Biochemical and Biophysical Research Communications 427 (2012) 785–789

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

Bénédicte Recolin [1], Domenico Maiorano *

Genome Surveillance and Stability Laboratory, Department "Molecular Bases of Human Diseases", CNRS-UPR1142, Institute of Human Genetics, 141 rue de la Cardonille, 34396 Cedex 5 Montpellier, France

ARTICLE INFO

Article history:
Received 27 September 2012
Available online 6 October 2012

Keywords:
ATR
Replication stress
Chk1
Replication protein A

ABSTRACT

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

1. Introduction

Checkpoints are feedback mechanisms that coordinate cell cycle phases thus ensuring that each phase of the cell cycle will be faithfully completed before the onset of the next one. DNA damage triggers activation of specific checkpoints that delay the cell cycle to facilitate DNA repair. Detection and signalling of DNA damage during S-phase is crucial in maintenance of genomic stability. This checkpoint, also known as replication checkpoint, depends upon the ATR kinase, a member of the PIKKK family ([1] for review). Inhibitors of replicative polymerases, such as aphidicolin, or inhibitors of nucleotide synthesis (hydroxyurea), inhibit the enzymatic activity of DNA polymerases, however the helicase activity is not affected. The result of this enzymatic uncoupling is the production of single-stranded DNA (ssDNA) generated by DNA unwinding by the helicase [2–5]. This DNA substrate is bound by the major eukaryotic ssDNA binding protein, the trimeric RPA complex, that stimulates replicative DNA polymerases to synthesise small replication intermediates that are binding sites for the essential checkpoint clamp, the 9–1–1 complex [1,4,6–9]. The interaction of 9–1–1 with ATR bound to its partner, the ATRIP protein, and the TopBP1 protein promotes ATR activation. Some types of DNA damage, such as interstrand crosslinks, natural replication forks barriers or specialized chromatin structures, also arrest the progression of the helicase, so that no replication fork uncoupling is produced [10]. In this situation checkpoint activation occurs in a single stranded-independent way since replication forks are not unwound. Both events lead to a more or less pronounced arrest of the replication fork, also known as replication stress.

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

* Corresponding author. Fax: +33 (0) 4 34 35 99 01.
E-mail address: domenico.maiorano@igh.cnrs.fr (D. Maiorano).
[1] Present address: Department of Experimental Oncology, University Medical Center Utrecht, Stratenum 2.241, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands.

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.09.139

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

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

2. Materials and methods

2.1. Xenopus egg extracts

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

2.2. DNA replication assay

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

2.3. Antibodies

Anti-RPA32. and RPA70 antibodies were previously described [7,11]. Human RPA32 and RPA70 antibodies were from AbCam. Histone H3 antibody was from Abcam. Anti-$^{P\text{-}S345}$Chk1 antibody was from Cell Signalling. ORC2 and Cdc45 antibodies were a kind gift of Marcel Méchali (Institute of Human Genetics). Proteins transferred to nitrocellulose membranes were probed with specific antibodies using the SNAPi.d.® Western blot system (Millipore). Detection of Western blot signals was obtained by enhanced chemio-luminescence (ECL) using the Luminata® reagent (Millipore).

