The EMBO Journal vol.11 no.6 pp.2177-2187, 1992

Cell cycle regulated phosphorylation of RPA-32 occurs within the replication initiation complex

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Communicated by H.Eisen

The transition from G_1 to S phase of the cell cycle may be regulated by modification of proteins which are essential for initiating DNA replication. One of the first events during initiation is to unwind the origin DNA and this requires a single-stranded DNA binding protein. RPA, a highly conserved multi-subunit single-stranded DNA binding protein, was first identified as a cellular protein necessary for the initiation of SV40 DNA replication. The 32 kDa subunit of RPA has been shown to be phosphorylated at the start of S phase. Using SV40 replication as a model, we have reproduced *in vitro* the S phase-dependent phosphorylation of RPA-32 and show that it occurs specifically within the replication initiation complex. Phosphorylated RPA-32 is predominantly associated with DNA. Phosphorylation is not a prerequisite for association with DNA, but occurs after RPA binds to single-stranded DNA formed at the origin during the initiation phase. The protein kinase(s) which phosphorylates RPA-32 is present at all stages of the cell cycle but RPA-32 does not bind to the SV40 origin or become phosphorylated in extracts from G_1 cells. Therefore, the cell cycle-dependent phosphorylation of RPA-32 may be regulated by its binding to single-stranded origin DNA during replication initiation.

Key words: cell cycle/DNA replication/phosphorylation/SV40/single-stranded DNA binding proteins

Introduction

Replication of DNA in the eukaryotic genome is a highly regulated event which is restricted to the S phase of the cell cycle. How this control is exerted is unclear. Cell fusion studies by Rao and Johnson (1970) demonstrated that fusion of a G_1 cell to an S phase cell prematurely induces DNA synthesis in the G_1 nucleus. These and other similar experiments have led to the conclusion that an inducer of DNA synthesis accumulates during G_1 and initiates synthesis at a certain threshold (Rao *et al.*, 1977; Rossow *et al.*, 1979). This inducer of DNA synthesis could be a replication factor but the level of many replication proteins remains unchanged from the G_1 to the S phase of the cell cycle (Wong *et al.*, 1986; Roberts and D'Urso, 1988; Wold *et al.*, 1988; Morris and Mathews, 1989; Din *et al.*, 1990). Alternatively, the replication proteins could be a target of this inducer. Current evidence suggests that this inducer might be the $p34^{cdc2}$ kinase or a closely related kinase and it might function by activating specific proteins involved in the initiation of DNA replication (McVey *et al.*, 1989; D'Urso *et al.*, 1990; Blow and Nurse, 1990; Fang and Newport, 1991).

Cell-free replication of SV40 DNA has proved to be a useful model for studying eukaryotic DNA replication and for characterizing the cellular proteins involved in replication (reviewed in Challberg and Kelly, 1989; Stillman, 1989). In this system, extracts from exponentially growing primate cells support the initiation and complete replication of DNA containing SV40 origin of replication in the presence of only one viral protein, the SV40 T antigen. Initiation of DNA replication at the SV40 origin involves multiple steps. First, SV40 T antigen binds to the sequences within the core origin using ATP or dATP as a co-factor (Dean *et al.*, 1987a,b; Deb and Tegtmeyer, 1987; Boroweic and Hurwitz, 1988). This results in the structural distortion and local untwisting of the DNA at the origin without hydrolysis of ATP and in the absence of single-stranded DNA binding proteins (Deb *et al.*, 1986; Boroweic and Hurwitz, 1988; Roberts, 1989; Parsons *et al.*, 1990; Dean and Hurwitz, 1991; for a review see Boroweic *et al.*, 1990). Subsequently, the DNA helicase property of SV40 T antigen leads to extensive unwinding of the origin in a process that requires hydrolysis of ATP, RPA (a single-stranded DNA binding protein) and topoisomerase I (Dodson *et al.*, 1987; Wold and Kelly, 1988; Kenny *et al.*, 1989; Tsurimoto *et al.*, 1989). Finally, polymerase α primase complex associates with the unwound origin and marks the direction of future forks. These events lead to the synthesis of RNA primers which are used for subsequent leading and lagging strand DNA replication (Tsurimoto *et al.*, 1989, 1990; Tsurimoto and Stillman, 1989). Unwinding of the origin of replication during initiation is a prerequisite for formation of primers and subsequent chain elongation (Wold and Kelly, 1988; Wold *et al.*, 1989; Tsurimoto *et al.*, 1990).

Kinetic analyses of SV40 replication *in vitro* reveals that a lag of 10-15 min precedes the start of DNA synthesis (Stillman and Gluzman, 1985; Wobbe *et al.*, 1985; Wold *et al.*, 1987; Tsurimoto *et al.*, 1989). The duration of this pre-synthesis phase is consistent with the time required for SV40 T antigen to bind to the origin and initiate events which lead to unwinding of the origin (Wobbe *et al.*, 1986; Tsurimoto *et al.*, 1989). Furthermore, pre-incubation of SV40 DNA with SV40 T antigen, RPA and ATP will eliminate the lag (Wobbe *et al.*, 1986; Wold *et al.*, 1987; Fairman and Stillman, 1988; Tsurimoto *et al.*, 1989). Unwinding of the origin of replication may therefore be the rate limiting step in initiating DNA synthesis and RPA is the only cellular protein known to be essential for this step. RPA is also required during elongation of SV40 DNA to stimulate the activity of polymerases α and δ in the presence of PCNA and RFC (Kenny *et al.*, 1989; Tsurimoto and Stillman, 1989, 1991).

Human RPA is composed of three tightly associated subunits, RPA-70, RPA-32 and RPA-11 (Wobbe *et al.*, 1987; Fairman and Stillman, 1988; Wold and Kelly, 1988).

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RPA-70 binds to single-stranded DNA but the function of other subunits is unknown (Wold *et al.*, 1989; Kenny *et al.*, 1990). The subunit structure and DNA binding properties of RPA are highly conserved in human (Erdile *et al.*, 1990, 1991; Heyer *et al.*, 1990), *Drosophila* (P.J.Mitis and I.R.Lehman, personal communication) and yeast (Brill and Stillman, 1989, 1991). In yeast, each subunit of RPA is encoded by a single gene, and co-ordinate expression of all three genes is essential for viability (Brill and Stillman, 1991). Biochemical analyses also suggest that all three subunits of RPA are important for DNA synthesis.

While the function of RPA-32 is not known, antibodies directed against RPA-32 inhibit DNA replication *in vitro* (Kenny *et al.*, 1990). RPA-32 is phosphorylated in a cell cycle-dependent manner in both yeast and human cells (Din *et al.*, 1990). In G_1, RPA-32 is unphosphorylated but becomes phosphorylated at the start of S phase. *In vitro*, RPA-32 is a substrate for $p34^{cdc2}$ kinase (A.Dutta and B.Stillman, personal communication). The requirement for RPA at a rate limiting step in the initiation of DNA replication and its phosphorylation in a cell cycle-dependent manner, makes it a likely candidate to play a key role in regulating DNA replication. To understand the significance of phosphorylation of RPA-32, our approach has been to examine the relationship between the association of RPA with DNA and the phosphorylation of RPA-32 during SV40 replication *in vitro*. We show that RPA-32 is phosphorylated during initiation of SV40 DNA replication *in vitro*, consistent with the S phase-dependent phosphorylation of RPA originally observed *in vivo*. The phosphorylated form of RPA-32 is predominantly associated with DNA. We also show that phosphorylation of RPA-32 is a consequence of and not a prerequisite for binding to DNA to form the initiation complex at the SV40 origin. Phosphorylation of RPA-32 therefore occurs within the initiation complex and may be essential to link initiation to the synthesis phase of DNA replication.

