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REVIEW ARTICLE

Multiple human single-stranded DNA binding proteins function in genome maintenance: structural, biochemical and functional analysis

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Abstract

DNA exists predominantly in a duplex form that is preserved via specific base pairing. This base pairing affords a considerable degree of protection against chemical or physical damage and preserves coding potential. However, there are many situations, e.g. during DNA damage and programmed cellular processes such as DNA replication and transcription, in which the DNA duplex is separated into two single-stranded DNA (ssDNA) strands. This ssDNA is vulnerable to attack by nucleases, binding by inappropriate proteins and chemical attack. It is very important to control the generation of ssDNA and protect it when it forms, and for this reason all cellular organisms and many viruses encode a ssDNA binding protein (SSB). All known SSBs use an oligosaccharide/oligonucleotide binding (OB)-fold domain for DNA binding. SSBs have multiple roles in binding and sequestering ssDNA, detecting DNA damage, stimulating strand-exchange proteins and helicases, and mediation of protein–protein interactions. Recently two additional human SSBs have been identified that are more closely related to bacterial and archaeal SSBs. Prior to this it was believed that replication protein A, RPA, was the only human equivalent of bacterial SSB. RPA is thought to be required for most aspects of DNA metabolism including DNA replication, recombination and repair. This review will discuss in further detail the biological pathways in which human SSBs function.

Keywords: *Homology-directed repair (HDR); nucleotide excision repair (NER); replication fork restart; translesion synthesis; checkpoint control*

Introduction

The exposure of ssDNA regions during normal cellular activity such as DNA replication and transcription is an essential process. However the generated ssDNA structure is less stable, is vulnerable to chemical and nucleolytic attack, can be bound by inappropriate enzymes, can form secondary structures masking the nucleotide group, can spontaneously re-anneal to its complementary DNA strand (or other homologous ssDNA strands) and if damaged has no complimentary strand for repair. The SSB family of proteins carry out the essential process of stabilizing ssDNA regions and preventing inappropriate reactions until the correct cellular processes can occur. However, in addition to binding ssDNA, it has been established that these proteins have the functional ability to recruit partner proteins and present the ssDNA substrate to them (Shereda et al., 2008). The SSB family of proteins are conserved in all three kingdoms of life (Forterre and Philippe, 1999). Their ubiquitous presence gives credence to the theory that these proteins evolved from RNA binding proteins, which would have been present in the last universal common ancestor (LUCA). With the evolution of DNA (uracil DNA) it seems likely that these proteins might have altered their function to protect ssDNA, in a manner similar to how they would have functioned to protect RNA in an RNA world.

The SSB family are characterized structurally by their oligonucleotide oligosaccharide-binding fold (OB fold), which binds to single stranded DNA (ssDNA) substrates.

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The SSB family can be subdivided into two distinct sub-groups: simple SSBs, typified by the *Escherichia coli* (*E. coli*) SSB, which contains a single OB-fold; and the higher ordered replication protein A (RPA) sub-group which contains multiple OB-folds (Iftode et al., 1999). Simple SSBs are structurally composed of a single polypeptide with either one or two OB-folds (Figure 1), although functionally this SSB sub-group bind ssDNA as homotetramers (*E. coli SSB*) *and* homodimers (*Deinococcus radiodurans and Thermus aquatics* SSB) (Shereda et al., 2008). The only exception to the oligomeric rule has been *Sulfolobus* SSB, which functions as a monomer (Kerr et al., 2003). The RPA sub-group is defined by the ordering of the OB folds, either multiply on one polypeptide or over two or more polypeptides such as RPA itself and Euryarchaeal SSBs (Figure 1).

RPA, however, like the simple SSBs, binds to ssDNA using four OB folds. Human RPA is a heterotrimeric polypeptide, widely believed to be a central component of both DNA replication and DNA repair pathways, yet it does not have any similarity in oligomeric structure to the bacterial SSBs. Eukaryotes also encode a mitochondrial SSB (mtSSB) within the mitochondrial genome. The eukaryotic mtSSB is a member of the simple SSB sub-group and has a number of conserved residues in the N-terminus that are shared with *E. coli* SSB (Tiranti et al., 1993). Recently two other chromosomally encoded members of the SSB family in human, named hSSB1 and hSSB2, have been identified (Richard et al., 2008). Their discovery challenges many of the established models of DNA transactions involving ssDNA and will provide an exciting area of research in the coming years. hSSB1 and 2 are structurally much more closely related to the bacterial and archaeal simple SSB sub-group than to RPA. They are composed of a single polypeptide containing an OB fold and a c-terminal tail predicted to be required for protein–protein interactions. hSSB1 has recently been described as having a central function in the repair of double strand DNA breaks (DSB) by homology directed repair (HDR) (Richard et al., 2008). Unlike RPA, however, hSSB1 appears to be dispensable for normal DNA replication, suggesting a real functional difference between hSSB1 and RPA. Biochemical analysis revealed that hSSB1 is dimeric in solution and binds to the classical ssDNA substrates. The function of hSSB2 however is not clear at this point. Expression data suggests that hSSB2 is predominantly expressed in lymphocytes and testes, indicating that it may have a role in class switch and meiotic recombination.

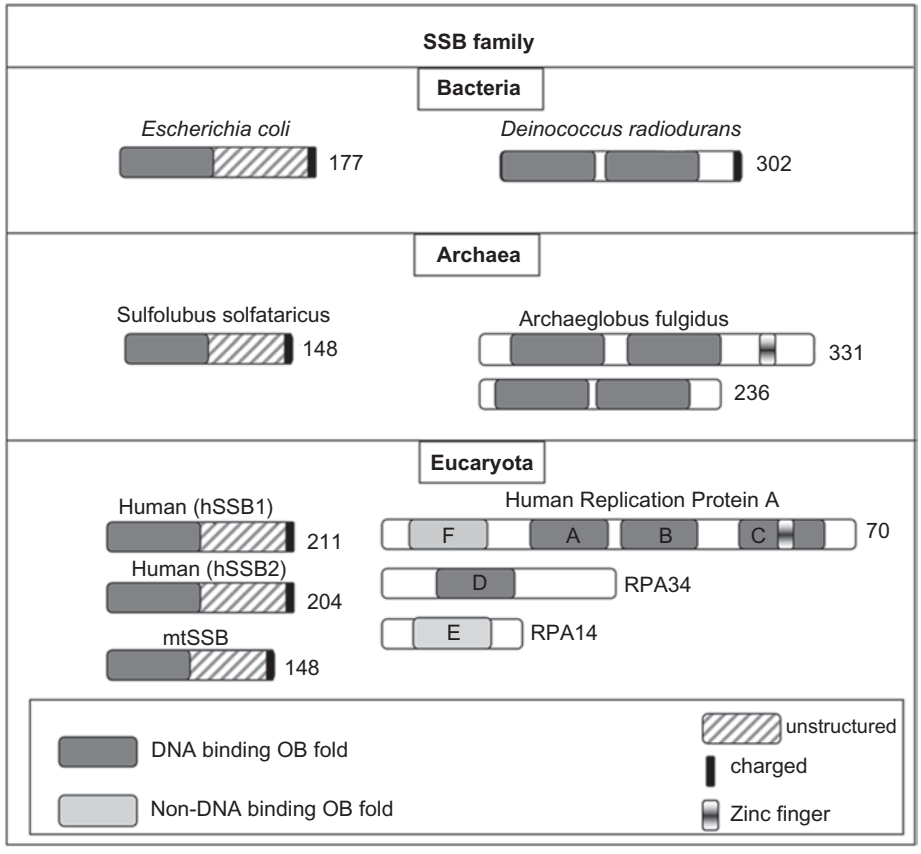

Figure 1. Schematic representation of the predicted domain architecture of single stranded DNA binding proteins (SSBs) in three domains of life including bacteria, archaea and eukaryote. The letters within the boxes represent individual OB folds. The domains are not drawn to scale.

SSBs: An overview

The ubiquitous nature of the SSB family of proteins suggests both a functional necessity for these proteins and a common ancestor. The sequence homology would suggest that all three domains of life share the same ancestral SSB and this most probably existed in the LUCA. The LUCA itself probably encoded its genetic information in the form of RNA; however, it was the evolution of UDNA (uracil containing DNA) that eventually gave rise to the three domains of life present today. It can perhaps therefore be assumed that the ancestor of the SSB proteins was in fact a RNA binding protein. During evolution the essential OB fold domain has remained essentially intact, while structural variations outside of this domain have resulted primarily as a means of adding additional control mechanisms to the function of the SSBs.

