Phosphorylation of Replication Protein A Middle Subunit (RPA32) Leads to a Disassembly of the RPA Heterotrimer

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Replication protein A (RPA), the major eukaryotic single-strand specific DNA binding protein, consists of three subunits, RPA70, RPA32, and RPA14. The middle subunit, RPA32, is phosphorylated in a cell cycle-dependent manner. RPA occurs in two nuclear compartments, bound to chromatin or free in the nucleosol. We show here that the chromatin-associated fraction of RPA contains the phosphorylated forms of RPA32. Treatment of chromatin with 0.4 M NaCl releases bound RPA and causes a separation of the large and the phosphorylated middle RPA subunit. Unmodified RPA in the nucleosolic fraction remains perfectly stable under identical conditions. Phosphorylation is most likely an important determinant of RPA desintegration because dialysis from 0.4 to 0.1 NaCl causes the reformation of trimeric RPA only under dephosphorylating conditions. Biochemical studies with isolated Cyclin-dependent protein kinases showed that cyclin A/CDK1 and cyclin B/CDK1, but not cyclin E/CDK2, can phosphorylate human recombinant RPA in vitro. However, only a small fraction of in vitro phosphorylated RPA desintegrated, suggesting that phosphorylation may be one, but probably not the only, determinant affecting subunit interaction. We speculate that phosphorylation and changes in subunit interaction are required for the proposed role of RPA during the polymerase switch at replication forks.

RPA, another is its ability to interact with a variety of proteins involved in DNA replication (6–8), DNA repair (9, 10), recombination (11, 12), and, possibly, transcription (13). Thus, RPA binding to single-stranded DNA and its interaction with DNA polymerase α-primase and with other replication factors are required for the establishment and the propagation of replication forks (14). In recombination, RPA stimulates DNA strand exchange between Rad51-coated single-stranded DNA and the double-stranded DNA substrate (11); and in nucleotide excision repair, binding of RPA may help to stabilize the locally unwound DNA and to localize the repair endonucleases at damaged DNA sites (15).

RPA32 is phosphorylated when cells transit from the G1 phase into the S phase of the cell cycle and remains so throughout S phase (16, 17). In addition, dramatic increases of RPA32 phosphorylation occur as a response to DNA damage in x-ray- and UV-irradiated cells (18–21) as well as during apoptosis (22). The enzymes responsible for RPA32 phosphorylation in intact nuclei have not yet been unambiguously identified although in vitro experiments show that RPA32 serves as a substrate for a cyclin-dependent kinase Cdc2-cyclin B as well as for the DNA-dependent protein kinase (DNA-PK). The enzymes recognize serine and threonine residues as phosphorylation sites in the amino-terminal region of RPA32 and cause a retardation in the electrophoretic mobility of RPA (23–25).

The physiological consequences of RPA32 phosphorylation are not clear yet. Mutations of the major phosphorylation sites of RPA32 cause no detectable phenotype in yeast (3). Mutant RPA, lacking the amino-terminal phosphorylation sites, binds well to single-stranded DNA (24), supports SV40 DNA replication in vitro, and appears to be active in nucleotide excision repair (26). Phosphorylation of RPA is suppressed in human cells lacking a functional DNA damage surveillance ATM gene product as well as in yeast cells without the MEC1 gene, a counterpart of the mammalian ATM gene (18, 27). The MEC1 gene product is known to be involved in S phase arrest of cells with damaged DNA (28). It is therefore possible that the phosphorylation of RPA32 has a function regulating the switch from replicative DNA synthesis to repair synthesis (26, 29). This is consistent with a report that damage-induced inhibition of DNA replication correlates with RPA32 phosphorylation in an in vitro system and can be reversed by the addition of purified unphosphorylated RPA (19).

In our previous studies with proliferating cultured human cells we observed that phosphorylated RPA32 may be less stably associated with the large RPA70 subunit than unmodified RPA32 (22). To further investigate this possibility we have performed the experiments reported in this communication. We could show that trimeric RPA complexes tend to desintegrate when subunit RPA32 is phosphorylated, whereas unmodified trimeric RPA remains perfectly stable under the same conditions. The results contribute to discussions on the role of RPA32 phosphorylation in proliferating cells and could explain...
earlier data of others who detected by immunological means that individual RPA subunits partition to different parts of the nucleus at some cell cycle stages (30).

