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Competition between the Rad50 Complex and the Ku Heterodimer Reveals a Role for Exo1 in Processing Double-Strand Breaks but Not Telomeres

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Department of Chemistry1 and Department of Biology,6 Shizuoka University, Shizuoka 422-8529, Department of Geriatric Research, National Institute for Longevity Science, Morioka, Ibaragi 376-0024,2 Graduate School of Integrated Science, Yokohama City University, Tsurumi-ku, Yokohama, Kanagawa 230-0045,4 and Genetic Research Unit Laboratory, RIKEN Institute, Wako, Saitama 351-0198,5 Japan, and Genome Damage and Stability Centre, University of Sussex, Brighton BN19RQ, United Kingdom3  
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The Mre11-Rad50-Nbs1(Xrs2) complex and the Ku70-Ku80 heterodimer are thought to compete with each other for binding to DNA ends. To investigate the mechanism underlying this competition, we analyzed both DNA damage sensitivity and telomere overhangs in Schizosaccharomyces pombe rad50-d, rad50-d pku70-d, rad50-d exo1-d, and pku70-d rad50-d exo1-d cells. We found that rad50 exo1 double mutants are more methyl methanesulfonate (MMS) sensitive than the respective single mutants. The MMS sensitivity of rad50-d cells was suppressed by concomitant deletion of pku70*. However, the MMS sensitivity of the rad50 exo1 double mutant was not suppressed by the deletion of pku70*. The G-rich overhang at telomere ends in taz1-d cells disappeared upon deletion of rad50*, but the overhang reappeared following concomitant deletion of pku70*. Our data suggest that the Rad50 complex can process DSB ends and telomere ends in the presence of the Ku heterodimer. However, the Ku heterodimer inhibits processing of DSB ends and telomere ends by alternative nucleases in the absence of the Rad50-Rad32 protein complex. While we have identified Exo1 as the alternative nuclease targeting DNA break sites, the identity of the nuclease acting on the telomere ends remains elusive.

While a DNA double-strand break (DSB) within a chromosome must be repaired to prevent cell death, a chromosome end is not recognized as DNA damage and is thus protected from the action of repair enzymes. It was therefore surprising when it was shown that several DNA repair proteins, including Mre11 and Ku, are involved in both DNA DSB repair and telomere maintenance (23, 24). Telomeres, the natural DNA ends of eukaryotic chromosomes (9), are stable and do not fuse with other chromosome ends. So that telomeres can be treated as specialized DNA structures and not as DNA damage, they are composed of repetitive DNA elements and associated with specialized proteins including human TRF1 and TRF2, Schizosaccharomyces pombe Taz1p or Saccharomyces cerevisiae Rap1 (4, 10, 11, 31, 62). Disruption of telomere architecture caused by the deletion of S. pombe taz1, for example, leads to massive telomere elongation and Ku-dependent end-to-end fusions (11, 20).

In eukaryotic cells, DSBs are mainly repaired either by homologous recombination (HR) or nonhomologous end joining (NHEJ) (12–14, 23, 24, 27). In the yeast S. cerevisiae, DNA DSBs are predominantly repaired by HR, which requires genes of the Rad52 epistasis group (50). The first step of HR is single-stranded DNA (ssDNA) end resection in a 5′ to 3′ direction to form long 3′ single-stranded tails (53). Although the Mre11-Rad50-Xrs2 complex is thought to participate in this step (29), Mre11 exhibits exonuclease activity of the opposite polarity under in vitro conditions, namely 3′ to 5′ exonuclease activity against both ssDNA and double-stranded DNA (dsDNA) (21, 40, 51, 61). Mre11 also displays ssDNA endonuclease activity. Consistent with this observation, mutants in the Mre11 nucleosome motif are not as sensitive to ionizing radiation (IR) as would be expected for mutants in an enzyme required for end processing (40). Furthermore, the observation that overexpression of exonuclease I (Exo1) partially suppresses the DNA damage sensitivity of Mre11 mutants (30, 41, 58) suggests that Exo1 acts redundantly with Mre11 in end processing.