Primarily the structural organization of the SSB family can be subdivided into the simple SSBs, which are represented by the bacteria, and RPAs, which are represented by the eukaryotes (Figure 1). Interestingly, archaea, which share the same evolutionary branch as eukaryotes, have examples of both SSB subdivisions. *Methanococus jannaschii* (Kelly et al., 1998), for example, has an RPA like SSB, while *Sulfolobus solfataricus* (Wadsworth and White, 2001) have a simple SSB. This may suggest either that RPAs existed prior to the divergence of eukaryotes and archaea, or archaea and eukaryotes evolved the RPA sub-domain independently. Until recently it was believed that higher eukaryotes had only the RPA sub-group; however, the human genome has now been shown to encode two functional members of the simple SSB sub-group (hSSB1 and hSSB2) (Richard et al., 2008). Intriguingly the presence of the simple SSB sub-group in eukaryotes is restricted to the higher eukaryotes, with no identifiable homologs in yeast, which may explain in part why it has remained undiscovered until recently. However, this raises the question of why yeast do not require a simple SSB. It is possible that in yeast RPA has taken over all the functions of the simple SSB and that evolution has removed the simple SSB gene. Alternatively, the mitochondrial SSB (mtSSB) in yeast, a form of simple SSB required for mitochondrial DNA replication, may also function in DNA damage processes within the yeast genome.

The importance of SSBs in biological processes can also be clearly demonstrated by many viruses. These viruses either highjack the cellular SSB, as with the Simian virus 40 (Dean et al., 1987; Wobbe et al., 1987; Fairman and Stillman, 1988; Wold and Kelly, 1988) or encode their own SSB as with the viral Φ29 bacteriophage (Gascon et al., 2000). The viruses primarily utilize the SSB to facilitate DNA replication; however the N4 virion requires SSB for activation of early transcription (Davydova and Rothman-Denes, 2003). Transcriptional activation by *Sulfolobus solfataricus* SSB has also been reported (Richard et al., 2004).

DNA binding

Essentially the SSB family have evolved as efficient DNA binding proteins. They are characterized by distinct OB folds, which are responsible mainly for their ssDNA binding. The OB fold itself is the canonical ssDNA binding domain (Arcus, 2002). The OB fold binds to its ssDNA substrate through a combination of base stacking with the nucleotides, and electrostatic forces with the phosphodiester backbone (Merrill et al., 1984; Casas-Finet et al., 1987a; 1987b; Khamis et al., 1987a; 1987b; Bujalowski et al., 1988; Lohman et al., 1988; Overman et al., 1988; Curth et al., 1993; Overman and Lohman, 1994; Raghunathan et al., 2000; Savvides et al., 2004). Most SSBs studied to date form various oligomeric arrangements for functionality: either hetero-oligomers, such as the eukaryotic RPA heterotrimer (Mitsis et al., 1993; Wold, 1997) and the euryarchaeal SSBs (Kelly et al., 1998), homotetramers such as *E. coli* SSB (Murzin, 1993; Shamoo et al., 1995; Bochkarev et al., 1997; Pfuetzner et al., 1997; Matsumoto et al., 2000) or homodimers such as the radio-resistant bacterium *Deinococcus radiodurans* (Bernstein et al., 2004). The only exception to the oligomeric rule has been the *Sulfolobus* SSB, which functions as a monomer (Kerr et al., 2003). The binding of *Sulfolobus* SSB occludes 5 nt per monomer of SSB and the binding has high density and is distributive; however unlike that of *E. coli* SSB it shows no evidence of cooperative binding. hSSB1 binds ssDNA with a high density. hSSB1 predominantly exists as dimers and a small fraction as tetramers in solution, as established by gel filtration and multi-angle laser light scattering (MALLS) (Richard et al., 2008). Isothermal titration calorimetry (by determining molar ratio of binding) and agarose gel shift analysis using virion phiX174 ssDNA as a substrate indicated that the binding of hSSB1 occludes 12 nt per dimer.

The eukaryotic RPA is typically heterotrimeric and is composed of six OB folds, only four of which bind to ssDNA. The four ssDNA binding OB folds of human RPA are arranged over two polypeptides with three OB folds on the RPA70 subunit and one on the RPA 34 subunit. RPA was originally identified as an essential component required for *in vitro* replication of Simian virus 40 (SV40). The identification of RPA provided the first confirmation of the presence of the SSB family in eukaryotes (Dean et al., 1987; Wobbe et al., 1987; Fairman and Stillman, 1988; Wold and Kelly, 1988; Wold et al., 1989). There have been extensive biochemical studies carried out on RPA. These have shown that, like its simple SSB cousins, RPA has a high affinity for exposed ssDNA, has

little affinity to double stranded DNA and reduced affinity to RNA (Wobbe et al., 1987; Fairman and Stillman, 1988; Wold and Kelly, 1988; Wold et al., 1989; Kim et al., 1992). Interestingly human RPA also contains a zinc finger motif. The zinc finger is not present in the simple SSBs and appears to add a level of regulation into RPA ssDNA binding. The role of the RPA70 zinc finger was not initially clear as deletion appeared to have little effect on RPA classical ssDNA binding (Kim et al., 1996), however a second study discovered that RPA ssDNA binding activity was regulated by reduction-oxidation (redox). In reducing conditions RPA binding to ssDNA was upregulated more than 10-fold and deletion of the zinc finger resulted in a loss of this upregulation (Park et al., 1999b). This interesting result gives rise to the possibility that RPA can sense and respond to certain oxidative stress conditions. This may be in the context of its role in DNA damage repair or its inactivation during the process of apoptosis.

The OB folds of RPA are homologous to the simple SSBs and are composed of five β-strands arranged in a β-barrel (Bochkarev and Bochkareva, 2004; Gomes et al., 1996). The main ssDNA binding activity of RPA is located within the RPA70 subunit. In RPA nomenclature each of the six OB folds are represented by the letters A–F, the order denoting their binding efficiencies to ssDNA. It is believed that RPA binds to ssDNA in a sequential manner. The binding of the high affinity RPA70 A fold results in the B fold being brought into close proximity with its ssDNA substrate. This allows the B fold to bind the ssDNA. A short flexible linker separates the A and B folds. The binding of the A and B folds now results in a conformational change to the RPA70 structure. Two finger-like loops from within each domain enclose the DNA, forming a stable interaction between RPA70 and the ssDNA. The next binding event is the C fold, which is also located on RPA70. Finally the D fold, which is located on RPA34, binds to the ssDNA. The sequential binding of the RPA OB folds represents distinct binding modes. The binding of the A and B folds occludes approximately 10 nt, the subsequent binding of the C fold occludes 12–23 nt, while the full binding mode occludes 28–30 nt (Fanning et al., 2006). RPA binds ssDNA with a 5′ to 3′ polarity, with RPA70 located 5′ to RPA 34 (de Laat et al., 1998; Iftode and Borowiec, 2000; Kolpashchikov et al., 2001; Bochkareva et al., 2002; Arunkumar et al., 2003; Wyka et al., 2003).

Both RPA and the simple SSBs show differing modes of ssDNA binding. The bacterial SSBs can bind to ssDNA in different modes depending on which OB folds are bound to the DNA (Griffith et al., 1984; Lohman and Overman, 1985; Bujalowski and Lohman, 1986; Ferrari et al., 1994; Lohman and Ferrari, 1994). However unlike RPA, one of the SSB binding modes shows cooperative binding allowing the formation of long SSB filaments (Lohman et al., 1986; Meyer and Laine, 1990; Ferrari et al., 1994; Lohman and Ferrari, 1994; Wold, 1997; Kumaran et al., 2006). The exact function of these filaments is not yet clear. Microscopic analysis of RPA reveals its different binding modes *in vitro*. These are visualized as different shapes which have been classified as globular, elongated contracted and elongated extended (Blackwell et al., 1996). Interestingly, these binding modes may be the result of different combinations of OB folds making contact with the ssDNA. It may also explain in part the differing affinities observed for RPA binding to ssDNA, with association constants ranging from 10^8 to 10^{11} M^{-1} (Kim et al., 1994; Kim and Wold, 1995; Liu et al., 2005). Interestingly, unlike bacterial SSBs, which wrap 65 nt of DNA around their homotetrameric structure, RPA alters its conformation allowing it to elongate along the ssDNA. Evidence also exists that RPA can bind to multiple ssDNA substrates at the same time, indicating that RPA may function to bridge ssDNA substrates or to stabilize ssDNA primers (Raghunathan et al., 1997; 2000; Bochkareva et al., 2002; Pestryakov et al., 2004). However one question that remains unanswered is how RPA is displaced from its ssDNA substrate. It may be that a post-translational modification occurs, stimulating the sequential displacement of the OB folds. RPA may be removed by a helicase or other enzymatic reaction. This clearly is an important area of research that should lead to a fuller understanding of how RPA binds and how it is regulated during DNA transactions.

