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Topoisomerase III Acts Upstream of Rad53p in the S-Phase DNA Damage Checkpoint

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Deletion of the *Saccharomyces cerevisiae TOP3* gene, encoding Top3p, leads to a slow-growth phenotype characterized by an accumulation of cells with a late S/G_2 content of DNA (S. Gangloff, J. P. McDonald, C. Bendixen, L. Arthur, and R. Rothstein, Mol. Cell. Biol. 14:8391–8398, 1994). We have investigated the function of *TOP3* during cell cycle progression and the molecular basis for the cell cycle delay seen in *top3*Δ strains. We show that *top3*Δ mutants exhibit a *RAD24*-dependent delay in the G_2 phase, suggesting a possible role for Top3p in the resolution of abnormal DNA structures or DNA damage arising during S phase. Consistent with this notion, *top3*Δ strains are sensitive to killing by a variety of DNA-damaging agents, including UV light and the alkylating agent methyl methanesulfonate, and are partially defective in the intra-S-phase checkpoint that slows the rate of S-phase progression following exposure to DNA-damaging agents. This S-phase checkpoint defect is associated with a defect in phosphorylation of Rad53p, indicating that, in the absence of Top3p, the efficiency of sensing the existence of DNA damage or signaling to the Rad53 kinase is impaired. Consistent with a role for Top3p specifically during S phase, *top3*Δ mutants are sensitive to the replication inhibitor hydroxyurea, expression of the *TOP3* mRNA is activated in late G_1 phase, and DNA damage checkpoints operating outside of S phase are unaffected by deletion of *TOP3*. All of these phenotypic consequences of loss of Top3p function are at least partially suppressed by deletion of *SGS1*, the yeast homologue of the human Bloom's and Werner's syndrome genes. These data implicate Top3p and, by inference, Sgs1p in an S-phase-specific role in the cellular response to DNA damage. A model proposing a role for these proteins in S phase is presented.

DNA topoisomerases play several important roles in DNA metabolism (61, 62, 67). These enzymes catalyze the interconversion of topological isomers of DNA and are required for the resolution of torsional stress in DNA and for the unlinking of topologically intertwined molecules. Budding yeasts express three topoisomerases, designated topoisomerases I, II, and III, all of which are highly conserved in mammalian cells. The role of yeast topoisomerase III (Top3p) in DNA metabolism has remained enigmatic, partly because this enzyme possesses only a very weak DNA relaxation activity on negatively supercoiled DNA and is therefore thought unlikely to participate in the maintenance of DNA supercoiling homeostasis (25, 61). It has been suggested that Top3p performs a nonessential role in the segregation of newly replicated DNA in *Saccharomyces cerevisiae*, although there is no direct evidence to support this proposal (61). Strains of *S. cerevisiae* that lack Top3p (*top3*Δ) show hyperrecombination in repetitive sequences and a severe slow-growth phenotype, which is due to an accumulation of cells in the late S/G_2 phase of the cell cycle (15). In the fission yeast *Schizosaccharomyces pombe*, the *top3*$^+$ gene is essential for viability, and in that organism there is direct evidence that *top3*Δ mutants display abnormal nuclear division (17, 38). The bacterial Top3p enzyme shows catalytic properties in vitro very similar to those of its eukaryotic counterparts and has been implicated in the unlinking of DNA strands during DNA replication to permit nascent chain elongation and the separation of daughter molecules (21). There are at least two homologues of the *TOP3* gene product in vertebrates (18, 24), one of which (Topo IIIα) has been shown to be essential for embryonic development in mice (30). *Drosophila* Topo IIIβ has been shown to efficiently relax hypernegatively supercoiled DNA (68), but the physiological role of this activity remains unclear.

One feature of the phenotypic effects of loss of Top3p function that has aroused considerable interest is the ability of the slow-growth and hyperrecombination phenotypes of *S. cerevisiae top3*Δ mutants to be suppressed by mutation of *SGS1* (15). This genetic interaction is conserved in *S. pombe*, in which loss of the RecQ helicase, Rqh1, suppresses deletion of *top3*$^+$ (17, 38). The *SGS1* gene encodes the sole member of the RecQ subfamily of DExH box-containing helicases in budding yeast, and deletion of *SGS1* leads to hyperrecombination (15, 63) and defects in chromosome segregation and sporulation (40, 64). Of the five known homologues of *SGS1* that exist in human cells, three are linked to disease conditions; mutations in the *BLM* gene give rise to the cancer-prone condition Bloom's syndrome (9); mutations in *WRN* lead to the premature-ageing condition Werner's syndrome (72); and mutations in *RECQ4* give rise to Rothmund-Thomson syndrome (27), which is associated with cancer predisposition as well as skin and skeletal abnormalities (58). Mutation of any one of the *SGS1*, *BLM*, *WRN*, and *RECQ4* genes leads to genomic instability and an

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abnormally high rate of genetic recombination events and/or chromosomal rearrangements (reviewed in references 5 and 23).

Cells respond to DNA damage or to an inhibition of DNA replication by delaying cell cycle progression in order to permit time to resolve the resulting abnormal DNA structures. These cell cycle checkpoint pathways are highly conserved in different eukaryotic species and act to preserve both genome integrity and cell viability (32, 36, 43, 47, 50, 65). The genetic and biochemical composition of a particular checkpoint pathway is determined, at least in part, by the point in the cell cycle at which the perturbation of DNA structure or function occurs. Entry into S phase is inhibited if cells incur DNA damage in G_1, such as through UV irradiation, and this G_1/S DNA damage checkpoint is dependent, among others, upon the *RAD9*, *RAD17*, *RAD24*, *MEC1*, *MEC3*, *DDC1*, and *RAD53* (also called *MEC2*, *SAD1*, or *SPK1*) genes in *S. cerevisiae* (2, 33, 34, 52–55). These genes, in conjunction with *PDS1*, are also required for the G_2/M checkpoint, which acts to inhibit entry into anaphase when DNA damage has not been repaired (66, 71). An additional DNA damage checkpoint that has been identified in budding yeast, termed the intra-S checkpoint, functions to slow the rate of progression through S phase in the presence of DNA damage (45). The majority of the G_1/S and G_2/M checkpoint genes play a minor role, at least in budding yeast, in the intra-S checkpoint (46, 47). Two exceptions to this are *MEC1* and *RAD53*, which are essential genes and are required for checkpoint pathways that operate in all phases of the cell cycle (2, 48, 52, 66). In addition to the "dedicated" checkpoint genes that play a role in S phase, there are certain gene products that function in S phase as part of the DNA replication machinery while also performing some as-yet-unidentified role in checkpoint surveillance of the genome. These include Pri1p (the catalytic subunit of DNA primase) and Rfc5p, which act in the intra-S checkpoint (39, 56), and replication protein A (RPA), which is a target for Mec1p in the cellular response to both DNA replication blockade and DNA damage (4).

In addition to subdividing checkpoints on the basis of the phase of the cell cycle in which they act, it is possible to categorize individual checkpoint proteins on the basis of whether they function in a DNA damage or abnormal structure "sensory" role, as signal transducers, or as targets of the signaling cascade (37). Thus, loss of a DNA damage sensor, such as Rad9p or Rad24p, causes failure to transduce the signal required for arrest of the cell cycle (26). Part of the signal transduction cascade that is absent in this class of mutants is the *MEC1*-dependent phosphorylation of Rad53p (7, 32, 37, 57, 65).

We have investigated the function of Top3p in *S. cerevisiae*. We show that deletion of *TOP3* causes sensitivity to a variety of DNA-damaging agents. While *top3*Δ mutants show proficiency in checkpoint responses to DNA perturbations occurring outside of S phase, they fail to adequately delay S-phase transit in the presence of DNA damage. This is the first indication that a eukaryotic topoisomersase is required for protection of cells against DNA-damaging agents. We present a revised version of the checkpoint response cascade that links the processing of DNA structural abnormalities to DNA repair.

