DNA polymerase stabilization at stalled replication forks requires Mec1 and the RecQ helicase Sgs1

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To ensure proper replication and segregation of the genome, eukaryotic cells have evolved surveillance systems that monitor and react to impaired replication fork progression. In budding yeast, the intra-S phase checkpoint responds to stalled replication forks by downregulating late-firing origins, preventing spindle elongation and allowing efficient resumption of DNA synthesis after recovery from stress. Mutations in this pathway lead to high levels of genomic instability, particularly in the presence of DNA damage. Here we demonstrate by chromatin immunoprecipitation that when yeast replication forks stall due to hydroxyurea (HU) treatment, DNA polymerases α and ε are stabilized for 40–60 min. This requires the activities of Sgs1, a member of the RecQ family of DNA helicases, and the ATM-related kinase Mec1, but not Rad53 activation. A model is proposed whereby Sgs1 helicase resolves aberrantly paired structures at stalled forks to maintain single-stranded DNA that allows RP-A and Mec1 to promote DNA polymerase association.

Keywords: DNA polymerase/Mec1/RP-A/Sgs1/stalled replication

Introduction

RecQ helicases are a universally conserved family of 3′–5′ DNA unwinding enzymes necessary for genomic stability in a wide range of organisms (reviewed in Karow et al., 2000; Enomoto, 2001). Mutations in three of the five human RecQ homologues, the Bloom’s, Werner’s and RECQL4 helicases, are implicated in heritable diseases associated with genomic instability and a predisposition to cancer. Budding yeast strains that lack their sole RecQ homologue (Sgs1) exhibit a well-documented array of defects, including hyper-recombination, chromosome loss and genomic rearrangements (Watt et al., 1996; Myung et al., 2001). These defects can be partially suppressed in sgs1-deficient yeast by expression of either the human BLM or WRN cDNAs (Yamagata et al., 1998; Heo et al., 1999). Like the human BLM helicase (Dutertre et al., 2000), yeast Sgs1 protein levels peak in S phase and the helicase co-localizes with Orc2p (Frei and Gasser, 2000). RecQ helicases are also known to interact with enzymes involved in DNA replication, such as topoisomerases I and II, proliferating cell nuclear antigen (PCNA), replication protein A (RP-A) and DNA polymerase δ (reviewed in Bjergbaek et al., 2002).

Unlike mutations in the presumed replicative helicase encoded by MCM genes (minichromosome maintenance, reviewed in Labib and Diffley, 2001), sgs1-deficient yeast cells divide with wild-type kinetics and show no significant delay in progression through an unperturbed S phase. On the other hand, the absence of Sgs1 enhances recombination rates and is synthetically lethal with null alleles of a second DNA helicase, Srs2 (Gangloff et al., 2000). Because this synthetic defect can be bypassed by eliminating Rad51, a strand exchange factor necessary for recombinational repair, the lethality of the sgs1 srs2 double mutant appears to result from unresolved recombination events, rather than defects in DNA replication per se (Gangloff et al., 2000). Similar synthetic slow growth or death could be demonstrated between deletions of sgs1 and genes encoding a variety of proteins implicated in recombination (Mus81, Mms4, Slx1, Slx4 and Rad50) and DNA repair (Rad27, Pol32 and Asf1; Tong et al., 2001), suggesting that Sgs1, like other helicases, is able to resolve a range of deleterious recombination structures. Intriguingly, impaired survival rates of WRN-deficient human cells (Saintigny et al., 2002), like the defect in sgs1 cells (Gangloff et al., 2000), are suppressed by eliminating Rad51-mediated homologous recombination.

A redundant role in the resolution of recombination intermediates is unlikely to explain the link between RecQ enzymes and heritable human syndromes (Karow et al., 2000). Nor does it account for the strongly synergistic increase in genome instability resulting from the combination of sgs1 with mutations in the ataxia telangiectasia-related checkpoint kinase, Mec1 (Myung and Kolodner, 2002). Genetic analyses in budding yeast argue that Sgs1 acts in parallel to the two-yeast ATM-like kinases, Mec1 and Tel1, to suppress an S phase-specific accumulation of gross chromosomal rearrangements, which occur even in the absence of DNA-damaging agents. Given its specificity for S phase, it has been proposed that RecQ helicases resolve or bypass aberrant structures at the replication fork, that might lead to unproductive recombination.