2.4. Immunological methods

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

2.5. Recombinant proteins

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

2.6. Subcellular fractionation procedures

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

3. Results and discussion

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

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

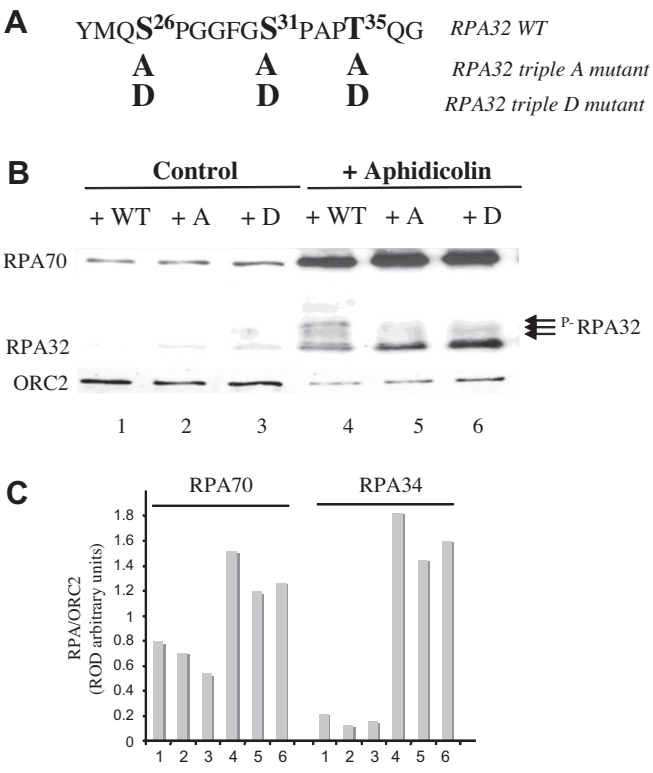

Fig. 1. RPA phosphorylation mutants do not affect the nucleation of RPA at arrested forks. (A) Schematic representation of the Xenopus RPA32 N-terminus wild-type, or triple phospho-deficient ("A" mutant) or phospho-mimetic ("D" mutant) mutants in the indicated residues. (B) Western blot of chromatin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

with Rad9, a subunit of the 9–1–1 checkpoint clamp, suggesting a role in checkpoint activation [8]. Xenopus RPA32 is rapidly phosphorylated upon replication fork stalling with aphidicolin (Fig. 1B, lane 4). We have then asked whether phosphorylation of RPA32 may have a role in activation of the replication checkpoint. Xenopus RPA32 phospho-mimetic (“D” mutant) or phospho-deficient forms (“A” mutant) previously described in Xenopus [21] as well as in human cells [19] were produced as recombinant proteins (Fig. 1A). These mutants were added into Xenopus egg extracts together with sperm DNA, and chromatin fractions were obtained by biochemical fractionation after a 90 min incubation. Proteins eluted from isolated chromatin were blotted with antibodies against RPA70, and an anti-RPA32 antibody that recognise both hypophosphorylated as well as phosphorylated RPA32 subunit [11]. After addition of aphidicolin both RPA70 and RPA32 accumulated onto chromatin as previously observed [2,7], and the RPA32 kDa subunits is specifically phosphorylated (Fig. 1B, ^{P}-RPA32, arrows). Addition of either the “A” or “D” mutant abolished the appearance of the phosphorylated forms of RPA32 on chromatin upon replication fork stalling by aphidicolin but did not alter the ability of RPA to nucleate at arrested forks (Fig. 1B, compare lanes 1–3 with lanes 4–6, and Fig. 1C). The ORC2 protein, a subunit of the origin recognition complex that binds replication origins, serves here as a chromatin loading control. Quantification of the Western blot signals of panel B confirms this conclusion and also shows that the RPA34 subunit accumulates at higher level than the RPA70 subunit, suggesting a possible difference in nucleation of these subunits onto single stranded DNA produced at arrested forks. These results suggest that both RPA34 mutants do not interfere with formation of RPA-coated single-stranded DNA, and therefore with replication fork uncoupling that generates ssDNA, an important requirement for checkpoint activation, thus suggesting that RPA phosphorylation at these sites is not implicated in regulating RPA nucleation onto single-stranded DNA.

3.2. Effect of RPA34 phosphorylation mutants on checkpoint activation

Next, we have analysed the effect of RPA32 phosphorylation mutants on activation of the S-phase checkpoint following aphidicolin addition into egg extracts. To this purpose we have analysed the ATR-dependent phosphorylation state of the Chk1 protein kinase, an important transducer of the checkpoint signalling pathway, at two different stages of DNA synthesis, initiation and elongation. As can be seen (Fig. 2A), addition of aphidicolin at the initiation stage ($t = 0$) resulted in complete inhibition of DNA synthesis, while addition of aphidicolin during ongoing DNA synthesis resulted in a quick (within 20 min) inhibition of DNA replication that remained at steady-state levels until the 90 min time point, showing that the aphidicolin treatment was indeed very effective. Analysis of Chk1 phosphorylation in extracts containing either wild-type or mutants forms of the RPA32 subunit shows that, only the RPA phospho-deficient mutant (+A) stimulated Chk1 phosphorylation, while the phospho-mimetic mutant (+D) did not do so (Fig. 2B and D). Quantification of the Western blot signals obtained with the anti-phospho Chk1 antibody confirms this

