suggested within human RPA2 but phosphorylation of RPA is not restricted to the N-terminus of RPA2 (Zernik-Kobak et al., 1997; Binz et al., 2004). Recent data has identified additional phosphorylation sites in response to DNA damage including multiple sites within the RPA1 both *in vitro* and *in vivo* (Nuss et al., 2005). These sites located in the OB-fold of the C-terminus of RPA1 (DBD-C) are likely to play a significant role in the duplex destabilisation activity of RPA (Nuss et al., 2005). Nuss et al. have suggested that these sites contribute to the decrease in affinity of phosphorylated RPA, in comparison to unphosphorylated RPA, for duplex DNA. A recent study has shown the

level of UV-induced RPA phosphorylation increases in the absence of Pol η and DNA-PK is responsible for this phosphorylation of RPA2 during UV-induced DNA damage response pathway (Cruet-Hennequart et al., 2008; for review see "DNA Polymerase η, a Key Protein in Translesion Synthesis in Human Cells" by Cruet-Hennequart et al., this book). The level of phosphorylation necessary to trigger the DNA damage response has yet to be determined but it is possible that the amount of RPA phosphorylated is proportional to the extent of DNA damage. In contrast, there may be a threshold of RPA phosphorylation required to promote down-regulation of DNA replication. It is apparent that the mechanism by which RPA is regulated differs between yeast and mammals, which underlines the importance of investigating RPA regulation in various organisms (Binz et al., 2004).

An Alternative Form of Replication Protein A

Recently a homolog to the RPA2 subunit was identified in human and was called RPA4 (Keshav et al., 1995; Mason et al., 2009). RPA4 shares 47% identity with RPA2 and it has been suggested that selective expression of RPA2 and RPA4 family may affect DNA repair, DNA replication and DNA recombination through regulation of both protein-protein interactions and post-translational modifications (Keshav et al., 1995). Recently RPA4 was expressed together with RPA1 and RPA3 subunit. RPA4 formed a heterotrimeric protein complex with RPA1 and RPA3 and it substituted for RPA2 in the purified complex. The purified complex composed of RPA1, RPA3 and RPA4 is capable of binding ssDNA in a way that is indistinguishable from canonical RPA since this newly established protein complex binds ssDNA with high affinity and low cooperativity (Keshav et al., 1995; Mason et al., 2009). Therefore, it was named as an alternative form of replication protein A, in short aRPA.

However, the RPA4-containing aRPA does not support SV40 DNA replication in vitro but actually inhibits the activity of canonical RPA during replication suggesting that this aRPA has a role in the regulation of human cell proliferation (Mason et al., 2009). Canonical RPA is essential in genome stability. Since the aRPA complex has similar properties in DNA-binding activity as the canonical RPA but seems to interfere with DNA metabolism there is a possibility that RPA4 has a function in maintaining the integrity of the cell or the regulation of pathway in the cellular DNA metabolism. Moreover, these studies revealed the potential for RPA4 as a therapeutic tool or target in preventing cell proliferation in cancer and also as antiviral replication agent through the prevention of viral replication (Mason et al., 2009).

Replication Protein A – The Cancer Link

RPA associates with the tumour suppressor protein p53 and it has been shown that this RPA-p53 interaction inhibits the functions of RPA in DNA replication (Dutta et al., 1993). Recent findings also suggest that RPA controls DNA recombination

and genome stability by associating with BRCA2, which might be involved in tumour suppression (Wong et al., 2003). RPA is essential to the viability of the cell (Wold, 1997). However, mutations in the RPA subunits, which do not interfere with the viability of an organism, can interfere with DNA damage pathways or increase chromosome instability (Mason et al., 2009; Wang et al., 2005). Mutations in RPA1 causes defective DSB repair (Wang et al., 2005; Wold, 1997). To study the role of RPA in human cancer and whether RPA1 is an essential factor in preventing tumour formation and maintaining cell survival and chromosomal stability in mammalian cells a mutation was introduced at nucleotide position 689 of RPA1 changing T to C, which yields a change of the amino acid L230P in one of the DNA binding domains, DBD-A, of RPA1. Heterozygous mice were viable but develop lymphoid tumours whereas mice, which are homozygous for this mutation, died during embryonic development. Analysis of the RPA1 mutation showed that primary heterozygous mutant mouse embryonic fibroblasts carrying it had defects in DSB repair showing chromosomal breaks and aneuploidy (Wang et al., 2005). These findings suggests that role of RPA1 in metabolism is vital for chromosome stability and tumour suppression and that RPA could be used to target tumour formation in humans and that RPA1 is a potential therapeutic tool in the treatment of cancer (Wang et al., 2005).