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Preparation of Chromatin Proteins, and Sucrose Gradient Centrifugation**—Human HeLa S3 cells (31) and Jurkat T lymphoma cells (32) were cultivated as described. For cell fractionation, nuclei were isolated using the digitonin procedure of Adam et al. (33) with 10 mM NaF and 20 mM β-glycerophosphate and the modifications described earlier (34). Chromatin was prepared as originally described by Hancock (35) with the modifications detailed in Ritzi et al. (31). For the preparation of chromatin-associated proteins, isolated chromatin was resuspended in hypotonic buffer (0.25 mM EDTA, pH 8) containing NaCl at concentrations specified in the text. Unsoluble nucleoprotein was removed by centrifugation at 12,000 × g. Supernatants were analyzed by centrifugation through linear 25 to 60% sucrose gradients in TE buffer (20 mM Tris-HCl, 5 mM EDTA, pH 7.8; with either 0.1 or 0.4 M NaCl) using the Beckman SW 40 rotor at 35,000 rotations/min and 4 °C for 60 h. The gradients were fractionated from the top into 0.6-ml aliquots. Samples from each fraction were analyzed by Western blotting for RPA.

**Analysis of RPA**—Recombinant human RPA was isolated from bacterial extracts as described previously (36). The purified protein was used as an antigen for the preparation of antisera in rabbits. RPA-specific antibodies were affinity-purified according to standard procedures (37). The antibodies were used for immunoblotting (Western blotting) after denaturing polyacrylamide gel electrophoresis (38) essentially as described by Towbin et al. (39). Immunoblots were visualized by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech).

**In Vitro Phosphorylation**—Active cyclin A/CDK1, cyclin B/CDK1, and cyclin E/CDK2 were isolated from Xenopus egg extracts according to Strausfeld et al. (40). For in vitro phosphorylation, 4 μg of recombinant human RPA was incubated with 500 ng of purified cyclin-dependent kinases in 15 μl of kinase buffer (20 mM Hepes, pH 7.5, 10 mM MgCl₂, 100 mM NaCl, 2 mM dithiothreitol, 0.02% Tween 20) with 0.2 mM ATP and, if required, 5 μCi of [γ-32P]ATP. Control phosphorylation assays were performed with 5 μg of histone H1 (Roche Molecular Biochemicals) instead of RPA as substrate. Aliquots from each assay mixture were analyzed by denaturing polyacrylamide gel electrophoresis and immunoblotting or autoradiography. Using frog enzymes in these reactions appears to be justified, since cyclin-dependent kinases are highly conserved as exemplified by the fact that human CDK1 can complement yeast strains mutated in the cdc2 gene (41).

**RESULTS**

**Phosphorylated RPA32 Occurs in the Chromatin-bound Fraction of HeLa Cell RPA**—About one third of RPA in cultured human cells is bound to chromatin while the remaining fraction of RPA occurs free in the nucleosol (34). To investigate whether phosphorylated RPA32 is present in the chromatin-bound fraction, in the nucleosol, or both, we extracted proteins from isolated HeLa cell nuclei under different salt conditions and probed the extracts with RPA-specific antibodies using an immunoblot (“Western”) procedure. The antibodies, prepared in rabbits against recombinant human RPA as an antigen, recognize the two largest RPA subunits, but not RPA14 (34) (Fig. 1A). Using these antibodies as a tool, we were able to identify subunits RPA70 and RPA32 in unfractonated total cell extracts (Fig. 1B, lane 1). We also detected these subunits in nuclear extracts and found that RPA32 subunits in the chromatin-bound fraction, but not in the nucleosol fraction, had retarded electrophoretic mobilities (indicated as bands p1 and p2 in Fig. 1B).

Identical results were obtained using fractionated nuclear extracts from cells of the human Jurkat lymphoma cell line (data not shown). To verify that the retarded electrophoretic mobilities were due to phosphorylation, we treated the nuclear extracts with alkaline phosphatase and found that the slower moving bands were converted to a single electrophoretic band corresponding to unmodified RPA32 (Fig. 1C). We conclude that most phosphorylated RPA32 occurs in the chromatin-bound fraction of RPA and could be in direct contact with DNA (34). This would be consistent with the results of biochemical experiments that had shown previously that DNA-bound RPA is a better substrate for protein kinases than free RPA (42).

To further investigate the chromatin-bound RPA fraction, we treated aliquots of isolated chromatin with buffers of increasing salt concentrations. We found that low salt concentrations cause a release of RPA with unmodified RPA32 subunits, whereas higher salt concentrations were necessary to also release RPA with phosphorylated RPA32 (Fig. 2). The data of Fig. 2 also indicate that more RPA70 subunits than RPA32 subunits appeared to be mobilized at 0.1 M NaCl. This has been noted before (see Fig. 5B in Ref. 34) and could mean that the subunits of chromatin-bound RPA separate upon release from their binding sites on chromatin. That would be surprising as isolated trimeric RPA complexes are known to be extremely stable under a variety of ionic conditions (2).