The role of SSBs in DNA replication

SSBs are central to DNA replication in all three domains of life. Replication protein A (RPA), as the name suggests, was originally identified as an essential component required for SV40 to replicate its DNA (Dean et al., 1987; Fairman and Stillman, 1988; Wobbe et al., 1987; Wold and Kelly, 1988). In the SV40 system the large T antigen coordinates assembly of the replisome through a direct physical interaction with RPA, DNA polymerase α/primase and topoisomerase (Borowiec et al., 1990; Hurwitz et al., 1990). As mentioned earlier this purification resulted in the identification of all three RPA subunits: RPA70, 34 and 14. So far in humans the data suggest that RPA is required for DNA replication, while no clear role in replication has been shown for either hSSB1 or 2 (Richard et al., 2008). In contrast to RPA, both hSSB1 and hSSB2 do not localize to replication foci in S-phase cells and their deficiency does not influence S-phase progression. Furthermore, these proteins fail to substitute for RPA in SV40-based *in vitro* replication assays (Ellen Fanning, personal communication). RPA functions at a number of steps of DNA replication, including at the origin of replication, during initiation of DNA replication,

and during elongation of the replication fork. In eukaryotes, DNA replication is initiated from multiple origins on each chromosome. These origins are bound by the origin recognition complex (ORC) which then recruits replication licensing factors Cdc6 and Cdt1, which together load minichromosome maintenance proteins (MCMs) forming the pre-initiation complex (Bell and Dutta, 2002).

The role of RPA in the pre-initiation of DNA replication in higher eukaryotes still remains a topic of debate. Currently sites of DNA replication are determined by the colocalization of RPA and DNA polymerase δ processivity factor PCNA (Adachi and Laemmli, 1992; Dimitrova et al., 1999). However while these two proteins colocalize into clear nuclear foci, other essential components of the pre-initiation complex do not colocalize with RPA (cdc6, ORC, MCMs). The initial evidence of RPA's involvement in replication initiation was conducted using the *Xenopus* system. Here, upon the incubation of sperm nuclei with egg extract, RPA can be seen to rapidly locate to the chromatin (< 20 min) and to form what is believed to be pre-initiation complexes (Adachi and Laemmli, 1992; Coue et al., 1996). These RPA foci form prior to nuclear membrane formation and approximately 20 min prior to DNA replication. However this differs from mammalian cells where no RPA foci are observed in normal G1 cells (Dimitrova et al., 1999; Dimitrova and Gilbert, 2000a; 2000b), but mainly in S-phase cells (Dimitrova et al., 1999). It has been speculated that the RPA foci observed in *Xenopus* are unrelated to DNA replication and represent non-specific storage of RPA prior to its requirement in replication (Francon et al., 2004).

There have been several studies that suggest a role for RPA phosphorylation in cellular DNA replication and repair. The N-terminus of RPA34 is phosphorylated by the Cdk2 family of kinases during the S and G2/M phase of the cell cycle (Din et al., 1990; Dutta and Stillman, 1992; Fang and Newport, 1993; Pan et al., 1994; Oakley et al., 2003; Anantha et al., 2008). The exact function of this phosphorylation event remains poorly understood as some studies were unable to show regulation of RPA ssDNA binding activity by changes in the pattern of RPA phosphorylation (Pan et al., 1995; Philipova et al., 1996). Further evidence also provided doubt on the relevance of the G1/S phosphorylation of RPA34, as the efficiency of DNA replication is not affected by Cdc2 (Henricksen and Wold, 1994). RPA34 is also hyperphosphoryled by Cdc2-cyclin B during mitosis (Fang and Newport, 1993). Interestingly this appears to be a mechanism of inactivating RPA, as hyperphosphorylated RPA is not bound to chromatin and the hyperphosphorylation disappears after mitotic exit. Consistent with this, purified Cdc2 added to purified interphase chromatin results in RPA foci disassembly (Cuvier et al., 2006). Intriguingly, the same study also demonstrated that the disassembly of RPA from chromatin during mitosis was not solely dependent on Cdc2, but also required ORC2, since the binding of Cdc2 to chromatin was impaired in ORC-deficient cells. Disassembly of RPA during mitosis appears to be an essential process, since a RPA34 variant, mutated at the Cdc2 sites to prevent phosphorylation, is defective in mitotic chromosome assembly. However this RPA variant still functions normally in DNA replication, suggesting that ORC2 is required for the phosphorylation of RPA34 by Cdc2 and this phosphorylation is dispensable for its association with replication centers (Cuvier et al., 2006).

The switch from hyper to hypo-phosphorylated RPA is likely to be catalysed by the 1A/2A phosphatases, as treatment with okadaic acid inhibits the dephosphorylation of RPA34 (Francon et al., 2004). This is further supported by the inhibition of DNA replication in *Xenopus* egg extracts immuno-depleted of phosphatase 2A (Lin et al., 1998). After mitosis hypophosphorylated RPA34 is the predominant form and remains until S-phase entry. It binds strongly to chromatin and forms discrete nuclear foci (Francon et al., 2004). Hyperphosphorylation of RPA34 also occurs after DNA damage including IR (Liu and Weaver, 1993), UV (Carty et al., 1994) or with radiomimetic agents such as camptothecin (Shao et al., 1999). These treatments result in the activation of the phosphatidylinositol 3-kinase related kinases, ATM, ATR and DNA-PK. These kinases can phosphorylate five of the potential seven sites on the N terminus of RPA34 (Fotedar and Roberts, 1992; Liu and Weaver, 1993; Brush et al., 1994; Pan et al., 1994; Niu et al., 1997; Zernik-Kobak et al., 1997; Gately et al., 1998; Chan et al., 2000; Wang et al., 2001). This phosphorylation has been shown to inhibit DNA replication *in vivo* and *in vitro* (Carty et al., 1994; Park et al., 1999a; Vassin et al., 2004); however, other studies have shown no effect of RPA phosphorylation on ssDNA binding or in SV40 DNA replication assays (Brush et al., 1994; Henricksen et al., 1996).

The association of RPAs at replication origins is surprisingly not dependent on ORCs. *Xenopus* extracts depleted of ORCs can still form RPA foci (Coleman et al., 1996). However it is still not clear whether immunodepletion of ORCs would be sufficient to prevent the formation of a pre-initiation complex, as ORCs are abundant and only a small amount of ORCs is required to form the replication complexes (Rowles et al., 1996; Walter and Newport, 1997). ORCs and RPA also bind to different elements in the origin so it may be possible that RPA locates to its element independently of ORC. Interestingly ORC itself may be able to displace RPA that has inappropriately bound to its element. ORCs are reported to bind ssDNA with a 3-fold higher affinity than RPA (Lee et al., 2000); however, ORCs have little affinity for ssDNA shorter than 85 bp. Supporting the independent nature of ORC and RPA assembly at the origin

of replication is the inhibition of RPA foci formation by p21, which is a cdk2/cyclin E inhibitor. This inhibition prevents the entry of cdc45 and DNA polymerase α, thus preventing initiation of DNA synthesis. However p21 does not prevent the formation of the pre-replicative complexes consisting of ORC, cdc6 and MCM (Yan and Newport, 1995; Hua and Newport, 1998; Mimura and Takisawa, 1998). These data would suggest that RPA functions independently of the pre-replication complexes, yet is required for their final activity. There have however been conflicting reports suggesting that RPA binds after cdc45 (Mimura et al., 2000; Walter and Newport, 2000). This model suggests that RPA binds less tightly prior to cdc45, with cdc45 being required for the unwinding of the DNA, exposing ssDNA to which RPA can bind tightly (Mimura et al., 2000). Both sets of data can however be taken together to propose a two stage binding function of RPA. Prior to initiation only a small region of ssDNA would be exposed at the origin, to which RPA can bind; following initiation, with cdc45 unwinding, larger tracks of ssDNA become available, allowing more RPA to bind. The helicase activity of the MCM proteins now allows nascent strand synthesis by the replicative polymerase.