Results

RPA-32 becomes phosphorylated during DNA replication in vitro

In both human and yeast cells, the 32 kDa subunit of RPA becomes phosphorylated at the G_1-S transition of the cell cycle (Din *et al.*, 1990). As a result of this phosphorylation, RPA-32 exhibits a reduced mobility on SDS polyacrylamide gels. To understand the significance of this phosphorylation, we first examined whether the same event occurred during replication of SV40 DNA *in vitro*. Figure 1A shows that 50% of the total RPA-32 showed a slower electrophoretic mobility following replication *in vitro* in comparison with the form of RPA-32 present in S-100 supernatants of hypotonic lysates from Manca cells. We also observed that the fraction of RPA-32 which became phosphorylated was directly proportional to the extent of DNA replication. For instance, when the amount of DNA synthesis increased 2-fold (compare Figure 1A to 1B), >95% of the RPA-32 became phosphorylated. Control reactions lacking DNA showed no change in the electrophoretic mobility of RPA-32 (Figure 1A).

The altered mobility of RPA-32 during DNA replication *in vitro* was due to phosphorylation. We observed that the slow mobility form of RPA-32 was labelled with ^{32}P when $[\gamma^{32}P]ATP$ was included in DNA replication reactions. The removal of ^{32}P upon treatment with alkaline phosphatase or protein phosphatase 1 resulted in faster migrating forms of RPA-32 (data not shown, see below). In the experiments described below, phosphorylation of RPA-32 refers only to the phosphorylation of RPA-32 which is associated with DNA replication and that leads to the 2−3 kDa shift in the mobility of RPA-32 upon polyacrylamide gel electrophoresis.

We used gel filtration chromatography to demonstrate that RPA binds to SV40 DNA during replication. DNA−protein complexes assembled on SV40 DNA during replication were isolated by fractionation of reaction products over a Sepharose 6B column. Most of the RPA-32 which was associated with DNA during replication was phosphorylated in comparison to RPA-32 from S-100 supernatant (Figure 1B). In the absence of SV40 DNA, none of the RPA-32 was detected in fractions where DNA−protein complexes eluted from the Sepharose 6B column (Figure 1B, bottom panel). Therefore the co-elution of RPA-32 with replicated DNA was due to its association with DNA. All the three subunits of RPA, RPA-70 (the single-stranded DNA binding subunit), RPA-32 and RPA-11 were associated with DNA (Figure 1C).

We have made the striking observation that the phosphorylation of RPA-32 was restricted to the fraction of RPA that associated with SV40 DNA during replication. As discussed above, under conditions where intermediate levels of replication occurred, ~50% of the RPA-32 in the reaction became phosphorylated. In comparison with the phosphorylated RPA-32 which was associated with DNA during replication, the RPA-32 in the free protein fractions was predominantly unphosphorylated (Figure 1D).

Phosphorylation of RPA-32 was dependent on DNA synthesis. Replication reactions were performed in the absence of SV40 T antigen (Figure 2A, top panel) or with DNA lacking the SV40 origin of replication (Figure 2A, bottom panel). Under both these conditions, RPA-32 did not associate with DNA and the free RPA-32 remained predominantly unphosphorylated.

Next we determined whether the phosphorylation of RPA-32 occurred during the initiation phase of DNA replication. Plasmid DNA containing the SV40 origin of replication was incubated for 15 min under conditions which only allow initiation to occur. For this purpose, extracts had been dialysed to remove deoxy- and ribonucleoside triphosphates to prevent the start of DNA synthesis (Wobbe *et al.*, 1986). ATP was added back to enable DNA to unwind, permitting the formation of the initiation complex. Under these conditions, no measurable DNA synthesis occurred but a fraction of RPA-32 associated with SV40 DNA and was phosphorylated (Figure 2B). Also, note that the remaining fraction of RPA-32 associated with DNA was initially unphosphorylated. This is consistent with our observation that the unphosphorylated form of RPA-32 can associate with DNA and suggests that phosphorylation of RPA-32 occurs after its association with single-stranded DNA (see below). No significant binding or phosphorylation of RPA-32 was detected with DNA lacking the SV40 origin (data not shown). These experiments showed that conditions required for the assembly of initiation complex were necessary and sufficient for RPA-32 to associate with DNA and become phosphorylated.

Besides functioning in the initiation of replication, RPA is also required during elongation. To determine if the rate of RPA-32 phosphorylation was increased when initiation and elongation were permitted as compared to initiation alone, reactions were started under replication initiation conditions as above. After 15 min deoxy- and ribonucleoside triphosphates were added to half the reaction to allow synthesis of DNA (Figure 3). After 30 min, RPA-32 from the replication initiation reaction was phosphorylated to a greater extent than RPA-32 from reactions in which DNA synthesis occurred. Therefore, once DNA synthesis began, the rate of phosphorylation of RPA-32 decreased. It was possible that the slow phosphorylation of RPA-32 which occurred during DNA synthesis was due to phosphorylation of RPA-32 located at the elongation fork. More likely, it was due to phosphorylation during initiation on new DNA templates as they were recruited into the pool of replicating molecules. In contrast, phosphorylation of RPA-32 in the initiation reaction continued at a rapid rate. Normally, unwinding of DNA in the presence of T antigen, RPA and topoisomerase I is coupled to the initiation of DNA synthesis. However, in the absence of DNA replication, the initiation process continues, resulting in the production of extensively unwound DNA (Bullock *et al.*, 1989; Tsurimoto *et al.*, 1989). Under these conditions the rapid phosphorylation of RPA-32 characteristic of the initiation phase is main-

