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HARP preferentially co-purifies with RPA bound to DNA-PK and blocks RPA phosphorylation

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Keywords: DNA-PK, HARP, RPA, SMARCAL1, annealing helicase

Introduction

The annealing helicase HepA-related protein (HARP; also known as SMARCAL1) is an ATP-dependent annealing helicase that is capable of rewinding DNA structures that are stably unwound due to binding of the single-stranded DNA (ssDNA)-binding protein Replication Protein A (RPA). HARP has been implicated in maintaining genome integrity through its role in DNA replication and repair, two processes that generate RPA-coated ssDNA. In addition, mutations in HARP cause a rare disease known as Schimke immuno-osseous dysplasia. While the molecular basis of SIOD is not fully understood, a potential link between an increase in chromosome breakage and mutations in HARP is consistent with a role of HARP in DNA repair.9

HARP binds preferentially and with high affinity to forked DNA structures (i.e., split ends), which are formed at the ends of ssDNA regions.1 HARP interacts directly with RPA through a conserved motif near the N terminus of HARP.5,7 While this interaction is dispensable for the annealing helicase activity of HARP, it helps localize HARP to sites of DNA replication and repair.5,7 In addition to the annealing helicase activity of HARP, which regulates the amount of RPA-bound ssDNA in the cell, RPA can be regulated through phosphorylation.10-15 RPA, a heterotrimer consisting of RPA1, RPA2 and RPA3, is preferentially phosphorylated on the N terminus of its RPA2 subunit. Several kinases phosphorylate RPA including cyclin-dependent kinase (CDK), ataxia-telangiectasia mutated (ATM), ATM and Rad3-related (ATR), and the DNA-dependent protein kinase (DNA-PK).10-15 Changes in RPA phosphorylation have been observed during cell-cycle progression and in response to DNA damage, which ultimately leads to hyperphosphorylation of RPA. The downstream effects of RPA phosphorylation depend on the residues that are phosphorylated and include checkpoint activation, the regulation of DNA repair, and the modulation of RPA-DNA interactions.16-19

Like RPA, HARP is also phosphorylated in response to replication stress and DNA damage,6,7,20,21 although the significance of HARP phosphorylation is less clear. ATM, ATR and DNA-PK are all capable of phosphorylating HARP.6,7,20,21 Despite the knowledge that HARP associates with RPA, and that both factors are substrates for multiple kinases, little is known about which factors form the preferred HARP complex. In this study, we found that, after RPA, DNA-PK is the most abundant factor in the HARP complex. A stable interaction of DNA-PK with HARP is mediated by RPA and suggests a tendency for HARP to associate with RPA bound by DNA-PK. Using DNA-PK purified from HeLa cells by a rapid DNA-affinity protocol, we found that while DNA-PK is able to phosphorylate RPA in vitro, it is unable to phosphorylate RPA in the presence of HARP. DNA-PK also phosphorylates HARP in vitro and the phosphorylation of HARP by DNA-PK does not significantly affect its annealing helicase activity. Together, our data suggest that HARP inhibits the function or activity of RPA by both eliminating ssDNA via...
the annealing helicase activity of HARP and by preventing RPA phosphorylation by DNA-PK.

**Results and Discussion**

As previously reported, we purified HARP from HeLa cells that constitutively express a FLAG-tagged copy of HARP by tandem-affinity purification. Following the purification, we observed that in addition to RPA, three other factors stably co-purify with HARP. These proteins were identified by mass spectrometry as the subunits of the DNA-PK heterotrimer (i.e., DNA-PKcs, Ku80, and Ku70). Other reports have identified DNA-PK subunits as part of a group of potential cofactors for HARP, but our analysis reveals that RPA and DNA-PK are the two most abundant factors that stably co-purify with HARP (Fig. 1A). Under our purification conditions, we did not detect WRN helicase, a factor previously reported to associate with HARP, suggesting that WRN may be part of a less abundant or less stable HARP complex.

