Molecular Cell
Article

Regulation of DNA Repair through DeSUMOylation and SUMOylation of Replication Protein A Complex

Hong Dou,[1] Chao Huang,[2] Melissa Singh,[3,4] Phillip B. Carpenter,[3] and Edward T.H. Yeh[1,2,*]
[1]Department of Cardiology, The University of Texas M.D. Anderson Cancer Center
[2]Texas Heart Institute/St. Luke's Episcopal Hospital
[3]Department of Biochemistry and Molecular Biology
The University of Texas Houston Health Science Center, Houston, TX 77030, USA
[4]Present address: Children's Cancer Hospital, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030, USA
*Correspondence: etyeh@mdanderson.org
DOI 10.1016/j.molcel.2010.07.021

SUMMARY

The replication protein A complex (RPA) plays a crucial role in DNA replication and damage response. However, it is not known whether this complex is regulated by the SUMOylation pathway. Here, we show that the 70 kDa subunit of RPA (RPA70) associates with a Sentrin/SUMO-specific protease, SENP6, in the nucleus to maintain RPA70 in a hypoSUMOylated state during S phase. Campothecin (CPT), an inducer of replication stress, dissociates SENP6 from RPA70, allowing RPA70 to be modified by a small ubiquitin-like modifier 2/3 (SUMO-2/3). RPA70 SUMOylation facilitates recruitment of Rad51 to the DNA damage foci to initiate DNA repair through homologous recombination (HR). Cell lines that expressed a RPA70 mutant that cannot be SUMOylated are defective in HR and have a marked increase in sensitivity to CPT. These results demonstrate that SUMOylation status of RPA70 plays a critical role in the regulation of DNA repair through homologous recombination.

INTRODUCTION

The small ubiquitin-like modifier (SUMO) has been shown to regulate cellular processes by controlling the localization, function, interaction, and stability of a large number of cellular proteins (Hay, 2005; Meulmeester and Melchior, 2008; Mukhopadhyay and Dasso, 2007; Yeh, 2009). SUMOylation is catalyzed by SUMO-specific E1, E2, E3s and is reversed by a family of Sentrin/SUMO-specific proteases, SENPs. In mammalian cells, six different SENPs belonging to three subfamilies have been identified. The first family, consisting of SENP1 and SENP2, has broad specificity for SUMO-1, -2, and -3 and localizes to the nucleus and nuclear envelope, respectively (Gong et al., 2000; Hang and Dasso, 2002; Zhang et al., 2002). The second family consists of SENP3 and SENP5 that favor SUMO-2/3 as substrates and are localized to the nucleolus (Di Bacco et al., 2006; Gong and Yeh, 2006; Yun et al., 2008). Members of the third family include SENP6 and SENP7; each has an additional loop inserted in the catalytic domain and also appears to prefer SUMO-2/3 (Mukhopadhyay and Dasso, 2007; Yeh, 2009). From an evolutionary standpoint, SENP1, SENP2, SENP3, and SENP5 are more closely related to the yeast Ulp1, whereas SENP6 and SENP7 are related to Ulp2 (Li and Hochstrasser, 1999, 2000; Mukhopadhyay and Dasso, 2007). Although deSUMOylation has been extensively studied in vitro, its in vivo functions have only begun to be understood.

We have previously shown that deletion of the murine *SENP1* gene leads to the development of severe fetal anemia as a result of erythropoietin (EPO) deficiency (Cheng et al., 2007). SENP1 regulates transcription of EPO through its ability to regulate the stability of hypoxia-inducible factor 1α (HIF1α). The in vivo functions of other SENPs are less well understood. Depletion of SENP3 by siRNA disrupts nucleolar ribosomal RNA processing, a phenotype similar to knockdown of NPM1 (Haindl et al., 2008). Knockdown of SENP5 by siRNA results in inhibition of cell proliferation and appearance of binucleate cells, suggesting that SENP5 may play a role in mitosis and/or cytokinesis (Di Bacco et al., 2006). Silencing of SENP6 causes redistribution of SUMO2 and SUMO3 into the PML bodies (Mukhopadhyay et al., 2006). Recently, an RNA interference-based screen showed that SENP6, but not other SENPs, functioned in cell proliferation (Kittler et al., 2007), but the mechanisms behind this were not reported.

