Replication protein A is sequentially phosphorylated during meiosis

George S. Brush*, Dawn M. Clifford, Suzanne M. Marinco and Amy J. Bartrand

Program in Molecular Biology and Genetics, Karmanos Cancer Institute, Wayne State University, 110 East Warren Avenue, Detroit, MI 48201, USA

Received August 13, 2001; Revised and Accepted October 5, 2001

ABSTRACT
Phosphorylation of the cellular single-stranded DNA-binding protein, replication protein A (RPA), occurs during normal mitotic cell cycle progression and also in response to genotoxic stress. In budding yeast, these reactions require the ATM homolog Mec1, a central regulator of the DNA replication and DNA damage checkpoint responses. We now demonstrate that the middle subunit of yeast RPA (Rfa2) becomes phosphorylated in two discrete steps during meiosis. Primary Rfa2 phosphorylation occurs early in meiotic progression and is independent of DNA replication, recombination and Mec1. In contrast, secondary Rfa2 phosphorylation is activated upon initiation of recombination and requires Mec1. While the primary Rfa2 phosphoisomer is detectable throughout most of meiosis, the secondary Rfa2 phosphoisomer is only transiently generated and begins to disappear soon after recombination is complete. Extensive secondary Rfa2 phosphorylation is observed in a recombination mutant defective for the pachytene checkpoint, indicating that Mec1-dependent Rfa2 phosphorylation does not function to maintain meiotic delay in response to DNA double-strand breaks. Our results suggest that Mec1-dependent RPA phosphorylation could be involved in regulating recombination rather than cell cycle or meiotic progression.

INTRODUCTION
Replication protein A (RPA) is an evolutionarily conserved single-stranded DNA (ssDNA)-binding protein that is required for DNA replication, repair and recombination in eukaryotic cells (1,2). The RPA complex consists of three polypeptides with molecular weights of ~70, ~30 and ~14 kDa, and the major ssDNA-binding activity of the protein resides in the largest of these subunits. While stabilization of ssDNA is central to its function, RPA also directly associates with other proteins that are involved in DNA replication, repair and recombination. These protein–protein interactions are thought to play a critical role in the initiation of DNA transactions, suggesting that RPA is an important regulatory target. In support of this hypothesis, the middle subunit of RPA becomes phosphorylated during progression through the mitotic cell cycle and also in response to genotoxic stress (3–7). While the effect of these post-translational modifications remains unclear, their conservation from yeast to humans provides strong evidence that RPA phosphorylation has a significant impact on the fitness of the eukaryotic cell.

One approach to understanding the function of RPA phosphorylation is to identify and characterize the RPA kinases that function in vivo. Our previous studies in the budding yeast Saccharomyces cerevisiae revealed that the protein kinase Mec1 is required for cell cycle-regulated phosphorylation of the RPA middle subunit (Rfa2) (7), which occurs during the S and G2 phases (3). We also demonstrated that various forms of genotoxic stress lead to Mec1-dependent phosphorylation of both Rfa2 (7) and the RPA large subunit (Rfa1) (8). Mec1 is a key regulator of the checkpoint responses that function to delay cell cycle and meiotic progression in response to cellular perturbations (9–13). Several lines of evidence indicate that Mec1 is also involved in recombination. During mitotic growth, mecl mutants exhibit increased spontaneous intergenic recombination (14), decreased spontaneous and DNA damage-induced intragenic recombination (15) and decreased non-homologous end joining (16). During meiosis, mecl mutants exhibit diminished homologous recombination (14,17) and enhanced ectopic recombination (17). Functional Mec1 is also required for redistribution of Sir3 from telomeres to DNA double-strand breaks (18,19), which are repaired by recombination (20), and for phosphorylation of the recombination proteins Rad55 and Srs2 (15,21). Finally, Mec1 directly phosphorylates of histone H2A in response to agents that cause DNA double-strand breaks, and mutation of the H2A phosphorylation site confers a defect in non-homologous end joining without affecting Mec1-mediated cell cycle delay (22).

Mec1 is structurally and functionally related to ATM, the protein kinase mutated in the pleiotropic human disorder ataxia telangiectasia (AT) (23). This disease is characterized by cerebellar degeneration, ocular and facial telangiectasia, immunodeficiency, infertility, premature aging and an increased risk of leukemias and lymphomas (24). AT cells are hypersensitive to killing by ionizing radiation (25) and, like mecl mutants, exhibit defects in both checkpoint-mediated cell cycle delay (26–28) and recombination (29–31). AT cells are also deficient in IR-induced phosphorylation of several proteins (32), including RPA (5). Therefore, an evolutionarily conserved pathway involving Mec1 in yeast and ATM in humans functions to modify RPA in response to genotoxic stress.