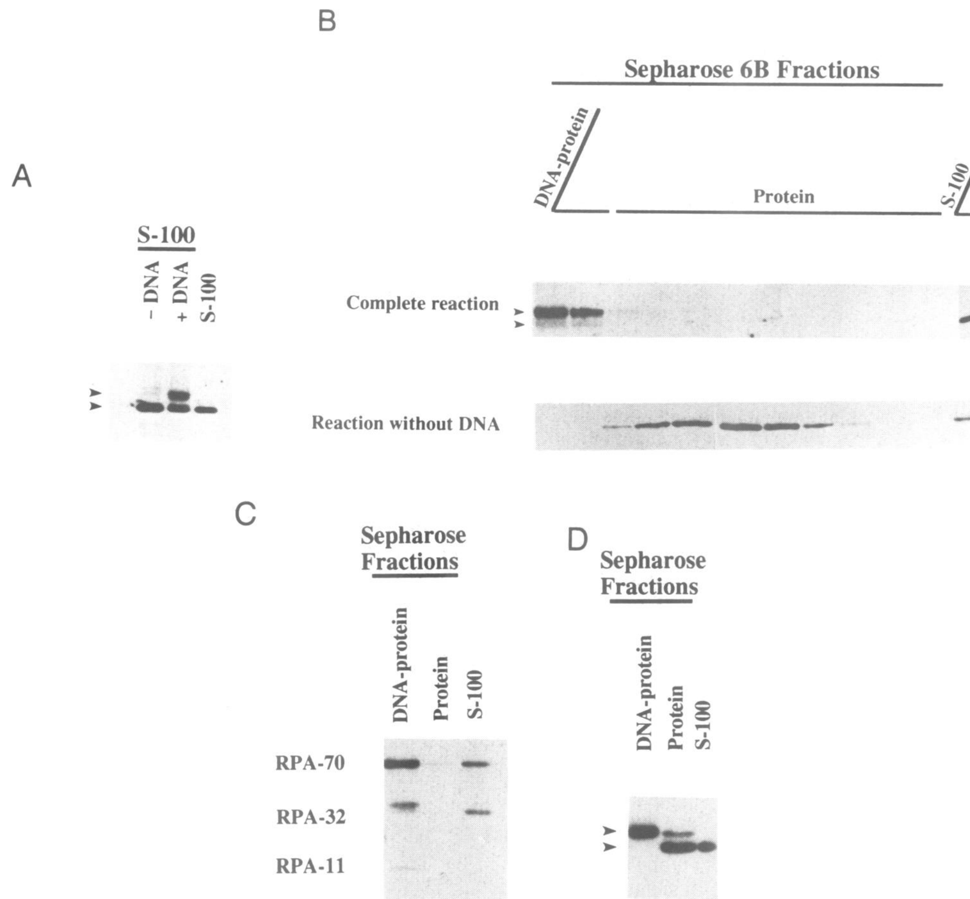

Fig. 1. (**A**) Conversion of RPA-32 from *in vitro* replication reactions into a slow mobility form. Reactions were performed under replication conditions in cell extracts from exponentially growing Manca cells in the absence (lane 1) or presence of SV40 ori DNA (lane 2) for 2 h. DNA synthesis in the absence and presence of SV40 ori DNA was 1.2 and 28 pmol dCMA incorporated/2 h respectively. Reaction products were resolved on 12% SDS–polyacrylamide gels and RPA-32 was detected by immunoblotting and monoclonal antibody against RPA-32. The S-100 supernatant from Manca cells was resolved in the lane labelled S-100. The positions of fast and slow mobility RPA-32 are indicated by arrowheads. (**B**) Association of the phosphorylated form of RPA-32 with DNA. Reactions were performed under replication conditions in extracts from S phase cells for 2 h in the presence (top panel) or absence of SV40 ori DNA (bottom panel). DNA synthesis in the absence and presence of SV40 ori DNA was 0.9 and 63 pmol dCMP incorporated/2 h, respectively. The reaction products from both sets of reactions were fractionated on Sepharose 6B columns. Fractions from the column containing DNA–protein complexes were pooled and are designated as DNA–protein in this and all other figures. The remaining fractions from the column which do not contain DNA but have protein, were also pooled and are designated as protein. All fractions were pooled in sets of two, proteins resolved on SDS–PAGE and detected by immunoblotting with monoclonal antibody against RPA-32. The S-100 supernatant from Manca cells (without fractionation over Sepharose 6B) was resolved in the lane labelled S-100. (**C**) Association of all three subunits of RPA with DNA during replication *in vitro*. Reactions were performed under replication conditions for 2 h in the presence of SV40 ori DNA. DNA synthesis in the presence of SV40 ori DNA was 20 pmol dCMP incorporated/2 h. Reaction products were fractionated as in B. Proteins resolved by SDS–PAGE were immunoblotted with antiserum against RPA which recognizes all the subunits. Free protein elutes in a volume three times greater than DNA–protein during fractionation. Therefore, since equal volumes of pooled fractions (DNA–protein or protein) were loaded on gels, the samples represent 5% of the DNA–protein fractions but only 1.6% of the free protein fractions, respectively. (**D**) Products from replication reactions (DNA synthesis; 24 pmol dCMP incorporated/2 h) were fractionated on Sepharose 6B columns. Proteins from these fractions were resolved by SDS–PAGE and detected by immunoblotting with monoclonal antibody against RPA-32. Samples representing equivalent percentages of DNA–protein and of free protein were loaded on the gel.

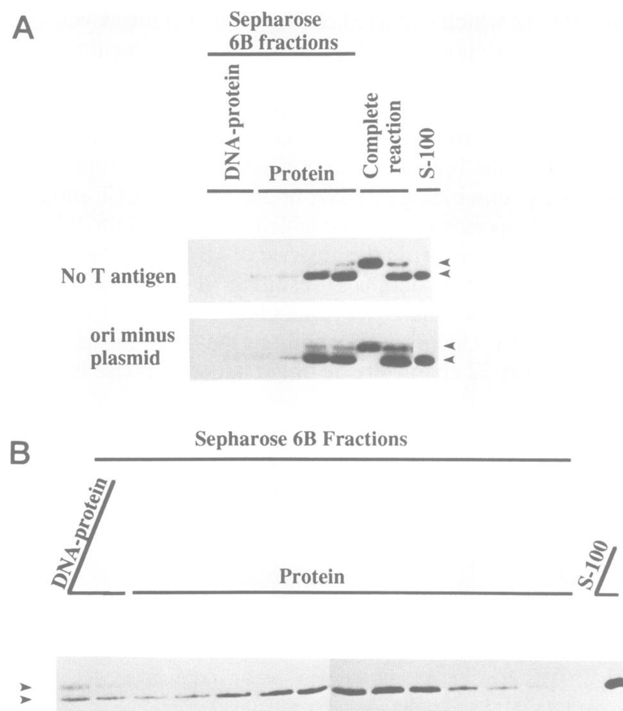

Fig. 2. (A) Dependency of phosphorylation of RPA-32 on DNA replication. Reactions were performed under replication conditions in the absence of SV40 T antigen (top panel) or in the presence of SV40 ori minus DNA (bottom panel) for 2 h. Reaction products were fractionated over Sepharose 6B column and the proteins resolved by SDS−PAGE. Proteins were detected by immunoblotting with monoclonal antibody against RPA-32. Control reactions performed under replication conditions in the presence of SV40 ori DNA (complete synthesis) were fractionated identically over Sepharose 6B column. (B) Phosphorylation of RPA-32 occurs during the initiation of DNA replication. Reactions were performed for 15 min under replication initiation conditions in the presence of SV40 ori DNA as described in Materials and methods. DNA synthesis in dialysed extracts without the addition of deoxy and ribonucleoside triphosphates was 1.8 pmol dCMP incorporated/2 h and upon addition of deoxy and ribonucleoside triphosphates was 42 pmol compared to 43.5 pmol dCMP incorporated/2 h in complete replication reaction without dialysed extracts. Reaction products were fractionated over Sepharose 6B columns as in Figure 1B and RPA-32 was detected by immunoblotting with monoclonal antibody against RPA-32.

tained. It therefore appeared that phosphorylation of RPA-32 may occur preferentially during the initiation phase of DNA replication.

RPA-32 is not associated with SV40 DNA nor is it phosphorylated in G_1 cell extracts

Replication of SV40 DNA occurs with a reduced efficiency *in vitro* in the presence of extracts from the G_1 as compared to the S phase of the cell cycle (Roberts and D'Urso, 1988). This effect is probably due to inefficient unwinding of DNA in G_1 extracts, which requires, in addition to SV40 T antigen, single-stranded DNA binding proteins. To examine further the relationship between phosphorylation of RPA-32 and assembly of replication initiation complexes on DNA, replication reactions were performed in extracts of G_1 cells. Under these conditions RPA-32 did not associate with SV40 DNA (Figure 4) although SV40 T antigen was bound to DNA in the same reactions (data not shown). Furthermore, the free RPA-32 remained largely unmodified. This experiment suggests that the inability to replicate DNA in extracts from G_1 cells results from the failure to assemble proteins at the replication origin to form a functional initiation complex.

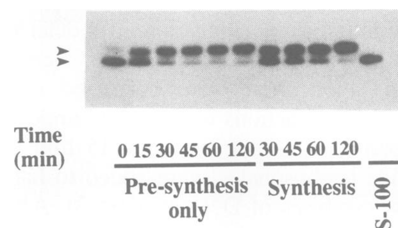

Fig. 3. Comparison of rate of phosphorylation of RPA-32 during initiation as opposed to initiation followed by elongation. Reactions were performed as described in Results. Aliquots removed at different time intervals were resolved by SDS−PAGE and RPA-32 was detected by immunoblotting with monoclonal antibody against RPA-32.

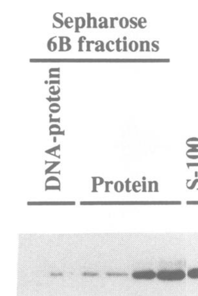

Fig. 4. Failure of RPA-32 to associate with SV40 ori DNA or become phosphorylated in G_1 extracts. Reactions were performed under replication conditions for 2 h and products fractionated over Sepharose 6B column as in Figure 1B. The proteins were detected by immunoblotting with monoclonal antibody against RPA-32.

Phosphorylation of RPA-32 is not essential for association with DNA

The above experiments raised the question as to whether phosphorylation of RPA-32 was a prerequisite for binding of RPA to DNA. Alternatively, phosphorylation of RPA-32 could occur within the initiation complex after RPA binds to DNA. Reactions were performed in the presence of dialysed extracts and dATP; conditions which allow initiation to occur. We used these conditions because SV40 T antigen can utilize the energy provided by hydrolysis of dATP to unwind the DNA at the origin (Goetz *et al.*, 1988; Roberts, 1989). We also assumed that in the absence of ATP, RPA-32 would not be phosphorylated. Under these conditions we found that the unphosphorylated form of RPA-32 was associated with DNA (Figure 5). We also noted that the amount of RPA-32 associated with DNA under these conditions was approximately the same as in the presence of ATP. Thus phosphorylation of RPA-32 was not required for its association with DNA to form the initiation complex. Furthermore these experiments suggested that in the presence of ATP, RPA-32 was probably phosphorylated after binding of RPA to DNA.