In contrast to the situation in yeast, the major mechanism for the repair of radiation-induced DSBs in higher eukaryotes is NHEJ. The Ku70-Ku80 heterodimer, DNA-PKcs, and a DNA ligase IV-Xrcc4 complex are all required for this process (19, 22, 63). NHEJ is not a major mechanism of DNA repair in S. cerevisiae, but yeast Ku70, Ku80, and Lig4 are essential for the repair of plasmid DSBs following the transformation of the linearized plasmid into cells (38, 60). In S. cerevisiae, the IR sensitivity of mre11 null mutants is partially suppressed by loss of YKU70 (7). Furthermore, the rate of 5′ to 3′ degradation of HO-induced DSBs is decreased by deletion of MRE11 and increased by deletion of YKU70. These data have led to the development of a model in which the Ku pathway competes with 5′ to 3′ exonuclease at DNA ends (29). However, it is
unclear which nuclease is competing with Yku70. In contrast to IR sensitivity, the methyl methanesulfonate (MMS) sensitivity of mre11 null mutants is not suppressed by the loss of YKU70 (38). The yku70 single mutant is not MMS sensitive, which indicates that Ku heterodimers play no role in the repair of MMS-induced DNA damage (52, 60). Ku has also been characterized in the fission yeast S. pombe. The Ku heterodimer is required for NHEJ of transformed linear plasmids and for the maintenance of correct telomere length (3, 32, 39). In contrast to linear plasmids and for the maintenance of correct telomere length, transformation of transformed cells with a tel 1 disruption fragment. The rad22 tel 1 mutant double mutant was constructed by transforming rad32-d cells with a tel 1 disruption fragment. rhp51-d cells were constructed by insertion of the ura4+ cassette in the NheI site of PlpR51. To tag R51 with the Myc epitope (50), the rad32 plasmid was amplified the rad32+ open reading frame by PCR with a primer set of Rad32T (S\textsuperscript{5}-GCATAACGGGGATCATCTAATTATTTGCTATCC-3\textsuperscript{3}) and Rad32B (S\textsuperscript{5}-GCATAACGGGGATCATCTAATTATTTGCTATCC-3\textsuperscript{3}), with wild-type genomic DNA used as a template. The Smal cut PCR fragment was then cloned into Smal cut pFA6a-Myc-kanMX6, which contained 13 copies of the Myc epitope and a kanMX6 marker. The resulting plasmid was transformed into 1.R. Pringle (University of North Carolina) (1). The resulting plasmid was linearized with BsrBI and used for transformation. Other double and triple mutants were constructed by genetic crossing.

**In-gel hybridization.** In-gel hybridization analysis was performed according to the protocol previously published (16), i.e., using a G-rich probe (5\textsuperscript{5}-GATCGGGTATCCAGTTAACACTTTAGCTGATC-3\textsuperscript{3}) and a C-rich probe (5\textsuperscript{5}-CGTGT AACCAGCTAACCTTGTAACCCGATC-3\textsuperscript{3}). A plasmid containing the telomere repeat sequence derived from pNSU70 (46) was used as a dsDNA and ssDNA control. For the dsDNA control, about 25 ng of Aprt-digested plasmid containing 300-bp-long telomere DNA was loaded. For the ssDNA control, the same amount of heat-denatured Aprt-digested plasmid was loaded. Using microgram amounts of genomic DNA was digested with EcoRI and electrophoresed on a 0.5% agarose gel at 0.5\texttimes TAE buffer (40 mM Tris-acetate [pH 8.0], 1 mM EDTA) with 0.01 mg of ethidium bromide per ml. The gel was vacuum dried at 45°C until it became thin and warm (about 45 min). Single-stranded telomere DNA probe was labeled with [\textgamma\textsuperscript{32}P]ATP (Amersham Pharmacia Biotech) using T4 polynucleotide kinase. The gel was prehydrated in hybridization buffer at 37°C for 15 min, and then 10 µl of probe was added and the incubation continued overnight at 37°C. The gel was washed with primary wash buffer at 37°C twice for 10 min and then washed with secondary wash buffer at room temperature three times for 5 min (AlkPhos Direct; Amersham Pharmacia Biotech). The gel was dried on the Whatman paper and exposed to X-ray film for about 2 days. To detect the double-stranded telomere DNA, the gel was treated with denaturing solution (0.5 M NaOH, 150 mM NaCl) for 25 min at room temperature. After that, the gel was treated with neutralizing solution (0.5 M Tris-HCl [pH 8.0], 150 mM NaCl) and reprobed with the same probe by in-gel hybridization.