Because Mec1 and ATM are required for normal checkpoint function, it is possible that RPA phosphorylation serves as a...
transducer of checkpoint signals that control cell cycle progression. However, experiments with mutants in the yeast Rad53 protein rule against this possibility, at least with respect to phosphorylation of the middle subunit. Rad53 is a protein kinase required for the DNA damage and DNA replication checkpoints in mitotic cells and its activation depends on Mec1 (9,10,33–36). In checkpoint-deficient rad53 mutants, Rfa2 phosphorylation still occurs during the normal cell cycle and in response to DNA damage or DNA replication inhibition (7), and Rfa1 phosphorylation still occurs in response to DNA replication inhibition (8). Therefore, RPA phosphorylation under these conditions does not correlate with checkpoint function. Consistent with these results, experiments in human cells have suggested that IR-induced RPA phosphorylation is not required for the S phase DNA damage checkpoint (37).

Nonetheless, Rfa1 phosphorylation is compromised in cells have suggested that IR-induced RPA phosphorylation is not required for the S phase DNA damage checkpoint (37). Nonetheless, Rfa1 phosphorylation is compromised in rad53 cells exposed to DNA-damaging agents during G1 (8), a condition under which Rfa1 is known to mediate cell cycle delay (38). Therefore, certain phosphoisomers of RPA might be involved in the checkpoint response during specific phases of the cell cycle.

Given that Mec1 and ATM also function in recombination, RPA phosphorylation could alternatively be involved in regulating recombination activities. To explore this possibility, we chose to examine RPA status in yeast cells undergoing meiosis, a process that includes a high level of programmed recombination. We now demonstrate that Rfa2 becomes hyperphosphorylated during meiotic progression through two distinct reactions. A primary Rfa2 phosphorylation event is induced early in meiosis that is independent of DNA replication and recombination and does not require Mec1. Subsequently, a secondary Rfa2 phosphorylation reaction is induced that requires initiation of recombination and is Mec1 dependent. Although Mec1 is necessary for the ‘pachytene checkpoint’ that halts meiotic progression when recombination is incomplete (12), we observe extensive Mec1-dependent Rfa2 phosphorylation in a checkpoint-defective meiotic recombination mutant. Based on these studies, we suggest that Mec1-dependent RPA phosphorylation is involved in recombination rather than maintenance of checkpoint delay.

MATERIALS AND METHODS

Yeast strains and growth

The following DSY strains were kindly provided by Drs David Stuart (University of Alberta) and Curt Wittenberg (The Scripps Research Institute) (13): DSY1089 (wild-type), MATα α ho::LYS2/" lys2/" leu2::hisG/" ura3/" his4B::LEU2/"his4X::LEU2-URA3 rad50KI81::URA3/", DKB159 (rad50S), MATα ho::LYS2 lys2 leu2::hisG ura3 his4B::LEU2 rad50K181::URA3; DKB330 (dmc1), MATα ho::LYS2/" lys2/" leu2::hisG/" ura3/" arg4-BglII/arg4-NspI his4B::LEU2/"his4X::LEU2-URA3 dmc1::LEU2/". YGB242 (rad50S mec1) was constructed in our laboratory employing strains DSY1031 and DKB159 and has the genotype MATα ho::LYS2/" lys2/" leu2::hisG/" ura3/" his4B::LEU2/"mec1-1/" smlx::rad50K181::URA3/". YGB138 (wild-type) is a diploid W303 strain (41): MATα ade2-1/" can1-100/"his3-11,115/" leu2-3,112/"trp1-1/"ura3-1/". With the exception of YGB138, all strains are derived from SK-1 (42).