MATERIALS AND METHODS

Yeast strains. All experiments were performed in the YP1 strain background except where stated. The following yeast strains were used: YP1 background, RKC1a (*MAT***a** *leu2*Δ *his4-R ura3-52 lys2 ade2-101 top3::LEU2*), JMK22d (*MAT***a** *leu2*Δ *his4-R ura3-52 lys2 ade2-101 sgs1::LYS2*), JMK253 (*MAT***a** *leu2*Δ *his4-R ura3-52 lys2 ade2-101 rad24::LYS2*), JMK469 (*MAT***a** *leu2*Δ *his4-R ura3-52 lys2 ade2-101 rad24::LYS2 top3::LEU2*), RKC1c (*MAT***a** *leu2*Δ *his4-R ura3-52 lys2 ade2-101 sgs1::LYS2 top3::LEU2*), and RKC 1d (*MAT***a** *leu2*Δ *his4-R ura3-52 lys2 ade2-101*); A364a background, RKC 31a (*MAT***a** *ura3 his3 trp1 leu2*), RKC 31b (*MAT***a** *ura3 his3 trp1 leu2 top3::G418*^{R}), AG1 (*MAT*α *ura3 his3 trp1 leu2 sgs1::G418*^{R}), JMK245 (*MAT***a** *ura3 his3 trp1 leu2 rad24::G418*^{R}), DLY264 (*MAT***a** *ura3 his3 trp1 leu2 mec2-1::URA3* [44]), and DLY 285 (*MAT***a** *ura3 his3 trp1 leu2 mec1-1::HIS3* [44]).

In the YP1 background, *TOP3* was disrupted using the *Not*I-digested plasmid pWJ258, kindly provided by R. Rothstein. This disruption contains the *LEU2* gene. Transformants were checked by Southern blot analysis and by PCR using primers flanking the *TOP3* gene and within *LEU2*. In both the A364a and the CG378 strains, *TOP3* was disrupted using pRKC20. In this plasmid, the full-length open reading frame of *TOP3* incorporating 5′ *Bam*HI and 3′ *Not*I sites was cloned into pT7. Blue (Invitrogen), and the coding sequence was disrupted by insertion of the KanMx:4 module (59) between the *Hpa*I and *Bst*XI sites. The product of a *Bam*HI and *Not*I digest was used for the transformation, and the resulting transformants were analyzed by PCR as described above but using internal KanMx primers. The phenotype of *top3*Δ was also confirmed genetically in all cases by the ability of an *sgs1*Δ mutation to rescue the slow growth of the *top3*Δ strains (15).

Flow cytometric analyses. Cells were grown in yeast extract-peptone-dextrose (YPD) medium, collected by centrifugation, and resuspended in 70% ethanol. The cells were then washed in 50 mM sodium citrate, pH 7.0, and resuspended in the same buffer containing 0.25 mg of RNase A/ml. The cells were sonicated and then incubated at 50°C for 60 min. Proteinase K was added to a final concentration of 1 mg/ml, and the cells were incubated at the same temperature for a further 60 min. The cells were then allowed to cool, and propidium iodide was added to a concentration of 8 μg/ml. Samples were analyzed using a Becton Dickinson FACScan machine incorporating LYSIS2 software. We confirmed that the peak shifting seen in flow cytometric analyses was a reflection of chromosomal DNA synthesis. The shift in the flow cytometric histograms from the G_1 to the G_2/M position was inhibited by α-factor, which induces a G_1 arrest, and by hydroxyurea (HU), which inhibits DNA synthesis.

Cell cycle blockade and irradiation procedures. All experiments were performed in YPD medium. For cell cycle blockade experiments, cultures were grown to early log phase (optical density, at 600 nm, 0.3 or 0.4). G_1 arrest was induced by adding α-factor to a final concentration of 20 μg/ml, and G_2/M arrest was achieved by adding nocodazole to a final concentration of 20 μg/ml. After the appropriate time intervals, the cells were checked microscopically and by flow cytometry to confirm the appropriate arrest phenotype. The method for UV irradiation (254 nm) has been described previously (1). Cell cycle delay during S phase was analyzed as described by Paulovich and Hartwell (45). Irradiation of nocodazole-arrested cells was done with 80 J of UV light/m^2 before the cells were washed and returned to drug-free medium for 90 min.

Determination of population doubling time and viability. The strains were grown overnight in YPD medium, diluted, and grown for several hours at 30°C. At time zero and at various times after dilution, the number of cells was determined with a Coulter Counter following brief sonication of the culture. To determine viability, a specific number of cells (usually 200 to 800) were spread on YPD agar, and the resulting colonies were counted after 3 days of incubation at 30°C.

Survival curves. (i) MMS. Cells were grown as described above before addition of methyl methanesulfonate (MMS) to concentrations of 0.005 to 0.03% for 60 min. The MMS was then inactivated by the addition of sodium thiosulfate to 5%, and the percent survival was determined in relation to untreated controls.

(ii) UV irradiation. Cells were grown to log phase, diluted at different concentrations in distilled H_2O, and spread on YPD agar. The plates were irradiated at the indicated doses with 254-nm-wavelength UV light, and 72 h later surviving colonies were counted. The percent survival was compared to that of an unirradiated control.

(iii) HU. Early-log-phase liquid cultures of the appropriate strains grown in minimal medium were exposed to 0.2 M HU for various times. The percent survival compared to that at time zero was determined by plating different dilutions of the culture on YPD agar and counting the resulting colonies 72 h later.

Northern blot analysis. Total cellular RNA was isolated using an RNeasy Mini kit (Qiagen). RNA was separated on glyoxal gels, transferred to nylon membranes (Hybond-N), hybridized, and probed as described previously (1). Northern blots were quantitated (PhosphorImager; Molecular Dynamics Ltd.), and the values were normalized to an *ACT1* loading control.

Preparation of yeast extracts and Western blot analysis. Total cellular protein extracts were prepared using a trichloroacetic acid extraction technique (10) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 6.5% acrylamide gels prepared at an 80:1 acrylamide-bisacrylamide ratio. A rabbit polyclonal antiserum to Rad53p (NLO16 [7]) was used at a final dilution of 1:10,000 in 1% fat-free milk in phosphate-buffered saline containing 0.02% Tween 20, with a primary incubation period of 12 h. Horseradish peroxidase-linked secondary antibody (Sigma) was used at 1:10,000 with a 60-min incubation period. Chemiluminescent detection was performed with an ECL kit (Amersham).

Chromosome preparation and electrophoretic separation. Intact yeast chromosomal-DNA samples were prepared from log-phase or arrested cells, as described previously (35). Briefly, samples representing approximately 10^7 cells were applied to a 1% agarose gel slab, and the chromosomes were separated using a Bio-Rad contour-clamped homogenous electric field apparatus. Separation was achieved in 24 h, with initial and final switching times of 60 and 90 s, respectively, at 200 V. The running buffer used was 0.5× Tris-borate-EDTA, which was maintained at a temperature of 11°C by rapid recirculation while the gel was run at an ambient temperature of 4°C. The DNA was then transferred to nylon membranes, and chromosome IV was detected with an *HO* gene probe, using standard protocols.

RESULTS

***top3*Δ cells demonstrate a cell cycle delay at G_2/M.** The cell cycle transit time of *S. cerevisiae top3*Δ mutants is approximately double that of isogenic wild-type strains (60). Previous microscopic studies of asynchronous *top3*Δ cultures revealed an accumulation of large-budded cells (≈70% of the total population) containing a single nucleus close to the neck of the mother cell (15), suggesting a delay in the cell cycle in the late S/G2 phase. In order to characterize this arrest phenotype in more detail, we performed flow cytometric analyses of wild-type and *top3*Δ cells at timed intervals following release from G_1 cell cycle arrest induced by the α-factor mating pheromone. Figure 1a shows that following release from this arrest, *top3*Δ cells progressed through S phase at a rate similar to that of wild-type cells but then delayed in the cell cycle at a point where they had a 2C content of DNA. Consistent with previous studies (15), we calculated that the extended cell cycle time in *top3*Δ strains was fully accounted for by this late S/G2 delay. Analysis of cell size using contour plots indicated that *top3*Δ cells showed a marked and progressive increase in size during the late S/G2 delay period, indicating that while cell division was blocked in *top3*Δ mutants, cellular growth (increase in mass) continued (Fig. 1c). It should be noted that the *top3*Δ cells emerged from an α-factor arrest about 10 min earlier than did wild-type cells, as assessed microscopically by the appearance of small-budded cells (data not shown). This probably reflects the larger size of the G_1-arrested *top3*Δ cells, which would influence the rate at which cells pass the cell size restriction point before Start (49). Synchrony experiments (not shown) confirmed previous results (15) showing that deletion of *SGS1* in a *top3*Δ background completely corrected the cell cycle defect associated with mutation of *TOP3*.