Following origin recognition and binding by the pre-initiation complex, DNA polymerase α/primase must be engaged. RPA interacts directly with both the DNA polymerase and the primase subunits and functions to stabilize the complex. RPA reduces the miss-incorporation rate of the polymerase, acting as a fidelity clamp (Frick and Richardson, 2001; Maga et al., 2001). In this complex the primase activity of DNA polymerase α functions to synthesize short RNA ~12 nucleotide primers which are then elongated by approximately 20 bases of DNA by DNA polymerase α (Conaway and Lehman, 1982a; 1982b). In addition to starting the leading strand synthesis, DNA polymerase α also initiates each of the approximately 20 million Okazaki fragments (Garg and Burgers, 2005). After synthesis of the RNA/DNA hybrid primers DNA polymerase α is then replaced by the more processive DNA polymerases δ and ε. This switch is mediated by replication factor C (RFC) which displaces DNA polymerase α from the primer, by competing for the binding of RPA (Maga and Hubscher, 1996). DNA polymerase ε synthesizes the leading strand while δ completes each Okazaki fragment synthesis initiated by α (Burgers, 2009).

RPA also functions in DNA synthesis to coordinate the removal of the initiator RNA primers of the Okazaki fragments by facilitating the sequential actions of helicase/nuclease (Dna2) and flap endonuclease (Fen1) in yeast (Bae and Seo, 2000; Bae et al., 2001). RPA has been shown to bind to the DNA polymerase δ displaced RNA–DNA flap and recruit Dna2. This recruitment stimulates the cleavage of this flap, leaving a shorter 5–7 nt flap which is then processed by Fen1. This leaves nicked duplex DNA which can be joined by DNA ligase (Bae and Seo, 2000; Bae et al., 2001). In the human SV40 system processing of nascent Okazaki fragments can be carried out by the Fen1 and RNaseH1 nucleases (Waga and Stillman, 1998).

SSBs and the link with homology directed repair (HDR)

HDR is one of the main pathways to repair DSBs in DNA. HDR can also repair a number of other substrates including ssDNA gaps, interstrand cross links, as well as being required for the recovery of stalled and collapsed DNA replication forks (Paques and Haber, 1999; Michel et al., 2004). HDR requires a sister chromatid to act as a DNA template for repair and this predominantly occurs in S and G2 cells. The DNA ends are first recognized and resected in the 5′ to 3′ direction by nucleases. The resulting 3′ single-stranded tails then invade the DNA double helix of a homologous, undamaged partner molecule to form an intermediate called a D loop (Figure 2). The invading strand is then extended by the action of a DNA polymerase, which copies information from the partner strand, whilst the second end is captured through annealing to the extended D loop. Following branch migration, the resulting DNA crossovers (Holliday junctions) are resolved to yield two intact DNA molecules.

One of the central complexes required for the initial stages of HDR is the MRN complex (de Jager et al., 2001; Lisby et al., 2004; Shroff et al., 2004; Stracker et al., 2004). The MRN complex is composed of three principle proteins: Mre11, Rad50 and Nbs1. This complex functions to process the double strand DNA break, exposing a length of ssDNA that will be the substrate for strand invasion of sister chromatids. Interestingly the complex exhibits both 3′ to 5′ exonuclease activity and ssDNA endonuclease activity (Krogh and Symington, 2004), with the endonuclease rather than exonuclease activity of MRN being required for resection (Williams et al., 2008). In human cells the interaction of the MRN complex with CtIP (C-terminal region of Adenovirus E1A binding protein (CtBP)-interacting protein), stimulates MRN endonuclease activity and end resection. The next step is thought to involve the binding of RPA to the resected 3′ tail. Consistent with this, RPA, like the MRN complex, localizes rapidly to sites of DSBs with activity of both CtIP and MRE11 being required for the formation of RPA foci (Chen et al., 2008; Jazayeri et al., 2006; Myers and Cortez, 2006; Sartori et al., 2007). Interestingly the retention of RPA at sites of DSBs also requires the presence of BRCA1 (Chen et al., 2008). This observation requires further investigation as it either implies that resection of the DSB by the MRN complex does not occur in the absence of BRCA1 or that RPA requires BRCA1 for loading or

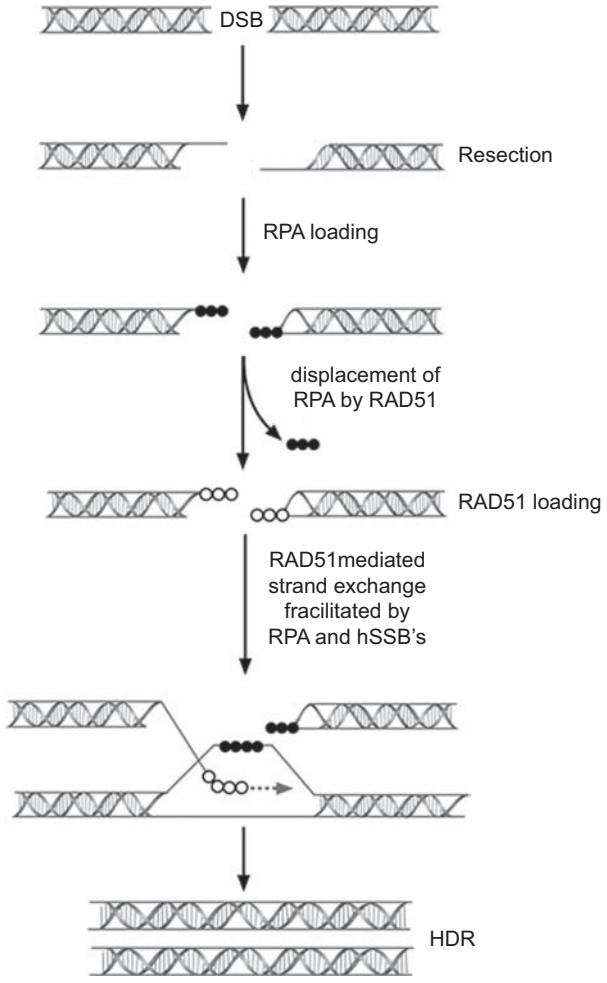

Figure 2. Homology directed repair (HDR) of double strand breaks (DSBs), possible involvement of RPA and hSSB1. HDR is primarily active in late S and G2 phases of the cell cycle due to Cdk-dependent resection of DSBs mediated by the MRN complex together with CtIP to form single stranded (ssDNA) ends. ssDNA is then bound by RPA followed by loading of Rad51 facilitated by various mediators (Rad52, Rad51 paralogs, and BRCA2). Rad51 then catalyzes invasion of the sister chromatid called D-loop formation. RPA and hSSB1 facilitate Rad51 mediated strand exchange *in vitro* (see text for details).

maintenance. Once loaded, RPA is believed to protect the naked ssDNA from attack by nucleases and free radicals as well as preventing formation of secondary structures and binding by inappropriate proteins.

RPA must be removed from the ssDNA strand to allow the loading of Rad51 recombinase (Figure 2). The exact mechanism by which this happens is not clear. It is known that Rad51 can bind the A-OB fold of RPA70 (Stauffer and Chazin, 2004). This interaction could compete for binding with ssDNA, and as such may be the mechanism by which RPA is displaced from DNA. Mutational analysis of Rad51 confirmed the importance of Rad51 interaction with RPA in HDR pathway. Loss of this interaction prevents the formation of the Rad51 nucleofilament (Stauffer and Chazin, 2004). A proposed mechanism of RPA displacement suggests that the Rad51 N-terminus captures RPA that has been displaced from a 3′ ssDNA overhang. The elongation of the Rad51 nucleofilament proceeds in this manner assisted by ATP hydrolysis, displacing RPA as it progresses (Stauffer and Chazin, 2004). Rad51 alone is not sufficient for displacement of RPA from ssDNA. Addition of saturating amounts of RPA prior to or at the same time as Rad51 is inhibitory to strand invasion (Sugiyama et al., 1997; Sung, 1997b; Symington, 2002; Krejci et al., 2003). However, RPA itself can facilitate the loading of Rad51 onto the ssDNA filament if added after the addition of Rad51 in the strand invasion reaction (Song and Sung, 2000; Sugiyama and Kowalczykowski, 2002; Symington, 2002). Another protein, Rad52, also acts to facilitate the loading of Rad51 and displacement of RPA. Rad52 can bind to both the RPA32 and RPA70 subunits (Davis and Symington, 2003; Jackson et al., 2002; Mer et al., 2000), although it is not clear if it can interact with both subunits simultaneously. Similarly, in yeast, Rad52, through its interaction with RPA, facilitates the loading of Rad51 onto DNA (Song and Sung, 2000; Sugiyama and Kowalczykowski, 2002; Symington, 2002). This occurs by accelerating the displacement of RPA from the DNA and facilitating presynaptic complex formation (Sugiyama and Kowalczykowski, 2002). Rad52 and Rad51 have been shown to interact directly although through different domains of RPA (Milne and Weaver, 1993; Shen et al., 1996). In higher eukaryotes it has been shown that Rad52 can function *in vitro* to stimulate Rad51 loading; however, the *in vivo* function of Rad52 is not required for Rad51 nucleofilament formation. Rad51 loading, is, however, mediated *in vivo* through Rad51 paralogs (Rad51B, Xrcc2 and Xrcc3) (Sung, 1997a; McIlwraith et al., 2000; Song and Sung, 2000; Sigurdsson et al., 2001; Sugiyama and Kowalczykowski, 2002; Cahill et al., 2006).