When budding yeast cells are exposed to high levels of hydroxyurea (HU), both the initiation of DNA replication at late-firing origins and spindle elongation are blocked in a Mec1- and Rad53- (CHK2) dependent manner (reviewed in Osborn et al., 2002). The checkpoint also is thought to ensure an efficient resumption of nascent strand elongation once stress is removed (Desany et al., 1998; Lopes et al., 2001; Tercero and Diffley, 2001). Previous studies have shown that RP-A (Tanaka and Nasmyth, 1998), DNA polymerase α/primase (pol α; Aparicio et al., 1999) and
DNA polymerase ε (pol ε; Masumoto et al., 2000) can be detected at stalled forks near early-firing origins in the presence of HU. Their presence presumably is needed for the resumption of strand elongation. These studies showed that checkpoint-deficient alleles of rad53 did not influence recoveries of DNA pol α or ε at stalled forks (Aparicio et al., 1999; Masumoto et al., 2000), although electron microscopy studies suggest that the frequency of ‘reversed fork’ structures in response to HU is higher in rad53 mutants (Sogo et al., 2002).

The budding yeast intra-S phase checkpoint response to HU requires DNA pol ε, RFC and Dpb11, while a parallel activation pathway responds to damage through the RFC-associated clamp-loader Rad24, and a PCNA-like complex of Rad17, Mec3 and Ddc1 (or 9-1-1; reviewed in Melo and Toczyński, 2002; Osborn et al., 2002). Both lead to Rad53 activation. Budding yeast cells deficient for sgs1 have a minor but reproducible defect in the intra-S phase checkpoint response, allowing spindle elongation on HU and fork progression in the presence of damage (Frei and Gasser, 2000). These defects are epistatic to pol2-11 and are strongly synergistic with mutations in the Rad24 pathway, which alone have only minor defects in the HU response. Combining sgs1 and rad24 deletions fully compromises HU-induced activation of Rad53 (Frei and Gasser, 2000). Surprisingly, the intra-S response to damage or fork arrest does not seem to bifurcate in Schizosaccharomyces pombe by 20 min after cells are released from G1 into HU-containing medium, DNA pol ε is seen to precipitate both the ARS607 and ARS305 fragments efficiently (bars m and o in Figure 1B and D). Very low signals are recovered at stalled forks in wild-type and sgs1Δ cells. Pheromone arrest and release into medium containing 0.2 M HU were performed at normal growth temperature (30°C), using cells that carry Myc-tagged DNA pol ε. Cells were fixed and lysed at regular intervals, pol ε was recovered by immunoprecipitation (IP), and quantitative real-time PCR was used to analyse the enrichment of fragments encompassing two early-firing origins, ARS607 and ARS305 (sites m and x, respectively, Figure 1A). Potential fork progression along the chromatin fibre was monitored with primer pairs up to 15 kb from the ARS consensus (n and o for ARS607, and y and z for ARS305), while a late-firing origin near telomere V (ARS501, l) and a late-replicating region near telomere VII-L (t) served as negative controls (Figure 1A). All data are presented as ratios of product accumulation (calculated from C_{T} values during real-time PCR) of DNA recovered by IP over that recovered on beads lacking antibody.

By 20 min after cells are released from G1 into HU-containing medium, DNA polymerase ε is seen to precipitate both the ARS607 and ARS305 fragments efficiently (bars m and x in Figure 1B and D). Very low signals are recovered in G1 phase, and the association of DNA pol ε coincides with the appearance of replication bubbles (Aparicio et al., 1999; see Supplementary figure 1 available at The EMBO Journal Online). The kinetics of DNA pol ε movement from ARS607 in HU show that the enzyme remains associated with the origin (m) or with a fragment 6 kb away (n) for nearly 80 min, consistent with the very slow fork progression predicted from electron microscopy studies (Sogo et al., 2002), or from density transfer experiments on methane methylsulfonate (MMS)-treated cells (Tercero and Diffley, 2001). Even 100 min after release, the polymerase does not reach the probe at 14 kb from the origin (o), nor do forks initiated from an adjacent origin (ARS606 at +17.4 kb). We find similar kinetics for pol ε binding at the early ARS305 (Figure 1D), although the 3.5 kb distal fragment is somewhat less enriched and the 10 kb distal probe slightly more (z). Given the distances of the fragments probed at the two origins, we conclude that in wild-type cells replication forks stall within 3–5 kb of an early-firing origin for ~60 min, and then progress slowly to 10 kb from the origin (Figure 1B and D). There is no polymerase association with late-replicating regions at these time points. The prolonged presence of pol ε (i.e. normal S phase is completed by 40 min; Figure 1F) could result from reduced dNTP levels and/or active polymerase stabilization.