It is not possible to distinguish between late S-phase and G_2/M cells by using flow cytometry, and therefore we considered the possibility that a residual level of unreplicated DNA exists in *top3*Δ cells that is sufficient to induce a late S-phase delay. To determine whether the late S/G2-arrested cells had completed DNA replication, intact chromosomes of log-phase cells were separated by pulsed field gel electrophoresis (Fig. 1b). In this assay, only fully replicated DNA enters the gel, whereas incompletely replicated DNA is retarded in the wells (20). As controls, wild-type cells were treated with HU (which blocks in S phase), α-factor (which blocks in G_1), or methyl benzimidazol-Zyl-carbamate (MBC) (which blocks in M). As expected, DNA from HU-treated cells failed to enter the gel, whereas the majority of the fully replicated DNA in α-factor- or MBC-treated cells entered the gel. In common with the findings for wild-type cells, the majority of DNA from log-phase *top3*Δ cells entered the gel (70.0% ± 5.1% of total DNA for wild-type cells versus 82.3% ± 4.2% for *top3*Δ cells). The latter figure was consistently greater than the percentage of both large-budded cells and cells with a 2C DNA content. We conclude that *top3*Δ cells are able to complete bulk DNA replication and arrest at a point in the cell cycle after S phase but prior to the onset of anaphase.

The progressive increase in cell size when *top3*Δ mutants attain a 2C content of DNA (Fig. 1c) would be consistent with activation of the G_2/M DNA damage checkpoint. To analyze this, we deleted separately the *RAD24* and *RAD9* DNA damage checkpoint genes in a *top3*Δ background and determined the cell cycle distribution of the resulting double mutants. An asynchronous population of *rad24*Δ *top3*Δ double mutant cells showed a marked reduction in the proportion of cells with a 2C content of DNA compared to an equivalent population of *top3*Δ cells (Fig. 1c). The proportions of cells gated for a 2C content of DNA were 61% (*top3*Δ) and 40.9% (*rad24*Δ *top3*Δ). The double-mutant cells with a 2C content of DNA were of a more uniform size than those of the *top3*Δ single mutant, also indicating that the double-mutant cells were progressing more rapidly through G_2/M (Fig. 1c). Similar results were seen with a *rad9 top3* double mutant (data not shown). Analysis of cell size and morphology by microscopy, coupled with DAPI (4′,6′-diamidino-2-phenylindole) staining of nuclear DNA, confirmed that following release from α-factor arrest, *top3 rad24* double mutants were smaller and of a more uniform size than *top3* mutant cells (data not shown). Taken together, these data suggest that the cell cycle delay in *top3*Δ cells is dependent on *RAD24* and *RAD9* and, by inference, on the G_2/M DNA damage checkpoint. Consistent with this suggestion, the cell synchronization experiment shown in Fig. 1c confirmed that deletion of *RAD24* in a *top3*Δ background had the effect of shortening the G_2/M phase. Thus, at 60 min following release from α-factor arrest, the *rad24*Δ *top3*Δ cells had begun to progress into the next cell cycle, whereas the *top3*Δ cells were still largely held at G_2/M even at the 100-min time point. These results were confirmed in two independent strain backgrounds.

Given that deletion of *RAD24* had the effect of shortening the period of G_2 arrest in *top3*Δ cells, we analyzed the effects that this had on growth rate and viability. We considered the possibility that the G_2/M checkpoint arrest that occurs in every cell cycle in *top3*Δ mutants could be important for their continued survival. Consistent with the fluorescence-activated cell sorter data presented above, *top3 rad24* double mutants showed a shorter doubling time than *top3*Δ mutants (120 min for *top3 rad24* cells compared to 210 min for *top3* cells). However, this more rapid proliferation did not appear to adversely affect survival, since *top3 rad24* double mutants and *top3* single

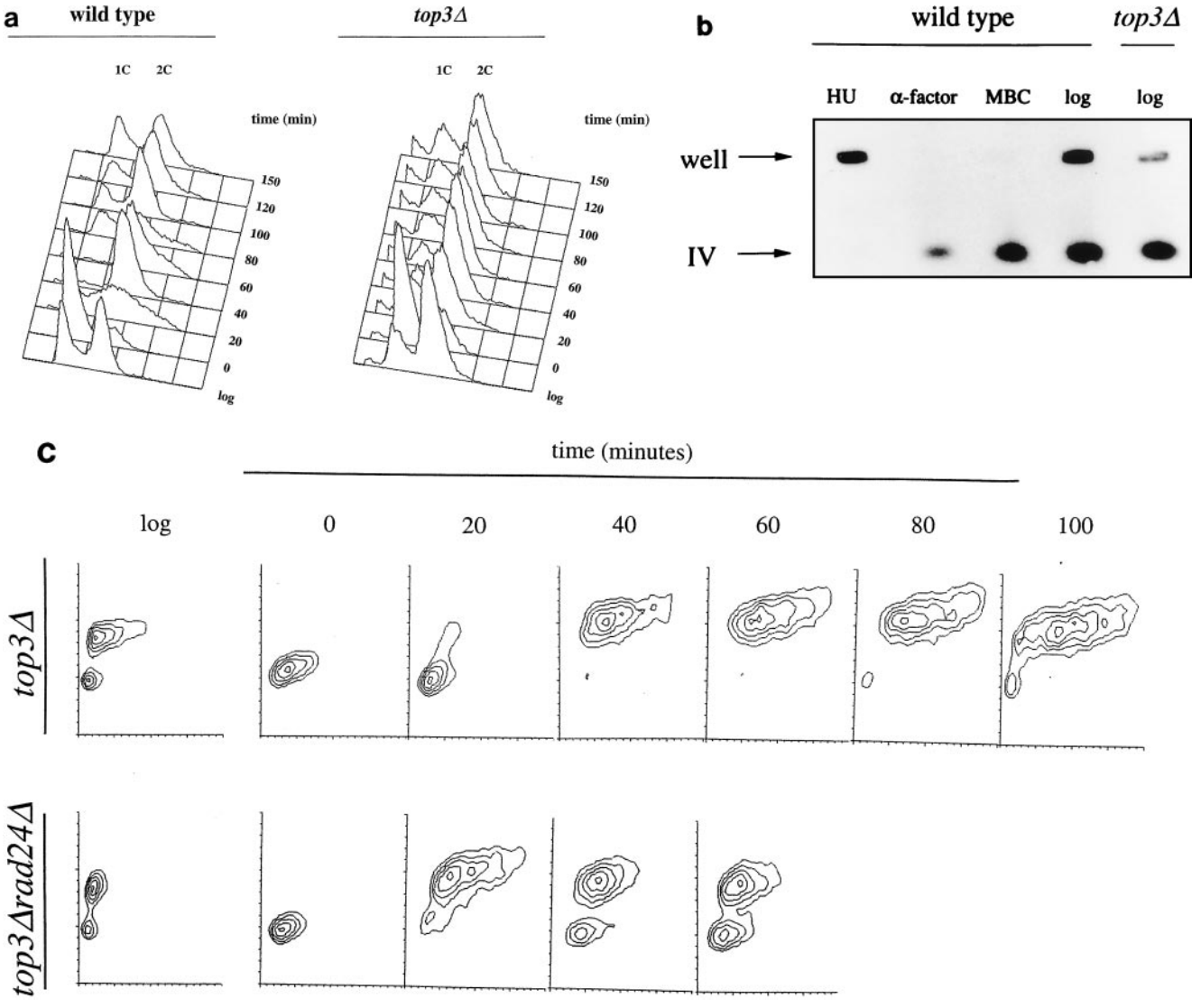

FIG. 1. *top3*Δ mutants show a G_2/M-phase delay. (a) Log-phase cultures of wild-type (RKC1d) and *top3*Δ (RKC1a) cells were arrested in G_1 with α-factor for 150 min, washed twice, and then released into fresh, warmed medium. Flow cytometric analysis was performed at timed intervals. Peaks representing cells with a 1C and a 2C content of DNA are indicated. (b) Intact chromosomal DNA from log-phase cultures of wild-type and *top3*Δ strains or wild-type cells arrested with HU, α-factor, or MBC was separated by pulsed-field gel electrophoresis. After transfer to a nylon membrane, the DNA was probed with the *HO* gene, which reveals chromosome IV. The positions of the wells and of chromosome IV that had migrated into the gel are indicated. (c) Flow cytometric analysis in the form of contour plots (with the DNA content on the vertical axis and forward light scatter, indicative of cell size, on the horizontal axis) of a *top3* mutant and a *top3 rad24* double mutant showing asynchronous log-phase cultures (log) in each case, α-factor-arrested cells (time zero), and cells at various times after release from α-factor arrest.

mutants showed comparable levels of overall cell viability (approximately 25%).