Replication protein A (RPA), the main eukaryotic ssDNA-binding protein complex, consists of three subunits, RPA1 (RPA70), RPA2 (RPA32), and RPA3 (RPA14). RPA70 is the major ssDNA-binding subunit and is involved primarily in interactions with other DNA metabolism proteins (Fanning et al., 2006; Zou et al., 2006). The RPA32 subunit has low affinity for ssDNA and utilizes its C-terminal α helix domain for other protein interactions. It has been suggested that hyperphosphorylation of RPA32 may redirect RPA from DNA replication to DNA repair (Zou et al., 2006). RPA14 does not exhibit affinity for ssDNA but is required for stable heterotrimer formation (Iftode et al., 1999; Wold, 1997).

In response to replication stress or fork stalling, the long stretches of RPA-coated ssDNA at DNA damage sites serve as a common intermediate structure for the assembly of two independent checkpoint apparatuses, 9-1-1/Rad17-Rfc2-5 and ATR-ATRIP complexes, that initiate the replication checkpoint

response (Zou and Elledge, 2003). RPA plays an important role in the repair of DSBs by homologous recombination (HR) through its ability to interact with Rad51, a recombinase (Galletto and Kowalczykowski, 2007). RPA stimulates DNA strand exchange by removing a DNA secondary structure that is inhibitory to contiguous filament formation. However, RPA inhibits DNA strand exchange when it saturates ssDNA before the addition of Rad51. During HR, cofactors (mediators), such as human FANCD1/BRCA2, and Rad51 paralogs overcome this inhibition and stimulate DNA strand exchange by recruiting Rad51 to replace RPA from ssDNA (Sugiyama and Kowalczykowski, 2002).

Here, we show that RPA70 is SUMOylated on lysine residues 449 and K577, with K449 being the major site. RPA70 is associated with SENP6 during S phase to maintain RPA70 in a hypoSUMOylated state. However, in response to replication-mediated DSBs, SENP6 is dissociated from RPA70, causing an increase of RPA70 SUMOylation that facilitates recruitment of Rad51 to initiate HR. This identifies a specific role of SENP6 in the regulation of RPA complex and reveals that SUMOylation is important in initiating Rad51-dependent HR.

RESULTS

SENP6 Is Associated with RPA70 during the S Phase

To gain further insight into the role of SENP6 in cellular function, we used a yeast two-hybrid screen to identify its potential substrates. RPA70, a key player in DNA replication and DNA damage responses (Fanning et al., 2006; Zou and Elledge, 2003), was isolated in this screen using a SENP6 catalytic site mutant (Cys_{1030}-Ser) as bait. Full-length SENP6 was able to coprecipitate with full-length RPA70 in vivo (Figure 1A). Truncation experiments showed that residues 629–777 of SENP6 were necessary for RPA70 binding (Figure 1A and Figure S1A available online). It has been shown that RPA70 is associated with chromatin and is colocalized with cholorodeoxyuridine (CldU) at the replication foci/fork only during S phase in cytoskeleton (CSK)-Triton-extracted cells, but not in G1 and G_2/M (Dimitrova and Gilbert, 2000). Treatment of CSK-Triton removes soluble RPA70 and retains chromatin-associated RPA70. Using the same treatment, we found that SENP6 colocalized with RPA70 and proliferating cell nuclear antigen (PCNA), a replication foci marker, during S phase (Figure 1B), suggesting that the association between SENP6 and RPA70 occurs at replication structures in S phase. Moreover, endogenous SENP6 coprecipitated with endogenous RPA70 during the S phase, but not in other cell-cycle phases (Figure 1C). This is not due to degradation of SENP6 at the G_2/M phase because endogenous SENP6 protein levels remain constant throughout the cell cycle (Figure 1C, bottom). This association is specific because nonspecific IgG could not precipitate RPA70 and SENP6 (Figure S1B) and is independent of DNA because DNase was included in the process of immunoprecipitation. Thus, the association between SENP6 and RPA70 appears to be cell cycle dependent.