We performed identical experiments on an isogenic sgs1 deletion strain synchronously released into HU. We see no shift in the timing or firing patterns of origins, with DNA pol ε being recruited to early-firing origins 20 min after release from G1 (Figure 1C and E). Consistently, the
efficiency of origin firing in sgs1 cells as monitored by two-dimensional gels, and the spacing of active origins as shown by a fibre spreading technique, are indistinguishable from those detected in wild-type cells (see Supplementary figure 1; Versini et al., 2003). Finally, neither the late origin ARS501 (l) nor the late-replicating Tel VII-L (t) region bind pol ε, confirming that there is neither premature initiation from late-firing origins nor activation of ‘cryptic’ pre-RC complexes in the sgs1Δ strain (compare m with l, and x with t, Figure 1C and E).

Fig. 1. Sgs1 helps stabilize DNA pol ε at replication forks on HU. (A) Primers amplify genome regions corresponding to two early-firing origins: ARS607 (m, black bars), and non-origin sites at +4 kb (n, light blue) and +14 kb (o, yellow), and ARS305 (x, grey), and non-origin sites +3.5 kb (y, green) and +10 kb (z, dark blue). Probes for the late-firing origin ARS501 (l, white) and the late-replicating Tel VII-L subtelomeric region (t, red) monitor background signals and late origin activation. Active and inactive origin positions (ARS) are described in Raghuraman et al. (2001) and Reynolds et al. (1989). ChIP was performed at 30°C on Myc-tagged DNA pol ε as described in Materials and methods for synchronized wild-type (GA-1296) or isogenic sgs1Δ (GA-2206) cells released into medium containing 0.2 M HU for the times indicated. The height of the bars represents the real-time PCR signal as fold increase of IP over beads alone control, for primers near ARS607 (B and C) and ARS305 (D and E). (F) FACS analysis shows that S phase is completed by 40 min at 30°C after release from pheromone for sgs1Δ (GA-2209) and isogenic wild-type cells (GA-893).
The fact that late origins do not fire is consistent with the fact that Rad53 is activated in the \( sgs1^D \) mutant by 1 h in 0.2 M HU (Frei and Gasser, 2000; Shimada et al., 2002).

In the HU-treated \( sgs1^D \) strain, the amount of DNA pol \( \varepsilon \) recovered at either ARS is less than half that detected in wild-type cells. This is true for both early origins and for the probes immediately adjacent to the origin fragment from 40 to 80 min (Figure 1C and E). We see a slight increase in the amount of polymerase recovered at the most distal fragments at the later time points (z and o, at ARS305 and ARS607, respectively, Figure 1C and E). Nonetheless, fluorescence-activated cell sorting (FACS) analysis indicates that progression through S phase in the presence of HU is nearly as slow in \( sgs1^D \) cells as in the wild-type background (Figure 1F).

One simple explanation for the reduced recovery of DNA pol \( \varepsilon \) in the \( sgs1^D \) mutant might be that helicase loss impairs the efficiency of origin firing, even without added HU. This was tested by performing ChIP for DNA pol \( \varepsilon \) and probing for its association with early ARS305 in both wild-type and \( sgs1^D \) cells synchronously traversing S phase in the absence of HU (Aparicio et al., 1999; Masumoto et al., 2000). We find that the kinetics and efficiency of pol \( \varepsilon \) association with ARS305 are indistinguishable between \( sgs1^D \) and its parental \( SGS1 \) strain (Figure 2A and B). At 40 min, we detect a higher signal in the \( sgs1^D \) mutant than in the wild-type strain for pol \( \varepsilon \) at the 10 kb distal fragment (z), and less at the proximal fragment (y). This may reflect a slight increase of fork rate in \( sgs1^D \)-deficient cells, as suggested by Versini et al. (2003), although FACS analysis in our hands does not detect significant differences in S phase length (Figure 2C).

**Sgs1 is present at DNA replication foci, associated with both stalled and normal forks**

Deletion of \( sgs1 \) could either have a direct effect at replication forks in S phase, or it might act indirectly, influencing repair or recombination near the fork. To see whether Sgs1 is present at normal replication forks, we repeated ChIP on a time course of untreated cells carrying a Myc-tagged Sgs1. We detect a low, but epitope-specific enrichment of Sgs1 at the origin and adjacent fragments (Figure 2D), which appears with the same kinetics as DNA pol \( \varepsilon \) (Figure 2A). We also co-localize Sgs1 and de novo DNA synthesis by immunofluorescence (Figure 2F). After a 30 min replication reaction in vitro, we see a significant co-localization of Sgs1 (in red) with some but not all foci of aphidicolin-sensitive DNA synthesis (green). We also confirm by Sgs1 ChIP that the helicase is present at both moving and HU-arrested forks (Figure 2E). Like the DNA pol \( \varepsilon \) enrichment (Figures 1D and 4A), the recovery of Sgs1 at ARS305 peaks by 40 min after release into HU (Figure 2E). The comparatively low enrichment of Sgs1 by ChIP, as compared with pol \( \varepsilon \), is probably due to the extreme lability of this protein and loss of its C-terminal epitope tag (data not shown).
Helicase activity is required for DNA pol ε stabilization by Sgs1