***top3*Δ cells are sensitive to DNA-damaging agents.** One explanation for the above-mentioned results is that *top3*Δ cells accumulate abnormal DNA structures in either S phase or G_2/M as a consequence of a defect in the processing and/or repair of DNA structural abnormalities. We therefore tested whether *top3*Δ mutants are sensitive to killing by DNA-damaging agents. *top3*Δ cells were found to be sensitive to the DNA-damaging agents MMS and UV light (Fig. 2a and b) and, to a lesser extent (approximately 1.5-fold), to γ-irradiation (data not shown). These results were confirmed in three independent strain backgrounds. The sensitivity of *top3*Δ strains to DNA-damaging agents was substantially suppressed by deletion of *SGS1* (Fig. 2a and b) or by ectopic expression of a wild-type *TOP3* gene (Fig. 2c).

***top3*Δ cells are defective in the intra-S checkpoint but not in the G_1/S or G_2/M DNA damage checkpoints.** The intra-S checkpoint acts to slow the rate of DNA synthesis when DNA is damaged during S phase, and it is dependent upon the products of a number of genes, including *MEC1*, *RAD53*, *RFC-5*, *PRI-1*, *RAD24*, and *RAD17* (45, 65). The data in Fig. 3a show that when *top3*Δ cells were released from an α-factor-induced G_1 arrest into medium containing 0.03% MMS, most cells achieved a 2C content of DNA after 120 to 150 min, whereas wild-type cells treated similarly progressed through S phase much more slowly and still had not attained a 2C content of DNA by 240 min. Indeed, wild-type cells had still not fully

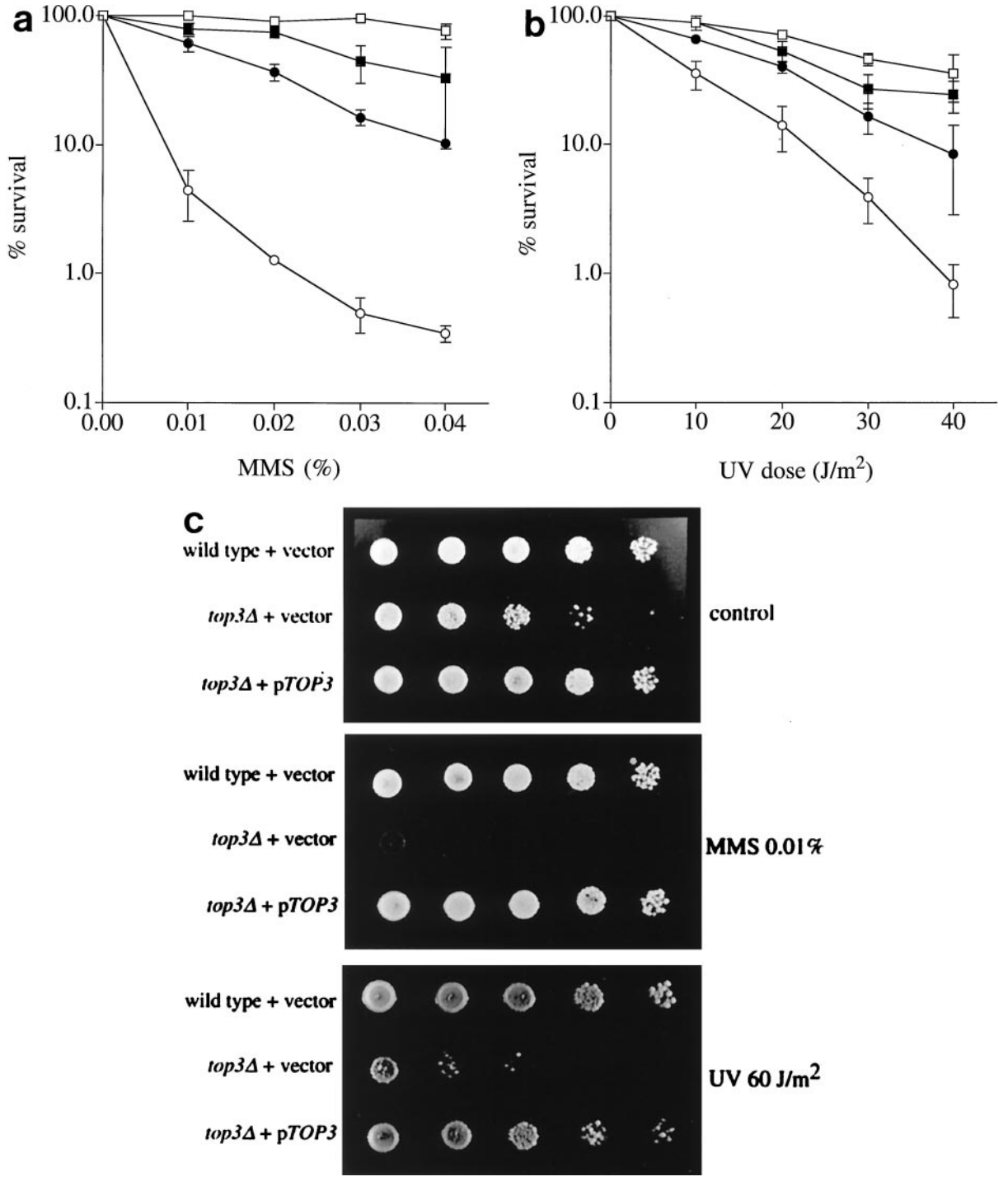

FIG. 2. *top3*Δ cells are sensitive to DNA-damaging agents. (a and b) Strains RKC1d (wild type; □), JMK22d (*sgs1*; ■), RKC1c (*sgs1 top3*; ●), and RKC1a (*top3*; ○) were grown to early log phase in YPD medium and then exposed to either MMS (a) or UV (b), and percent survival was determined. The means and standard errors of three independent experiments are shown. (c) Tenfold serial dilutions of exponentially growing yeast cultures from wild-type cells harboring an empty vector, a *top3*Δ strain harboring an empty vector, or a *top3*Δ strain harboring plasmid-encoded *TOP3* (p*TOP3*), as indicated on the left. The cells on the upper plate were untreated, those on the middle plate were treated with 0.01% MMS, and those on the lower plate were irradiated with 60 J of UV light/m^2, as indicated on the right.

completed DNA replication by 360 min (data not shown). Control experiments showed that wild-type cells and *top3*Δ mutants each progressed through S phase in approximately 60 min in the absence of MMS (data not shown). To analyze this apparent intra-S checkpoint defect in *top3*Δ mutants further, the rate of S-phase progression in the presence of MMS in *top3* cells was compared directly to the previously reported partial defect of *rad17*Δ mutants and the complete defect of *mec1*