**DNA damage sensitivity assay.** Clonogenic cell survival after MMS treatment was determined as described previously (38). Logarithmically growing cells were plated directly onto solid medium containing 0.002% MMS. Colonies formed at 30°C, and the surviving fraction was calculated. For the spot assay, 4 µl of 10-fold dilutions of log-phase cells (0.5 \times 10\textsuperscript{7} cells/ml) was spotted onto a YEA (2% agar) plate or YEA plate containing the indicated concentration of MMS. For IR survival, logarithmically growing cells were irradiated by using a \textgamma\textsuperscript{32}Cs source at a dose rate of 12.5 Gy/min. For UV survival, a germicidal lamp (FUJI UV-LINKER, FS-800) was used at a dose rate of 50 to 100 J/m\textsuperscript{2} per min. Irradiated and unirradiated cells were plated on YPAD medium plates and incubated at 30°C for 4 days. All experiments were repeated at least twice.

**Indirect immunofluorescence microscopy.** Indirect immunofluorescence microscopy was performed according to the protocol previously described (8) with the following change: anti-H1Rad51 (Santa Cruz H-92) was diluted 1:100. To determine the percentage of cells showing nuclear foci, we visually scored 1,000 cells for each sample.

**Immunoprecipitation.** For immunoprecipitation, immunoglobulin G (IgG)-conjugated magnetic beads were produced with Tosylactivated Dynabeads M-280 (DYNAL) and mouse IgG according to the manufacturer’s instructions. Twenty microtiter of Dynabeads was added to 12 mg of total proteins in 400 µl of buffer (50 mM HEPESKOH [pH 7.5], 140 mM NaCl, 300 mM (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 0.01% (wt/vol) bovine serum albumin, protease inhibitor cocktail (Roche), 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). This mixture was incubated for 2 h at 4°C. After extensive washing, the beads were suspended in 50 µl of sodium dodecyl sulfate sample buffer. Ten microtiter of the suspension was analyzed by Western blotting. The anti-Myc-Tag 9B11 antibody (Cell Signaling) and anti-protein A antibody (Sigma) were used for detection of proteins.

**Chromatin immunoprecipitation.** The chromatin immunoprecipitation (ChIP) assay described by Takahashi et al. (55) was adopted with modification. Cells grown in 100 ml of YPD culture at 30°C were fixed with formaldehyde. For immunoprecipitation, anti-Myc-Tag 9B11 antibody (Cell Signaling) and protein G Dynabeads (DYNAL) were used. Immunoprecipitated DNA was extracted and suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA). PCRs used the following primers to amplify the telomeric DNA (top, 5\textsuperscript{5}-CCGGCTGAC

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**MATERIALS AND METHODS**

**Growth medium.** S. pombe cells were grown in YPD medium (1% yeast extract, 2% polypeptide, 2% glucose, 20 µg of adenine per ml), YES medium (0.5% yeast extract, 3% glucose, 20 µg of adenine per ml) or Edinburgh minimal medium with required supplements (42).

**Strain construction.** The rad50\textsuperscript{+} gene was disrupted by replacing the region between the first HindIII restriction site and second HindIII site (nucleotide positions 529 to 1158, relative to initiation codon) with either the ura4\textsuperscript{+} or the LEU2\textsuperscript{+} gene (Table 1). Standard methods were used to create the disrupted constructs, and linear fragments were transformed into a wild-type strain (JY741) (42). rad32-d, trt1-d and tel 1-d cells were constructed by insertion of the ura4\textsuperscript{+} or LEU2\textsuperscript{+} cassette into the HindIII, BglII and PstI sites, respectively. To construct pka70-d, pka80-d and lqg-d cells were constructed by insertion of the LEU2\textsuperscript{+} cassette into the EcoRV, EcoRV and NcoI sites, respectively. To construct pka70-jen2/ade6\textsuperscript{+} cells, the ade6\textsuperscript{+} cassette was inserted into the EcoRV site in the LEU2 gene in the pka70-LEU2 disruption fragment. rad50 tu1 and rad50

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TABLE 1. S. pombe strains used in this study

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GGTTGGGGAATCCATA-3'; bottom, 5'-AGGTGGAATTTGATATGGTGA-3'; or the ade6-6' DNA (top, 5'-AGGTGGAATTTGATATGGTGA-3'; bottom, 5'-CAAGGCATACAGTGTGAAAC-3').