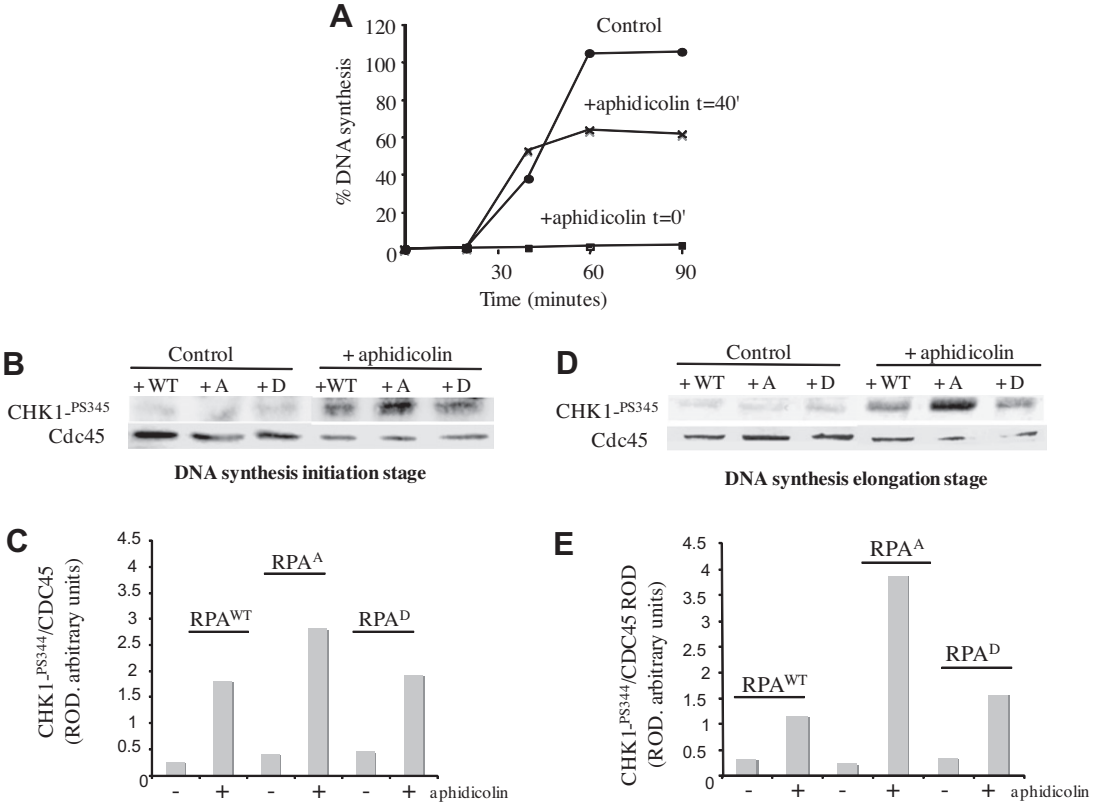

Fig. 2. Checkpoint activation by RPA phosphorylation mutants. (A) Kinetics of DNA synthesis of Xenopus egg extracts in the absence (control) or presence of aphidicolin added at the initiation ($t = 0$) or elongation ($t = 40$ min) stage of DNA synthesis. (B) Detection of Chk1 phosphorylation by Western blot with an anti-phospho ^{S345}Chk1 antibody with (+) or without (control) aphidicolin at the initiation stage of DNA synthesis ($t = 0$). Cdc45 serves here as a loading control. (C) Quantification of P-^{S345}Chk1 signals observed in the experiment shown in panel B. Western blot signals were quantified by densitometry scanning and expressed as relative optical density (ROD) compared to the Cdc45 signal as loading control. (D) Detection of Chk1 phosphorylation by Western blot with an anti-phospho ^{S345}Chk1 antibody with (+) or without (control) aphidicolin added at the elongation stage ($t = 40$ min). (E) Quantification of P-^{S345}Chk1 observed in the experiment shown in panel D. Western blot signals were quantified by densitometry scanning and expressed as relative optical density (ROD) compared to the Cdc45 signal as loading control.