YPD and YPG media for yeast growth were prepared as described (43). YPA for presporulation growth consisted of 1% yeast extract, 2% bactopeptone and 2% potassium acetate. Sporulation medium (SPM) for SK-1 cells included 0.3% (w/v) potassium acetate, 0.02% (w/v) raffinose, 2.5 × 10–5% (w/v) leucine, and each of arginine, histidine, tryptophan and uracil at 5 × 10–6% (w/v). An established sporulation protocol was employed to allow for synchronous progression of SK-1 cells through meiosis (13,44). Diploid cells were first streaked from a frozen stock onto a YPG plate and incubated at 30°C. A single colony was selected and grown in YPD medium overnight at 30°C and the YPD culture was used to inoculate YPA medium. The YPA culture was incubated in an air shaker at 30°C, 200 r.p.m. for 12 h from a starting OD600 of ~0.2 or for 16 h from a starting OD600 of ~0.1. The cells were harvested by centrifugation at 1900 g for 5 min at room temperature, washed with SPM, resuspended in SPM at the approximate density of the overnight YPA culture and then incubated at 30°C, 200 r.p.m. A similar protocol was employed for the YGB138 time course. Where indicated, cells were resuspended in SPM with hydroxyurea (HU) (Toronto Research Chemicals) added to a final concentration of 0.1 M from a 1 M stock; an equal volume of water was added to controls.

Western blot analysis

Cells (0.5–1.0 ml) were harvested by centrifugation at 14 000 g for 5 min at 4°C, washed with 1 ml of cold distilled water and stored at ~70°C. To prepare denatured extract, cells were resuspended with 50 ml of sample buffer for SDS–PAGE and then subjected to three cycles of freezing (dry ice or liquid nitrogen for 2 min) followed by heating (~95°C for 2 min). The extracts were spun at 14 000 g for 5 min at room temperature and aliquots of the supernatant were analyzed by western blotting. Proteins were resolved by SDS–PAGE employing a 12% polyacrylamide gel (150:1 w/w acrylamide:bisacrylamide) and then transferred to nitrocellulose in either 25 mM Tris, 192 mM glycine, 20% methanol or 10 mM CAPS–NaOH, pH 11, 10% methanol. Immobilized RPA subunits were detected by autoradiography after incubation with anti-Rfa1 and anti-Rfa2 polyclonal primary antibodies, horseradish peroxidase-linked goat anti-rabbit secondary antibody (Pierce Chemical Co.) and chemiluminescence reagents (Pierce Chemical Co.). Antiserum included our previously described anti-Rfa1 and anti-Rfa2 preparations (8) and an anti-Rfa2 antiserum kindly provided by Dr Steven Brill (Rutgers University). For the western blot analyses shown in Figures 1–3 and 5, an equal volume of denatured extract was loaded in each lane. In the other experiments, we attempted to equalize the detectable
RPA by loading reduced volumes of the 0 h sample or both the 0 and 2 h samples.

Protein phosphatase treatment

Wild-type cells (DSY1089) undergoing synchronous sporulation were harvested at 6 h, resuspended in buffer containing 50 mM Tris–HCl, pH 7.5, 0.5 M NaCl, 2 mM Na$_2$EDTA, 10 mM β-mercaptoethanol, 20% (v/v) glycerol, 0.2 mM PMSF and vortexed with glass beads at 4°C (10 repeats of 30 s vortexing followed by 30 s on ice). The resulting suspension was centrifugally filtered through cheese-cloth at 1900 g for 2 min at 4°C and the supernatant of the filtrate was used for protein phosphatase studies. Reaction mixtures (20 µl) containing crude extract were assembled on ice and included 50 mM Tris–HCl, pH 7.5, 0.1 mM Na$_2$EDTA, 5 mM DTT, 0.01% Brij 35, and 2 mM MnCl$_2$. Where indicated, 400 U bacteriophage λ protein phosphatase (λPPase) (New England Biolabs) and 10 mM sodium orthovanadate were added and the mixtures were incubated at 30°C for either 0.5 or 2 h. Vanadate was included for its λPPase inhibitory activity (45) and the longer incubation time was employed to completely convert Rfa2 to the unphosphorylated form. Reactions were terminated by addition of Na$_2$EDTA to 12 mM and RPA in the reaction mixtures was visualized by western blot analysis.

Analysis of DNA content

Cells were harvested by centrifugation at 14 000 g for 5 min at 4°C, resuspended in 70% ethanol and stored at 4°C. Aliquots of the fixed cells were washed once with 1 ml of 50 mM Tris–HCl, pH 7.5, and resuspended in 1 ml of the same buffer. Samples were then treated with 250 µg proteinase K for 1 h at 37°C followed by 250 µg proteinase K for 1 h at 37°C. The digested samples were incubated with either 10x SYBR Green I (Molecular Probes; Figs 1, 4, 6 and 7) or 50 µg/ml propidium iodide (Figs 2 and 3) at 4°C over 1–3 nights, sonicated briefly and analyzed by flow cytometry with a Becton Dickinson FacsCalibur. DNA content histograms were generated using WinMDI software.