RESULTS

The DNA damage sensitivity of rad50-d cells is suppressed by deletion of pku70+. The Rad50-Mre11-Nbs1(Xrs2) complex and the Ku heterodimer are thought to compete with each other for binding to DSB ends (29). To investigate the mechanism underlying this competition, we analyzed the DNA damage sensitivities of rad50-d cells and rad50 pku70 double mutants in S. pombe. We found that the γ-ray sensitivity of rad50-d cells was suppressed by deletion of pku70+ (Fig. 1A). By analogy to the mechanism proposed for S. cerevisiae, in which the IR sensitivity of mre11 null mutants is partially suppressed by the loss of YKU70 (7), our results suggest that deletion of pku70+ in S. pombe cells lacking a functional Rad50-Rad32 protein complex improves the efficiency of HR repair by enhancing the ability to process DSB ends. In S. cerevisiae, the MMS sensitivity of mre11 mutants is not suppressed by the loss of YKU70 (38). However, we found that the MMS, UV, and HU sensitivities of rad50-d cells were suppressed by concomitant deletion of pku70+ (Fig. 1B through D). These results strongly suggest that S. pombe Ku70 plays an important role in the repair of MMS-, UV-, and HU-induced DNA damage, probably by inhibiting HR repair in the absence of the Rad50 complex. As rad50+ is epistatic to rad32-2 for DNA damage sensitivity, we examined whether rad50-d cells and rad32-d cells display the same phenotype with respect to the suppression of DNA damage sensitivity. Spot assays revealed that deletion of pku70+ suppressed the sensitivity of both rad50-d and rad32-d cells to MMS and HU (data not shown).
Because the function of Rad50 is thought to be in DNA damage processing, upstream of Rad51, the suppression of DNA damage sensitivity presumably reflects the enhancement of DSB end processing. In this case, suppression should be limited to the early stages of HR. We therefore asked whether the MMS sensitivity of \textit{rhp51-d} cells, which are defective in later steps in HR (43), is also suppressed by the deletion of \textit{pku70}\/. The survival of \textit{rhp51-d} cells at 0.002\% MMS (0.1\% 0.01\%) was almost same as that of \textit{rhp51 pku70} double mutants (0.1\% 0.01\%) (Fig. 2). These results indicate that the suppression of DNA damage sensitivity occurs at an early stage in HR, probably before strand invasion, which requires Rad51 (Rhp51).

Similarly, Ku70/Ku80 is thought to function at an early stage of NHEJ and Lig4 at a later stage (3, 39). We investigated whether the DNA damage sensitivity of \textit{rhp51-d} cells was suppressed by the deletion of either \textit{pku80}+ or \textit{lig4}+. The MMS sensitivity of \textit{rhp50-d} cells was suppressed by the concomitant deletion of \textit{pku80}+ but was not significantly suppressed by the concomitant deletion of \textit{lig4}+ (Fig. 2). These results indicate that the DNA binding of the Ku70/80 heterodimer plays an important role in the suppression of DNA damage sensitivity.

Evidence that Exo1 resects DNA DSB ends in the absence of Rad50 and Ku70. The data presented above suggest that the Ku heterodimer represses a repair function early in HR, perhaps directly at DNA ends, and the data also suggest that the derepression of this function can partially overcome the loss of Rad50. Because Mre11-Rad50 is known to encode an endonuclease, we hypothesized that the repair function that is derepressed when Ku is lost could be provided by an unknown nuclease that resects DNA DSB ends in the absence of Ku and Rad50. Exo1 is a good candidate for such a nuclease, because overexpression of \textit{EXO1} can suppress the DNA damage sensitivity of \textit{mre11} disruptants in \textit{S. cerevisiae} (30, 41, 58). Thus, if \textit{S. pombe} Exo1 can resect DSB ends independently of the Rad50-Rad32 protein complex, then \textit{rad50 exo1} double mutants should be more sensitive to DNA damage than the single mutants. To test this, we examined the MMS and the IR sensitivity of \textit{rad50 exo1} double mutants. As shown previously (54), \textit{exo1} single mutants were not significantly IR sensitive (Fig. 3A). However, the \textit{rad50 exo1} double mutant was significantly more IR and MMS sensitive than the single \textit{rad50} mutant (Fig. 3A and B). These results suggest that Rad50 and Exo1 can function independently. Similar results have been reported in \textit{S. cerevisiae}, in which \textit{exo1 mre11} double mutants become more MMS sensitive than either of the single mutants (41, 58).