Given that Sgs1 is at the replication fork, it was of interest to determine if its helicase activity was required for DNA pol ε stabilization or if, instead, Sgs1 acts as a 'scaffold' to recruit components of the replication and/or checkpoint machinery. To test this, we introduced a helicase-deficient mutant of Sgs1 (sgs1-hd) into the sgs1Δ background expressed from its natural SGS1 promoter. This mutation alters a critical lysine in the Walker A motif (Figure 3A), eliminating 3' to 5' helicase activity. The mutant polypeptide is synthesized normally and partially suppresses the lethality of a top1Δ sgs1Δ double mutant (Lu et al., 1996). We compare isogenic sgs1Δ and SGS1 strains carrying empty vectors, as well as the sgs1Δ mutant expressing full-length SGS1 (Sgs1-FL). As a control, we show that plasmid-borne Sgs1-FL restores viability on HU and MMS to ~80% of wild-type values, while the sensitivity of cells expressing sgs1-hd resembles that of the null background (Figure 3B). This correlates with the inability of sgs1-hd to suppress the sgs1 hyper-recombination phenotype (Onoda et al., 2000; Safﬁ et al., 2000). We note that Sgs1 function is very sensitive to its expression level, since the doubling of SGS1 copy number in a wild-type strain slightly reduces viability on HU and MMS, and its overexpression has strong dominant-negative effects (data not shown). This could account for the reduced complementation by Sgs1-FL.

Following G1 arrest and release, we monitored the abundance of DNA pol ε at ARS305, conﬁrming that the presence of DNA pol ε at the origin fragment (x) is abrogated by the sgs1Δ mutation (Figure 3C). As in Figure 1, pol ε is less abundant at the fork in the sgs1Δ deletion strain. Expression of the helicase-dead sgs1 fails to restore DNA pol ε stability, while Sgs1-FL does (Figure 3C). This correlates with the drop in cell survival in the presence of the helicase-dead mutant (Figure 3B). These data strongly implicate Sgs1 helicase activity in the stable association of DNA pol ε at stalled forks.

DNA pol ε binding is compromised in a mec1 mutant

Mec1 is a central kinase in all DNA damage response checkpoints in yeast, and directly activates Rad53. Because the effects of mec1 mutations on cell survival in the presence of HU are more debilitating than those of rad53 alleles (Weinert et al., 1994; Paulovich and Hartwell, 1995; Desany et al., 1998), it was suggested that Mec1p plays additional, possibly direct, roles to ensure recovery of replication forks after HU arrest. To see if this is reﬂected in the stabilization of DNA pol ε, we performed the same ChIP assay described above in the mec1-1 strain and its isogenic parental background treated with HU (Figure 4A and B). The mec1-1 allele is the most potent of the 12 alleles originally isolated, rendering yeast highly sensitive to all types of genetic insult (UV,
γ-irradiation, alkylating agents and HU) and impairing Rad53 activation (Weinert et al., 1994; Paulovich et al., 1997).

Indeed, the mec1-1 mutation leads to a highly significant reduction in the recovery of DNA pol ε at ARS305, at 20, 40 and 60 min time points after release into HU (Figure 4A and B). Previous studies have shown that initiation occurs with normal kinetics in both mec1Δ (Tercero and Diffley, 2001) and mec1-1 mutant cells (Santocanale and Diffley, 1998; Shirahige et al., 1998). We confirm that spindle elongation proceeds in the presence of HU, and that late G2-length spindles appear by 75 min after pheromone release, even though continuous DNA synthesis is impaired (Figure 4D and E; Weinert et al., 1994). Aberrant nuclear division begins at 2 h after release from G1 in this allele, consistent with a compromised checkpoint response (Figure 4D). As expected, we detect increased levels of DNA pol ε at the late-firing origin ARS501 by 75 min (Figure 4B), reflecting loss of the checkpoint-induced repression of late origin firing. This also indicates that the mec1-1 mutation does not interfere in an artefactual way with DNA pol ε level or recovery by ChIP.