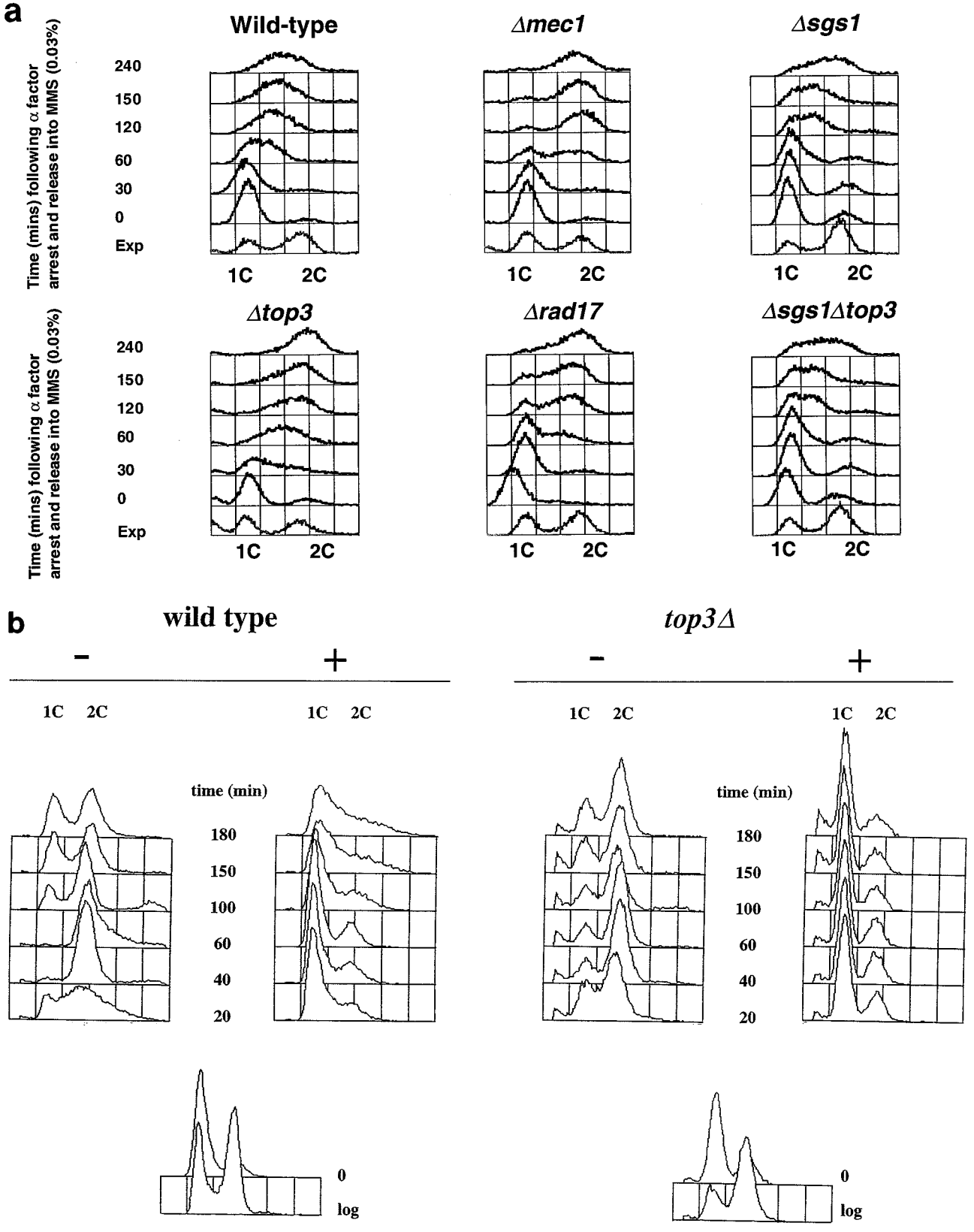

FIG. 3. *top3*Δ cells are defective in the intra-S checkpoint but proficient in the G_1/S DNA damage checkpoint. (a) To assess the intra-S checkpoint, A364a strains (as indicated above each panel) were grown in YPD medium to early log phase and then arrested in G_1 with α-factor for 150 min. The cells were then washed twice with fresh warmed medium (time zero) and released into medium containing 0.03% MMS for the times indicated on the left. The DNA content was assessed by flow cytometry, and the peaks representing cells with a 1C or a 2C content of DNA are indicated. The sample denoted Exp represents a control log-phase cell population to show the positions of the 1C and 2C peaks. (b) To assess the G_1/S checkpoint, cells were arrested as above and then irradiated with 80 J of UV light/m^2 (+) or mock treated (−) before being washed twice and released into fresh medium. Similar results were obtained from four independent experiments.

mutants in arresting S phase in the presence of MMS (45). As shown in Fig. 3a, the magnitude of the intra-S checkpoint defect in *top3*Δ cells was comparable to and consistently a little more severe than that observed in a *rad17*Δ mutant (and in *rad24*Δ cells [not shown]) but less severe than that seen in a *mec1* mutant. In contrast, both *sgs1*Δ and *sgs1*Δ *top3*Δ mutants behaved essentially as wild type, indicating that deletion of *SGS1* in a *top3*Δ background restores a largely functional intra-S checkpoint.

In contrast to the above results, cell cycle checkpoint responses to DNA damage occurring outside of S phase were apparently unaffected by deletion of *TOP3*. Figure 3b shows that when α-factor-arrested *top3*Δ cells were UV irradiated and then released into fresh medium, they showed a marked delay in the rate of progression through the G_1/S phase transition compared to nonirradiated controls. The cell cycle delay seen in *top3*Δ strains after UV irradiation in G_1 was dependent upon functional *RAD24* (data not shown). Hence, we conclude that the G_1/S DNA damage checkpoint is intact in *top3*Δ mutants. The data presented in Fig. 1 indicate that *top3*Δ cells delay transiently at a G_2/M checkpoint in the absence of exogenously added DNA-damaging agents. Consistent with this, when *top3*Δ cells were arrested in G_2/M with nocodazole and then UV irradiated, the extents of subsequent delay in the cell cycle following removal of nocodazole were comparable in wild-type and *top3*Δ cells (data not shown). This confirms that the G_2/M DNA damage checkpoint is intact in *top3*Δ mutants.

Rad53p phosphorylation is defective in *top3*Δ mutants following DNA damage in S phase in *top3*Δ cells. Checkpoint proteins can be categorized on the basis of whether they function as sensors of abnormal DNA structures, signal transducers, or targets of the signaling apparatus (37, 65). Loss of sensory function might be expected to result in an absence of all downstream responses following DNA damage or replication inhibition, including a failure to phosphorylate Rad53p.

In order to position Top3p function in relation to this broad outline of the checkpoint pathway, we studied Rad53 phosphorylation in wild-type and *top3*Δ cells following release from G_1 arrest into medium containing MMS. Figure 4a shows that phosphorylation of Rad53p (as indicated by the appearance of slower-migrating species on SDS-PAGE) was significantly impaired in *top3*Δ cells following exposure to MMS. Indeed, the phosphorylation of Rad53p in *top3*Δ cells was quantitatively and qualitatively different from that seen in wild-type cells. In MMS-treated wild-type cells, all of the Rad53p was fully phosphorylated within 100 min, as evidenced by the Rad53 species running with a substantially slower migration than the unphosphorylated Rad53p extracted from untreated cells. In contrast, in *top3*Δ cells, only a small fraction (approximately 25%) of the total Rad53p was phosphorylated, and the degree to which this modified Rad53 had a retarded migration was far less marked that that seen in wild-type cells. These data indicate that Top3p acts at an early step in the checkpoint cascade, upstream of Rad53p, and that in the absence of Top3p there is a diminution in signal transduction. Consistent with the observed restoration of the intra-S checkpoint by deletion of *SGS1* in a *top3*Δ background (Fig. 3), an *sgs1 top3* double mutant showed an apparently normal degree of Rad53p phosphorylation following MMS treatment in S phase (Fig. 4a).

To confirm that *top3*Δ mutants did not show a general defect in signaling during a check point-mediated cell cycle arrest, we studied Rad53 phosphorylation during a G_2/M checkpoint arrest induced after UV irradiation of nocadazole-treated cells (Fig. 4b). Figure 4c shows that comparable levels of Rad53p phosphorylation were seen in wild-type and *top3*Δ strains following irradiation, confirming that signaling to Rad53 in the G_2/M DNA damage checkpoint is intact in *top3*Δ strains.

Genetic interactions between *top3* and other checkpoint-deficient mutants. The *MEC1* and *RAD53* genes encode essential proteins involved in the signal transduction pathway that is activated during S phase by inhibition of DNA replication and during all phases of the cell cycle by DNA damage (2, 52, 66). Combination of a *top3*Δ mutation in the A364a strain with alleles of *MEC1* (*mec1-1*) and *RAD53* (*mec2-1*) resulted in synthetic lethality (either microcolonies that could not be propagated or no visible colony) after sporulation of the appropriate heterozygous diploids (in each case, at least 40 tetrads were dissected). In an effort to characterize genetic interactions between *top3*Δ and other mutations that disable potential targets of the Mec1/Rad53 signal transduction pathway, we examined the effect of combining deletion of *TOP3* with conditional mutations in *RFA2*, which encodes the 34-kDa subunit of the heterotrimeric single-stranded DNA binding protein RPA, and *PRI1*, which encodes the large subunit of DNA primase. Rfa2p has roles in both DNA replication and DNA repair in budding yeast. Rfa2p is phosphorylated in a MEC1-dependent manner in response to DNA replication block or DNA damage (4). Combination of *top3*Δ with either of two independent *rfa2* alleles (*rfa2-1* and *rfa2-2*) resulted in synthetic lethality, either at the permissive temperature for *top3*Δ *rfa2-1* double mutants (25°C; $n = 20$ tetrads dissected) or at the semipermissive temperature for *top3*Δ *rfa2-2* double mutants (30°C; $n = 20$). Similarly, combination of a *top3*Δ mutation with the *pri1-M4* mutation, which itself leads to a defect in S-phase checkpoint responses to DNA damage (39), resulted in synthetic lethality at the permissive temperature for the *pri1-M4* strain (25°C; $n = 20$). One interpretation of these data is that deletion of *TOP3* is lethal in combination with any mutation that perturbs DNA replication. However, this proved not to be the case, since a combination of *top3* with *pol2-12* (42) produced viable spore colonies for the double mutant (dissection of 40 tetrads).