RPA displacement may also be mediated by BRCA2. The crystal structure of the BRCA2 c-terminal region revealed five domains (Yang et al., 2002). The first domain is a helical domain predicted to be involved in protein–protein interactions; following this domain are three distinct OB folds, named OB1, OB2 and OB3. These BRCA2 OB folds bind to ssDNA in a manner similar to RPA (Yang et al., 2002). The fourth domain within OB2 forms a protruding tower-like structure referred to as a tower domain. The tower domain consists of a pair of long anti-parallel α-helices supporting a three helix bundle. Interestingly this study also found that the tower like structure binds to double stranded DNA junctions found at the processed DSBs. The importance of this domain is clear as four of the seven most common missense mutations of BRCA2 occur in this c-terminal domain. The BRCA2 OB folds also binds the DSS1 protein, the product of a gene mapped to a region lost in the deleted in split foot/split hand

syndrome (Marston et al., 1999; Yang et al., 2002). The crystal structure of DSS1 bound BRCA2 suggests that DSS1 may mimic a small region of ssDNA, and thus play a role in regulating DNA binding by BRCA2. It has also been shown that RPA interacts with BRCA2 and that a common cancer predisposing mutant of BRCA2 fails to interact correctly with RPA (Wong et al., 2003). Taken together, these studies suggest that the BRCA2 OB folds therefore may compete with and displace RPA from ssDNA.

BRCA2 is also thought to be involved in Rad51 loading; this is backed by evidence that BRC motifs in BRCA2 directly interact with Rad51 (Bork et al., 1996; Wong et al., 1997; Chen et al., 1998). Consistent with this, cells defective for BRCA2 exhibit gross chromosomal instability and are sensitive to a wide range of DNA damaging agents and fail to form Rad51 foci (Godthelp et al., 2002; Yuan et al., 1999). The crystal structure of BRCA2 BRC4 bound to Rad51 revealed a region within BRC4 that mimics the interaction interface between two Rad51 molecules preventing them from oligomerizing, suggesting that BRCA2 might hold Rad51 molecules in the monomer state, leaving them primed for loading onto ssDNA at processed DSBs (Pellegrini et al., 2002). Together these studies suggest that BRCA2 functions in the displacement of RPA and in the loading of Rad51.

Notably, hSSB1-deficient cells are defective in repair of double strand DNA breaks (DSBs) by the HDR pathway. hSSB1 accumulates at the sites of DSBs and co-localizes with other repair proteins (Richard et al., 2008). hSSB1 functions both at early and late stages of HDR where it is required for efficient signaling responses to DSBs. Like RPA, hSSB1 functions *in vitro* to stimulate Rad51-mediated strand exchange (Richard et al., 2008) and Rad52 and polymerase eta-mediated second-end capture and DNA synthesis reactions (our unpublished data). Consistent with this, loss of hSSB1 results in spontaneous chromosomal aberrations and a significant increase in radiation-induced chromosomal aberrations (Richard et al., 2008). Taken together these data suggest that, like RPA, hSSB1 plays a crucial role in HDR. It is unclear at present why both RPA and hSSB1 are needed for HDR and what distinguishes the roles of these two proteins. High-resolution wide-field microscopy has demonstrated that radiation-induced RPA and hSSB1 foci do not co-localize directly but are proximal to each other, forming touching foci. This may suggest that their functions are distinct but that they act within the same repair centers. Future study of these two proteins will define more clearly their cellular roles. It will also be of interest to determine whether hSSB1 and RPA co-operate together in HDR or if each has a distinct role.

The role of SSBs in the repair of stalled replication forks

DNA damage during S-phase can be caused by a number of endogenous or exogenous factors and poses a major threat to the accuracy and efficiency of DNA synthesis. DNA lesions that interfere with the progress of the replication fork may lead to fork blockage and collapse producing gaps and one-sided DSBs (Li and Heyer, 2008). DSBs can arise either as a consequence of DNA replication fork collapse and replisome disassembly or as an intermediate to restart stalled or collapsed replication forks. Moreover, genetic experiments have identified natural DNA sequences that are difficult to replicate and lead to fork stalling and fork breakage; these are designated as natural pause sites. These processes have significant ramifications for human disease, as they involve fragile sites, which represent a common form of genomic instability in humans (Freudenreich, 2007). Furthermore, aberrant resolution of replication stress is a major cause of genomic instability in yeast (Kolodner et al., 2002).

There are two main mechanisms which lead to restarting of the replication fork (Figure 3); homology directed repair (HDR) and translesion synthesis (TLS) (Budzowska and Kanaar, 2009). As mentioned earlier in this article HDR utilizes a homologous region of DNA to repair or bypass the lesion and is therefore less prone to errors. In contrast, TLS bypasses the damage using low-fidelity polymerases and is consequently more error-prone. Both these repair mechanisms require the generation of ssDNA and also the recruitment of RPA. This will be discussed in further detail below.

HDR at the stalled fork

The type of damage sustained by the DNA in S-phase influences the mechanism by which it is repaired. Whether it is the leading or lagging strand of the DNA that is damaged also has an affect. These two factors influence the structures that arise at stalled replication forks and the sub-pathways that are required to resolve them. For example, a bulky lesion induced by UV light is too large to be processed by a replicative DNA polymerase and will stall DNA synthesis. If the lesion occurs in the lagging strand the next Okazaki fragment upstream may be utilized for repair. However, experiments in *E. coli* have shown that if the lesion occurs in the leading strand replication forks can be re-established downstream of the lesion (Heller and Marians, 2006a). Repair of replication forks in both the leading and lagging strands results in a ssDNA gap behind the fork, which is repaired by either HDR or the cooperative action of TLS and replicative polymerases (Figure 3).

