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 same 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 Toczyski, 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* (Marchetti *et al.*, 2002), and a role for its RecQ homologue, Rqh1, in checkpoint activation has been questioned. Nonetheless, the *rqh1* mutants recover poorly from HU-mediated arrest (Stewart *et al.*, 1997).

Here we examine the presence of DNA polymerases at stalled replication forks, and test whether this association is influenced by either the Sgs1 helicase or Mec1 kinase, which serves both as a 'sensor' for checkpoint activation and as a signal-transducing kinase (reviewed in Melo and Toczyski, 2002). Quantitative real-time PCR on DNA co-immunoprecipitated with DNA pol ϵ and pol α implicates both Mec1 and Sgs1 activities in the stable binding of polymerases at stalled forks (here called stabilization), providing a mechanistic basis for the inability of these mutants to recover from prolonged fork arrest.

Results

Chromatin immunoprecipitation (ChIP) assays have shown that Cdc45 and RP-A load at origins of replication just prior to the coordinate binding of DNA pol α , Dpb11 and DNA pol ϵ during the synchronized progression of cells into S phase (Tanaka and Nasmyth, 1998; Aparicio *et al.*, 1999; Masumoto *et al.*, 2000). Low temperatures (16°C) were used to capture polymerases at the forks and, depending on the length of the pheromone treatment, replication initiated between 40 and 60 min after release from G₁ arrest. In the presence of HU, all three complexes were detected at early- but not late-firing origins, while in *rad53* point mutants RP-A and the polymerases both remained bound at early-firing origins and appeared at the late origin, ARS501 (Tanaka and Nasmyth, 1998; Aparicio *et al.*, 1999; Masumoto *et al.*, 2000). To date, no mutation has been shown to destabilize DNA polymerases on HU, although stalled forks appear to collapse when checkpoint

activation is compromised (Lopes *et al.*, 2001; Tercero and Diffley, 2001; Sogo *et al.*, 2002). Given the above, we reasoned that mutations that compromise fork stability were likely to act upstream of Rad53.

Stable binding of DNA pol ϵ at a stalled replication fork is compromised by loss of Sgs1

Mutations in DNA pol ϵ and in Sgs1 helicase are epistatic for recovery from the intra-S phase checkpoint (Frei and Gasser, 2000), leading us to compare the stability of pol ϵ at stalled forks in wild-type and *sgs1* Δ 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 chromatid 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 pol 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

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).

efficiency of origin firing in *sgs1Δ* 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. 2. Sgs1p is associated with replication forks. (A) Time course ChIP was performed on Myc-tagged DNA pol ϵ for wild-type (GA-1296) and (B) isogenic *sgs1* Δ (GA-2206) cells after synchronization and release into YPAD without HU at 16°C. Primer sites are described in Figure 1. Quantitation by real-time PCR is described in Materials and methods and Figure 1. (C) FACS analysis shows similar progression through S phase for wild-type and *sgs1* Δ strains in the absence of HU at 25°C (for more extensive analyses of such profiles see Versini *et al.*, 2003). (D) ChIP was performed at the indicated time points at 16°C for Myc-tagged Sgs1 from GA-1699 cells, as in (A). Amplified ARS305 fragments, quantitation, primers and colour coding are described in Figure 1. (E) As (D), except that cell growth was at 30°C and cells were released from G₁ into 0.2 M HU. (F) Isolated S phase nuclei from GA-1699, carrying Myc-tagged Sgs1, were incubated in an S phase nuclear extract supplemented with nucleotides and digoxigenin-derivatized dUTP (Pasero *et al.*, 1999). After 30 min incorporation at 25°C, fixed nuclei were probed with mAb 9E10 (Sgs1, in red) and rabbit anti-digoxigenin to detect newly synthesized DNA (green). On average, 48% of the Sgs1 signal co-localizes with Dig-dUTP (see arrows).

The fact that late origins do not fire is consistent with the fact that Rad53 is activated in the *sgs1* Δ mutant by 1 h in 0.2 M HU (Frei and Gasser, 2000; Shimada *et al.*, 2002).

In the HU-treated *sgs1* Δ strain, the amount of DNA pol ϵ 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* Δ cells as in the wild-type background (Figure 2C).

One simple explanation for the reduced recovery of DNA pol ϵ in the *sgs1* 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 ϵ and probing for its association with early ARS305 in both wild-type and *sgs1* Δ 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 ϵ association with ARS305 are indistinguishable between *sgs1* Δ and its parental *SGS1* strain (Figure 2A and B). At 40 min, we detect a higher signal in the *sgs1* mutant than in the wild-type strain for pol ϵ 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*-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 ϵ (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 ϵ 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 ϵ , is probably due to the extreme lability of this protein and loss of its C-terminal epitope tag (data not shown).