Nuclear staining

Cells that had been fixed with 3.7% formaldehyde (final concentration) were washed twice with water, resuspended in water and sonicated briefly. Aliquots were stained with an equal volume of 100 µg/ml 4,6-diamidino-2-phenylindole (DAPI) prepared in mounting medium (Sigma Chemical Co.). The cells were observed by fluorescence microscopy and nuclear bodies were scored in at least 100 unbudded cells for each sample. Budded cells were observed in only a subset of experiments and at a very low percentage of the total cells in these cases.

RESULTS

RPA becomes phosphorylated during meiosis

Studies employing vegetative yeast cells have indicated that RPA phosphorylation is catalyzed during normal growth and also in response to various forms of genotoxic stress (3,7,8). Because advancement through meiosis includes both a DNA replication phase and a subsequent recombination phase initiated by DNA double-strand breaks (44,46), we investigated the possibility that RPA also becomes phosphorylated during this specialized developmental pathway. When wild-type SK-1 cells are subjected to conditions that result in synchronous meiotic progression, two Rfa2 isoforms are generated that have reduced mobility during SDS–PAGE (Fig. 1A). Note that a lighter exposure of the data presented in Figure 1A reveals that the majority of Rfa2 at 0 h migrates with the faster mobility (data not shown, but see Fig. 4A). The reduced mobility species are abolished upon treatment of native extract with protein phosphatase (Fig. 1B), indicating that both forms are Rfa2 phosphoisomers. The two phosphorylated forms of Rfa2 are also detected in wild-type W303 cells (data not shown), indicating that the pattern of RPA phosphorylation during meiosis is not influenced by strain background. In addition, we have observed that the general pattern of mitotic RPA phosphorylation is not altered by differences in strain background (data not shown).

Although SDS–PAGE detection of phosphorylated species does not provide an exact measure of stoichiometry, we can conclude from our data that both a primary phosphorylated and a secondary hyperphosphorylated species of Rfa2 are generated during meiosis. We compared the timing of Rfa2 phosphorylation and hyperphosphorylation with the timing of various meiotic events (Fig. 1A, C and D). Primary Rfa2 phosphorylation is initially observed early in meiosis, leading to modification of ~50% of the detectable Rfa2 by 3 h (Fig. 1A). At this time point, very little DNA has been synthesized as measured by flow cytometric analysis of DNA content (Fig. 1C). Therefore, the degree of primary Rfa2 phosphorylation does not correlate with the extent of DNA synthesis. In fact, primary Rfa2 phosphorylation occurs even in the absence of DNA replication (see below). Further meiotic progression leads to quantitative Rfa2 phosphorylation and to Rfa2 hyperphosphorylation, which coincides with induction of his4 heteroallelic recombination as measured by detection of histidine prototrophs in a ‘return-to-growth’ assay (47,48; Fig. 1A and D). Therefore, the appearance of neither phosphorylated nor hyperphosphorylated Rfa2 correlates directly with initiation of DNA synthesis, as has been suggested for Rfa2 phosphorylation during unperturbed vegetative growth (3). Rfa2 dephosphorylation also occurs in a stepwise manner, proceeding from the hyperphosphorylated to the phosphorylated form when recombination is complete and from the phosphorylated to the unphosphorylated form later in meiosis (see 28 h time points in Figs 2A and 3A).

Primary meiotic RPA phosphorylation is independent of DNA replication

To gain insight into the role that DNA synthesis plays in meiotic RPA phosphorylation, we analyzed a strain containing deletions of the clb5 and clb6 genes. This double mutant is incapable of replicating DNA during meiosis but continues through later meiotic events due to a checkpoint defect (13,49). As shown in Figure 2, clb5 clb6 cells support the early meiotic Rfa2 phosphorylation reaction with kinetics similar to wild-type despite the defect in meiotic DNA replication. Therefore, the initial Rfa2 phosphorylation event is independent of DNA replication. These results further indicate that a cyclin-dependent kinase activated by either the Clb5 or Clb6 B-type cyclin does not catalyze this Rfa2 phosphorylation reaction. In contrast to primary Rfa2 phosphorylation, secondary Rfa2
phosphorylation is significantly delayed in the clb5 clb6 mutant. It is likely that the absence of normal Rfa2 hyperphosphorylation in these cells results from a recombination deficiency (50; see below).