To gain insight into the stability of the HARP-RPA-DNA-PK interactions, we resolved the complex by glycerol gradient sedimentation. We collected fractions off the top of the gradient and analyzed them by SDS-PAGE and silver staining. We found that the DNA-PK heterotrimer is present in the same fractions that contain both HARP and RPA (Fig. 1B, fractions 7 to 11), suggesting that HARP, RPA, and DNA-PK associate in a single, stable complex.

To examine how DNA-PK affects the activity of HARP, we purified the DNA-PK heterotrimer from HeLa cells using a one-step DNA-affinity protocol (Fig. 2A). We first crosslinked sonicated poly-[dI:dC] DNA fragments to Sepharose beads and then used the free DNA ends to purify DNA-PK from HeLa nuclear extracts. We initially prepared nuclear extracts from 4 L of HeLa cells and pre-incubated the extracts with plasmid DNA (which has no free DNA ends) to remove non-specific DNA-binding proteins. We then incubated the pre-cleared extracts with the DNA-resin that contains free DNA ends. The beads were then washed to remove the unbound proteins, and the bound proteins were eluted using a step gradient of increasing NaCl. Under these conditions, the collected fractions consisted almost entirely of the DNA-PK heterotrimer, which eluted from the resin in the fractions containing between 0.3 to 0.4 M of NaCl (Fig. 2B). The identity of the eluted proteins was confirmed by mass spectrometry. A scaled-down version of the protocol was used to extract DNA-PK from HeLa cells from a single 15 cm dish, showing that small-scale preparations of DNA-PK can be rapidly made (Fig. 2C).

Using the native DNA-PK purified from HeLa cell extracts, we first asked whether DNA-PK interacts directly with HARP or associates with the complex through an interaction with RPA. Following several independent purifications of the HARP complex, we noticed that the DNA-PK is often less abundant than HARP and RPA. We considered the possibility that DNA-PK, which is known to associate with RPA, is present in the HARP complex through a direct interaction with RPA. To determine whether RPA is required for DNA-PK to stably associate with the HARP complex, we tested whether a mutant version of HARP, which contains a 10 amino acid substitution in the N-terminal RPA-interaction motif, can interact with DNA-PK. We incubated purified RPA, DNA-PK with either wild-type or mutant HARP, immunoprecipitated the HARP proteins using anti-FLAG antibodies, and analyzed the co-precipitating factors by western blotting (Fig. 3A and B). As previously reported, we found that wild-type but not mutant HARP interacts with RPA. Moreover, DNA-PK readily co-precipitates with wild-type HARP but not the mutant HARP, suggesting that a direct interaction between HARP and DNA-PK is not stable. A similar finding was observed using a truncated version of HARP. We cannot rule out the possibility that RPA and DNA-PK compete for the same binding site on HARP and that the mutant HARP fails to interact with both RPA and DNA-PK. However, we think this is unlikely as our gradient analysis suggests that HARP, DNA-PK, and RPA all appear to stably interact in one single complex. Our findings suggest that HARP exhibits a preference, or tendency, to interact with RPA bound to DNA-PK.