The Human ssDNA-Binding Protein hSSB1

Recently new human SSBs known as hSSB1 and hSSB2 were discovered with hSSB1 binding characterised in more detail (Richard et al., 2008). Similarly to RPA, hSSB1 binds specifically to ssDNA substrates, particularly to polypyrimidines. hSSB1 is highly conserved in metazoa and contains an OB-fold domain, which is followed by a carboxy-terminal region. The binding affinity of hSSB1 is enhanced with increasing length of its DNA substrate. Upon activation of ATM activity in response to DSBs, hSSB1 is phosphorylated along with several other proteins. The failure to activate ATM activity in A-T (Ataxia telangiectasia) cells results in the inability to stabilise hSSB1 after ionizing radiation in these cells. These findings suggest that ATM activity, which is crucial for cellular signalling in response to DSBs, regulate the function of hSSB1. One result of these analyses is that in an ATM-dependent manner DNA damage triggers the accumulation of hSSB1 in the nucleus, which yields distinct foci that co-localize with several other repair proteins (Richard et al., 2008). hSSB1 is found to form foci at sites of DNA replication and DNA damage. In cells containing both RPA and hSSB1 foci, Richard et al. observed low instances of co-localisation between these human SSBs (<5%). However, RPA foci were shown to be in close proximity hSSB1 suggesting a dual function in DNA repair (Richard et al., 2008). hSSB1 acts as a substrate for ATM and influences diverse endpoints in the DNA damage response including cell cycle checkpoint activation, recombination-based repair and in maintaining genomic stability. Like RPA, it also contributes to HR by promoting Rad51-mediated strand repair. hSSB1 may be

associated with the prevention of tumourigenesis, altering the response of tumours to radiotherapy and DNA-damaging chemotherapies. Although hSSB1 is not essential cells lacking in hSSB1 show signs of increased genome instability, defects in checkpoint activation and are more sensitive to radioactivity (Richard et al., 2008).

Mitochondrial SSBs

Nuclear SSBs (RPA and hSSB1) do not resemble eubacterial SSBs in sequence or structure. However, mtSSBs share a number of conserved regions with *E.coli* but differ otherwise (Ghrir et al., 1991; Wong et al., 2009). mtSSBs are evolutionarily conserved proteins found in all eukaryotes from yeast to humans (Maier et al., 2001). mtSSB binds to ssDNA and its main function is to stabilise the single-stranded regions of mtDNA in the displacement loops (D-loop) (van Tuyle and Pavco, 1985). Depletion of mtDNA has been linked to a number of inherited human diseases. In the absence of mtSSB in *Drosophila*, the majority of mitochondria loose respiratory function due to a loss of mtDNA (Maier et al., 2001). Human mtSSB is a tetramer consisting of two dimers that interact head-to-head and is in D_2 symmetry (Yang et al., 1997). Yang et al. proposed that during binding, ssDNA wraps around mtSSB through four electropositive channels, which is conserved between *Ecoli* SSB and human mtSSB, guided by flexible loops.

Human mtSSB and p53

Mutations in mtDNA are commonly observed in cancer patients. p53 is a key player in maintaining mitochondrial genomic stability through its ability to translocate to mitochondria and physically interact with Pol γ, mitochondrial DNA polymerase (for more details see "The Mitochondrial DNA Polymerase in Health and Disease" by Copeland, this book), in response to mtDNA damage caused by endogenous and exogenous insults such as ROS (reactive oxygen species) (Achanta et al., 2005). Recently Wong et al. (2009) identified human mtSSB as a novel binding partner of tumour suppressor p53 and a component of DNA mitochondrial replisome *in vitro*. p53 interacts with human mtSSB physically through its transactivation domain. Depletion of p53 results in an increase of mtDNA mutation. Therefore, p53 has been implicated in DNA repair in mitochondria during oxidative stress (Wong et al., 2009). Moreover, human mtSSB modestly stimulates 3′ to 5′ exonuclease activity of p53, which is an intrinsic to the protein and is able to excise 8-oxodG, suggesting a role for p53 and the mtSSB-p53 complex in genome stability including in mitochondria (Wong et al., 2009).

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