Fig. 3. DNA pol ϵ stability requires Sgs1 helicase activity. (A) Map of the Sgs1 helicase domain (grey), Walker A motif (black) and point mutation K₇₀₆A (Lu *et al.*, 1996). (B) Wild-type (GA-1296) cells bearing pRS415 vector (WT, black), or *sgs1*Δ cells (GA-2206) bearing either pRS415 vector (Δ, white), pRS415 with full-length *SGS1* (FL, dark grey) or pRS415-*sgs1*-hd, expressing helicase-dead protein (hd, light grey), were grown into SC-Leu, synchronized and released into either 0.2 M HU for the indicated times (upper panel) or increasing amounts of MMS for 1 h for viability assays (see Materials and methods). (C) Time course ChIP for DNA pol ϵ was performed with strains described in (B), as described in Figure 1. Upper and lower graphs show data for ARS305 (probe x) and Tel VIII (probe t), respectively.

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 *sgs1*Δ 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 *top1*Δ *sgs1*Δ double mutant (Lu *et al.*, 1996). We compare isogenic *sgs1*Δ and *SGS1* strains carrying empty vectors, as well as the *sgs1*Δ 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 *sgs1*Δ mutation (Figure 3C). As in Figure 1, pol ϵ is less abundant at the fork in the *sgs1*Δ 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,

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 *smi1Δ*, 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.

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 *smi1* deletions to maintain viability; Zhao *et al.*, 1998). Consistent with results from point-mutated alleles, *rad53*

Fig. 5. Loss of *sgs1* impairs DNA pol α association at a stalled replication fork. Time course ChIP for DNA pol α was performed as described in Figure 1 for (A) wild-type (GA-1296) and (B) *sgs1Δ* (GA-2206) cells. In (C), ChIP for Orc2 was performed in wild-type (GA-893) and *sgs1Δ* (GA-2209) isogenic strains. Primer sites and colour coding for fragments are described in Figure 1A and, in (C), wild-type data are introduced for the ARS305 region (labelled x^{WT}) as stippled open bars. (D) Cultures of GA-1759 (expressing Myc-tagged Sgs1 and HA-tagged Rpa1) and GA-1699 (expressing Myc-tagged Sgs1) were arrested in G₁ with α -factor and released into S phase in either the presence or absence of 0.2 M HU. Detection of the indicated proteins in WCEs is shown in the top panel. WCEs were used for IP by incubating with Dynabeads coupled to anti-pol α , anti-HA or anti-Myc (see Materials and methods). Stringent washing was followed by elution and western blot analysis with the monoclonal antibodies 24D9 (pol α), 9E10 (Myc for Myc-Sgs1) or 12CA5 (HA for HA-Rpa1). Probing the Sgs1 IP for Rfc3 was also negative (data not shown).

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 G_1 are lower with anti-pol α). In the *sgs1 Δ* 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 30 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 *sgs1 Δ* 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 subunits 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 confirm 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 *sgs1 Δ* strains (see Supplementary figure 1). In addition to confirming that initiation is not altered either spatially or temporally in the *sgs1 Δ* 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; Pelliccioli *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* cells. This is consistent with the fact that Rad53 is activated in response to HU in an *sgs1 Δ* 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 *rqh1* 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

Fig. 6. Proposed mechanisms for stabilizing polymerases at stalled replication forks. When replication stalls, the integrity of the replisome must be maintained to ensure the resumption of fork elongation. Potential inappropriate strand pairing between nascent strands or within a repeat-containing nascent strand might be reversed by Sgs1p helicase. This could suppress recombination and provide ssDNA for Mec1 kinase binding. We do not exclude the participation of other factors, but note that genomic instability increases 1000-fold upon mutation of both *sgs1* and *mec1* (Myung and Kolodner, 2002).

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 (Pelliccioli *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