We next used the purified DNA-PK to investigate the phosphorylation of HARP and RPA. In order to examine whether HARP is a substrate for DNA-PK and whether HARP affects the phosphorylation of RPA by DNA-PK, we performed in vitro kinase assays. We incubated DNA-PK with HARP, the RPA heterotrimer, or both in the presence of double-stranded plasmid DNA and [32P]-ATP. We then resolved the proteins by SDS-PAGE, dried and scanned the gel using a phosphorimager. We found that both HARP and RPA can be independently phosphorylated in vitro by DNA-PK (Fig. 3C). As previously reported, DNA-PK preferentially phosphorylates the RPA2.
subunit of RPA. When both HARP and RPA are present in the reactions, the phosphorylation of HARP and RPA2 by DNA-PK is substantially reduced (Fig. 3C). In fact, we were unable to detect any RPA phosphorylation in the presence of HARP. One potential mechanism by which HARP could block the phosphorylation of RPA is by binding and masking the phosphorylation sites of RPA2. This idea is supported by the fact that the N terminus of HARP contains an RPA2-interacting motif. On the other hand, it is also possible that HARP interferes with RPA2 phosphorylation through a more subtle mechanism, such as by interfering with the kinase activity of DNA-PK or by weakening DNA-PK-RPA2 interactions. In order to address these potential models, we compared the levels of phosphorylation of RPA2 by DNA-PK in the presence of wild-type and mutant HARP proteins. The mutant HARP does not stably interact with RPA and should be less likely to mask RPA2 phosphorylation sites. We found that both wild-type and mutant HARP proteins are able to block RPA2 phosphorylation by DNA-PK (Fig. 3D). In addition, while the levels of phosphorylation of wild-type HARP are reduced in the presence of RPA, the levels of phosphorylation of mutant HARP remain high in the presence of RPA. These findings suggest that HARP does not simply mask the phosphorylation sites of RPA through the stable interaction of the two factors. It is likely that a more complex mechanism is behind the ability of HARP to block the phosphorylation of RPA2. For example, in addition to the stable interaction between HARP and RPA that is mediated by the conserved N terminus of HARP, multiple weaker interactions may exist between other regions of HARP and RPA. These interactions may be sufficient for both wild-type and mutant HARP to block the phosphorylation of RPA2. Furthermore, interactions between HARP and DNA-PK may exist that are not strong enough for the mutant HARP to co-purify with DNA-PK, but are strong enough for HARP to affect the ability of DNA-PK to phosphorylate RPA. In contrast, the observed reduction in the phosphorylation of HARP by DNA-PK and in the presence of RPA may be explained by the masking of phosphorylation sites on HARP by RPA. This would explain why mutant HARP, which fails to stably interact with RPA, does not show the reduced levels of phosphorylation seen with wild-type HARP.

The role of phosphorylation in the regulation of the activities of RPA is not entirely understood. Some studies have reported that DNA-PK preferentially phosphorylates RPA that is bound to ssDNA near junctions with dsDNA, such as at fork DNA, which are also the preferred binding sites for HARP. This suggests that HARP, RPA, and DNA-PK may function together at these unique DNA sites. Our in vitro pull-down experiments were performed in the absence of DNA and demonstrate that HARP, RPA, and DNA-PK do not require DNA for their interaction (Fig. 3B). Whether significant amounts of HARP, RPA, and
DNA-PK are interacting without DNA in the nucleus is unclear. The purification of the HARP complex may result in extraction of the three factors off of the DNA. Our study also found that HARP interferes with RPA phosphorylation both by its annealing helicase activity, which limits the amount of ssDNA in the cell, and by preventing phosphorylation of RPA by protein:protein interactions. Thus, it appears that HARP plays a multifaceted role in regulating the function of RPA. The connection between mutations in HARP and SIOD, a disease with a pleiotropic phenotype, is not fully understood. The diverse roles of HARP and RPA in modulating the levels of ssDNA in the cell likely impact many processes such as DNA replication, repair, and transcription, ultimately contributing to the complex nature of SIOD.

**Materials and Methods**

Native HARP complex

The native HARP-containing complex was purified from HeLa cell nuclear extracts by tandem-affinity purification as previously reported. The glycerol gradients were performed by loading the purified HARP complex onto a 4 ml, 10 to 30% linear gradient in HARP buffer (20 mM HEPES, pH 7.6, 1.5 mM MgCl2, 10% glycerol, 0.05% NP40, 1 mM DTT, 0.2 mM PMSF) + 0.1 M KCl. The gradients were centrifuged at 170,000 × g for 3 h at 4 °C.