RPA-32 associated with single-stranded DNA is phosphorylated

Our results suggested that phosphorylation of RPA-32 required a functional SV40 T antigen complex at the SV40

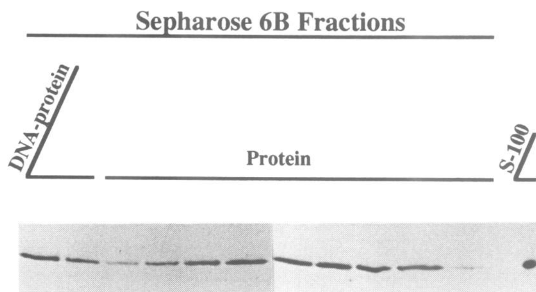

Fig. 5. Phosphorylation of RPA-32 is not a prerequisite for association with DNA. Reactions were performed under replication initiation conditions for 30 min with the exception that ATP was replaced by dATP as described in Results. Products were fractionated over a Sepharose 6B column and the proteins were detected by immunoblotting with monoclonal antibody against RPA-32.

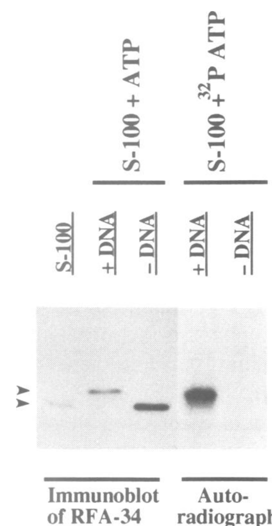

Fig. 6. Association of phosphorylated RPA-32 with SV40 ori minus single-stranded M13 DNA. Reactions were performed for 1 h in the presence or absence of single-stranded M13 DNA under conditions described in Materials and methods. The reactions were also performed in the presence of [γ^{32}P]ATP. The products from both these sets of reactions were fractionated over Sepharose 6B column and proteins were detected by immunoblotting with antiserum raised against RPA or by autoradiography. All the RPA-32 in the reaction was phosphorylated and associated with DNA. Therefore the remaining fractions from the column are not shown.

origin. One function of SV40 T antigen in this complex is to untwist the DNA at the origin of replication to generate single strands. RPA is a single-stranded DNA binding protein and the phosphorylation of RPA-32 occurred only under conditions where single-stranded DNA was generated. Therefore we reasoned that in the presence of single-stranded DNA, RPA-32 may associate with DNA and become phosphorylated. Consistent with this hypothesis, we found by immunoblotting and by incorporation of the ^{32}P label that RPA-32 associated with SV40 origin minus single-stranded M13 DNA and quantitatively converted into the phosphorylated form (Figure 6). In the absence of DNA, RPA-32 was not phosphorylated, either in its slow or fast mobility form. Similar results were obtained if phosphatase inhibitors were included in the reactions (data not shown). We repeatedly observed that all the RPA-32 in the reaction was associated with single-stranded M13 DNA and phosphorylated. Therefore in subsequent experiments we did not fractionate the reaction products on Sepharose 6B columns.

To determine whether the phosphorylated form of RPA-32 observed *in vitro* in the presence of single-stranded M13 DNA was the form previously described *in vivo* as undergoing phosphorylation at the G_1-S transition, hypotonic extracts were prepared from Manca cells labelled with [^{32}P]orthophosphate. RPA-32 from both *in vitro* and *in vivo* labelling reactions was immunoprecipitated with anti-RPA-32 monoclonal antibody (Din *et al.*, 1990). The phosphorylated derivative of RPA-32 *in vivo* co-migrated with the phosphorylated form of RPA-32 from *in vitro* reactions containing DNA (Figure 7A). Phosphopeptides generated by partial proteolysis of the slow migrating form of RPA-32 with *Staphylococcus aureus* V8 protease showed that phosphorylation of RPA-32 *in vivo* and *in vitro* was indistinguishable (Figure 7B). Chymotryptic peptide mapping of ^{32}P-labelled RPA-32 confirmed that most but not all phosphopeptides were shared by the *in vivo* and *in vitro* phosphorylated forms of RPA-32 (Figure 7C). However, additional phosphopeptides are present in the RPA-32 phosphorylated *in vivo*. This suggests that other kinases, in addition to the one which phosphorylates RPA-32 upon binding to single-stranded DNA, also phosphorylate RPA-32 (see Discussion).

We also tested the effect of various phosphatases on the phosphorylated form of RPA-32. For this, RPA-32 was labelled with ^{32}P during incubation of Manca cell extracts with single-stranded M13 DNA, immunoprecipitated with an anti-RPA-32 monoclonal antibody and then treated with various phosphatases. Upon treatment with protein phosphatase 1, protein phosphatase 2A and alkaline phosphatase, RPA-32 was dephosphorylated and simultaneously converted from the slow mobility form of RPA-32 into the less phosphorylated fast mobility forms (Figure 8A). Treatment with protein tyrosine phosphatase, however, had no effect on phosphorylation (Figure 8A, lane 5). This is consistent with the observed phosphorylation on serine residues in the phosphorylated form of RPA-32 both *in vivo* (Din *et al.*, 1990) and *in vitro* (data not shown). The effect of protein phosphatase 2A was inhibited by the presence of okadaic acid and that of alkaline phosphatase by *p*-nitrophenylphosphate. These changes in the mobility of RPA-32 in the presence of protein phosphatase 2A and alkaline phosphatase were therefore specifically due to the phosphatase activity of these proteins. We also used immunoblotting to show that digestion with alkaline phosphatase results in a quantitative conversion of the phosphorylated form of RPA-32 into fast mobility forms (Figure 8B).

The protein kinase(s) responsible for the phosphorylation of DNA associated RPA-32 is not cdc2 kinase and is present at all stages of the cell cycle

RPA is a substrate for p34^{cdc2} kinase *in vitro* in the absence of DNA (A.Dutta and B.Stillman, personal communication; R.Fotedar, unpublished data). We investigated whether p34^{cdc2} or a related kinase was responsible for the phosphorylation of RPA-32 which occurs upon association with single-stranded DNA. To perform this experiment, Manca cell extracts were depleted of p34^{cdc2} and most of

the related kinases by incubation with p13–Sepharose (Brizuela *et al.*, 1987; Dunphy *et al.*, 1988; Dunphy and Newport, 1989). Following depletion with p13–Sepharose, no protein could be detected in the supernatant with antiserum to PSTAIRE peptide (Figure 9A, left panel). Anti-PSTAIRE antibody detects $p34^{cdc2}$ and most known members of *cdc2* gene family (Lee and Nurse, 1987). Cell extracts treated identically with control Sepharose retain $p34^{cdc2}$ and related proteins in the supernatant (Figure 9A). Both control and p13-depleted cell extracts were then incubated with single-stranded M13 DNA. RPA-32, from both the p13–Sepharose supernatant and the control Sepharose supernatant, was associated with single-stranded DNA and phosphorylated (Figure 9B). Similarly, when the p13-depleted extracts were used in reactions which were performed under initiation conditions with SV40 origin containing DNA and the reaction products were fractionated on a Sepharose 6B column, RPA-32 was associated with DNA and was phosphorylated (Figure 9C). The kinetics of phosphorylation of RPA-32 in the presence of single-stranded DNA remain unchanged after depletion of at least 90% of the $p34^{cdc2}$ from cell extracts with p13–Sepharose compared to the control Sepharose (Figure 10). Therefore it did not seem likely that $p34^{cdc2}$ or a related kinase was required for the phosphorylation of RPA-32 associated with DNA. This experiment did not exclude the possibility that phosphorylation of RPA-32 by $p34^{cdc2}$ kinase may contribute to its activity (see Discussion).