Mec1 is required for the secondary meiotic RPA phosphorylation reaction

Because RPA phosphorylation is dependent on Mec1 during mitosis (7,8), we analyzed Rfa2 status in a mecl mutant undergoing meiosis. Although the primary Rfa2 phosphorylation reaction is unaffected in this mutant, the secondary reaction leading to Rfa2 hyperphosphorylation is completely abolished (Fig. 3A). Consistent with previous reports (12,13), the mecl mutant used in our study proceeds through meiotic S phase with normal kinetics (Fig. 3B) and is capable of forming mature asci at later time points (data not shown).

Mutants in mecl are unable to arrest cell cycle progression when DNA replication is inhibited during either mitosis or meiosis (9,13). Therefore, we compared RPA phosphorylation in wild-type and mecl cells that had entered meiosis in the presence of hydroxyurea (HU), an inhibitor of DNA replication. Wild-type cells exhibit substantial primary Rfa2 phosphorylation in the presence of HU, although the extent of this reaction is compromised relative to that of normal meiotic progression (Fig. 4A). Interestingly, hyperphosphorylated Rfa2 is not generated to a significant extent in response to HU, although we observe a low level of this Rfa2 phosphoisomer at later time points (Fig. 4A). The pattern of Rfa2 phosphorylation is similar in HU-exposed wild-type and mecl cells (Fig. 4C), indicating that Rfa2 phosphorylation in the presence

Figure 1. Rfa2 phosphorylation during meiotic progression. (A) Western blot analysis of Rfa2 in synchronously sporulating DSY1089 cells (SK-1 wild-type). In this time course and all others represented below, 0 h is the time of transfer from YPA to SPM. (B) Phosphatase treatment of native extract from DSY1089 cells harvested 6 h after transfer to SPM. As indicated, reaction mixtures were incubated for either 0.5 or 2 h. λPPase, bacteriophage λ protein phosphatase. (C) DNA content analysis by flow cytometry of the DSY1089 culture examined in (A). (D) Recombination and chromosome segregation analysis of the DSY1089 culture examined in (A). Recombination was measured by scoring histidine prototrophs and progression into meiosis II (MII) was measured by scoring cells with three or four DAPI staining bodies. cfu, colony forming units.

Figure 2. Primary meiotic Rfa2 phosphorylation in the absence of DNA replication. (A) Western blot analysis of Rfa2 in DSY1089 (wt) and DSY984 (clb5 clb6) cells induced to enter meiosis. P-Rfa2, phosphorylated Rfa2. (B) DNA content analysis of cultures examined in (A).
of HU is equivalent to the Mec1-independent primary Rfa2 phosphorylation observed during normal meiotic progression. Therefore, HU treatment allows for a significant level of primary but not secondary Rfa2 phosphorylation. Although Mec1 is required for delay of meiotic progression in response to HU exposure (13), the paucity of HU-induced Rfa2 hyperphosphorylation suggests that Mec1-dependent Rfa2 phosphorylation does not play an important role in the meiotic DNA replication checkpoint.

We previously reported that Rfa1 becomes phosphorylated in response to various forms of genotoxic stress (8). Although meiotic progression leads to the generation of DNA double-strand breaks as a prerequisite to recombination (44,46), we have not detected phosphorylated Rfa1 under these conditions. However, an Rfa1 phosphoisomer with reduced electrophoretic mobility is detectable at 2 h upon HU exposure, a time at which untreated cells are beginning to enter S phase in this experiment (Fig. 4A and B). Similar to Rfa1 phosphorylation in vegetative cells, this Rfa1 phosphorylation reaction is dependent on Mec1 (Fig. 4D). Therefore, genotoxic stress during meiosis, but not the DNA double-strand break ‘damage’ that is naturally induced during meiotic progression, leads to detectable Mec1-dependent Rfa1 phosphorylation.

Secondary meiotic RPA phosphorylation occurs upon initiation of recombination

The DNA double-strand breaks required for initiation of meiotic recombination are catalyzed by Spo11 (51). Interestingly, the absence of recombination initiation in spo11 mutants is accompanied by a defective pachytene checkpoint (52). Our analysis of Rfa2 in spo11 cells reveals normal primary Rfa2 phosphorylation but a severe defect in the secondary reaction (Figs 5 and 6A). Therefore, meiotic Mec1-dependent Rfa2 phosphorylation is dependent on Spo11, most likely due to its essential function in initiating recombination.

