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Interplay of DNA damage and cell cycle signaling at the level of human replication protein A

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ABSTRACT

Replication protein A (RPA) is the main human single-stranded DNA (ssDNA)-binding protein. It is essential for cellular DNA metabolism and has important functions in human cell cycle and DNA damage signaling. RPA is indispensable for accurate homologous recombination (HR)-based DNA double-strand break (DSB) repair and its activity is regulated by phosphorylation and other post-translational modifications. HR occurs only during S and G2 phases of the cell cycle. All three subunits of RPA contain phosphorylation sites but the exact set of HR-relevant phosphorylation sites on RPA is unknown. In this study, a high resolution capillary isoelectric focusing immunoassay, used under native conditions, revealed the isoforms of the RPA heterotrimer in control and damaged cell lysates in G2. Moreover, the phosphorylation sites of chromatin-bound and cytosolic RPA in S and G2 phases were identified by western and IEF analysis with all available phosphospecific antibodies for RPA2. Strikingly, most of the RPA heterotrimers in control G2 cells are phosphorylated with 5 isoforms containing up to 7 phosphates. These isoforms include RPA2 pSer23 and pSer33. DNA damaged cells in G2 had 9 isoforms with up to 14 phosphates. DNA damage isoforms contained pSer4/8, pSer12, pThr21, pSer23, and pSer33 on RPA2 and up to 8 unidentified phosphorylation sites.

1. Introduction

Human cells continuously encounter DNA double-strand breaks (DSBs) that must be repaired for cells to survive and for human health [1,2]. DSBs are repaired by highly complex, multi-step processes involving large protein–DNA complexes. When cells respond to DNA damage, repair proteins are regulated by the action of kinases and phosphatases. The phosphorylation patterns of proteins differ based on the type of DNA repair needed [3,4]. DSBs are repaired by non-homologous end joining (NHEJ) and homologous recombination (HR) pathways [5]. Of interest to this research is HR which includes two pathways: genetic conversion (GC) and single-strand annealing (SSA). In human GC, after the recognition and initial maturation of the ends of a DSB, replication protein A (RPA) binds to the resulting single-stranded DNA (ssDNA). In the next step, the breast cancer type 2 susceptibility protein, BRCA2, displaces RPA from the ssDNA sequences at the DSB ends and loads RAD51 recombinase onto the ssDNA [6]. Alternatively RPA and RAD52 can repair DSBs using the more error-prone SSA pathway [7]. The research reported here is focused on the phosphorylation of RPA in response to DSBs that are relevant to both GC and SSA pathways.

The cell cycle position of cells when a DSB occurs defines which DNA repair mechanisms is available for DSB repair. NHEJ is used in all phases of the cell cycle whereas HR-based repair can only take place in S and G2 since HR pathways use DNA sequences on the

Abbreviations: AT, ataxia telangiectasia; ATM, ataxia telangiectasia mutated kinase; ATR, ataxia telangiectasia and Rad3-related kinase; ATRIP, ATR-interacting protein; CDK, cyclin-dependent kinase; CIP, calf intestinal phosphatase; DDR, DNA damage response; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; DSB, DNA double-stranded break; GC, genetic conversion; HR, homologous recombination; IR, ionizing radiation; NHEJ, non-homologous end joining; NT, N-terminus; PIKK, phosphatidylinositol 3-kinase-related kinase; RPA, replication protein A; SSA, single-strand annealing; ssDNA, single-stranded DNA; TOPBP1, topoisomerase II-binding protein 1.

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sister chromatid for faithful repair of DNA lesions [8,9]. To understand the regulation of DSB repair in S and G2 phases it is important to know the phosphorylation status of repair proteins. Although RPA and its post-translational regulation are crucial for DNA repair in response to DSBs, the knowledge concerning the phosphorylation patterns of RPA during S or G2 phase is still incomplete [10–13].

In response to the threat of damage to their genetic material, eukaryotes have evolved the DNA damage response (DDR) [2]. Ataxia telangiectasia (AT), a rare, neurodegenerative, inherited disease that leads to an increased risk of cancer, was a key component for discovering the kinases governing the DDR in eukaryotic cells [14,15]. At the apex of the DDR there are three related kinases belonging to the phosphatidylinositol 3-kinase-related kinase (PIKK) protein family: AT-mutated (ATM), AT and Rad3-related (ATR), and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) [16–18]. Activated ATM and ATR are thought to be the main regulators of HR whereas DNA-PKcs appears to be more important for NHEJ. DNA damage activates ATM through auto-phosphorylation and acetylation that facilitates the disassociation of the ATM dimer and the formation of highly active kinase monomers [19–21]. In addition, RPA binding to nuclease-resected DSB sites recruits and activates ATR via the ATR-interacting protein (ATRIP) [22]. Then ATR remains at resected sites and is further activated by protein–protein interactions with topoisomerase II-binding protein 1 (TOPBP1) [23]. This activation of the DDR is followed by the induction of a kinase cascade where a multitude of proteins are phosphorylated including cell cycle checkpoint kinases CHK1, and CHK2 that further amplify the signal [24]. Phosphatases also play an important role in the DDR and keep the levels of phosphorylation in balance for DSB repair [25,26]. Loss of phosphatases has been reported to inhibit HR-based DNA repair [27,28] and dephosphorylation of RPA is required for cells to restart the normal cell cycle following repair [29].