The phosphorylated form of RPA-32 is first detected *in vivo* at the G_1–S transition and it persists until G_2–M, at which time RPA-32 is dephosphorylated (Din *et al.*, 1990). To test if the protein kinase(s) which phosphorylates RPA-32 was similarly cell cycle regulated, extracts from cells at different stages of the cell cycle were incubated with single-stranded M13 DNA. RPA-32 from all these cell extracts was associated with single-stranded DNA as a phosphorylated form (Figure 11). Therefore, although the phosphorylation of RPA-32 is cell cycle-dependent, the kinase(s) which phosphorylates RPA-32 is present at all stages of the cell cycle. However, we have observed recently that the rate of phosphorylation of RPA-32 is slower in extracts of G_1 phase cells compared to S phase or mitotic cell extracts. This raises the possibility that the ability of RPA-32 to serve as a substrate or the activity of the kinase phosphorylating RPA-32 may increase when the cells enter S phase.

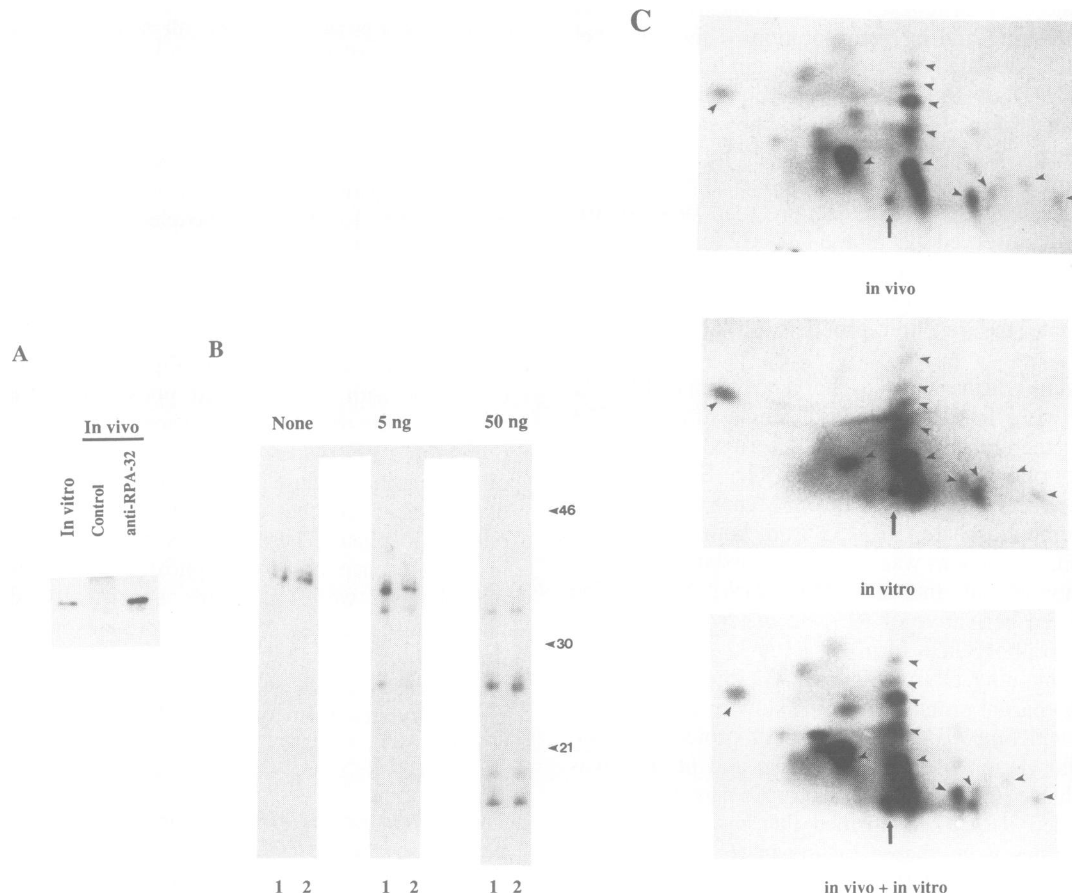

Fig. 7. Comparison of *in vitro* and *in vivo* phosphorylated RPA-32. *In vitro* reactions with M13 single-stranded DNA were performed in the presence of [γ^{32}P]ATP and S phase extracts as in Figure 6. Extracts were also prepared from Manca cells labelled with [^{32}P]orthophosphate as described in Materials and methods. (**A**) Proteins were immunoprecipitated with a monoclonal antibody against RPA-32 (lanes 1 and 3) or with a negative control monoclonal antibody (lane 2), resolved by SDS–PAGE and detected by autoradiography. (**B**) Comparison of peptide maps generated by partial *S.aureus* V8 protease digestion of the *in vivo* (lane 1) and *in vitro* (lane 2) ^{32}P phosphorylated slow migrating form of RPA-32 excised from gels. (**C**) Comparison of chymotryptic phosphopeptide maps of RPA-32 phosphorylated *in vivo* and *in vitro*. Origins are indicated by arrows. Electrophoresis in the first dimension was performed at pH 1.9 in the horizontal direction with the cathode on the right. Thin layer chromatography was performed from bottom to top. The phosphopeptides that are shared by *in vivo* and *in vitro* phosphorylated RPA-32 are indicated by arrowheads.

Discussion

Cell cycle regulated phosphorylation of RPA-32

Our approach to understanding the G_1-S phase transition of the cell cycle has been to examine the regulation of proteins involved in initiation of DNA replication. SV40 T antigen and RPA form a DNA–protein complex at the origin which is a prerequisite for initiation of DNA synthesis. Previous studies have shown that the 32 kDa subunit of RPA, a single-stranded DNA binding protein, is phosphorylated at the G_1-S transition *in vivo* (Din *et al.*, 1990) suggesting that this modification may play a role in regulating the start of DNA synthesis. Using the SV40 replication system we have reproduced *in vitro* the S phase-dependent phosphorylation of RPA-32. Since we show that the phosphorylation of RPA-32 occurs specifically during the formation of a replication initiation complex, our results offer an explanation for the phosphorylation of RPA-32 at the G_1-S transition. Phosphorylation of RPA-32, however, is not a prerequisite for its association with DNA to form the replication initiation complex, since the unphosphorylated form can also associate with DNA. Rather, phosphorylation of RPA-32 may occur shortly after it associates with DNA within the initiation complex. Under these conditions all three subunits of RPA are associated with DNA. Although our experiments deal exclusively with SV40 replication, we speculate that similar events may occur at cellular replication origins.

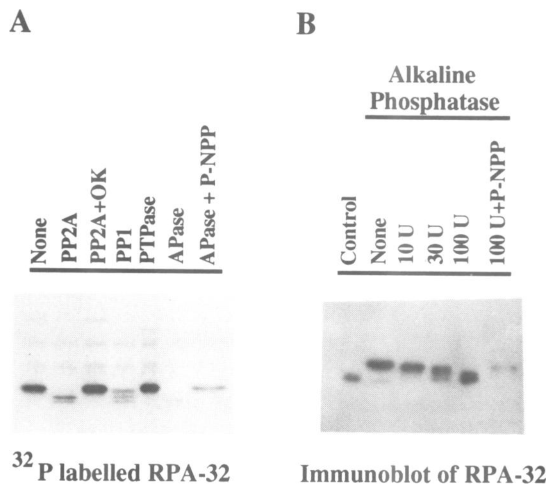

Fig. 8. (**A**) Sensitivity of the phosphorylated form of RPA-32 to specific phosphatases. Reactions were performed with M13 DNA in the presence of [γ^{32}P]ATP. Labelled RPA-32 was immunoprecipitated, treated with various phosphatases as described in Materials and methods, resolved by SDS–PAGE and the proteins were detected by autoradiography. (**B**) Immunoblot of RPA following phosphatase treatment. Reactions were performed as in A in the absence of ^{32}P and the products were treated with alkaline phosphatase. RPA-32 was detected by immunoblotting with monoclonal antibody to RPA-32.