Importantly, the MMS sensitivity of the \textit{rad50} mutant was suppressed by the deletion of \textit{pku70}+ (Fig. 2). However, the
MMS sensitivity of the rad50 exo1 double mutant was not suppressed by the deletion of pku70 (Fig. 3B). Similar results were obtained in the spot tests when the rad32 mutant instead of the rad50 mutant was used (Fig. 3C). These results indicate that the Ku heterodimer may prevent DSB resection by Exo1 in the absence of the Rad50-Rad32 protein complex. In agreement with our hypothesis that both Rad50 and Exo1 can act in an early step in HR, the rad50 exo1 double mutant was as IR sensitive as the rhp51 single mutant.

Rad50 and Exo1 function independently upstream of Rad51. Treatment of wild-type S. pombe cells with 500 Gy of γ rays resulted in the formation of Rad51 (Rhp51) foci in almost 100% of cells within 1 h after irradiation (8). If Rad50 and Exo1 function independently and upstream of Rad51, IR-induced Rad51 focus formation should be significantly compromised in rad50 exo1 double mutants compared to the respective single mutants. Indeed, we find that rad50-d cells and exo1-d cells showed only a moderate reduction in the focal assembly of Rhp51 following IR (rad50-d, 50% of cells with foci; exo1-d, 80% of cells with foci), whereas rad50 exo1 double mutant cells were strongly impaired in Rhp51 focus formation 1 h after irradiation (rad50-d exo1-d, 8% of cells with foci) (Fig. 4). These data are consistent with a model in which Rad50-Rad32 and Exo1 process DNA DSB ends in a redundant manner upstream of Rad51.

Rad50-Rad32 is involved in the production of G-strand overhang in taz1-d cells. Our genetic data suggest that the reason that the S. pombe Rad50-Rad32 complex is required for DNA damage resistance is most probably because of its role in the processing of DSB ends. The Rad50 complex has also been implicated in the processing of telomere ends (15, 25, 49), and we therefore examined whether these interactions were reflected in the processing of these specific DNA structures. Asynchronous wild-type S. pombe cells contain only very small amounts of the G-rich overhang at telomeres (2), making it difficult to evaluate the role of Rad50-Rad32 in the processing of telomere ends in wild-type cells (Fig. 5B, top panel, lane 2). However, using an in-gel hybridization assay (16), we observed strong signals corresponding to the G-strand overhang in asynchronous taz1-d cells (Fig. 5A, top panel, lane 1). We therefore constructed taz1 rad50 double mutants and taz1 rad32 double mutants and examined the extent of the single-stranded overhang at the telomeres. Intriguingly, both taz1 rad50 double mutants and taz1 rad32 double mutants lacked the G-strand overhang (Fig. 5A, top panel, lanes 2 and 3). These results suggest that the Rad50-Rad32 complex is required either for degradation of the corresponding C-rich strand in taz1-d cells or for elongation of the G-rich strand. If Rad32 and Rad50 are required for elongation of the G strand of telomeres, the elongation of telomeres themselves in taz1 rad32 double mutants would be less efficient than that in taz1 single mutants. However, the lengths of telomeres themselves in a taz1 rad32 double mutant, which was constructed by deletion of taz1/H11001 in rad32-d cells, was identical to that seen in the taz1 single mutant (Fig. 5A, bottom panel, lanes 1 and 3), suggesting that the Rad50-Rad32 complex is not required for G-strand elongation in taz1-d cells.

To exclude the possibility that the G-strand overhang in taz1-d cells is telomerase dependent, we also created a taz1 trt1 double mutant that lacked active telomerase. As shown previously (46, 47), taz1 trt1 double mutants lost telomeres very rapidly (Fig. 5B, bottom panel, lane 1); however, the signals corresponding to the G-rich overhang were still detected (Fig. 5B, top panel, lane 1). These results indicate that the G-rich...
overhang in _taz1-d_ cells can be generated without telomerase activity, probably through degradation by the Rad50-Rad32 complex. Although degradation of the C-rich strand at the telomere ends in _taz1-d_ cells seems to be fully Rad50-Rad32 dependent, DNA ends made by HO endonuclease are still processed in _mre11_ mutants in _S. cerevisiae_ (29). This difference suggests that telomere ends may be highly protected from degradation even in the absence of the Mre11 complex.