**Separation of RPA Subunits**—To further investigate the possibility that phosphorylated RPA could dissociate into its sub-
units, HeLa cell chromatin was treated with 0.4 M NaCl, a salt concentration that mobilizes a large fraction of non-histone chromatin proteins, including most of the bound RPA (Fig. 2). Chromatin proteins were then fractionated through sucrose gradients at 0.4 M NaCl. Controls were investigated in parallel tubes of the same sedimentation run. One control was recombinant human RPA, and a second control was the soluble nucleosolic fraction that contains unmodified RPA as shown in lane 2 of Fig. 1B. We confirmed that recombinant human RPA remained stable under the experimental conditions of 0.4 M NaCl, since all three RPA subunits sedimented together in a symmetrical peak with a sedimentation rate just ahead the bovine serum albumin marker with 4.2 S (Fig. 3A). Unphosphorylated RPA in the nucleosolic fraction had the sedimentation properties of recombinant RPA (Fig. 3B), suggesting that all three RPA subunits remained in trimeric complexes although the properties of the antibodies precluded a determination of the smallest subunit, RPA 14 (see Fig. 1A).

In contrast, the RPA fraction, prepared from chromatin at 0.4 M salt, behaved quite differently: the RPA70 subunits and the phosphorylated RPA32 subunits sedimented as independent peaks (Fig. 3C). Again, we could not determine the distribution of the RPA14 subunit in the gradient. We can therefore not say whether RPA14 remained bound to RPA70, to RPA32, or to both subunits. In either case, the data clearly show that the large and the phosphorylated middle subunit of RPA dissociate when extracted from chromatin at 0.4 M NaCl. The sedimentation analysis revealed an unusual distribution of released RPA70. While RPA70 subunits appeared in symmetric peaks when they remain in intact trimeric RPA (Fig. 3, A and B), released RPA70 exhibited a leading shoulder (Fig. 3C), suggesting that RPA70 tends to form aggregates with itself or with unidentified chromatin proteins upon release from RPA32. In the particular experiment, leading to Fig. 3C, a minor fraction of unphosphorylated RPA32 sedimented together with the main RPA70 peak in the gradient. This is an

![Fig. 2. Mobilization of chromatin-bound RPA.](image)

**FIG. 2. Mobilization of chromatin-bound RPA.** HeLa cell chromatin was prepared according to Hancock (35) with the modifications described in Ritzi et al. (31). Equal aliquots of chromatin were resuspended in hypotonic buffer with different salt concentrations as indicated. After 5 min on ice, insoluble nucleoprotein was removed by centrifugation, and the supernatants were investigated by denaturing polyacrylamide gel electrophoresis and Western blotting. C, control without centrifugation.

![Fig. 3. Analysis of RPA by sucrose gradient centrifugation.](image)

**FIG. 3. Analysis of RPA by sucrose gradient centrifugation.** A, recombinant human RPA; B, nucleosolic soluble RPA (see Fig. 1B, lane 2); C, chromatin-bound RPA, released from isolated chromatin at 0.4 M NaCl (see Fig. 2, lane 5). Sucrose gradients in hypotonic buffer contained 0.4 M NaCl. Sedimentation is from the left to the right. Gradient fractions in A were analyzed by Laemmli gel electrophoresis and Coomassie Blue staining. Gradient fractions in B and C were analyzed by Western blotting.

![Fig. 4. Reformation of RPA.](image)

**FIG. 4. Reformation of RPA.** Chromatin-bound RPA was prepared as in Fig. 3C and dialyzed for 8 h at 4 °C against hypotonic buffer with 0.1 M NaCl in the absence (A) or presence (B) of phosphatase (see Fig. 1C). Sucrose gradient centrifugation was performed as in Fig. 3 except that the gradient contained 0.1 M NaCl.
interesting internal control showing again that the association of unphosphorylated RPA32 and RPA70 was not disrupted under the experimental conditions.