RPA70 Is Modified by SUMO-2/3 In Vivo

Because SENP6 is a SUMO-specific protease (Mukhopadhyay et al., 2006), we asked whether RPA70 is a substrate of SENP6. For this purpose, we knocked down endogenous SENP6 by siRNA. The knockdown was highly efficient, as shown in Figure S2A. As expected, SENP6 knockdown induced the accumulation of higher molecular weight forms of endogenous RPA70 (Figure 2A). To confirm that these higher molecular weight bands were due to SUMOylation, endogenous RPA70 was immunoprecipitated and blotted with anti-SUMO-1 or anti-SUMO-2/3 antibodies. These higher molecular weight bands could readily be detected with anti-SUMO-2/3 (Figure 2B), but not with anti-SUMO-1 antibody (Figure 2B) that had been used to detect endogenous SUMO-1 (Cheng et al., 2007; Häkli et al., 2005; Yamaguchi et al., 2005) or control IgG (Figure 2B). The observation that endogenous RPA70 is modified by endogenous SUMO-2/3 is consistent with the known substrate preference of SENP6 for polySUMOylated SUMO2/3 species (Mukhopadhyay et al., 2006). SUMOylation of RPA70 was further confirmed by cotransfection of Myc-tagged RPA70 and HA-tagged SUMO-2 constructs in COS-1 cells. In the presence of HA-SUMO-2 plasmids, two SUMOylated bands were generated that migrated slower than that of unmodified Myc-tagged RPA70 (Figure 2C). Overexpression of SENP6 decreased the intensity of these two slower-migrating bands, but a SENP6 catalytic mutant did not reduce these bands (Figure 2D). The ability of SENP6 to regulate RPA70 SUMOylation is biologically relevant because endogenous RPA70 SUMOylation is increased following the dissociation of RPA70 and SENP6 in the G_2/M phase (Figure S2B and Figure 1C). The higher molecular weight band shown in Figure S2B was RPA70 modified by SUMO2/3; this was demonstrated by an IP-western experiment (Figure S2C). Taken together, these results demonstrate that SENP6 regulates the SUMOylation status of RPA70 in vivo.

RPA70 contains three consensus SUMOylation motifs, centered on K273, K449, and K577. We tested the ability of these sites to be SUMOylated in vivo by introducing various combinations of K273R, K449R, and K577R point mutations into RPA70. The results showed that RPA70-SUMO-2 conjugates were virtually absent in the RPA70 (K449, 577R) double mutant, RPA70(ΔSUMO) (Figure 2E). The number of SUMOylated conjugates in cells with the K449R mutant was significantly reduced, but in contrast, the K577R mutant only showed a small effect. This indicates that RPA70 is modified in vivo on lysine residues 449 and K577, with K449 being the major site of SUMOylation.

Knocking Down SENP6 Induces Replication Defects that Result in DNA Breaks

To study the relevance of RPA70 and SENP6 association in S phase, we examined the cell-cycle profile of SENP6 knockdown cells. Knocking down SENP6 in HeLa cells caused a delay in the S phase and accumulation of cells in the S and G_2/M phases after release from the second thymidine block (Figure S3A). This cell-cycle defect can be repaired by a siRNA-resistant SENP6, but not by the catalytic inactive SENP6 (Figure S3B). Furthermore, DNA synthesis was markedly reduced, as the number of cells incorporating BrdU dropped from 36.2% (control cells) to 6.4% (SENP6 knockdown cells) (Figure S3C). Furthermore, SENP6 knockdown induced colocalization of RPA70 and SUMO-2/3 at many punctate foci (Figure S3D). Moreover, many of the SUMO-2/3 foci also colocalized with γ-H2AX foci,