FIG. 3. _rad50 exo1_ double mutants become more sensitive to DNA damage than each single mutant. (A) The sensitivities to γ rays of wild-type cells, _rad50-d_ , _exo1-d_ , _rad50-d exo1-d_ , and _rhp51-d_ cells. The percent survival of wild-type cells (700), _rad50-d_ (701), _exo1-d_ (702), _rad50-d exo1-d_ (703), and _rhp51-d_ cells (704) is plotted against the γ ray dose. (B) Cell survival frequencies on 0.002% MMS-containing plates versus control plates for wild-type cells (JY741), _exo1-d_ (KTH00g), _rad50-d_ (KTH02), _rad50-d exo1-d_ (KTH02g), and _pku70-d rad50-d exo1-d_ (KTH12g5) cells. Standard deviations are shown by error bars (for two to four independent experiments). (C) The MMS sensitivity of wild-type cells (119), _rad32-d_ (324), _rad32-pku70-d_ (315), _rad32-d exo1-d_ (403), _pku70-d rad32-d exo1-d_ (407), _pku70-d_ (319), _exo1-d_ (302), and _exo1-d pku70-d_ (394) cells was assayed by the spot test. The cells were grown in YEA (0.5 × 10⁶ cells/ml), serially diluted (1:10) with sterilized water, and spotted (4 μl of each dilution was spotted onto the MMS plates).
Interestingly, as we observed for the sensitivity to DNA damage, inactivation of the Ku heterodimer could overcome the loss of Rad50-Rad32 and restore the G-strand overhang at telomere ends. In a taz1 rad50 pku70 triple mutant, a significant amount of G-rich overhang was detected (Fig. 5A, top panel, lanes 4 and 5). This again can be interpreted to indicate that an unknown nuclease activity can digest the corresponding C-rich strand to produce the G-rich overhang in the absence of the Rad32-Rad50 complex and to indicate that this nuclease activity is inhibited by the presence of Ku70. However, unlike the situation deduced from the DNA damage sensitivity analysis presented above, this nuclease activity cannot be attributed exclusively to Exo1 activity, since we still detected significant levels of G-rich overhang in the taz1 rad50 pku70 exo1 and taz1 rad32 pku70 exo1 quadruple mutants (Fig. 5A, top panel, lanes 6 and 7). These data suggest that telomere ends are processed by a different mechanism from those of DSB ends.

rad32 nuclease domain mutants possess G-strand overhang in taz1-d cells. Our data suggest a model in which the Rad50-Rad32 protein complex is involved in end-processing at DSBs and at telomeres and further suggest that the Ku heterodimer negatively influences exonuclease 1, which can act on DSBs but not on telomeric ends in the absence of Rad50-Rad32. To test whether the nuclease activity of Rad32 is indeed required for resection of telomeres, we made a rad32-D25A taz1 double mutant. Aspartate 25 in the S. pombe Rad32 protein corresponds to the catalytically important aspartate residue 8 in the P. furiosus Mre11 protein (18). This aspartate coordinates two...
Mn\(^{2+}\) atoms that are located in the active site and are required for the endo- and exonuclease activity of P. furiosus Mre11 (26). A mutation of the same protein in S. cerevisiae, Mre11 D16A, does not possess 5\(^{\prime}\) to 3\(^{\prime}\) exonuclease activity in vitro (21). S. cerevisiae mre11-D16A mutants are as MMS and HU sensitive, but this sensitivity is about 10-fold weaker than that of a null mutant (21). Consistent with the important role of this aspartate, rad32-D25A mutant strains exhibit MMS sensitivity, but this sensitivity is about 10-fold weaker than that of wild-type Rad32 (21).

Interestingly, taz1-d rad32-D25A double mutants contained a significant level of G-strand overhang (Fig. 5C, lanes 3 and 4) (two independent clones), indicating that the Rad32 nuclease domain is not required for degradation of the C-rich strand. Our results suggest that, at the telomere ends, the Rad50 complex does not act as a nuclease itself but probably recruits an unknown nuclease activity to telomeres. To ascertain if this nuclease is independent of Exo1, we created taz1-d exo1-d double mutants. These taz1-d exo1-d double mutants contained a significant G-rich overhang (data not shown), which suggested that the recruited nuclease activity is not exclusively due to Exo1. In S. cerevisiae, some of the Mre11 nuclease domain mutants do not form a complex with Rad50 (28). Thus, we tested the interaction between Rad32-D25A and Rad50 by coimmunoprecipitation experiments. We tagged the N terminus of Rad50 with a tandem affinity purification (TAP) tag (64) and tagged the C terminus of Rad32 with the Myc tag (1). Cells expressing both tagged proteins were lysed, and Rad50 was affinity precipitated from the soluble lysate with IgG-conjugated magnetic beads (see Materials and Methods). As expected, Rad32-Myc was coprecipitated with Rad50 (Fig. 5D).