To demonstrate that phosphorylation was responsible for the dissociation of RPA, we dialyzed released chromatin proteins against buffer with 0.1 m NaCl in the presence or absence of phosphatase. The structure of RPA was then determined by sucrose gradient centrifugation and Western blotting as shown above. We found that the removal of phosphate groups resulted in reformation of RPA complexes as shown by a cosedimentation of subunits RPA70 and RPA32 exactly as normal trimeric RPA (Fig. 4A). However, when not dephosphorylated, RPA32 failed to combine with RPA70 during dialysis against 0.1 m NaCl (Fig. 4B). We conclude that phosphorylation is a major determinant causing the separation of RPA70 and RPA32 in chromatin-bound RPA. Immunoprecipitation of chromatin-bound RPA showed indeed that phosphorylated RPA32 could be immunoprecipitated with polyclonal antibodies to the total RPA complex (data not shown), but a monoclonal RPA70 antibody failed to coimmunoprecipitate the phosphorylated RPA32, in agreement with previous reports (20, 22).

While necessary, RPA32 phosphorylation may not be sufficient for RPA desintegration, and we cannot exclude the possibility that an additional chromatin function may be required for the separation of RPA70 and RPA32. As a first attempt to investigate this possibility we used recombinant human RPA as substrate for cyclin-dependent protein kinases in in vitro phosphorylation experiments.

In Vitro Phosphorylation of RPA—Previous work had shown that isolated RPA serves as an in vitro substrate for DNA-PK and for cyclin-dependent protein kinases (CDK) (24, 26, 43). Since DNA-PK has a major function in DNA damage recognition and repair (20), and the present research deals with undamaged cycling cells, we decided to investigate the effects of CDK on the stability of isolated RPA. For that purpose, we used CDK1 in association with cyclin A and cyclin B, or cyclin E/CDK2 as kinase activities and recombinant RPA as a substrate. While cyclin A/CDK1, cyclin B/CDK1, and cyclin E/CDK2 efficiently phosphorylated the common substrate histone H1, only cyclin A/CDK1 and cyclin B/CDK1 could phosphorylate RPA32 causing a electrophoretic mobility shift characteristic for phosphorylated RPA32 (Fig. 5A). The kinetics of the reaction showed that incubation times of 30–60 min were sufficient to obtain a maximal transfer of phosphate groups to RPA and that longer incubation had no additional effects. We also observed a low degree of phosphate transfer to the large subunit, RPA70 (Fig. 5B).

To determine whether CDK-mediated phosphorylation caused a separation of RPA subunits, we performed sucrose gradient centrifugation at 0.1 and 0.4 m NaCl as described above under Fig. 3. In four independent experiments, we obtained ambiguous results: most RPA subunits remained together in one complex as shown by a cosedimentation of the middle and the large RPA subunits, of which some were degraded during incubation (Fig. 6). However, a minor fraction of RPA32 sedimented slower than the main peak of RPA. Interestingly, the fraction of slower sedimenting RPA32 appeared to be enriched for the phosphorylated P1 and P2 forms of RPA32 (Fig. 6), showing a partial dissociation from the RPA complex. Similar results were obtained by centrifugation at 0.1 m NaCl (data not shown). In addition, we observed in these experiments a wide distribution of RPA70 reaching into fractions close to the top of the sucrose gradient. The reason for this is not known, but could be due to an adherence of CDK-treated RPA to the walls of the centrifuge tubes. A trailing of RPA or its subunits was not observed in control experiments using RPA, incubated with phosphorylation buffer in the absence of CDK (not shown) or with RPA in nuclear extracts (see Figs. 3 and 4).

To summarize the in vitro phosphorylation experiments, we note that recombinant human RPA serves as a substrate for cyclin A/CDK1 and for cyclin B/CDK1, but even incubation times of up to 60 min lead to an incomplete phosphorylation of RPA32 as shown by the fact that only a minor fraction of
CDK-treated RPA showed the electrophoretic retardation characteristic for highly phosphorylated RPA32. The reasons for this behavior of RPA in biochemical phosphorylation assays remain to be investigated. However, the interesting point here is that whenever a high degree of in vitro phosphorylation was achieved, the large and the middle subunit of RPA tended to dissociate.