The premature initiation of replication at late-firing origins clearly distinguishes the defects correlated with mec1 and sgs1Δ mutants: Rad53 is activated and efficiently represses late-firing origins in sgs1Δ, but not in mec1-1, cells. This indicates that the effects of sgs1 deletion on pol ε stability cannot be due to impaired Rad53 activation. To test whether the mec1-1 effects on polymerase recovery act through Rad53, we completely deleted RAD53 in the same background (both contain snn1 deletions to maintain viability; Zhao et al., 1998). Consistent with results from point-mutated alleles, rad53

Fig. 4. DNA pol ε is lost from stalled forks in a mec1 mutant. Time course ChIP for DNA pol ε was performed on isogenic wild-type (A, GA-1296), mec1-1 (B, GA-1306) and rad53Δ (C, GA-2208) cells as described in Figure 1. Both mutants carry snn1Δ, which has no effect on S phase checkpoints (Zhao et al., 1998). Primer sites and colour coding for fragments are described in Figure 1A. (D) Spindle length and nuclear separation were monitored with TAT1 antibody and DAPI as described (Shimada et al., 2002) for isogenic wild-type (GA-1296) and mec1-1 (GA-1306) cells. Following G1 arrest and release into HU, 200 cells were measured at each indicated time point. (E) FACS analysis at 30°C on wild-type and mec1-1 cells after α-factor block and release into 0.2 M HU.
deletion does not impair DNA pol ε stability at the stalled ARS305 fork (Figure 4C). As expected, the late-firing origin (ARS501) is activated due to the absence of active Rad53 (Figure 4C), and ARS501 firing is more efficient in rad53Δ than in mec1-1 cells, possibly reflecting a partial activation of Rad53 by Tel1 in the mec1-1 strain.

**Stable binding of DNA pol α is also compromised in an sgs1Δ strain**

The polymerization activity of DNA pol ε is not essential for viability in yeast, presumably because DNA pol δ can replace its gap-filling and lagging strand functions (Dua et al., 1999; Kesti et al., 1999). Nonetheless, deletion of its N-terminal domain is lethal for yeast, and pol ε is the only replicative polymerase directly implicated in the intra-S phase checkpoint response. Since the association of Dpb11 and DNA pol ε with stalled forks does not require DNA pol α (Masumoto et al., 2000), we entertained the thought that the sgs1 and mec1 effects reflect a mechanism linked to the checkpoint function of pol ε, and not its role within the replisome.

We therefore checked whether the destabilizing effect of the sgs1 mutation would also affect DNA pol α. Using the same arrest–release protocol, we performed ChIP for the large subunit of DNA pol α on cells released into HU. Like pol ε, pol α remains stably associated with ARS305 for 30–50 min as forks stall in wild-type strains (Figure 5A). The enrichment is consistently lower than for Myc-tagged DNA pol ε, possibly because anti-Myc is precipitating a multimerized epitope that enhances the
avidity of binding (note that background signals in G1 are lower with anti-pol α). In the sgs1Δ strain, the amount of pol α at ARS305 is reduced by 3- to 4-fold at 30 and 50 min after release into HU (grey bars, Figure 5B). Although pol α levels are also reduced at the +10 kb site (z) by 50 min in HU, slightly more is recovered at this site at 50 min, compared with wild-type cells (Figure 5B). This is again consistent with the proposal of a slightly faster fork rate in the sgs1 mutant (Versini et al., 2003), although other explanations are possible.

To check that we are not simply observing a drop in the efficiency of pre-replication complex formation, which is a prerequisite for both RP-A and pol α binding, we performed ChIP for a subunit of the ORC complex in wild-type and sgs1Δ cells under identical conditions. In both, we recover an identical complement of ORC bound to the ARS305 origin fragment (Figure 5C). This is consistent with data showing that there is no alteration in the genome-wide origin firing patterns in sgs1-deficient cells (B.Brewer, personal communication) and two-dimensional gels that show normal initiation patterns (see Supplementary figure 1). We conclude that Sgs1 influences the stability of both DNA pol ε and pol α at stalled forks, possibly by stabilizing the entire replication complex.