Next, we tested the interaction between Rad32-D25A and Rad50. Although the efficiency of protein binding was lower than that of wild-type Rad32, Rad32-D25A retained the ability to interact with Rad50 (Fig. 5D). We also tested the interaction between Rad32-D25A and telomere by ChIP assay. As reported previously (48), telomeric DNA was specifically amplified from Rad32-Myc immunoprecipitate (Fig. 5E). We also found that Rad32-D25A-Myc can bind to telomere ends (Fig. 5E). These two results strongly suggest that the nuclease mutant Rad32-D25A forms a complex with Rad50 on the telomere DNA. This is consistent with a model in which Rad23-D25A can recruit the unknown nuclease to telomere ends.

**DISCUSSION**

The roles of the Rad50-Rad32 complex and Ku70 complex at DSB ends. S. pombe Rad50 and Rad32 are required for efficient HR repair, but their exact roles at DSB ends remain unclear (25, 32, 56, 65, 66). Unlike for S. cerevisiae, a method to analyze the rate of degradation of DSB ends has not been developed for S. pombe. This makes it difficult to study the roles of the Rad32-Rad50 complex at DSB ends. We have demonstrated that rad50 exo1 double mutants are more IR sensitive than the respective single mutants and are as sensitive as cells lacking Rad51 (Rhp51) (Fig. 3A). Consistent with this observation, a rad50 exo1 double mutant was strongly impaired in its ability to form Rhp51 foci after irradiation (Fig. 4). These results are fully consistent with a model in which the Rad50-Rad32 complex and Exo1 can independently and redundantly act on DNA DSB ends to generate the substrates for Rhp51 filament formation (Fig. 6A).

We also found that both the MMS and IR sensitivities of rad50-d cells were suppressed by concomitant deletion of pku70', which encodes the Ku70 protein required for an early step in NHEJ (Fig. 1). However, the MMS sensitivity of the rad50 exo1 double mutants was not suppressed by deletion of pku70' (Fig. 3B). In S. cerevisiae, it has been suggested that Ku competes with 5\(^{\prime}\) to 3\(^{\prime}\) exonucleases at DNA ends (29). However, the nuclease competing with Yku70 has not been identified. Our results strongly suggest that the nuclease competing with Ku70 at DSB ends is Exo1 (Fig. 6 A). In contrast to the competition between Ku and Exo1, the Rad50 complex can process DSB ends in the presence of Ku heterodimer. pku70-d cells are not IR sensitive (32), which suggests that Ku70 does not recruit the Rad50 complex to the DNA ends. Although the biological significance of the Exo1 pathway for HR repair is not clear, Ku might be removed in a controlled manner and protect
DSBs from nonspecific degradation in the absence of certain activities.

Is a DNA DSB produced following exposure to UV, MMS, and HU? The sensitivities of rad50-d cells to IR, UV, MMS, and HU were all suppressed by concomitant deletion of pku70 (Fig. 1A through D). This suggests that, in the absence of rad50-d, γ rays, UV light, MMS, and HU cause the generation of similar DNA structures, probably DSBs, that may be bound by the Ku70-Ku80 heterodimer. However, the suppression of the UV sensitivity is potentially confusing because UV light produces primarily cyclobutane-pyrimidine dimers (CPDs) or 6-4 photoproducts, not DSBs. Recombination is much more important for UV survival in S. pombe than in budding yeast, as shown by the fact that rad22, rhp51, and rhp54 mutants are all significantly UV sensitive (36). To initiate homologous recombination, DNA DSBs should be generated. Interestingly, in S. pombe, CPDs and 6-4 photoproducts are not only repaired by the nuclear excision repair pathway but also by the UV damage endonuclease pathway, which facilitates homologous recombination (36). Furthermore, unrepaired UV-induced lesions are thought to become substrates for HR-based postrepllication repair processes when encountered by a replication fork. Therefore, DNA DSBs could be produced as a secondary lesion during the recombinitive recovery from UV damage. A similar mechanism probably underlies the HU and MMS sensitivity of rad50-d cells. Both HU and MMS can stall the replication fork, and such stalled forks can result in DSBs, as shown in Escherichia coli (27, 37). It is now becoming clear that in many eukaryotes, including S. pombe, DNA DSBs can be produced by replication arrest and that HR is required for their repair (44, 45). In S. pombe, a Holliday junction formed at a stalled or collapsed replication fork is thought to either be reversed by Rqh1 helicase in a nonrecombinogenic pathway or resolved by a Mus81-Eme1-dependent endonuclease (potentially via a recombinogenic pathway) (5, 17). In the latter case, DSBs are suggested to be produced to initiate homologous recombination.