A mechanism for replication-dependent phosphorylation of RPA-32

We show that the association of RPA with DNA and its phosphorylation require factors which are essential for unwinding the DNA, such as SV40 T antigen and SV40 origin of replication. Furthermore, we have noted that the concentration of ATP and the time required to initiate phosphorylation of RPA-32 in an SV40 ori- and T antigen-dependent manner were similar to that reported for SV40 T antigen to function in origin unwinding (R.Fotedar, unpublished data). In addition, RPA-32 did not associate with SV40 DNA or become phosphorylated in extracts from G_1 cells (this work) which are deficient in supporting SV40 T antigen-dependent unwinding (Roberts and D'Urso, 1988). Since phosphorylation of RPA-32 occurs only under conditions where single-stranded DNA was generated, we have reasoned that its association with single-stranded DNA may be necessary for its phosphorylation. This conclusion is supported by our finding that RPA-32 becomes associated and appropriately phosphorylated in the presence of (SV40 ori minus) single-stranded M13 DNA. The phosphopeptide maps of phosphorylated RPA-32 associated with single-stranded DNA are similar to that of RPA-32 phosphorylated *in vivo* during the S phase. We therefore propose that the RPA-32 becomes a target for phosphorylation after it binds to single-stranded DNA generated by SV40 T antigen during the formation of initiation complex at the origin of replication.

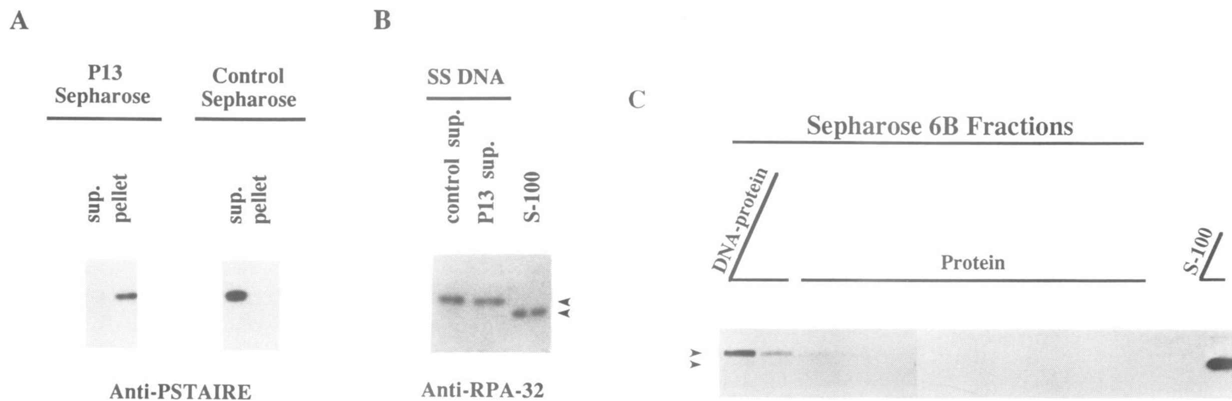

Fig. 9. (**A**) Depletion of $p34^{cdc2}$ and related kinases from S phase extracts. S phase extracts were depleted of $p34^{cdc2}$ and related kinases by incubation with p13–Sepharose (left panel). As a control, extracts were identically treated with Sepharose 4B (right panel). Proteins in both of the supernatants and pellets after incubation were resolved by SDS–PAGE and immunoblotted with antiserum against PSTAIRE peptide. (**B**) $p34^{cdc2}$ or a related kinase is not responsible for the phosphorylation of DNA-associated RPA-32. p13 and Sepharose 4B treated extracts were incubated with single-stranded M13 DNA as in Figure 6. Reaction products were loaded on gels and immunoblotted with monoclonal antibody against RPA-32. (**C**) p13 and Sepharose 4B treated extracts were incubated with SV40 ori DNA under replication conditions for 30 min. Reaction products were fractionated over Sepharose 6B column and proteins were detected by immunoblotting with monoclonal antibody against RPA-32.

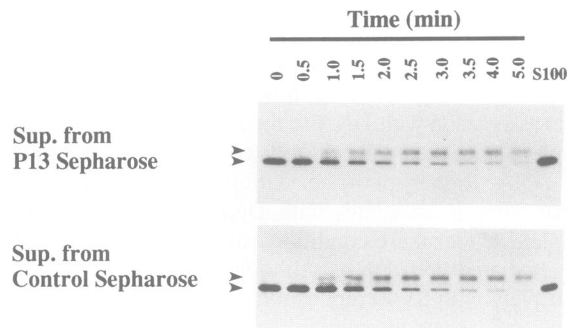

Fig. 10. Kinetics of phosphorylation of RPA-32 remain unchanged after depletion of p34^{cdc2} and related kinases from S phase extracts. Extracts were depleted of p34^{cdc2} and related kinases as in Figure 9A. p13 and Sepharose 4B treated extracts were incubated with single-stranded M13 DNA under conditions described in Materials and methods and the reaction was stopped at the indicated times. Reaction products were loaded on gels and immunoblotted with monoclonal antibody against RPA-32.

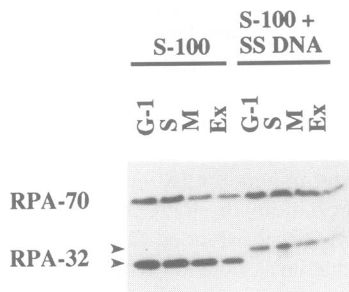

Fig. 11. Presence of the protein kinase responsible for the phosphorylation of DNA associated RPA-32 at all stages of the cell cycle. Manca cell extracts from G_1 phase (G−1), S phase (S), nocodazole blocked (M) and exponentially growing cells (Ex) were prepared and incubated either without or with single-stranded M13 DNA as described in Materials and methods.

There are several ways to explain the phosphorylation of RPA-32 associated with single-stranded DNA. One possibility is that the dephosphorylation of RPA-32 is decreased upon binding single-stranded DNA. The binding of RPA-70 to DNA could have an allosteric effect protecting the phosphorylated sites on RPA-32 from the action of protein phosphatases. This model seems unlikely since the addition of phosphatase inhibitors to cell extracts does not result in phosphorylation of RPA-32 in the absence of DNA (R.Fotedar, unpublished results). Alternatively, the protein kinase(s) responsible for phosphorylation of RPA-32 could be activated by single-stranded DNA. If this were true the protein kinase should be able to phosphorylate RPA-32 regardless of whether RPA-32 is associated with DNA. However, we find that only the DNA associated RPA-32 is phosphorylated and the free RPA-32 remains predominantly unphosphorylated. We therefore favour a model in which the binding of RPA-70 to single-stranded DNA is accompanied by a change in the conformation of the RPA complex, making the potential phosphorylation sites on RPA-32 accessible to a constitutively active protein kinase. At present we cannot, however, rule out the possibility that the kinase may be activated by single-stranded DNA.

We do not know the identity of the kinase which phosphorylates RPA-32 during DNA replication. Our preliminary observations suggest that the protein kinase(s) responsible for phosphorylation of RPA-32 is associated with DNA. RPA-32 associated with DNA during initiation of replication in the presence of dATP could be phosphorylated by incubation of the DNA−protein complex with ATP (R.Fotedar, unpublished results). The replication-dependent phosphorylation of RPA-32 does not appear to be DNA-PK, a kinase reportedly requiring double-stranded linear DNA for its activity *in vitro* (for a review see Lees-Miller and Anderson, 1991) since we show that phosphorylation of RPA-32 is stimulated in the presence of single-stranded DNA and not by double-stranded circular or linear DNA (R.Fotedar, unpublished results). RPA-32 is phosphorylated by p34^{cdc2} kinase *in vitro* in the absence of DNA (A.Dutta and B.Stillman, personal communication). Similarly, we have observed that immunoprecipitated RPA-32 can be phosphorylated by purified cyclin A−p34^{cdc2} kinase, but this phosphorylation did not alter the mobility of RPA-32 on polyacrylamide gels by 2−3 kDa (R.Fotedar, unpublished data). Our experiments clearly show that the phosphorylation of RPA-32 which occurs within the initiation complex is not mediated by p34^{cdc2} or a related kinase. First, RPA-32 from S phase cell extracts depleted of p34^{cdc2} and related proteins, associates with both single-stranded M13 DNA and the SV40 origin during initiation, and becomes phosphorylated. Secondly, we show that RPA-32 from G_1 cell extracts, which lacks p34^{cdc2} kinase activity, is competent to associate with single-stranded DNA and become phosphorylated. It is nonetheless likely that RPA-32 is phosphorylated by other kinases, such as p34^{cdc2} kinase, which do not require association of RPA-32 with DNA. Additional studies will be necessary to determine whether phosphorylation by p34^{cdc2} kinase, in addition to the phosphorylations described here, are necessary for RPA function.