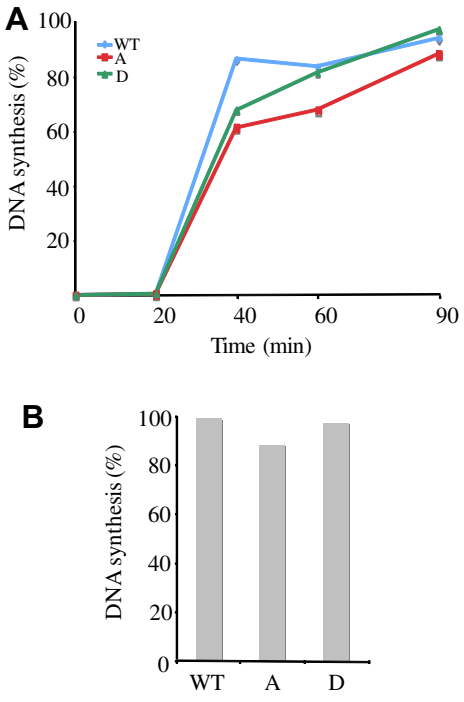

Fig. 3. RPA32 phosphorylation mutants do not stimulate DNA synthesis. (A) Kinetics of DNA synthesis of Xenopus egg extracts in the presence of either wild-type (WT), phospho-deficient (A) or phospho-mimetic (D) recombinant mutant proteins. (B) Quantification of DNA synthesis obtained upon 90 min of the experiment shown in panel A.

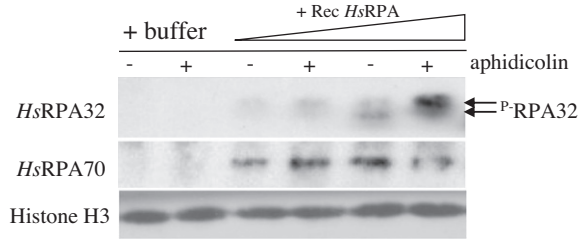

Fig. 4. Human RPA32 is phosphorylated in egg extracts treated with aphidicolin. Chromatin fractions obtained upon incubation of sperm chromatin in RPA-depleted extracts in the absence (+buffer) or presence of increasing concentrations of a recombinant human RPA complex (Rec*Hs*RPA).

conclusion (panels C and E). The stimulation effect on the checkpoint of the "A" mutant was much more pronounced when aphidicolin was added during the elongation stage of DNA synthesis (Fig. 1D). This is very likely due to the higher number of replication origins present when aphidicolin is added once DNA synthesis has already started, since addition of aphidicolin at the very early stages of DNA synthesis activates the ATR-dependent checkpoint that inhibits further origin firing [23,24].

Because DNA synthesis is an important requirement for checkpoint activation [6,25] we have checked that stimulation of checkpoint activation by the RPA phospho-deficient mutant is not due to a super activation of replication origins that can explain higher levels of Chk1 phosphorylation. As can be seen (Fig. 3A), neither the "A" mutant, nor the "D" mutant increased DNA replication in egg extracts compared to addition of wild-type recombinant RPA32 protein since the total amount of DNA synthesized at the end of the replication reaction is very similar for all reactions (panel B), thus excluding a positive effect on DNA synthesis. Taken together these results suggest that phosphorylation of RPA32 upon aphidicolin treatment is not implicated in activation of the replication checkpoint. These findings are consistent with the observation that the replication-competent hypophosphorylated form of RPA32 [26] plays a role in checkpoint activation [7], while the phosphorylated form associates with repair foci [19], and interacts with Rad50, Mre11 and Rad51 proteins [14,19]. Thus, stimulation of checkpoint activation by RPA is very likely due to its function in DNA replication. Previous *in vitro* studies have shown that phosphorylated RPA32 has less DNA double stranded destabilising activity, suggesting a switch of RPA from a replication to a repair mode [27]. To determine whether RPA32 phosphorylation at unwound forks is also conserved in evolution, we next asked whether phosphorylation of human RPA32 could be observed in Xenopus egg extracts upon replication forks stalling with aphidicolin. To this end, we added human recombinant RPA complex to egg extracts. We have recently shown that this complex is fully functional since completely rescues the DNA synthesis defect observed upon removal of XlRPA [7]. As can be seen (Fig. 4) only the human RPA32 and not the human RPA70 subunit was specifically phosphorylated upon aphidicolin treatment in egg extracts, as observed with the Xenopus RPA 32 and 70 subunits (Fig. 1B). The result of this experiment then shows that mechanisms of RPA32 subunit phosphorylation upon replication fork stalling are conserved from Xenopus to human cells.