To see if the effects of Sgs1 are due to direct interaction with components of the pol α complex, we screened for stable or HU-induced binding between Sgs1 and the large subunit of either RP-A or DNA pol α, all expressed from their genomic loci at wild-type levels. Exponentially growing or HU-arrested cultures of a strain carrying Myc-tagged Sgs1 and haemagglutinin (HA)-tagged Rpa1 were lysed, and magnetic beads coated with anti-pol α, anti-Myc or anti-HA were used to precipitate complexes. Western blots were performed on the whole-cell extract (WCE) and precipitates (IP) washed under stringent conditions (0.25 M NaCl, 1% NP-40 and 2.5 mM deoxycholate). Western blots on total extracts for both tagged proteins and the catalytic subunit of pol α confirmed that the antibodies recognize single bands, although the Myc-tagged Sgs1 shows some degradation under all conditions tested (top panel, Figure 5D). Using either anti-HA or anti-Myc, we are able to precipitate efficiently a complex of Sgs1 and Rpa1, which is not detected if beads lacking antibodies or a strain lacking the HA epitope is used (Figure 5D). There is no difference in the interaction upon treatment with HU. Neither the Myc-tagged Sgs1 nor the HA-tagged Rpa1 binds stably to pol α, although RP-A associates transiently with the holo-polymerase in vivo. This underscores the specificity and strength of the Rpa1–Sgs1 interaction, and is consistent with evidence showing that the human RP-A complex binds and stimulates recombinant human WRN helicase activity in vitro (Brosh et al., 1999).

We have examined the pattern of origin firing and fork progression using neutral–neutral two-dimensional gels on HU-treated populations of wild-type and sgs1Δ strains (see Supplementary figure 1). In addition to confirming that initiation is not altered either spatially or temporally in the sgs1Δ mutant, we note the absence of a ‘cone’ signal extending beyond the top of the Y arc in sgs1 samples, as predicted for the accumulation of reversed forks seen in rad53 cells (Sogo et al., 2002). Although we cannot rule out that these structures are selectively depleted during purification, this result may also indicate that such structures are resolved rapidly by Rad53-induced mechanisms, acting independently of Sgs1.

Discussion

It is thought that a key element of the intra-S phase checkpoint response is the stabilization of the replication fork such that elongation can resume once genotoxic stress is alleviated (Desany et al., 1998; Melo and Toczyski, 2002). It has been shown by several groups that DNA polymerases and RP-A can be detected at stalled forks (Tanaka and Nasmyth, 1998; Aparicio et al., 1999; Masumoto et al., 2000). DNA polymerase was also detected at an early-firing origin in rad53 mutants that compromise the downstream checkpoint response. The lack of a requirement for Rad53 seemed to be at odds with the notion that the DNA replication checkpoint response is required to ensure resumption of elongation after an appropriate cell cycle delay, and it was unclear whether maintenance of the replisome required active intervention or was a default state.

Replication polymerase stability at stalled forks requires Sgs1 and Mec1

The detailed real-time ChIP results presented here provide a kinetic and genetic analysis of DNA polymerases at stalled replication forks. Polymerases remain bound within 3 kb of the early-firing origins for 40–60 min on HU, after which forks progress slowly along the chromosomal fibre, remaining within 5–10 kb by 80 min. We find that Rad53 (CHK2), the major downstream effector in the intra-S phase checkpoint, is not necessary for the stabilization of DNA pol ε at stalled forks (Figure 4). This, of course, does not exclude that Rad53 kinase activation is essential for other events critical for recovery from replicative stress (see Weinert et al., 1994; Pellicioli et al., 1999).

Importantly, we find that the loss of either the RecQ homologue, Sgs1p, or the Mec1 kinase reproducibly reduces the recovery of DNA polymerases at origin-proximal fragments in HU (Figures 1 and 4). In the case of Sgs1, the effect is partial, yet it requires the enzyme’s helicase activity and is more severe in cells grown under nutrient-limiting conditions (Figure 3). This drop in polymerase recovery is not an artefact of inefficient initiation nor does it reflect activation of alternative or ‘cryptic’ origin sites. Moreover, when HU is added, late-firing origins remain repressed in sgs1 mutant. This is consistent with the fact that Rad53 is activated in response to HU in an sgs1Δ strain, unless the Rad24 pathway of checkpoint activation is also compromised (Frei and Gasser, 2000). This loss of polymerase binding is consistent with genetic arguments showing that the recovery from HU arrest is defective both in sgs1-deficient budding yeast strains (Frei and Gasser, 2000; Saffi et al., 2000) and in the fission yeast rgh1 mutant (Stewart et al. 1997).

We also note that DNA pol ε is displaced from stalled forks when the function of the ATR orthologue, Mec1, is impaired (Figure 4). This is consistent with the slow progression made by replication forks in mec1 mutants exposed to HU or MMS, and with the observation that a
mec1 allele can alter the efficiency of fork progression through certain chromosomal domains (Cha and Kleckner, 2002). We favour the hypothesis that Mec1 kinase acts directly at the fork to stabilize the polymerase and/or its associated factors by phosphorylation, although we cannot rule out indirect effects of an unidentified kinase cascade. The stable binding of pol ε, on the other hand, does not depend on Rad53 activation.