A good candidate protein kinase for the replication-dependent phosphorylation of RPA-32 is the product of *Saccharomyces cerevisae CDC7* gene. The *CDC7* gene product is a serine/threonine protein kinase localized in the nucleus (Paterson *et al.*, 1986; Bahman *et al.*, 1988; Hanks *et al.*, 1988; Hollingsworth and Sclafani, 1990; Yoon and Campbell, 1991) whose activity is required at the last genetically defined step before initiation of DNA synthesis (Hartwell, 1973; Byers and Goetsch, 1974; Hereford and Hartwell, 1974). Furthermore, yeast RPA-36 is not phosphorylated in cells containing a *cdc7^{ts}* allele at the nonpermissive temperature but becomes phosphorylated when the cells are returned to the permissive temperature (Din *et al.*, 1990). It is interesting to note that *CDC7* is also required for the commitment of meiotic recombination and induced mutagenesis (Simchen, 1974; Schild and Byers, 1978; Njagi and Kilby, 1982), two processes modulated by RPA. A homologous-pairing protein, HPP-1 in humans (Moore *et al.*, 1991) and SEP1 in yeast (Heyer *et al.*, 1990) is stimulated by RPA and replicative excision repair reactions in crude human extracts also require RPA (Coverly *et al.*, 1991).

Function of replication-dependent phosphorylation of RPA-32

Although we show that the phosphorylation of RPA-32 occurs during initiation phase of DNA replication, it is

unlikely that this phosphorylation facilitates the origin unwinding function of RPA. First, several single-stranded DNA binding proteins can substitute for RPA in origin unwinding reactions (Dean *et al.*, 1987a; Wold *et al.*, 1987; Goetz *et al.*, 1988; Brill and Stillman, 1989; Kenny *et al.*, 1989). Secondly, we find that the efficiency with which RPA binds to DNA is not reduced under conditions in which RPA-32 remains unphosphorylated. Thirdly, we observe that the complete phosphorylation of RPA-32 which is associated with SV40 DNA takes longer than the time required to unwind the origin of replication. Finally, RPA-32 is quantitatively phosphorylated in the presence of single-stranded DNA, a product of the unwinding reaction. Our observations therefore suggest that phosphorylation of RPA may be important in the steps downstream of DNA unwinding.

During initiation, RPA is required for the SV40 T antigen-dependent unwinding of the origin presumably to stabilize the unwound DNA (Wold and Kelly, 1988; Tsurimoto *et al.*, 1990). Indeed other single-stranded DNA binding proteins can substitute for RPA in the origin unwinding reaction (Dean *et al.*, 1987a; Wold *et al.*, 1987; Goetz *et al.*, 1988; Brill and Stillman, 1989; Kenny *et al.*, 1989), as can RPA-70 alone (Erdile *et al.*, 1992). None of these proteins can, however, substitute for RPA in complete replication reactions, suggesting that RPA is also required for other events during DNA replication (Wold and Kelly, 1988; Brill and Stillman, 1989; Matsumoto *et al.*, 1990; Erdile *et al.*, 1991). This idea is supported by the observation that RPA can stimulate human DNA polymerase α-primase while other prokaryotic and viral single-stranded proteins cannot (Kenny *et al.*, 1989; Tsurimoto and Stillman, 1989; Erdile *et al.*, 1992). In *Escherichia coli*, bacteriophage (T4 and T7) and eukaryotic (adenovirus, herpes simplex virus) viral DNA replication systems, single-stranded DNA binding proteins stimulate homologous DNA polymerases through specific protein–protein interactions (Cha and Alberts, 1988; Huber *et al.*, 1988; Chiou *et al.*, 1985; Lindenbaum *et al.*, 1986; O'Donnell *et al.*, 1987). These observations might explain why other single-stranded DNA binding proteins cannot substitute for RPA in complete reactions.

Multiple phosphorylations on RPA-32 might be necessary to induce a conformation change which allows RPA to interact with other proteins that initiate DNA synthesis, such as polymerase α-primase and SV40 T antigen. This process may thereby direct the assembly of polymerase α-primase to the replication fork and couple the unwinding of the origin with the start of DNA synthesis. In prokaryotic replication systems, protein–protein interactions play an important role in coupling the formation of pre-initiation complexes with synthesis of DNA (for a review see Bramhill and Kornberg, 1988). In the SV40 system, physical interactions among RPA, SV40 T antigen and DNA polymerase α-primase have all been observed *in vitro* (Smale and Tjian, 1986; Gough *et al.*, 1988; Gannon and Lane, 1990; Dornreiter *et al.*, 1990, 1992). The demonstration that these interactions occur *in vivo* is, however, lacking.

Replication-dependent phosphorylation of RPA-32 modifies only the RPA which participates in the initiation complex. If phosphorylation of RPA-32 negatively regulates its ability to support unwinding, then reinitiation of DNA replication would be prevented until RPA-32 was dephosphorylated. This could limit the use of each replication origin to once per cell cycle. The two proposed roles of phosphorylated RPA-32, one in directing the assembly of polymerase α-primase to the replication origin to couple unwinding of the origin with the start of DNA synthesis and the other in restricting the use of each origin once per cell cycle, are not mutually exclusive.

Another protein probably phosphorylated upon binding to DNA is the mammalian transcription factor, Sp1 (Jackson *et al.*, 1990). DNA containing specific Sp1 binding sites is more effective in promoting phosphorylation than nonspecific DNA. In this case, the kinase itself has been shown to be associated with DNA. Recently, it was shown that the phosphorylation of GAL4 depends upon its having both a functional DNA binding and a transactivation domain (Sadowski *et al.*, 1991). Like RPA-32, the phosphorylation of GAL4 may require binding to DNA to become a substrate for its kinase. The DNA binding-dependent phosphorylation of regulatory proteins, such as described here for RPA-32, may be a mechanism to ensure that the proteins are activated at the site where they are likely to be used. Only appropriately phosphorylated DNA bound protein will be able to interact with other proteins while free unphosphorylated protein cannot. Such a mechanism will prevent the nonproductive sequestration of a limiting protein by other proteins present in excess (Gill and Ptashne, 1988). Phosphorylation has also been implicated in modulating activity of a variety of transcription factors such as c-jun, c-myb, HSF, SRF, E2F, E4F, CREB, Oct-2 (for a review see Bohmann, 1990). Whether a subset of these transcription factors may be phosphorylated upon binding to DNA is unknown. Phosphorylation might be a general mechanism to co-ordinate the assembly of multiprotein–DNA complexes such as those involved in transcription and replication.

Materials and methods

Cell culture and extracts

Manca cells were grown in spinner flasks with RPMI-1640 containing 5% calf bovine serum (CBS) and 2 mM L-glutamine. S-100 supernatants containing 100 mM NaCl were made from hypotonic lysates of exponentially growing cells as described elsewhere (Stillman, 1986). To obtain Manca cells in S phase, exponentially growing cells at $5-6 \times 10^5$/ml were synchronized with 2 mM hydroxyurea (0.15 g/l, Sigma) for 10–12 h, washed with Tris-buffered saline and resuspended at 6×10^6/ml in RPMI containing 5% CBS and 2 mM L-glutamine. Four hours after release, cells were collected (250 *g* for 7 min in Beckman GPR centrifuge), washed three times with cold phosphate-buffered saline and resuspended at $2-2.5 \times 10^8$ cells/ml in hypotonic buffer (20 mM HEPES–KOH, pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$ and 0.1 mM DTT). S-100 supernatants were made as described above. To obtain G_1 cells, 6 l of Manca cells at 5×10^5/ml were blocked in mitosis with 0.33 μM Nocadozole (Sigma). After 10 h, cells were washed and released in fresh medium (1–2 h) until 50% of the cells had a cell volume corresponding to the G_1 cells as determined by a Coulter Counter and Channelizer. These cells, collected by centrifugation, were then resuspended in medium and subjected to centrifugal elutriation (JE 6-B Beckman centrifuge, using a JE 5 rotor and a 30 ml chamber). An aliquot was taken from pooled fractions for analysis by flow cytometry (Marraccino *et al.*, 1992). Extracts from cell preparations containing 93–95% G_1 cells, as determined by flow cytometric analysis of DNA content after staining with propidium iodide, were used for subsequent experiments. The mitotic cell extracts were made after blocking cells in nocodazole for 10 h. Mitotic index of such a preparation was typically up to 80%, with $<5\%$ of the cells in G_1 or S phase.