In this work we have explored the role of RPA32 phosphorylation at CDK and ATR sites and propose that phosphorylation of the RPA32 subunit is dispensable for checkpoint activation induced by replication stress with aphidicolin. Very recent work in human cells suggests that RPA32 phosphorylation by DNA-PK, a PIKKK family member, functions in activation of the checkpoint at forks stalled with DNA damaging agents that do not induce strong replication fork uncoupling (e.g. without extensive formation of ssDNA), such as etoposide [28]. Altogether these data, suggest that phosphorylation of RPA32 at forks unwound by aphidicolin may occur in preparation of replication forks restart after a long term arrest, for instance by homologous recombination, a process that may implicate the participation of DNA-PK but relying on previous phosphorylation of RPA32 by ATR [28]. Hence, it is likely that post-translation modification of the RPA32 subunit mediated by the ATR kinase during the DNA damage response may function in DNA repair and/or recombination. In the future it will be interesting to determine the localization of both hypophosphorylated and hyperphosphorylated forms of RPA32 at arrested forks.

Note added in proof

While this work was in the writing process a study in a human *in vitro* system that reproduces checkpoint activation reached similar conclusions [29].

Acknowledgments

This work was supported by grants from ARC (personal grant to B.R. and grant 3156 to D.M.) and FRM ("Equipes"). We are grateful to M. Méchali for reagents and helpful discussions. We also wish to thank H. Ubscher (Institute of veterinary Medicine, Switzerland) for the generous gift of recombinant human RPA.

References

[1] K.A. Cimprich, D. Cortez, ATR: an essential regulator of genome integrity, Nat. Rev. Mol. Cell Biol. 9 (2008) 616–627.
[2] J. Walter, J. Newport, Initiation of eukaryotic DNA replication: origin unwinding and sequential chromatin association of Cdc45, RPA, and DNA polymerase alpha, Mol. Cell 5 (2000) 617–627.
[3] T.S. Byun, M. Pacek, M.C. Yee, J.C. Walter, K.A. Cimprich, Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint, Genes Dev. 19 (2005) 1040–1052.