Several arguments suggest that Mec1’s role at replication forks is linked to the affinity of the Mec1–Ddc2 complex for single-stranded DNA (ssDNA). It has been demonstrated that the Mec1–Ddc2 complex is recruited to double-strand breaks and other DNA lesions to form large repair foci (Kondo et al., 2001; Melo et al., 2001). Recent data using a quantitative PCR-based detection of ssDNA show that this correlates with the 5′ to 3′ resection of the damage, occurs upstream of checkpoint activation and is influenced by a mutation in the large subunit of RP-A (K.Dubrana, F.Hediger and S.M.Gasser, submitted). Work in Xenopus egg extracts also suggests that ssDNA is the signal for activation of ATR when replication is impaired, leading to a block in initiation (Costanzo et al., 2003). Thus we suggest that the effects we see on replisome stability reflect the association of Mec1 with ssDNA, which accumulates at stalled forks (Sogo et al., 2002). It is noteworthy that in addition to Rad53, Mec1 phosphorylates two subunits of RP-A independently of Rad53 (Brush et al., 1996; Brush and Kelly, 2000). If RP-A modification helps stabilize pol α–DNA interactions, this could improve polymerase recovery at the fork. We note that the phosphorylation of Rpa1 by Mec1 is lost in the mec1-1 mutant used here (Brush and Kelly, 2000), as is the modification of pol α itself by Rad53 (Pellicioli et al., 1999).

**Sgs1 is present at replication forks and binds RP-A**

ChIP and immunofluorescence confirm that Sgs1 is present at a large fraction of replication forks even in the absence of genotoxic stress. This supports data showing that eukaryotic RecQ helicases interact with a number of proteins critical for DNA replication, including RP-A (Figure 5; Brosh et al., 1999). Moreover, sgs1 deletion is synthetically lethal with mutations in several components of the replication machinery (Murray et al., 1997; Tong et al., 2001). Finally, recent data suggest that under unperturbed conditions, replication forks proceed slightly faster in sgs1-deficient strains (Versini et al., 2003). This link to replication must be considered in view of the fact that the family of RecQ helicases are generally regarded as enzymes affecting recombination. Indeed, cell lethality due to the loss of RecQ helicases can be complemented by the suppression of homologous recombination (Gangloff et al., 2000; Saintigny et al., 2002). While these results provide strong evidence that RecQ helicases function downstream of strand exchange events, they do not contradict our proposal that they help stabilize polymerases at replication forks as well. Loss of either function could lead to chromosomal rearrangements and loss of heterozygosity.

Both the recombination- and replication-linked functions of Sgs1 may reflect its unusual substrate preference. It has been shown that RecQ helicases prefer 3- or 4-way junctions, G quartets, hairpin or other unusually paired structures in vitro, preferentially catalysing the unpairing or separation of strands (Bennett et al., 1998). Thus it is commonly thought that Sgs1 functions at stalled forks to reverse unwanted strand pairing or recombination intermediates that form between nascent strands at stalled forks (Figure 6). To relate this to polymerase stability, we propose that the unwinding of paired structures at stalled forks leads to the maintenance of ssDNA, which in turn is necessary for normal fork morphology. The suppression of recombination, moreover, may favour RP-A binding over that of Rad51 or Rad52.

The highly synergistic drop in chromosome stability in sgs1 mec1 double mutants suggests that the two proteins stabilize replication forks through complementary pathways (Myung and Kolodner, 2002). As shown in Figure 6, one possible scenario is that Sgs1 counteracts the tendency for inappropriate pairing at stalled forks, while Mec1 plays...
a more direct role, by binding ssDNA and directly modifying replisome-associated targets. Since Sgs1 binds tightly to Rpa1, and Mec1 modifies the RP-A complex, we suspect that RP-A will play a key role in this particular response to HU.

**Is polymerase stabilization by Sgs1 a checkpoint function?**

Formally speaking, the effect of the sgs1 mutant on the polymerase stability at stalled forks can be considered as completely independent of the checkpoint activation pathway, i.e. Sgs1 contributes to DNA pol ε recovery independently of Rad53 activation. The **sgs1Δ** strain maintains a functional Rad24 pathway for Rad53 activation, and many aspects of the intra-S phase checkpoint are intact. Rad53 becomes phosphorylated and late-firing origins are repressed on HU in this mutant (Figure 1; see also Frei and Gasser, 2000). Thus, the loss of DNA polymerase stability seen in sgs1 cells is not due to an effect on Rad53.