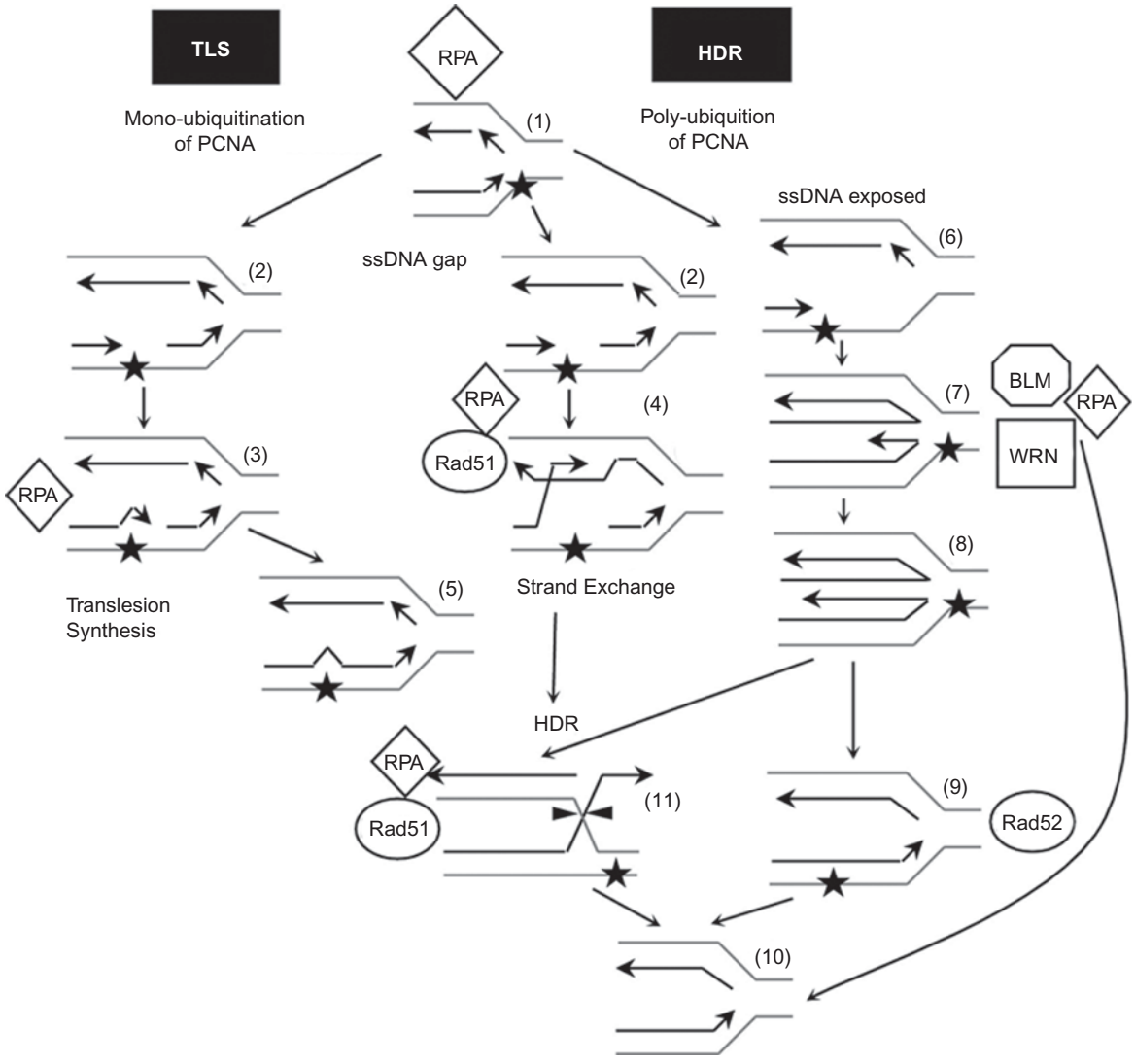

Figure 3. RPA regulated repair of stalled replication forks: (1) when a replication fork collides with a DNA lesion it may stall. (2) DNA synthesis may be reinitiated downstream of the lesion, resulting in a ssDNA gap. The ssDNA gap may be repaired by TLS (3), which requires recruitment of a TLS polymerase or HDR via template switching (4), which requires recruitment of Rad51. Both these mechanisms allow bypass of the lesion (5). The replication fork stalled at the lesion (6) can also regress (7), (8), which requires the action of helicases stimulated by RPA. (9), if the fork regresses far enough the replication fork will reinitiate past the damage and replication can continue (10). Alternatively the regressed replication fork may be resolved by HDR (11), which requires RPA-dependent Rad51 recruitment.

When the lesion occurs in the leading strand, replication can continue temporarily on the lagging strand. This does not disrupt the action of the helicase, which can continue to unwind the DNA. The fork may then regress to form a "chicken foot" structure, which resembles a four-way Holliday junction intermediate. This structure avoids breakage of the replication fork and can be resolved via template switching which is likely to be independent of Rad51, but still requires RPA and Rad52-dependent DNA annealing (Heller and Marians, 2006b). Single-ended DSBs may arise when the replication fork collides with a nick in the DNA or from specific endonucleolytic processing at stalled forks. These single-ended DSBs are specifically repaired by HDR. Similar to conventional HDR described above, HDR of stalled replication forks requires RPA to promote ATRIP:ATR loading onto DNA (Ball et al., 2005). RPA has been shown to be essential for Rad51 foci formation in response to hydroxyurea, which leads to replication fork stalling (Sleeth et al., 2007). Rad51 foci at stalled replication forks are indicative of HDR. In addition, RPA has been shown *in vitro* to be required for Rad51 loading onto DNA structures that mimic stalled replication forks. RPA also relocalizes to DNA damage repair foci at stalled replication forks, along with other DNA proteins including members of the MRN complex (Robison et al., 2004; Manthey et al., 2007). RPA also interacts with Mre11 following replication fork stalling

induced by hydroxurea (Robison et al., 2004). NBS1, another member of the MRN complex, has been shown, along with the DNA damage kinase ATR, to be required for the hyperphosphorylation of RPA once replication forks are stalled. This phosphorylation event inhibits RPA's destabilization activity of DNA, but does not alter its DNA binding activity (Binz et al., 2003; Oakley et al., 2003).

RPA also co-localizes with members of the RecQ family of helicases including Werner's syndrome protein (WRN) and Bloom's syndrome protein (BLM) at stalled replication forks (Constantinou et al., 2000; Sanz et al., 2000; Sakamoto et al., 2001). Deficiency in BLM or WRN proteins leads to cancer-prone syndromes in humans, known as Bloom's and Werner's syndromes, respectively (Epstein et al., 1966; Chaganti et al., 1974). Cells from patients with these syndromes display genomic instability. Both WRN and BLM possesses ATP-dependent 3′-5′ helicase activity (Karow et al., 1997). RPA has also been shown to interact with both WRN and BLM and is proposed to stimulate their recruitment to stalled replication forks (Brosh et al., 1999; Shen et al., 2003; Doherty et al., 2005). It has been suggested that the primary role of the RecQ helicases is to maintain the integrity of replication forks and to restart stalled forks, as deficient cells have severe replication defects. These defects include hypersensitivity to agents that stall replication forks, defective restart of stalled replication forks, prolonged S-phase and diminished proliferation (Martin et al., 1970) and accumulation of abnormal DNA structures (Fukuchi et al., 1989). It has been suggested that the RecQ helicases may inhibit Rad51-dependent strand exchange and contribute instead to the restart of stalled replication forks via fork regression (Bugreev et al., 2007; Wu, 2008). This is supported by evidence that conventional HDR is elevated in BLM deficient cells and implicates RecQ helicases as negative regulators of HDR (Chaganti et al., 1974; Luo et al., 2000). Interestingly RPA has been shown *in vitro* to promote the helicase activity of BLM along long stretches of duplex DNA through a direct interaction (Brosh et al., 2000; Doherty et al., 2005). A recent study also provided evidence that BLM could unwind short DNA duplexes resembling stalled replication fork structures and that this activity was also stimulated to some extent by RPA (Yodh et al., 2009). RPA also stimulates the helicase activity of WRN (Brosh et al., 1999).

Another group of proteins implicated in replication fork recovery are the Fanconi anemia (FA) proteins. Like deficiencies in WRN and BLM, deficiencies in the FA proteins lead to a genome instability syndrome in humans called Fanconi's anemia, characterized by chromosomal instability (Grompe and D'Andrea, 2001). A total of 13 complementation groups of FA proteins have been discovered and many of these proteins have been implicated in DNA repair, including replication fork stabilization and recovery. FA proteins are recruited to stalled replication forks in an RPA-dependent manner in *Xenopus* extracts, again implicating RPA as an important regulator for stabilizing stalled forks (Wang et al., 2008). The FA downstream effector FANCD2 also co-localizes with and interacts with BLM at stalled replication forks (Pichierri et al., 2004). RPA interacts with the FANCJ helicase following replication fork stalling and RPA increases the processivity of FANCJ helicase activity (Gupta et al., 2007). It is suggested that FANCJ may function on short DNA duplexes that can arise during replication restart and that RPA may cooperate to allow further processing of longer substrates.

Since RPA interacts with many helicases at the site of stalled replication forks it is difficult to dissect its precise role in the repair of damaged and stalled forks; however, it appears likely that RPA may be required to exchange helicases at different points in repair. Different interactions may also cause conformational changes in RPA that alter its binding properties to ssDNA. Taken together, it appears that RPA is required to place helicases onto the DNA but helicases may also influence RPA binding to ssDNA, suggesting that the dependence might be mutual (Fanning et al., 2006). It would also be of considerable interest to determine whether hSSB1 and hSSB2 function in HDR in response to stalled replication fork.

Translesion synthesis

TLS employs a specialized set of DNA polymerases to continue replication through damaged DNA. The common characteristic of these polymerases is generally more open active sites, which enables them to accommodate bulkier DNA templates (Yang, 2003). These specialized polymerases also often lack proofreading activity, allowing them to copy past lesion-containing DNA, and consequently making them intrinsically more error-prone (Kunkel et al., 2003). The choice of whether a lesion is repaired by HDR or TLS is believed to be regulated by PCNA. In response to DNA damage the ubiquitin-ligase Rad18/Rad6 catalyses the mono-ubiquitination of PCNA. If PCNA is mono-ubiquitinated the lesion is repaired by TLS; in contrast if PCNA is poly-ubiquitinated by another E3 ligase, Mms/Ubc13, the lesion is repaired by HDR (Kannouche et al., 2004). RPA was found to interact with Rad18 in both yeast and mammalian cells and purified RPA can recruit Rad18 to ssDNA *in vitro* (Davies et al., 2008). This suggests that RPA may regulate the switch between HDR and TLS at stalled replication forks. There is also some recent evidence that RPA may stimulate TLS through an interaction with DNA polymerase λ (Crespan et al., 2007; Maga et al., 2007; Krasikova et al., 2008). Although it is unclear how each specific polymerase is selected and recruited,

it is suggested that RPA together with PCNA may have a role in this process (Maga et al., 2007).