The duplex ends resulting from Spo11-mediated DNA breakage are processed to generate 3′ single-stranded tails that can effectively initiate strand exchange. We analyzed meiotic RPA phosphorylation in response to HU exposure. (A) Western blot analysis of Rfa1 and Rfa2 in DSY1089 cells induced to enter meiosis in the absence or presence of HU. For this experiment, a single population was resuspended in SPM (0 h) and then divided into untreated (–HU) and treated (+HU) cultures. P-Rfa1, phosphorylated Rfa1; P-Rfa2, phosphorylated Rfa2. (B) DNA content analysis of cultures examined in (A). Identical 0 h data are presented in the two histograms. (C) Western blot analysis of Rfa2 in DSY1089 (wt) and DSY1057 (mec1) cells induced to enter meiosis in the presence of HU. (D) Western blot analysis of Rfa1 in 6 h samples from the cultures analyzed in (C). The asterisk indicates an immunoreactive band that is presumably an Rfa1 degradation product.

RPA phosphorylation in rad50S and dmc1 mutants, which are defective for the resection and strand exchange steps, respectively (39,46,53). As with the spo11 strain, primary Rfa2 phosphorylation appears to be unaffected in either rad50S or dmc1 cells (Fig. 6A). Note that the slight delay in induction of primary Rfa2 phosphorylation in the mutant strains is likely explained by their slight delay in meiosis entry as determined...
by DNA content analysis (Fig. 6B). We conclude that the timing of primary Rfa2 phosphorylation is consistent in all strains employed.

In contrast to the primary reaction, secondary Rfa2 phosphorylation is significantly altered in rad50S and dmc1 cells relative to the wild-type, as both mutants accumulate a high level of hyperphosphorylated Rfa2 (Fig. 6A). Although induction of the secondary reaction appears to be delayed in rad50S and dmc1 cells, the differences in early meiotic progression between the mutants and wild-type are likely to account for this effect (Fig. 6B). Despite the similar Rfa2 hyperphosphorylation in the rad50S and dmc1 strains, rad50S cells accumulate unresected DNA double-strand breaks while dmc1 cells generate DNA ends with long ssDNA tails (39,46,53). In addition, rad50S mutants in the SK-1 background are reported to progress into meiosis I with either normal or delayed kinetics, while dmc1 mutants arrest at pachytene in a Mec1-dependent manner (12,52–54). The strains used in our study exhibit the predicted phenotypes, as multinuclear bodies indicative of chromosome segregation are observed in spo11 and rad50S cells, but not in dmc1 cells (Fig. 6C). Because a rad50S mec1 mutant generates very little hyperphosphorylated Rfa2 (Fig. 7), we conclude that Mec1 is required for the vast majority of secondary Rfa2 phosphorylation in rad50S cells. Taken together, these studies indicate that the Mec1-dependent Rfa2 phosphorylation reaction activated upon initiation of recombination is not sufficient to maintain delay of meiotic progression.

**DISCUSSION**

During meiosis, DNA is replicated and recombined in reactions that transiently generate ssDNA. Our studies have revealed that the cellular ssDNA-binding protein RPA becomes phosphorylated in two successive steps during this process. These phosphorylation reactions exhibit differences in both timing and genetic dependencies, indicating that RPA is modified in a highly controlled fashion during meiosis. Based on the central role of RPA in DNA replication and recombination and the strictly ordered manner by which these processes occur during meiosis, it is likely that RPA phosphorylation plays a regulatory role in meiotic DNA metabolism.