On the other hand, we cannot exclude a role for Sgs1 in other aspects of the Rad53-mediated response. Sgs1 is able to bind Rad53 both in vitro and in vivo (M.Tsai-Pflugfelder, J.A.Cobb, L.Bjerregaard and C.Frei, in preparation), and could recruit or position this kinase for phosphorylation of stalled forks. It will be of interest to determine to what degree this reflects cooperation with Sgs1’s universally conserved partner, topoisomerase III.

**Materials and methods**

**Cell cultures and plasmids**

All strains used are either W303 or A364a background and are listed in Table I. Growth was at 30°C in YPD unless otherwise indicated. POL2-13

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA-893</td>
<td>MATa, ade2-1, trpl-1, his3-11, -15, ura3-1, leu2-3, -112, can1-100, orc2::ORC2-9Myc::LEU2 (W303 background)</td>
</tr>
<tr>
<td>GA-904</td>
<td>MATa, his3, leu2, ura3, trpl, mec1-1 sm1l (A364a background) (Paulovich et al., 1997)</td>
</tr>
<tr>
<td>GA-1020</td>
<td>MATa, ade2-1, trpl-1, his3-11, -15, ura3-1, leu2-3, -112, con1-100 pep4::LEU2 (W303 background)</td>
</tr>
<tr>
<td>GA-1296</td>
<td>MATa, his3, leu2, ura3, trpl, POL2-13Myc::KanMX (A364a background)</td>
</tr>
<tr>
<td>GA-1306</td>
<td>GA-904 with POL2-13Myc::G418</td>
</tr>
<tr>
<td>GA-1699</td>
<td>GA-1020 with SGS1-3Myc::HIS3</td>
</tr>
<tr>
<td>GA-1759</td>
<td>GA-1699 with RPA1-3HA::URA3</td>
</tr>
<tr>
<td>GA-1923</td>
<td>MATa ade2-1, trpl-1, his3-11, -15, ura3-1, leu2-3, -112, URA3::GPD-TK3, RAD53-13Myc::G418 (W303 background)</td>
</tr>
<tr>
<td>GA-1925</td>
<td>GA-1923 with sgls1::TRP1</td>
</tr>
<tr>
<td>GA-2206</td>
<td>GA-1296 with sgls1::TRP1</td>
</tr>
<tr>
<td>GA-2208</td>
<td>MATa, his3, leu2, ura3, trpl, rad53::TRP1 sm1l POL2-13Myc::KanMX (A364a background)</td>
</tr>
<tr>
<td>GA-2209</td>
<td>GA-893 with sgls1::TRP1</td>
</tr>
</tbody>
</table>

**Replication in vitro and IF**

Isolated S phase nuclei from GA-1699 were incubated in S phase extracts containing digoxigenin-derivatized dUTP for 30 min to allow aphidicolin- and ORC-dependent replication as described (Pasero et al., 1999). Nuclei were spun onto a coverslip and fixed with 1% formaldehyde, prior to reaction with anti-Myc (9E10) and goat anti-digoxigenin, and appropriately derivatized secondary antibodies. Visualization and quantitation of signal overlap by Imaris software (Bitplane, Zurich, Switzerland) are as described (Frei and Gasser, 2000).
Co-immunoprecipitation

GA-1759 (Myct-Sgs1, HA-Rpa1) or GA-1699 (Myct-Sgs1) cells were grown to 0.5 × 10^6 cells/ml, arrested with α-factor for 1 h and released to enter S phase with or without 0.2 M HU. Cell pellets were resuspended in 0.4 ml of lysis buffer (50 mM HEPEs pH 8.0, 140 mM NaCl, 1 mM EDTA and 1% Triton X-100, with a final concentration of protease inhibitors: 300 μg/ml benzamidine, 1 μg/ml pepstatin, 2 μg/ml antipain, 0.5 μg/ml leupeptin, 100 μg/ml TPCK and 50 μg/ml TLCK), and were lysed by vortexing with glass beads at 4°C. The supernatant was removed to clean tubes, and the resulting WCE was incubated with either Myc-, HA- or anti-Po1-coupled Dynabeads M-450 (Dynal A.S., Norway) for 2 h at 4°C. The Dynabeads were washed twice with lysis buffer, twice with wash buffer (10 mM Tris pH 8.0, 250 mM NaCl, 1 mM EDTA, 1% NP-40, 2.5 mM deoxycholate and the indicated protease inhibitors), and resuspended in 40 μl of wash buffer, to which SDS sample buffer is added. Eluted proteins were analysed by SDS-PAGE and western blotting.

Supplementary data

Supplementary data are available at The EMBO Journal Online.

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References


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