SSBs and involvement in nucleotide excision repair (NER)

The NER pathway plays an important role in the maintenance of genome stability by eliminating a large variety of structurally different lesions that are formed in the genome as a result of exposure to genotoxic agents within the environment or exerted by endogenous processes. The NER pathway is a highly sophisticated and conserved mechanism, which can recognize and remove an array of different nucleotide damage events. However the primary targets of this pathway are ultraviolet (UV) light-induced cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts. How the NER pathway recognizes its diverse substrate base may not lie specifically in the damage but in the distortion caused to the helix. NER can be split into two pathways: global genomic NER (GG-NER), which removes DNA damage from entire genome, and transcription coupled NER (TC-NER) which corrects lesions located on actively transcribed genes. Defects in the NER pathway are associated with severe photosensitivity with predisposition to skin cancers and premature aging. Xeroderma pigmentosum is a repair syndrome with seven complementation groups with most groups having defects in both GG-NER and TC-NER. Cockayne syndrome is specific to TC-NER and has two complementation groups designated as CSA and CSB.

The role of RPA in the NER pathway has been firmly established (Coverley et al., 1991; 1992; Guzder et al., 1995; Mu et al., 1995). RPA functions in the NER pathway both to stabilize the ssDNA products of the process and to recruit and retain proteins at the repair site. Its role in early stages of NER was first suggested when requirement of RPA for NER was seen to be bypassed by the *Escherichia coli* UvrABC, a complex involved in the incision of the DNA (Coverley et al., 1992). The RPA34 subunit interacts directly with the XPA protein and the RPA-XPA complex is thought to play a role in damage recognition (He et al., 1995; Lee et al., 1995; Li et al., 1995; Matsuda et al., 1995; Stigger et al., 1998) however, this role is contentious, based on recent data showing the assembly of the NER complex using normal and repair-deficient cells (Batty and Wood, 2000; Batty et al., 2000; Volker et al., 2001). The damage recognition protein appears to be XPC, which forms a complex with hHR23B, and *in vivo* XPC is essential for the recruitment of all subsequent NER factors including XPA (Figure 4). Furthermore, XPA is recruited relatively late in the process after unwinding of the lesion by XPB and XPD, the ATPases/helicases of the transcription and repair factor TFIIH. XPA together with RPA then assist in the expansion of the DNA bubble and the full opening of the lesion around the damage site (Evans et al., 1997; Mu et al., 1997). It is believed that RPA binds to the undamaged DNA strand (de Laat et al., 1998) occluding an area of approximately 30 nt (the optimal size for one RPA heterotrimer binding to DNA). As mentioned previously RPA binds ssDNA with a defined polarity. This is particularly relevant in NER as the 5′ end of RPA interacts with XPG and the 3′ end of RPA interacts with ERCC1-XPF (He et al., 1995; Matsunaga et al., 1996; Bessho et al., 1997;

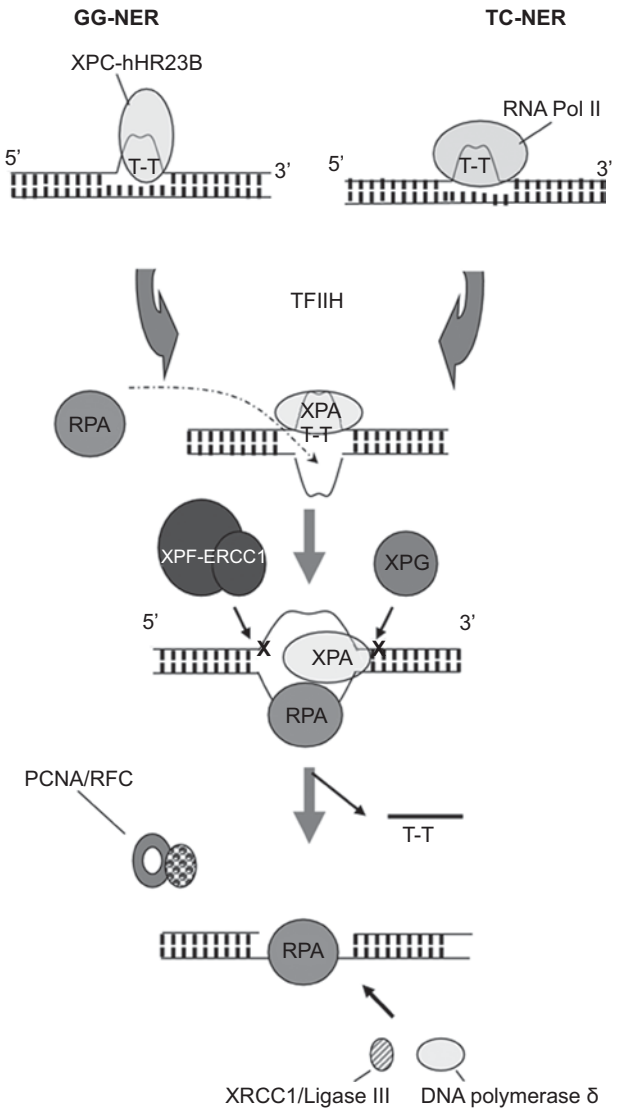

Figure 4. The possible roles of RPA in nucleotide excision repair (NER): The damage recognition complex XPC-hHR23B (for global genomic (GG) NER) and stalled RNA Pol II (for transcription coupled NER) allows for the recruitment of core NER factors including TFIIH, XPA and RPA which open up the DNA around the site of damage. XPA-RPA complex then recruits the ERCC1-XPF complex. The ERCC1-XPF and XPG endonucleases incise the damaged DNA strand both 5′ and 3′ of the lesion respectively, followed by gap filling and ligation.

de Laat et al., 1998). RPA not only defines the orientation with which these nucleases bind, but it also functions to protect the correct strand from nuclease attack, directing and stimulating cleavage by ERCC1-XPF to the damaged DNA strand (de Laat et al., 1998). As well as stimulating and directing the NER nuclease, RPA also may function to stimulate the DNA polymerases involved in gap filling (Figure 4). DNA polymerases δ and ε are implicated in NER and both enzymes are stimulated by the presence of RPA; however, this stimulation is not as the result of protein:protein interactions as RPA may be replaced by other SSBs (Kenny et al., 1989; 1990; Tsurimoto et al., 1989). Therefore RPA has multiple roles to play in the NER pathway. After UV damage, XPA and RPA interact to fully open the lesion around damaged DNA; RPA then recruits ERCC1-XPF, which then cleaves 3′ of RPA. XPG is bound to the 5′ end of RPA, which then cleaves the flap structure. RPA then stimulates the DNA polymerases, which fill in the gap prior to ligation.

The RPA34 subunit of RPA is phosphorylated in response to UV in an ATR-dependent manner and this phosphorylation is required for suppression of DNA synthesis after UV-light exposure (Olson et al., 2006). Consistent with this, the addition of purified recombinant RPA to UV irradiated Hela cell extracts has been shown to restore normal levels of DNA synthesis using an SV40 DNA-based *in vitro* replication assay (Carty et al., 1994). However, phosphorylated and non phosphorylated RPA interact equally as well with XPA and both forms support NER to similar levels (Stigger et al., 1998). Notably, like RPA, hSSB1 is stabilized after exposure of cells to UV damage (Richard et al., 2008) and recruited to sites of UV-induced lesion. Therefore it would be of interest in the future to examine whether hSSB1 plays a role in NER.

SSBs and DNA damage checkpoint response

DNA damage checkpoints are biochemical pathways that delay cell cycle entry in response to DNA Damage (Zhou and Elledge, 2000). In mammals ataxia-telangiectasia mutated (ATM) and ATM and Rad3 related (ATR) protein kinases function as critical regulators of DNA damage checkpoints (Abraham, 2001; Khanna and Jackson, 2001). Both are members of the PI3 kinase-related kinases (PIKK) family. ATM is crucial for cellular responses to DSBs whereas ATR functions as a critical regulator of replication stress induced by DNA-damaging agents or replication inhibitors. There is accumulating evidence to support the role of RPA in checkpoint response through assembly of two independent checkpoint complexes, 9-1-1/Rad17-Rfc2-5 and ATR-ATRIP, at the sites of DNA damage (Zou and Elledge, 2003; Zou et al., 2003). The 9-1-1 complex is analogous to the replicative sliding clamp, PCNA (proliferating cell nuclear antigen), and is loaded on to DNA by the specialized RFC (replication factor C) complex containing the human Rad17 protein. In human cells, RPA interacts with the 9-1-1 complex and facilitates the loading of the complex to gapped and primed structures *in vitro*. The loaded 9-1-1 complex recruits an ATR activator, topoisomerase binding protein 1 (TopBP1), which directly binds and stimulate the kinase activity of ATR-ATRIP complex (Figure 5) (Kumagai et al., 2006).