[4] L. Zou, S.J. Elledge, Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes, Science 300 (2003) 1542–1548.
[5] V. Costanzo, D. Shechter, P.J. Lupardus, K.A. Cimprich, M. Gottesman, J. Gautier, An ATR- and Cdc7-dependent DNA damage checkpoint that inhibits initiation of DNA replication, Mol. Cell 11 (2003) 203–213.
[6] C. Van, S. Yan, W.M. Michael, S. Waga, K.A. Cimprich, Continued primer synthesis at stalled replication forks contributes to checkpoint activation, J. Cell Biol. 189 (2010) 233–246.
[7] B. Recolin, S. Van der Laan, D. Maiorano, Role of replication protein A as sensor in activation of the S-phase checkpoint in Xenopus egg extracts, Nucleic Acids Res. 40 (2012) 3431–3442.
[8] X. Wu, S.M. Shell, Y. Zou, Interaction and colocalization of Rad9/Rad1/Hus1 checkpoint complex with replication protein A in human cells, Oncogene 24 (2005) 4728–4735.
[9] B. Shiotani, L. Zou, Single-stranded DNA orchestrates an ATM-to-ATR switch at DNA breaks, Mol. Cell 33 (2009) 547–558.
[10] S. Lambert, A.M. Carr, Checkpoint responses to replication fork barriers, Biochimie 87 (2005) 591–602.
[11] Y. Adachi, U.K. Laemmli, Study of the cell cycle-dependent assembly of the DNA pre-replication centres in Xenopus egg extracts, EMBO J. 13 (1994) 4153–4164.
[12] S. Mimura, T. Masuda, T. Matsui, H. Takisawa, Central role for cdc45 in establishing an initiation complex of DNA replication in Xenopus egg extracts, Genes Cells 5 (2000) 439–452.
[13] M.S. Wold, Replication protein A: a heterotrimeric, single-stranded DNA-binding protein required for eukaryotic DNA metabolism, Annu. Rev. Biochem. 66 (1997) 61–92.
[14] J.G. Robison, J. Elliott, K. Dixon, G.G. Oakley, Replication protein A and the Mre11.Rad50.Nbs1 complex co-localize and interact at sites of stalled replication forks, J. Biol. Chem. 279 (2004) 34802–34810.
[15] M.P. Carty, M. Zernik-Kobak, S. McGrath, K. Dixon, UV light-induced DNA synthesis arrest in HeLa cells is associated with changes in phosphorylation of human single-stranded DNA-binding protein, EMBO J. 13 (1994) 2114–2123.
[16] V.F. Liu, D.T. Weaver, The ionizing radiation-induced replication protein A phosphorylation response differs between ataxia telangiectasia and normal human cells, Mol. Cell. Biol. 13 (1993) 7222–7231.
[17] R.G. Shao, C.X. Cao, H. Zhang, K.W. Kohn, M.S. Wold, Y. Pommier, Replication-mediated DNA damage by camptothecin induces phosphorylation of RPA by DNA-dependent protein kinase and dissociates RPA:DNA-PK complexes, EMBO J. 18 (1999) 1397–1406.
[18] J.E. Nuss, S.M. Patrick, G.G. Oakley, G.M. Alter, J.G. Robison, K. Dixon, J.J. Turchi, DNA damage induced hyperphosphorylation of replication protein A. 1. identification of novel sites of phosphorylation in response to DNA damage, Biochemistry 44 (2005) 8428–8437.
[19] V.M. Vassin, M.S. Wold, J.A. Borowiec, Replication protein A (RPA) phosphorylation prevents RPA association with replication centers, Mol. Cell. Biol. 24 (2004) 1930–1943.
[20] A.W. Murray, Cell cycle extracts, Methods Cell Biol. 36 (1991) 581–605.
[21] O. Cuvier, M. Lutzmann, M. Mechali, ORC is necessary at the interphase-to-mitosis transition to recruit cdc2 kinase and disassemble RPA foci, Curr. Biol. 16 (2006) 516–523.
[22] E. Olson, C.J. Nievera, V. Klimovich, E. Fanning, X. Wu, RPA2 is a direct downstream target for ATR to regulate the S-phase checkpoint, J. Biol. Chem. 281 (2006) 39517–39533.
[23] D. Shechter, V. Costanzo, J. Gautier, ATR and ATM regulate the timing of DNA replication origin firing, Nat. Cell Biol. 6 (2004) 648–655.
[24] K. Marheineke, O. Hyrien, Control of replication origin density and firing time in Xenopus egg extracts: role of a caffeine-sensitive ATR-dependent checkpoint, J. Biol. Chem. 279 (2004) 28071–28081.
[25] W.M. Michael, R. Ott, E. Fanning, J. Newport, Activation of the DNA replication checkpoint through RNA synthesis by primase, Science 289 (2000) 2133–2137.
[26] P. Francon, J.M. Lemaitre, C. Dreyer, D. Maiorano, O. Cuvier, M. Mechali, A hypophosphorylated form of RPA34 is a specific component of pre-replication centers, J. Cell Sci. 117 (2004) 4909–4920.
[27] S.K. Binz, Y. Lao, D.F. Lowry, M.S. Wold, The phosphorylation domain of the 32-kDa subunit of replication protein A (RPA) modulates RPA-DNA interactions. Evidence for an intersubunit interaction, J. Biol. Chem. 278 (2003) 35584–35591.
[28] S. Liu, S.O. Opiyo, K. Manthey, J.G. Glanzer, A.K. Ashley, C. Amerin, K. Troksa, M. Shrivastav, J.A. Nickoloff, G.G. Oakley, Distinct roles for DNA-PK, ATM and ATR in RPA phosphorylation and checkpoint activation in response to replication stress, Nucleic Acids Res. (2012).
[29] L.A. Lindsey-Boltz, J.T. Reardon, M.S. Wold, A. Sancar, In vitro analysis of the role of RPA and RPA phosphorylation in ATR-mediated checkpoint signaling, J. Biol. Chem. (2012).