Further evidence for an S-phase role for Top3p. Thus far, our data implicate Top3p in an S-phase-specific role in response to DNA perturbations. To gain further evidence to substantiate this suggestion, we studied whether *top3*Δ strains are sensitive to the ribonucleotide reductase inhibitor HU, which inhibits DNA replication, and whether the pattern of *TOP3* gene expression was indicative of a role in S phase. Figure 5a shows that *top3*Δ mutants are highly sensitive to killing by HU compared to wild-type control cells and that this sensitivity is substantially suppressed by deletion of *SGS1*. To assess cell cycle regulation of *TOP3* gene expression, wild-type cells were arrested in G_1 with α-factor and then released into fresh medium. RNA samples were prepared at timed intervals for Northern blot analysis, and the cell cycle position was assessed in parallel by analysis of both the budding index and DNA content by flow cytometry. In the representative experiment shown in Fig. 5b, synchrony was maintained until the middle of the second cycle. The level of *TOP3* mRNA peaked

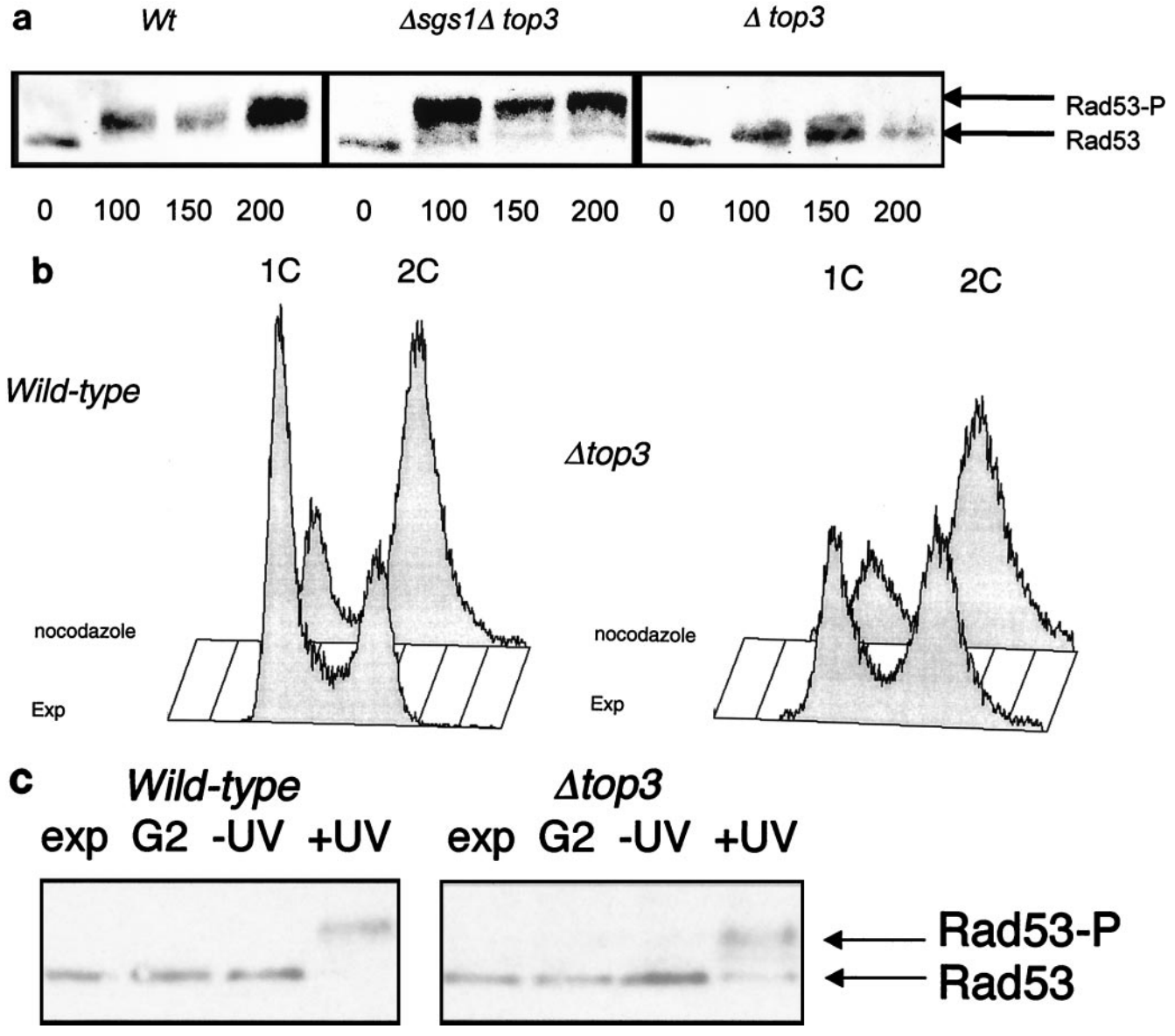

FIG. 4. Rad53 phosphorylation in *top3*Δ mutants. (a) Wild-type (Wt), *top3*, and *top3 sgs1* cells were grown in YPD medium to early log phase and then arrested in G_1 with α-factor for 150 min. The cells were then released into YPD medium containing 0.03% MMS. Protein extracts derived from cells removed at timed intervals (as indicated in minutes below the lanes) were then separated by SDS-PAGE, transferred to nylon, and immunoblotted with an anti-Rad53 antibody. A representative Western blot for extracts from wild-type, *top3 sgs1*, and *top3* cells is shown. The positions of unphosphorylated Rad53 and phosphorylated Rad53 (Rad53-P) are shown on the right. (b) Flow cytometric analysis to confirm the arrest of wild-type (left) and *top3* (right) strains after exposure to nocodazole for 90 min. A population of exponentially growing cells (EXP) is shown in each case for comparison. Peaks representing cells with a 1C and a 2C DNA content are indicated. (c) Phosphorylation of Rad53 in wild-type and *top3* strains (as indicated above the lanes) following UV irradiation in G_2/M. Lanes: Exp, unirradiated exponentially growing culture; G2, nocodazole-arrested culture; −UV, cells released from nocodazole arrest, mock irradiated, and incubated for 90 min; +UV, cells released from nocodazole arrest, UV irradiated, and incubated for 90 min. The positions of the nonphosphorylated and phosphorylated bands of Rad53 protein, detected by Western blotting as for panel a, are indicated on the right.

20 min after release from α-factor arrest at a level 11-fold higher than that in the arrested cells (Fig. 5c). This time point coincided with the onset of the decline of G_1 cells, as measured by flow cytometry, but was prior to the appearance of budded cells. In the second cycle, the peak in *TOP3* mRNA levels was coincident with the rise in the proportion of G_1 cells. We estimated the size of the *TOP3* transcript to be ≈2.5 kb. This pattern of transcription was confirmed in a second strain background, and the 2.5-kb transcript was undetectable in RNA derived from *top3*Δ cells (data not shown). Thus, it appears that *TOP3* transcripts are few in early G_1, appear abruptly around Start, and then decline during late S/G_2.

DISCUSSION

We have shown that *S. cerevisiae top3*Δ mutants are sensitive to DNA-damaging agents and HU and have an abrogated intra-S checkpoint response to DNA damage. These defects