Cell labelling

Manca cells grown in RPMI as described above were washed twice with phosphate-free DMEM. Approximately 5×10^7 cells were incubated with 10 ml phosphate-free DMEM containing 5% CBS and 2 mM L-glutamine

for 2 h. Cells were pelleted and labelled with 20 mCi [^{32}P]orthophosphate (ICN 64013) in 10 ml of phosphate-free DMEM containing 5% CBS and 2 mM L-glutamine for 2 h. S-100 supernatants were prepared as described above.

Replication reactions and single-stranded M13 assay

Replication reactions were performed for 2 h at 37°C as described earlier (Stillman and Gluzman, 1985) with the exception that 100 ng DNA was used in 50 μl reactions. Replication reactions performed in the presence of SV40 DNA routinely contained [^{32}P]dCTP to allow estimation of DNA synthesis and to follow DNA–protein complexes fractionated on Sepharose 6B columns. The amount of DNA synthesis was quantified by measuring the incorporation of [^{32}P]dCMP into trichloroacetic acid precipitable counts. In all reactions the protein concentration of extracts was adjusted to 100 μg per 50 μl reaction. Reactions performed under replication initiation conditions only, utilized the same buffer conditions as replication reactions, except dialysed S-100 extracts were used and the reactions lacked all deoxy- and ribonucleoside triphosphates but contained 3 mM ATP or dATP. Dialysed extracts were tested for depletion of deoxy- and ribonucleoside triphosphates by their ability to replicate SV40 DNA. In the presence of exogenously added deoxy- and ribonucleoside triphosphates, the levels of replication in dialysed extracts were equal to untreated extracts. Reactions containing single-stranded M13 DNA were performed in the presence of 40 mM HEPES–KOH pH 7.5, 8 mM $MgCl_2$, 0.5 mM DTT, 300 mM ATP and 100 ng single-stranded M13 DNA (USB) in 50 μl. Reactions were incubated at 37°C for 1 h and stopped by the addition of EDTA to 20 mM. RPA-32 was labelled with ^{32}P by including 10 μl of [γ^{32}P]ATP (NEN, 3000 Ci/mmol) per 50 μl reaction and reducing ATP to 50 mM. For experiments in which the rate of phosphorylation of RPA-32 was measured, the reaction was performed with 50 μg protein/50 μl reaction volume at 30°C for the indicated time.

Isolation of DNA–protein complexes

DNA–protein complexes assembled on SV40 DNA or single-stranded M13 DNA were isolated by fractionation of reaction products over a Sepharose 6B column as described earlier by Fotedar and Roberts (1992). The Sepharose 6B fractions were boiled in Laemmli SDS sample buffer and the proteins resolved on 12% SDS–polyacrylamide gels.

Immunoprecipitation

Anti-serum against all three subunits of RPA as well as monoclonal antibodies against the 70 and 32 kDa subunits of RPA were a gift from B.Stillman and Salah-u-Din. Five reactions containing DNA–protein complexes were adjusted to 0.1% SDS in RIPA buffer described by Din *et al.* (1990) and incubated at 4°C for 30 min. All the steps were performed at 4°C. 100 μl of anti-RPA-32 antibody was added and the mix incubated for 2 h. The immune complexes were precipitated with 150 μl of protein A–Sepharose (Sigma) that had been pre-incubated with 12 μl of rabbit anti-mouse antibody (DAKO) for 1 h and washed three times with RIPA buffer. After 1 h, the immunocomplexes were washed six times with RIPA buffer without SDS. The immunoprecipitates were used directly for phosphatase treatment. Where necessary, the proteins were eluted by boiling beads in 100 μl of sample buffer and an aliquot was loaded onto 12% SDS–polyacrylamide gels.

Phosphatase treatment

Protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A) and protein tyrosine phosphatase (PTPase) were kindly provided by N.Zander and E.H.Fischer. Immunoprecipitates were treated with 10 U each of PPI, PP2A and PTPase in 25 mM imidazole pH 7.2, 0.1% 2-mercaptoethanol and 1 mg/ml BSA in a final volume of 30 μl for 30 min at 37°C. Immunoprecipitates were also treated with 10 U of alkaline phosphatase (Boehringer) in 20 mM Tris, 0.1 mM EDTA, pH 8.5. Reactions were terminated by the addition of Laemmli sample buffer. One unit of PP1, PP2A or PTPase removed 1 nmol phosphate/min for phosphorylase a (PP1, PP2A) or lysozyme (PTPase) used as a substrate. Okadaic acid (Gibco) and *p*-nitrophenylphosphate (Sigma) were used at 1 μM and 200 μM final concentration, respectively.

Digestion with V8 protease

Bands containing the phosphorylated form of RPA-32 were excised from SDS–polyacrylamide gels and subjected to proteolysis during gel electrophoresis using 5 or 50 ng of *S.aureus* V8 protease as described by Cleveland *et al.* (1977). The peptides were resolved on 15% SDS–polyacrylamide gels.

Peptide maps

RPA-32 was immunoprecipitated from extracts of Manca cells labelled with [^{32}P]orthophosphate. RPA-32 was similarly immunoprecipitated from *in vitro* reactions containing Manca cell extracts, single-stranded M13 DNA and [γ^{32}P]ATP. The proteins were resolved on 12% SDS–polyacrylamide gels and visualized by autoradiography. Bands containing the ^{32}P-labelled RPA-32 were excised from the gel and digested with chymotrypsin as described by Beemon and Hunter (1978). The phosphopeptides were separated on cellulose thin layer plates by electrophoresis in the first dimension at pH 1.9 in (v/v) formic acid: glacial acetic acid: water (22.5:77.5:900) for 15 min at 1.6 kV. The second dimension was performed at pH 5.2 in (v/v) *n*-butanol: pyridine: acetic acid:water (375:250:75:300) for 7–8 h.

Immunoblotting

Proteins were resolved by SDS–PAGE and immunoblotted as described earlier (Fotedar and Roberts, 1992) except for the following changes. The filters were not washed before overnight incubation with antibody. Filters were washed three times with wash buffer, incubated with rabbit anti-mouse antibody (DAKO, 1:1000). After 1 h, filters were washed three times and incubated with 0.1 mCi [^{125}I]protein A/ml/5 cm^2 (NEN, NEX-146L) for 1 h. After three washes the filters were dried and autoradiographed.

Depletion of p34^{cdc2} by p13–Sepharose

Depletion was performed as described in Fotedar and Roberts (1992).

Acknowledgements

We are grateful to Salah-ud-Din, Anindhya Dutta and Bruce Stillman for providing antibodies to RPA and for sharing their results. We thank Norbert Zander and Edmond H.Fischer for providing protein phosphatase 1, protein phosphatase 2A and protein tyrosine phosphatase, Robert L.Margolis for helpful suggestions and critical reading of the manuscript, Steve Kussick and Alasdair MaCauley for their advice on performing V8 protease and peptide maps, Timothy Day for preparing gels and Tina Loucks for typing the manuscript. J.M.R. is a Lucille P.Markey Scholar in Biomedical Sciences and this work was supported by a grant to J.M.R. from NIH and Lucille P.Markey Charitable Trust. R.F. is the recipient of a fellowship from Leukemia Society of America.

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Received on November 22, 1991; accepted on March 17, 1992