RPA is a heterotrimeric protein complex that binds ssDNA with high affinity and is essential for DNA replication, recombination and repair [3,4,23,30–33]. RPA is highly regulated by protein–protein interactions and post-translational modifications. RPA's protein binding partners that are relevant to HR and SSA include RAD51 and RAD52 [34,35]. RPA is known to have binding sites for RAD52 on the 70 kDa (RPA1) and 32 kDa (RPA2) subunits [36] and these interactions are important in RAD52 activity. RPA plays a direct role in the assembly of RAD51 and RAD52 proteins during HR and these interactions are affected by checkpoint signaling [37]. Phosphorylation of RPA by DNA-PKcs induces a conformational change in RPA, involving a DNA binding domain in RPA1, and phosphorylated RPA is more resistant to proteases, indicating less intrinsic disorder [38]. Thus, phosphorylation appears to change the stability of RPA/DSB repair complexes and to alter the structure of RPA.

The N-terminus (NT) of RPA2 is known to be phosphorylated in response to DNA damaging events as well as in a cell cycle-dependent manner (Supplementary Table S1) [3,4]. Residues Ser23 and Ser29 of RPA2 are known cyclin dependent kinase (CDK) sites, and Ser29 has been shown to be mitotically phosphorylated whereas Ser23 phosphorylation has been observed in both mitosis and in S phase [13,39]. There are at least five other sites (Ser4, Ser8, Ser12, Thr21, and Ser33) that are known to be phosphorylated in response to DNA damage by the PIKKs [12,40–43]. RPA2-NT phosphorylation follows preferred pathways with Ser33 phosphorylation by ATR stimulating subsequent phosphorylation at the other sites [44–46]. The RPA2-NT sites also show reciprocal priming effects (e.g. Thr21Ala mutation reduces Ser4/8 phosphorylation and vice versa) [40,45,46]. Phosphorylation of Ser12 occurs at later time points than the other RPA2-NT sites [40]. It has been reported that phosphorylated RPA2 facilitates chromosomal DNA repair [45] and that phosphorylation prevents RPA from associating with replication centers in human cells [47]. The RPA2-NT is phosphorylated in response to ionizing radiation (IR) [13,32,48] and is delayed in cells with inactive ATM kinase [10,42]. Phosphorylated RPA preferentially localizes to DSB repair complexes as shown by enhanced co-immunoprecipitation with RAD51 and RAD52 and colocalization with RAD52 and ATR in nuclear foci [49]. Additionally, we have demonstrated that phosphorylation of RPA regulates the transfer of ssDNA from RPA to RAD52 [50]. In summary, though these findings provide evidence that RPA2 phosphorylation is involved in the HR-relevant DNA damage response to IR, the present knowledge is incomplete.

All three subunits of RPA are phosphorylated but functional studies of these sites have not been reported (Supplementary Tables S1 and S2). In yeast, the ATR and ATM homologs, Mec1 and Tel1, respectively, phosphorylate all three subunits of RPA [51,52]. In the PHOSIDA and PhosphoSitePlus databases human RPA1, RPA2 and RPA3 had 28, 19 and 4 phosphorylation sites, respectively, that were measured at least once by mass spectrometry [53,54]. These sites included six phosphorylated Tyr (underlined in Table S2). In an in vitro study RPA1 was found to be just as hyperphosphorylated as RPA2 [55]. The patterns of RPA phosphorylation differ depending on the type of DNA damaging agent (e.g. UV, hydroxyurea or IR) [55–57]. It is noteworthy that the databases also list a multitude of RPA acetylation and ubiquitination sites. Also, after DNA damage RPA1 can be modified by a 60 kDa polymer of SUMO-2/3 (small ubiquitin-like modifier 2/3) and after heat stress it can be modified with diSUMO-2 [58,59]. Taken together, these data indicate the complexity of RPA's regulation and demonstrate the importance of defining the exact pattern of DSB-induced phosphorylation sites on all three subunits of RPA.

To this end, we have extended these studies by further defining the phosphorylation of the RPA2-NT and the RPA heterotrimer as a whole, during S and G2 phases of the cell cycle, and we have observed the remodeling of these phosphorylation sites upon induction of DNA damage.

2. Materials and methods

2.1. Cell line selection and growth

The UM-SCC-38 WT RPA2 (human squamous carcinoma) cell line was used for all experiments. This cell line has endogenous RPA2 knocked down with shRNA, stably expresses C-terminally HA-tagged RPA2 and allows for efficient isolation of trimeric RPA [40]. Cells were maintained at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (Valley Biomedical), 100 U/mL penicillin (Invitrogen), 100 μg/mL streptomycin (Invitrogen), 20 μg/mL hygromycin B (Cellgro) and 150 μg/mL G 418 (Sigma–Aldrich).

2.2. Antibodies

A table summarizing the primary antibodies used, the companies they were purchased from and their dilutions for western blot and capillary isoelectric focusing is included in Supplementary Table S3. Anti-mouse, anti-rat and anti-rabbit secondary antibodies conjugated with Infrared Dye 800CW (LI-COR) or Infrared Dye 680LT (LI-COR) were used to detect primary antibodies in western blot analysis. Goat secondary antibodies against rabbit and mouse for IEF immunoassays were conjugated to horseradish peroxidase (HRP) and purchased from ProteinSimple. Goat anti-Rat-HRP was purchased from Santa Cruz Biotech.

2.3. Subcellular fractionation

The subcellular fractionation protocol was adapted from Mendez and Stillman [60]. To detect nuclear and cytosolic RPA,