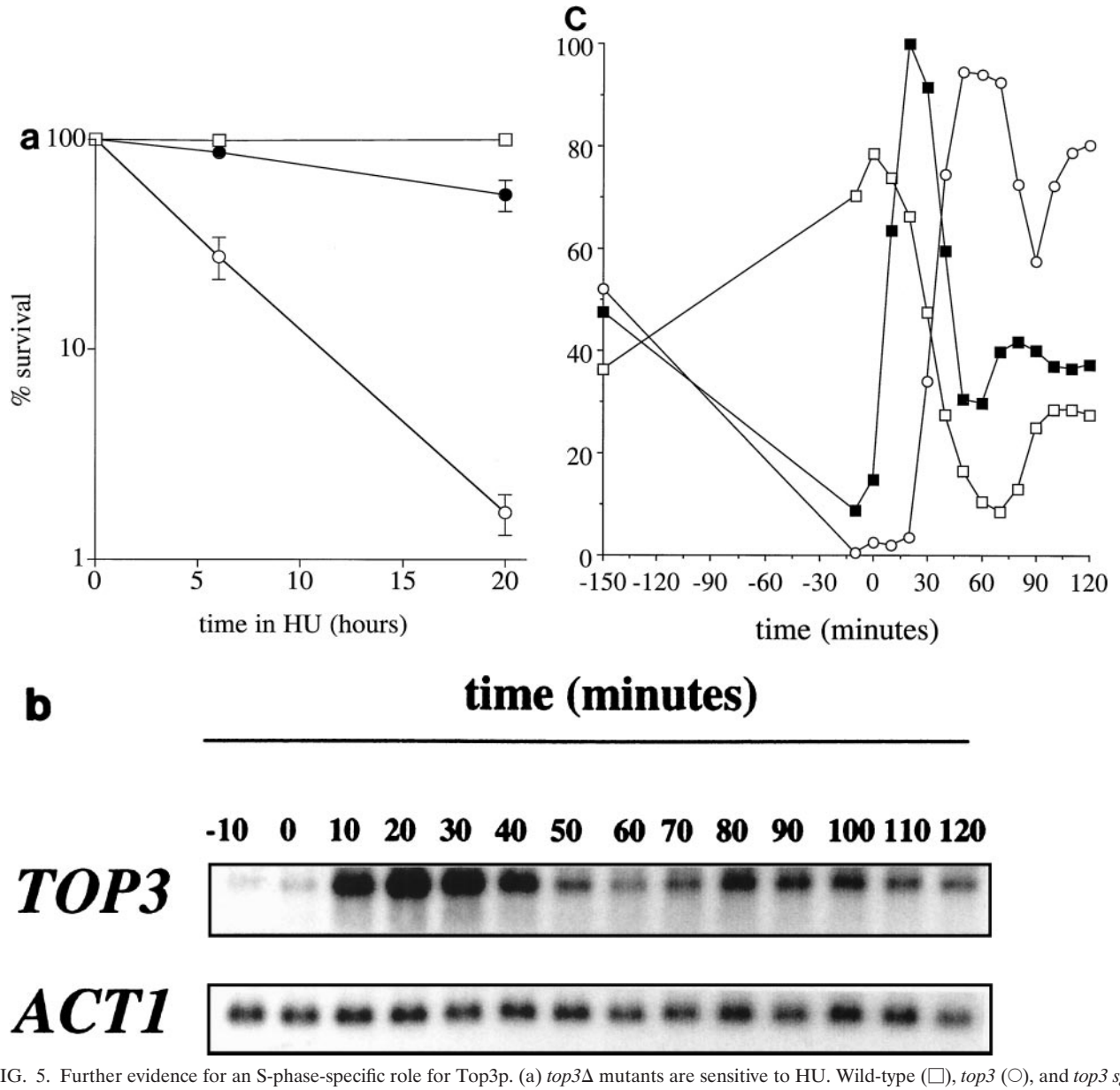

FIG. 5. Further evidence for an S-phase-specific role for Top3p. (a) *top3*Δ mutants are sensitive to HU. Wild-type (□), *top3* (○), and *top3 sgs1* (●) strains (as indicated in Fig. 2) were exposed to 0.2 M HU for the times indicated, and percent survival was determined. The means and standard errors of three independent experiments are shown. (b and c) Cell cycle regulation of *TOP3* mRNA. Wild-type cells (RKC1d) were grown to early log phase, arrested in G_1 as described in the legend to Fig. 4, and then released into fresh warmed medium. The cells were removed at timed intervals for RNA extraction, measurement of the budding index, and flow cytometric analysis of the DNA content. (b) Levels of the *TOP3* and *ACT1* transcripts at timed intervals prior to and following release from G_1 arrest. (c) The percentage of budded cells (○) and cells gated with a G_1 content of DNA (□) plotted relative to *TOP3* transcript levels (■) quantified from the data in panel a. The maximal transcript level is given an arbitrary value of 100%. Note that cells lose synchrony at about the midpoint of the second cycle.

can be rescued by deletion of the *SGS1* gene. In contrast, *top3*Δ mutants are proficient in DNA damage checkpoint responses that operate in the G_1 or G_2 phase of the cell cycle. We have also provided evidence that Top3p lies upstream of the Mec1-Rad53-dependent signal transduction cascade in the cellular response to DNA structural perturbations occurring within S phase.

***top3*Δ strains are defective in cell cycle progression in the absence of exogenous DNA-damaging agents.** Previous work has shown that the extended doubling time of *top3*Δ strains is a result of an accumulation of cells in the late S/G2 phase of the cell cycle (15). We have shown here that these cells have completed bulk DNA replication and are arrested at the G_2/M DNA damage checkpoint. The most economical explanation for these findings is that some form of abnormal DNA structure and/or DNA lesion is generated during the process of DNA replication and that while this is not sufficient to prevent completion of DNA synthesis, it nevertheless is recognized by the G_2/M DNA damage checkpoint machinery as abnormal. Although *top3*Δ strains delay at the G_2/M checkpoint for an

extended period, when this checkpoint is disabled by deletion of *RAD24*, the already-reduced viability of *top3*Δ strains is not further decreased. Indeed, the doubling time of the double mutant is shortened compared to that of *top3*Δ mutants. This might indicate that a substantial fraction of the DNA lesions that lead to induction of the G_2/M checkpoint arrest in *top3*Δ mutants are either irreparable, at least in G_2/M, or can be tolerated in subsequent cell cycles.

***top3*Δ strains are sensitive to several classes of DNA-damaging agents and replication inhibitors.** Mutations in a wide variety of genes encoding DNA repair enzymes or checkpoint proteins confer sensitivity to DNA-damaging agents. For example, mutation of nucleotide excision repair genes leads to sensitivity to UV light, while mutation of recombinational repair genes confers sensitivity primarily to ionizing radiation and MMS. *top3*Δ mutants are unusual in being sensitive to MMS and UV light, but not markedly to γ rays, suggesting that the Top3 protein probably does not play a dedicated role in one of the major pathways for the repair of specific DNA lesions but instead operates more generally in the cellular response to DNA damage. To our knowledge, this represents the first evidence in eukaryotes that a topoisomerase can protect cells from the cytotoxic effects of DNA-damaging agents. Given the intact nature of DNA damage checkpoint responses occurring in G_1 and G_2, but a failure to adequately invoke the intra-S DNA damage checkpoint, it would appear that DNA damage arising during S phase presents the most (or possibly only) serious challenge to *top3*Δ mutants. A major goal for the future is to identify the abnormal DNA structures that might occur during progression through S phase in these mutants. It is known that aberrant replicative structures resembling recombination intermediates or late Cairns-type structures can be observed on two-dimensional gels following drug-mediated inactivation of topoisomerase I or II in budding yeast (29). However, we have shown that DNA samples derived from asynchronous cultures of wild-type and *top3*Δ strains exhibit no consistent differences on two-dimensional gels (our unpublished data). We conclude that any putative abnormal replication intermediates that might arise in *top3*Δ strains either fall outside the group of structures detectable by this method or are accumulated at levels below the detection limit. We have shown that *top3*Δ strains are highly sensitive to the ribonucleotide reductase inhibitor HU. This sensitivity is suppressed by deletion of *SGS1*. Some of the genes required for protection against HU, such as *MEC1* and *RAD53*, are required to prevent mitosis from occurring during arrest in S phase (loss of the so-called S/M checkpoint) (7, 32, 37, 57, 65). Our recent work indicates that HU-treated *top3*Δ cells do not obviously enter mitosis directly from an early S-phase arrest, as evidenced by the fact that there is no progressive elongation of the mitotic spindle. Nevertheless, during exposure to HU, *top3*Δ strains do show a progressive increase in the percentage of cells displaying aberrant nuclear DNA staining, including cells with marked DNA fragmentation (unpublished data). Further work will be required to characterize the terminal phenotype of HU-treated *top3* mutants.

***TOP3* is a putative new member of the SCB box-containing family of genes.** The proposal that Top3p has a role in S phase is supported by our data showing that *TOP3* transcript levels are cell cycle regulated, arising in G_1 and declining in late S/G2. A genome-wide transcript analysis also indicated that the *TOP3* mRNA is induced in G_1 (6). A number of genes are transcribed exclusively in the late G_1 phase or at the G_1/S boundary, including the G_1 cyclins and certain genes required for DNA synthesis (reviewed in reference 51). These late-G_1-activated genes can be classified into two groups on the basis of *cis*-acting sequences found within their promoter sequences. The first group of genes includes the DNA metabolism genes (e.g., *RFA1-3*, *POL1-3*, and *DBF4*) and the *CLB5* and *CLB6* cyclin genes, and the promoter regions of these genes contain an element similar to the Mlu1 cell cycle box (MCB element). The second group of genes, including *CLN1*, *CLN2*, and *HCS26*, contain a promoter motif, termed the SCB element, which acts as a late-G_1-specific upstream activating sequence and binds the Swi4-Swi6 complex (44). Following S phase, transcription of both of these groups of genes is down-regulated. In this context, we have identified a potential SCB element in the 5′ flanking region of *TOP3* (CGCGAAA, at positions −130 to −124 from the ATG start codon), suggesting that *TOP3* is a new member of the group of genes regulated by the Swi4-Swi6 complex. While this finding would be consistent with the G_1 activation of *TOP3* gene expression, it should be noted that the minimal promoter region and the positions of any potential transcription start sites in the *TOP3* gene have yet to be characterized.