A striking feature of primary Rfa2 phosphorylation is that nearly all of the detectable Rfa2 becomes modified. The extent of this reaction is particularly evident in the mec1 mutant, which is devoid of secondary Rfa2 phosphorylation. The timing of the primary reaction suggests that an early meiotic gene encodes an RPA kinase. This hypothesis is supported by the HU-dependent attenuation of the primary reaction, as a recent study has shown that early meiotic genes are down-regulated by HU treatment (55). Regardless of mechanism, the complete conversion of Rfa2 to a modified form would suggest that primary Rfa2 phosphorylation plays a critical role in...
meiosis. It is doubtful that the reaction functions in a meiotic checkpoint process related to DNA metabolism because primary Rfa2 phosphorylation is not significantly compromised in the checkpoint-deficient clb5 clb6, mec1, spo11, rad50S or rad50S mec1 strains. An alternative possibility is that the primary Rfa2 phosphorylation reaction is required for proper DNA replication during meiosis. Although the same origins of DNA replication and many of the same replication proteins are employed in the mitotic and meiotic S phases, regulation of these two processes is different and meiotic DNA replication proceeds at a significantly slower pace than its mitotic counterpart (56,57). DNA replication during mitotic growth serves to duplicate the genetic material prior to chromosome segregation, while meiotic DNA replication leads to a recombination phase that precedes two rounds of chromosome segregation. Studies have shown that meiotic recombination depends on meiotic DNA replication (50,58) and that the meiotic recombination proteins Spo11 and Rec8 are involved in regulating meiotic DNA replication (59). Therefore, the sequential processes of DNA replication and recombination are tightly coupled during meiosis. Differences in mitotic and meiotic DNA replication, and perhaps RPA phosphorylation, may be related to differences in the events that directly follow the two S phases, and it is possible that primary Rfa2 phosphorylation serves as a molecular switch that converts RPA from a mitotic to a meiotic DNA replication protein.

There is now strong evidence that yeast RPA plays a role in transcription as well as DNA metabolism. RPA was purified from yeast cells as a factor that binds to the *URS1* transcriptional repression element (60,61) and has been shown to bind *in vitro* to *URS* sites found upstream of various genes involved in DNA metabolism (62). A recent report has indicated that a Mec1-independent, Rad53-dependent Rfa2 phosphorylation event, which is induced in cells lacking the Set1 chromatin regulator, decreases RPA–URS interaction and leads to increased expression of certain DNA repair genes (63). In addition to its function in transcriptional repression, *URS1* serves as an upstream activation sequence of several genes that are induced early in meiosis (57). Therefore, it will be interesting to determine whether primary RPA phosphorylation, which occurs early in meiosis and is Mec1 independent, functions in *URS1*-mediated transcriptional activation.

In contrast to the primary reaction, secondary Rfa2 phosphorylation is dependent on Mec1 and therefore resembles the Rfa2 phosphorylation reaction that is initially induced during DNA replication in mitotic cells (3,7). We demonstrate here that the meiotic Mec1-dependent Rfa2 phosphorylation reaction is more closely associated with recombination than DNA replication. First, induction of secondary Rfa2 phosphorylation occurs after the bulk of meiotic DNA replication has been completed and coincides with induction of recombination. Secondly, Rfa2 hyperphosphorylation requires Spo11, an enzyme that catalyzes DNA double-strand break formation during initiation of meiotic recombination (51). Finally, mutants that generate unresolved meiotic recombination intermediates accumulate hyperphosphorylated Rfa2 well after meiotic DNA synthesis is complete. These results indicate that Mec1-dependent Rfa2 phosphorylation is not likely to function in meiotic DNA replication. It is also unlikely that Mec1-dependent Rfa2 phosphorylation functions in the meiotic DNA replication checkpoint, which requires Mec1 (13), because Rfa2 hyperphosphorylation does not occur to a significant extent when wild-type cells are treated with HU. Nonetheless, Mec1-dependent Rfa1 phosphorylation is observed upon HU exposure and the timing of this reaction coincides with inhibition of DNA replication. Therefore, it remains possible that Rfa1 phosphorylation is involved in regulating meiotic progression through S phase.

Our results are consistent with a model in which DNA structures formed during the process of meiotic recombination lead to Mec1 activation. Such a mechanism has also been proposed for the Mec1-dependent phosphorylation of Mek1 (64), a homolog of Rad53 that is required for meiotic recombination and the associated pachytene checkpoint (52,65). We previously demonstrated that DNA containing both double- and single-stranded regions efficiently activates human RPA phosphorylation catalyzed by the DNA-activated protein kinase catalytic subunit (DNA-PKcs) (66), a structural homolog of Mec1 (67). In addition, a combination of double-stranded and single-stranded DNA molecules stimulates ATM-catalyzed RPA phosphorylation (68,69). Therefore, DNA resembling recombination intermediates supports RPA phosphorylation catalyzed by two Mec1-like enzymes from human cells. Several reports have indicated that Mec1 contains a protein kinase activity (70–72) and it is possible that Mec1 catalyzes RPA phosphorylation during meiosis through a mechanism similar to that of either DNA-PKcs or ATM. RPA readily forms DNA double-strand break-dependent foci in wild-type, *rad50S* and *dmc1* cells undergoing meiosis and it is likely that RPA binds to ssDNA generated at DNA double-strand breaks (73). Binding of Mec1 to DNA ends, analogous to DNA-PKcs (74) or ATM (75), would lead to co-localization of Mec1 and RPA and efficient Rfa2 phosphorylation. In support of such a model, ATM and RPA have been reported to co-localize on synapsed meiotic chromosomes in mouse spermatocytes (76).