**Single-step purification of DNA-PK**

The native DNA-PK heterotrimer was purified by a single step DNA-affinity chromatography method. Poly-[dI:dC] (Amersham) was resuspended in H2O and briefly sonicated (three times for 2 s each) to fragment the polymers into smaller fragments. The fragments were then crosslinked to CNBr-(three times for 2 s each) to fragment the polymers into smaller fragments. The fragments were then crosslinked to CNBr-activated Sepharose (GE) according to the manufacturer protocol. Nuclear extracts were prepared from HeLa cells using 0.42 M activated Sepharose (GE) according to the manufacturer protocol. The mix was poured into a 10 ml disposable column and the resin was allowed to settle. The column was drained and the resin washed four times with Binding buffer and the proteins eluted with 10 μl of anti-FLAG resin for 3 h at 4 °C. The resin was then centrifuged at 16,000 × g for 10 min to remove precipitations. The clarified extract was then incubated with the crosslinked Sepharose beads for 10 min at 4 °C on a rotator. The mix was poured into a 10 ml disposable column and the resin was allowed to settle. The column was washed 4 times with 5 column volumes of HARP Buffer + 0.1 M NaCl. The bound proteins were eluted by a step gradient of increasing NaCl.

**Pull-down and in vitro kinase assays**

Recombinant human HARP was purified by baculovirus expression as previously described. The human RPA used in all of the assays was expressed and purified from bacteria as previously described. The pull-down experiments were performed essentially as described. HARP (0.5 μg), RPA (0.5 μg), DNA-PK (0.1 μg) were incubated in 20 μl of Binding buffer (20 mM Hepes [K+], pH 7.6, 0.1 M KCl, 5 mM MgCl2, 3% [v/v] glycerol, 0.25 mg/ml BSA, 0.05 mM EDTA, 0.5 mM DTT, 0.01% (v/v) NP-40) for 20 min at 30 °C. The reactions were then diluted to 200 μl with Binding buffer and incubated with 10 μl of anti-FLAG resin for 3 h at 4 °C. The resin was washed three times with Binding buffer and the proteins eluted with SDS-sample buffer. For the in vitro kinase assays, HARP (0.4 μg), RPA (0.5 μg), DNA-PK (0.3 μg) were incubated in 20 μl of Binding buffer with plasmid DNA (60 ng) and [32P]-ATP and plasmid DNA. The proteins were then resolved by SDS-PAGE and analyzed by phosphorimaging analysis. The in vitro kinase assays were performed in the presence of equal amounts of WT or Mut HARP proteins.

**Figure 3. Interaction and kinase activity of DNA-PK with HARP and RPA.** (A) Purified wild-type (WT) or mutant (Mut) HARP proteins were purified by a baculovirus system and incubated with bacterially expressed RPA and native DNA-PK. (B) The HARP proteins were then immunoprecipitated using anti-FLAG antibodies, and the immunoprecipitated material was analyzed by western blotting. (C) In vitro kinase assays were performed by incubating purified DNA-PK with HARP, RPA, [32P]-labeled ATP and plasmid DNA. The proteins were then resolved by SDS-PAGE and analyzed by phosphorimaging analysis. (D) The in vitro kinase assays were performed in the presence of equal amounts of WT or Mut HARP proteins.
Figure 4. The presence of DNA-PK does not significantly affect the annealing helicase activity of HARP. Annealing helicase assays were performed in the presence or absence of DNA-PK using a plasmid partially loaded with primers. For the reactions that contain both DNA-PK and HARP, the two factors were preincubated in the presence or absence of ATP prior to addition of the RPA-bound DNA substrate. Following the reactions, the purified DNA was analyzed by agarose gel electrophoresis and visualized by ethidium bromide (EtBr) staining. The migration positions of supercoiled and relaxed DNA are indicated.

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Acknowledgments

We thank James T Kadonaga and Patricia Szajner for critical reading of this manuscript. This work was supported by the Dana-Farber Cancer Institute and the Ellison Medical Foundation. Initial studies were also supported with the NIH grant RO1GM058272 to James T Kadonaga.