**DISCUSSION**

Phosphorylation of the RPA32 subunit has been observed in normally proliferating cells at the time of genome replication and in cells with damaged DNA during DNA repair synthesis (see Introduction). Here we have investigated RPA32 phosphorylation in proliferating transformed human HeLa and Jurkat T lymphoma cells in tissue culture. We confirmed our previous results showing that the majority of RPA occurs in the nucleolus, while only about one-third of RPA is associated with chromatin (34). The physiologic reason for this distribution is not clear. RPA in the soluble nuclear fraction could function as a back-up system for situations with high demands for RPA such as repair synthesis. Another possibility is that soluble RPA forms a nucleosolic pool or reservoir where phosphorylated RPA from replicated DNA is dephosphorylated and recycled. In this regard it is interesting to note that most soluble RPA is unphosphorylated, whereas chromatin-bound RPA contains phosphorylated RPA32. This suggests that RPA32 is phosphorylated as a consequence of the functions that RPA performs at replication forks. These functions could include an orchestration of the “polymerase switch” occurring when DNA polymerase α has finished the synthesis of short RNA-DNA primers and DNA polymerase δ takes over to continue chain elongation synthesis (14, 44). While the large RPA subunit probably resides on and protects the exposed single-stranded DNA template, subunit RPA32 contacts the 3’-ends of DNA primers (29). After the polymerase switch RPA32 changes its position and can no longer be detected at the 3’-ends of the more advanced products of DNA synthesis. RPA cycling at replication forks may require changes of the RPA70/RPA32 interface and flexible subunit interactions.

These conformational changes could be regulated by phosphorylation and may facilitate the separation of the large and the middle RPA subunits at the experimental conditions described above (Fig. 3). This does not necessarily imply that subunits RPA70 and the RPA32 physically separate when bound to their sites at replication forks. It is in fact quite possible that RPA desintegration is induced upon its release from chromatin at 0.4 mM NaCl. We note, however, that a reduction of salt concentration by dialysis from 0.4 to 0.1 mM NaCl leads to a reformation of stable trimeric RPA only when dialysis is accompanied by dephosphorylation of RPA32 (Fig. 4). Furthermore, Murti et al. (30) have shown by immunostaining of fixed intact HeLa cells that RPA70 binds to spindle poles and RPA32 to chromosomes during mitosis. Cardoso et al. (45) found that RPA70, but not RPA32, localizes at replication foci in mouse myotube cells. These earlier studies are consistent with the possibility that a separation of the RPA subunits could occur in vivo, at least in some cell types and during certain cell cycle stages.

While our data clearly indicate that the mode of interaction between RPA70 and RPA32 is strongly affected by phosphorylation, we can say yet which enzymes are responsible for RPA32 phosphorylation and how their function is regulated. Biochemical studies had shown that DNA-PK as well as cyclin A- or cyclin B-dependent kinases phosphorylate RPA32 in vitro at defined serine and threonine side chains in an amino-terminal section of RPA32 (17, 46). DNA-PK has a major function in DNA damage repair (2). Therefore, CDKs are more likely candidates for RPA phosphorylation in normally proliferating cells as studied in this communication. For this reason, we have used cyclin A/CDK1 and cyclin B/CDK1 for in vitro phosphorylation experiments with recombinant RPA as a substrate. Niu et al. (25) have shown that cyclin B/CDK1 efficiently transfers one phosphate group to a serine residue at amino acid position 29 of RPA32 and other phosphates with reduced efficiency to additional unmapped RPA32 sites. This leads to two electrophoretic bands of phosphorylated RPA32, termed P1 with one, and P2 with two or maybe more, phosphate groups per molecule. In agreement with these results, we find that a treatment of RPA with either cyclin A/CDK1 or cyclin B/CDK1 predominantly produces the P1 form of phosphorylated RPA32 (Fig. 5). However, even after long incubation times, in vitro phosphorylation never reached the level of RPA32 phosphorylation in vivo as judged from the relative abundance of the phosphorylated RPA forms P1 and P2. Thus, CDKs are either more active in vivo, or the DNA-PK together with other unidentified protein kinases are responsible for the major phosphorylation of chromatin-bound RPA in vivo. In either case, only a partial fraction of the most highly phosphorylated form of CDK-treated RPA desintegrated in vitro (Fig. 6), supporting the notion that phosphorylation plays a major role in changing the interaction of the RPA subunits. A possible explanation for the ambiguous results could be the use of Xenopus CDKs to phosphorylate human RPA in vitro, although CDKs are known to be highly conserved from yeast to human and are functionally exchangeable (41). However, it could be possible that there exist important differences in the phosphorylation pattern of RPA32 in vivo and in vitro. Further work has to identify the amino acid residues in RPA32 whose phosphorylation is responsible for the changes in RPA complex formation that we have observed.

The important point emerging from the experiments reported in this communication is that phosphorylation changes the interaction and affinity between the large and the middle RPA subunits, resulting in their separation at least under in vitro conditions, but possibly also in intact nuclei.

**Acknowledgment—**We thank C. Eckerich for critical reading of the manuscript.

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