The RPA-coated single-stranded DNA (ssDNA) is also believed to be an important signal for localization of the ATR-ATRIP complex at the site of damage (Zou and Elledge, 2003). The ATRIP N-terminus binds directly to the N-terminal OB-fold domain of the RPA70 subunit. ATR is recruited by the ATR-interacting protein (ATRIP), which binds the RPA-ssDNA that accumulates at DNA lesions. Binding of ATR:ATRIP to the RPA coated ssDNA results in the activation of ATR, which then phosphorylates downstream targets including Chk1 and Rad17 (Zou et al., 2003). Consistent with this, chromatin association and nuclear foci formation of ATR is dependent on RPA, after exposure to DNA damage. Recent work has also shown that the MRN complex creates regions of ssDNA by an ATM and CDK-dependent process in S/G2 phase cells following exposure to ionizing radiation (Figure 5). These ssDNA regions subsequently bind RPA and serve as a signal to activate ATR, providing evidence that, in this situation, ATM functions upstream of ATR (Jazayeri et al., 2006). Despite the crucial requirement of RPA coated ssDNA for the localization of ATRIP:ATR to DNA breaks, their involvement in ATR activation remains contentious, as disruption of the RPA–ATRIP interaction reduces but does not eliminate the ability of ATR-ATRIP complex to localize to sites of DNA damage and results only in mild defects in ATR phosphorylation of Chk1 (Ball et al., 2005), suggesting additional means of ATR-ATRIP recruitment are sufficient to allow activation of ATR. Consistent with this, the recruitment of ATR to ssDNA, although dependent on RPA, is also known to require the cofactors claspin and Cut5 (Kumagai et al., 2004; Lin et al., 2004). Furthermore, it will be important to determine whether recently identified hSSB1 provides an alternative mechanism of recruitment of the ATR-ATRIP complex to ssDNA (Richard et al., 2008). Interestingly in that regard, hSSB1 modulates ATM activation and activity and therefore influences activation of multiple checkpoints in response to ionizing radiation (Figure 5; Richard et al., 2008). hSSB2, on the other hand, is dispensable for ATM activation and ionizing radiation-induced checkpoint arrest (our unpublished data).

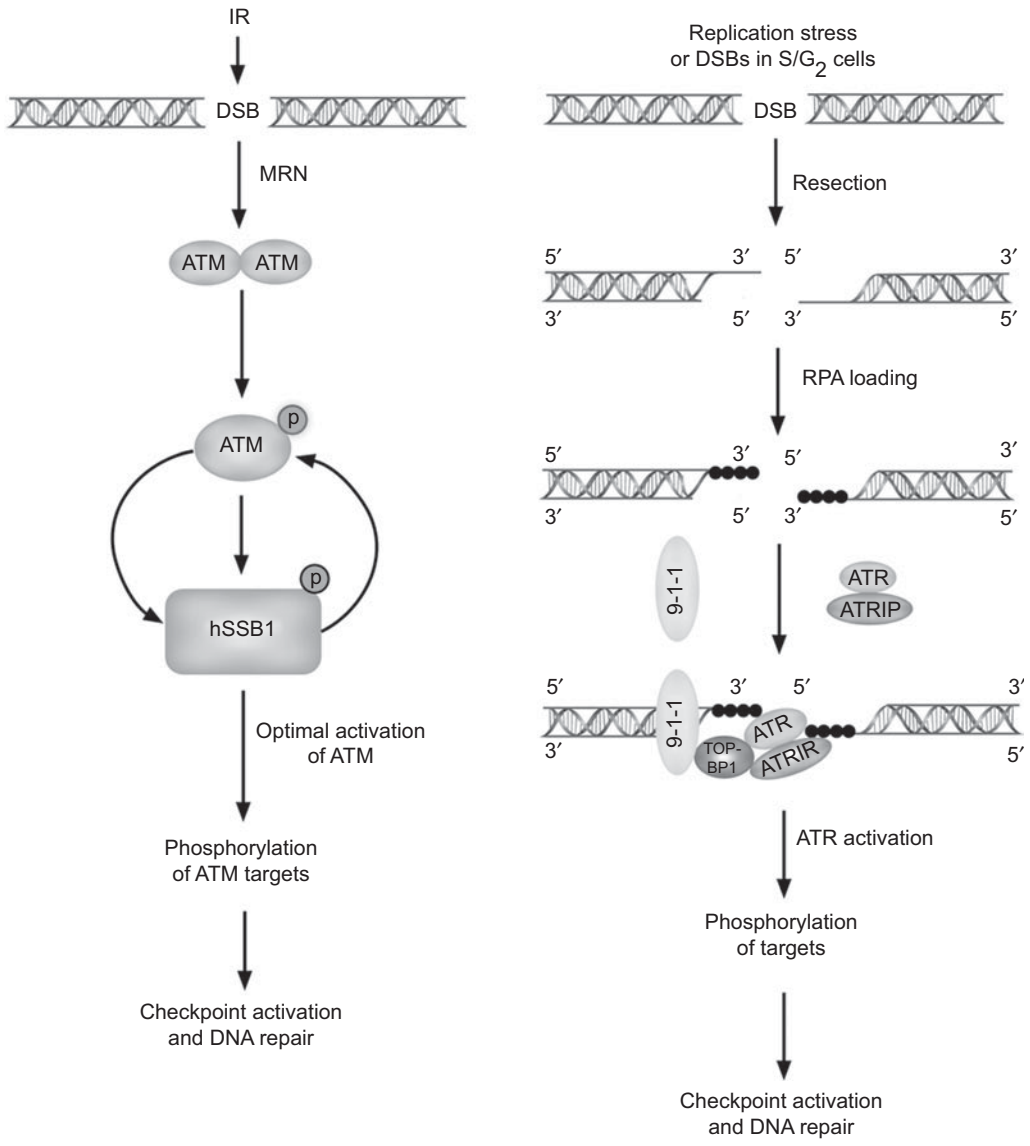

Figure 5. Involvement of hSSB1 and RPA in the checkpoint response to DNA damage and replication stress. (A) In this model hSSB1 is required for optimal ATM activation and activity. The MRN complex anchors ATM to lesion and converts it from a dimer to monomer active form, which phosphorylates hSSB1. A positive feedback loop is then set up by phosphorylated hSSB1 resulting in further stimulation of ATM activation and activity and the consequent activation of checkpoints and DNA repair. (B) In this model RPA coated ssDNA contribute to independent recruitment of ATR-ATRIP complex and 9-1-1 checkpoint clamp to sites of damage. 9-1-1 recruits Top-BP1 and stabilizes the interaction between Top-BP1 and ATR-ATRIP, resulting in activation of ATR, phosphorylation of a number of substrates involved in checkpoint control and DNA repair.

Summary

SSBs are clearly an evolutionarily important family of proteins, which have roles in numerous cellular processes where ssDNA is exposed. RPA not only functions to sequester the exposed ssDNA, protecting it from incorrect processing or degradation, but also to recruit repair proteins and coordinate its correct processing. This review has outlined in part our understanding of RPA function. RPA is clearly a central component of many processes and as such has the ability to alter many aspects of its function. This includes its modular conformation, which sets it aside from the bacterial SSBs, its ability to alter the mode by which it binds ssDNA, its functional modulation by post-translational modifications and its ability to interact with its protein partners. Although the field has progressed remarkably towards understanding the functions of RPA, many questions have now been raised by the discovery of two new simple SSBs in humans (hSSB1 and hSSB2). Future investigations should address why different SSBs are needed, what the functional differences between them are, what they interact with, and whether they have some overlapping and unique functions. It can be implied for

now that hSSB1 and hSSB2 must have important cellular functions due to the level of conservation through evolution. Their discovery challenges existing models of DNA transactions involving ssDNA. Deciphering the role of these two new hSSBs will not only allow us to understand the cellular functions of RPA more clearly but also help in the development of new models of DNA damage repair, and may ultimately result in the development of novel cancer therapeutics.

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