How does loss of Top3p activity result in loss of checkpoint proficiency? The *TOP3* gene shows genetic interactions with *SGS1*, and biochemical analyses have shown that the products of these genes physically associate (3, 15). Moreover, we and others have shown that topoisomerase IIIα and BLM also physically interact in human cells, confirming that the association between the topoisomerase III and RecQ helicase enzymes is highly conserved (22, 69). It is not unreasonable to assume, therefore, that Sgs1p and Top3p act in concert while performing many, if not all, of their cellular functions. However, in contrast to *top3*Δ mutants, cells lacking Sgs1p show only a modest growth defect, are only slightly more sensitive to DNA-damaging agents than are wild-type cells, and are reported to have only a minor intra-S checkpoint defect (see the discussion below). How can the above-mentioned findings be incorporated into a model that explains the role of Top3p during normal DNA replication or when replication is perturbed by DNA damage? Following DNA damage, replication forks can stall, and we suggest that Sgs1p-Top3p is involved in the processing of the resulting abnormal DNA structures or lesions (Fig. 6). The suggestion that the enzymatic activity of Top3p is required for function is consistent with the finding that the sensitivity of *top3*Δ mutants to DNA-damaging agents cannot be corrected by expression of Top3p which has been mutated at its catalytic active site (our unpublished data). The Sgs1p-Top3p complex could serve two functions, which are not mutually exclusive, in the cellular response to S-phase perturbation. First, it could act in the generation of DNA structures that are a necessary intermediate in the activation of the checkpoint cascade. Second, it could prepare the damaged DNA for the DNA repair machinery. Specifically, we propose that during S phase this repair could exploit the availability of the genetic information on the intact sister chromatid and therefore proceed via the Rad52-dependent recombinational-repair pathway. Murray et al. (41) have suggested a similar role for

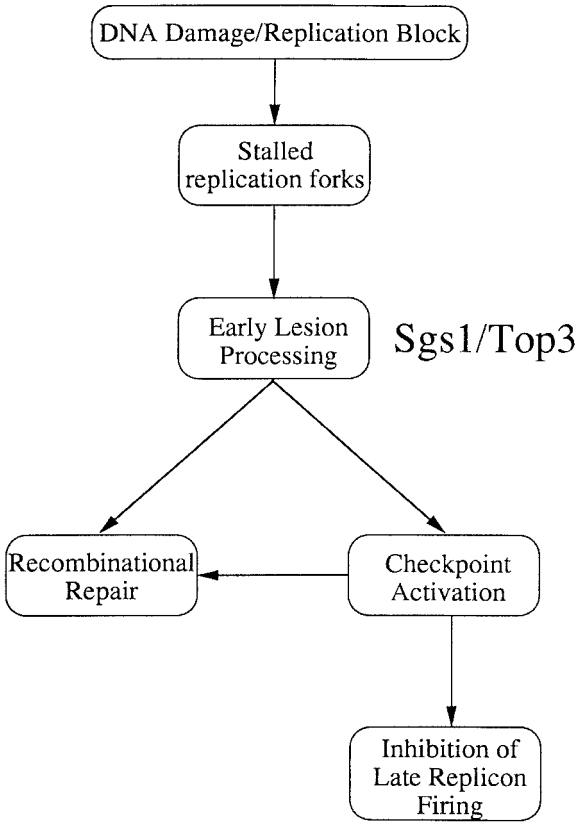

FIG. 6. Model for the role of Top3p in mediating S-phase checkpoint responses. We propose that the Sgs1-Top3 complex is involved in the early processing of aberrant structures arising at blocked or stalled replication forks. The complex may play at least two roles: preparation of lesions for repair by the recombinational repair pathway and activation of the checkpoint that leads to an inhibition of cell cycle progression. In the absence of Top3, there is a failure to adequately integrate the cellular response to the disturbance of replication.

fission yeast *rqh1*$^+$ in preparing DNA lesions at blocked replication forks for the recombinational repair machinery. Consistent with this proposal, we have shown recently that Sgs1p interacts with the Rad51 recombinase (70). This model would also be consistent with the proposed role of the RecQ protein, the *Escherichia coli* homologue of Sgs1p, which in concert with RecA can initiate homologous recombination and disrupt joint molecules formed by aberrant recombination (19). In further support of this general concept are the observations that recombination intermediates (Holliday junctions) can be detected in yeast during S phase and that perturbation of replication leads to an elevation in their frequency (73).

Deletion of *SGS1* at least partially suppresses all of the studied phenotypes in *top3*Δ mutants, including the intra-S checkpoint defect. One explanation for this could be that the phenotype of *top3*Δ mutants relates primarily to a deregulation of Sgs1p enzymatic activity. Such deregulated activity could interfere, either directly or indirectly, with the checkpoint machinery. Alternatively, deletion of *SGS1* could permit the utilization of a redundant pathway that could lead to activation of the S-phase checkpoint. It has been suggested previously that a key role for checkpoint proteins is to process certain DNA structural abnormalities in readiness for their repair by dedicated repair proteins (reviewed in reference 65). If Top3p were to participate in such a role, one implication would be that some degree of lesion processing by the Sgs1p-Top3p complex is required in order for a robust S-phase checkpoint response to be invoked. This putative role would likely require the catalytic activity of Top3p to resolve structures created by the Sgs1 helicase. Evidence in support of the concept that some processing of lesions is required to invoke certain checkpoint responses comes from the finding that in DNA repair-deficient *rad14* mutants, the UV-induced G_1/S checkpoint is not *RAD9* dependent, as it is in wild-type cells (53). Whether Top3 performs roles independent of Sgs1 will require additional studies. This suggestion is not unreasonable, however, given that *sgs1 top3* mutants grow more slowly and are more UV/MMS sensitive than are *sgs1* mutants.

Recent data indicate that deletion of *SGS1* leads to a partial defect in the intra-S checkpoint, which is not associated with an alteration in phosphorylation of Rad53p after DNA damage (12, 13). However, in combination with deletion of *RAD24*, loss of Sgs1 function leads to some attenuation in the extent of Rad53 modification. Further, Sgs1p and Rad53p have been shown to colocalize in S-phase-specific foci (12). Our results are consistent with those of Frei and Gasser (12) in that we have shown that Top3p acts upstream of Rad53 in the S-phase response to DNA damage. However, our data show that *top3* mutants have a much more severe S-phase checkpoint defect than do *sgs1* mutants and, moreover, that deletion of *SGS1* has the effect of strongly suppressing the defects in *top3* mutants. Indeed, at least in the strain background that we analyzed, any effects of an *sgs1* mutation alone or the combination of an *sgs1* and a *top3* mutation on intra-S checkpoint proficiency and Rad53 phosphorylation were not obvious. A second DNA helicase in *S. cerevisiae,* Srs2p, is required for normal activation of Rad53p during S phase, and *srs2*Δ strains show a defect in the intra-S checkpoint (31). In combination with deletion of *SGS1* or *TOP3*, *srs2*Δ strains show very low viability, which is apparently associated with an accumulation of aberrant genetic recombination structures (8, 16, 28). Interestingly, recent data indicate that defects in a recently identified protein, Tof1p, which was first identified through interactions with topoisomerase I, have phenotypic consequences similar to those reported here for *top3*Δ strains. (11). These similarities include sensitivity to DNA-damaging agents and HU and an S-phase-specific defect in DNA damage checkpoint signaling to Rad53p. Further work will be required to assess whether Top1p is functionally connected with Sgs1-Top3p

In summary, we have shown that functional topoisomerase III is required for the normal response of *S. cerevisiae* cells to DNA-damaging agents. A recent report indicated that expression of a truncated form of human topoisomerase IIIα can partially reverse certain phenotypes associated with ataxia telangiectasia cells, which are defective in the response to DNA damage (14). Since the expression of hTOP3α was shown to correct both radio-resistant DNA synthesis (analogous to the intra-S checkpoint in yeast) and hyperrecombination, it is possible that the general model described here is also applicable in certain circumstances to higher eukaryotes.

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