The dependence of secondary Rfa2 phosphorylation upon initiation of recombination implies that Mec1-dependent RPA phosphorylation has a recombination function. Although Mec1 is required for the pachytene checkpoint that delays meiotic progression when recombination is incomplete (12), our analysis provides evidence that RPA hyperphosphorylation is not involved in meiotic delay. Consistent with previous reports (52–54), we have observed significant chromosomal segregation in the recombination-defective *rad50S* mutant. The inappropriate meiotic progression of these cells is accompanied by extensive accumulation of hyperphosphorylated Rfa2, arguing against a checkpoint function for the secondary Rfa2 phosphorysomer. It is noteworthy that *rad50S* cells retain a residual checkpoint activity that delays meiotic progression relative to wild-type cells (52,54). Both Mec1 and Tel1 are required for this characteristic meiotic delay (54) and we have shown that secondary Rfa2 phosphorylation in *rad50S* cells is also Mec1 dependent. Therefore, we cannot formally exclude the possibility that secondary Rfa2 phosphorylation is involved in the signal that initiates meiotic delay. Nonetheless, our data clearly demonstrate that secondary Rfa2 phosphorylation is not involved in maintaining the pachytene checkpoint.

In the absence of a checkpoint function, an attractive possibility is that Mec1-dependent Rfa2 phosphorylation is directly involved in regulating the process of meiotic recombination. A number of studies have suggested that the phosphorylation state of RPA can affect DNA metabolism, as RPA from human
cells exposed to DNA damaging agents is hyperphosphorylated and does not support efficient SV40 DNA replication in vitro (6,77,78). Interestingly, our previous studies employing the same cell-free SV40 system revealed a connection between RPA phosphorylation and recombination. We demonstrated that DNA replication-dependent RPA phosphorylation is catalyzed by DNA-PKcs (66), an enzyme that is required for DNA double-strand break repair and V(D)J recombination (79–82). Although our studies suggested that RPA phosphorylation occurs as a consequence of DNA replication (66), recombination is intimately involved with replication during mitotic S phase (83,84) and it might be difficult to distinguish a replication effect from a recombination effect. Therefore, RPA phosphorylation during mitotic S phase could resemble the meiotic reaction by requiring initiation of recombination rather than DNA replication and could also be involved in regulating recombination. It will be important to determine whether RPA phosphorylation affects any of the functional interactions between RPA and other meiotic and recombination proteins (85,86).

A comparison of the predominant forms of phosphorylated RPA that are observed in untreated and HU-treated wild-type yeast is depicted in Figure 8. As shown, each condition leads to the generation of a different RPA phosphoisomer. It is intriguing that different recombination mechanisms are known to be favored under different conditions. For example, mitotic recombination preferentially involves sister chromatids while meiotic recombination involves homologs, and crossing over occurs much more frequently in meiosis than mitosis (20,87). Determining whether RPA phosphorylation is involved in controlling the relative recombination activities of mitotic versus meiotic and unperturbed versus stressed yeast cells will require genetic analysis of RPA phosphorylation site mutants and biochemical characterization of the various RPA phosphoisomers. These studies will help to define the contribution of defects in RPA phosphorylation to the mec1 phenotype and, ultimately, to the AT disease state.

ACKNOWLEDGEMENTS

We thank Drs David Stuart, Curt Wittenberg and Douglas Bishop for providing yeast strains and Dr Steven Brill for providing antiserum. We also thank Dr Grant Brown and Dagmawi Iyasu for critical review of the manuscript. This work was supported by grant RPG-00-211-01-CCG from the American Cancer Society and by funds from the Karmanos Cancer Institute.

REFERENCES


Figure 8. Schematic representation of RPA phosphorylation states. Note that HU-induced RPA phosphorylation in mitotic cells also involves the Mec1 homolog Tel1 (7,8).