Table I. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype
GA-893	MATa, <i>ade2-1, trp1-1, his3-11, -15, ura3-1, leu2-3,-112, can1-100, orc2::ORC2-9Myc::LEU2</i> (W303 background)
GA-904	MATa, <i>his3, leu2, ura3, trp1, mec1-1 sml1</i> (A364a background) (Paulovich <i>et al.</i> , 1997)
GA-1020	MATa, <i>ade2-1, trp1-1, his3-11, -15, ura3-1, leu2-3,-112, can1-100 pep4::LEU2</i> (W303 background)
GA-1296	MATa, <i>his3, leu2, ura3, trp1, POL2-13Myc::KanMX6</i> (A364a background)
GA-1306	GA-904 with <i>POL2-13Myc::G418^r</i>
GA-1699	GA-1020 with <i>SGS1-13Myc::HIS3</i>
GA-1759	GA-1699 with <i>RPA1-3HA::URA3</i>
GA-1923	MATa, <i>ade2-1, trp1-1, his3-11,-15, ura3-1, leu2-3, -112, URA3::GPD-TK_{7X}, RAD53-13Myc::G418^r</i> (W303 background)
GA-1925	GA-1923 with <i>sgs1::TRP1</i>
GA-2206	GA-1296 with <i>sgs1::TRP1</i>
GA-2208	MATa, <i>his3, leu2, ura3, trp1, rad53::TRP1 sml1 POL2-13Myc::KanMX6</i> (A364a background)
GA-2209	GA-893 with <i>sgs1::TRP1</i>

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 *sgs1* Δ 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.Bjergbaek and C.Frei, in preparation), and could recruit or position this kinase for fork-related checkpoint functions. Thus while Sgs1's role in fork stabilization is independent of Rad53 activation, it is still possible that in some conditions polymerase binding itself contributes to Rad53 activation.

Cancer cells show increased genome rearrangements, which can be induced by many different defects. Mutations in RecQ-like DNA helicases, such as BLM and WRN, are thought to increase genome instability, although the exact DNA metabolic defects that underlie such genome instability are obscure. Given that most spontaneous genome rearrangements result from dysfunction during replication, we propose that mammalian RecQ helicases may also help stabilize DNA polymerases at 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*

Myc::G418^r and *SGS1-13Myc::HIS3* strains were constructed by PCR-based tagging as previously described (Goldstein and McCusker, 1999) using plasmid pFA6a. The Myc-tagged strains grow with kinetics identical to parental strains. The disrupted allele *sgs1::TRP1* (designated *sgs1* Δ), is identical to the *sgs1-3* allele (Lu *et al.*, 1996), which produces no detectable Sgs1 protein (Frei and Gasser, 2000). Empty plasmid pRS415, and pRS415 expressing either full-length (*SGS1*-FL) or a helicase-deficient allele (*sgs1*-hd; K 704 to A; Lu *et al.*, 1996) from the *SGS1* promoter were introduced into wild-type and mutant cells (GA-1296 and GA-1761). The overnight selective cultures were diluted into YPAD for two generations prior to synchronization with α -factor (Lipal Biochem, Zürich, Switzerland). Viability measurements were determined for cultures blocked in SD-Leu pH 5.0 with α -factor, released into fresh SD-Leu and treated with 0.2 M HU for the indicated times or for 1 h with the indicated amounts of MMS (v/v) as described (Frei and Gasser, 2000).

Chromatin immunoprecipitation

ChIP was performed as described (Martin *et al.*, 1999), except that IP wash buffers contained 0.5 M NaCl. The amplified DNA regions were quantified by real-time PCR performed using the Perkin-Elmer ABI Prism 7700 Sequence Detector System and software. Primers that amplify regions in the *Saccharomyces cerevisiae* genome correspond to ARS305 and sites 3.5 and 10 kb away from ARS305, ARS607 and sites 4 and 14 kb away from ARS607, ARS501, and a late-replicating Tel VII-L subtelomeric region (see Figure 1A; sequences available upon request). Cultures were grown to 10⁶ cells/ml prior to pheromone synchronization in G₁ (blocked 90 min or one generation time; Frei and Gasser, 2000). Cells were sedimented, washed and released into pre-warmed YPAD medium containing 0.2 M HU, prior to fixation with 1% formaldehyde at the indicated time points. Monoclonal antibody against Myc (9E10) for the Myc-tagged proteins or against pol α (24D9) were bound to Dynabeads. Dynabeads without antibody were used for background controls.

ChIP data are averaged over two or three independent experiments with real-time PCR performed in triplicate (standard deviation is shown by error bars). Real-time PCR monitors the 'threshold cycle (C_T)' at which the exponential curve of accumulated product passes a threshold. The height of the bars represents the ratio of the difference of signal accumulation rates for IP versus input, divided by the difference in rates for Dynabeads alone (background) versus input. Each C_T determination is performed in triplicate. Assuming the efficiency of probe accumulation to be 2, the simple formula used is: fold increase = $2^{(C_{Tinput} - C_{TIP})} / 2^{(C_{Tinput} - C_{Tbkg})}$.

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 (Myc-Sgs1, HA-Rpa1) or GA-1699 (Myc-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 μ g/ml benzamidine, 1 μ g/ml pepstatin, 2 μ g/ml antipain, 0.5 μ g/ml leupeptin, 100 μ g/ml TPCK and 50 μ g/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-Pol α -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 μ l 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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