Given that IR, UV, MMS, and HU could all cause DSBs in S. pombe, suppression of the sensitivity of rad50-d cells to all these agents by the concomitant deletion of pku70 could be explained by a model in which the Ku heterodimer has to be removed from the break site in the absence of Rad50 to allow end processing by alternative nucleases (Fig. 6A).

**Roles of Rad32-Rad50 complex and Ku70 at the telomere ends in taz1-d cells.** Both the Rad50-Rad32 protein complex and the Ku heterodimer are involved not only in the processing of DSBs but also in telomere maintenance (25, 66). However, it is unknown how these proteins act to regulate telomere length. Asynchronous taz1-d cells contain extensive G-rich single-stranded 3’ overhangs at telomere ends (Fig. 5A, top panel, lane 1), thereby making it possible to study the roles of Rad50, Ku70, and Exo1 at these telomeres. As shown in Fig. 5B (top panel, lane 1), the generation of the G-rich overhang in taz1-d cells occurs in the absence of telomerase activity, suggesting that the G-rich overhang is generated by degradation of the C-rich strand. Our results suggest that this nuclease step is dependent on the Rad32-Rad50 complex without utilizing its nuclease activity, because the G-rich overhang in taz1-d cells disappeared upon deletion of either rad50-d or rad32+ (Fig. 5A, top panel, lanes 2 and 3) but not upon mutation of the nuclease domain of Rad32 (Fig. 5C, top panel, lanes 3 and 4). To explain the physical requirement for Rad32, we propose that (in taz1 disruptants) the Rad50-Rad32 complex recruits an unknown nuclease, which contains 5’ to 3’ exonuclease activity, to the telomere (Fig. 6B). We do not know which nuclease is recruited. However, we can exclude the major activity being due to Exo1, because taz1-d exo1-d double mutants contained significant G-rich overhangs (data not shown). Although the biological significance of the Rad50–Rad32-dependent generation of G-rich overhang in taz1-d cells is not clear, Taz1 may be detached from telomeric DNA during telomere elongation. Therefore, our results may reflect the function of these proteins during telomere elongation.

Recently, it has been suggested that Exo1 is required for both ssDNA generation at telomeres and the subsequent cell cycle arrest of yku70 mutants in S. cerevisiae (34). Our results in S. pombe are not consistent with this—we found no detectable role for Exo1 at telomere ends. It is possible that there are significant differences between S. pombe and S. cerevisiae or that the different assays used affect competing activities in distinct ways.

**The roles of Rad32-Rad50 at DSB ends.** The importance of nuclease activity in Rad32 for the processing of DSB ends remains clear. Although Mre11 has a 3’ to 5’ exonuclease and endonuclease activity in vitro (51, 57), in vivo observations suggest that Mre11 is required for the oppositely oriented (i.e., 5’ to 3’) exonuclease activity (29). There are two possible models to explain this discrepancy. (i) A long 3’ ssDNA is generated by the endonuclease activity of Rad32 combined with unidentified helicase components. (ii) An unidentified 5’ to 3’ exonuclease is recruited to DSB ends by a Rad32 (Mre11) complex. At this point it is difficult to distinguish these two possibilities, and further studies are required to resolve this discrepancy. It has been reported that in vivo 5’ to 3’ resection of DNA ends is strongly dependent upon the successful formation of the Mre11 protein complex, perhaps along with other as yet unidentified components (28). We found that Rad32 nuclease domain mutants were significantly MMS sensitive (data not shown). However, we cannot conclude that the nuclease activity in Rad32 is required for the processing of DSB ends, because the interaction between Rad32-D25A and Rad50 were less efficient than the interaction between Rad32 and Rad50 (Fig. 5D). The reduced stability of Rad32-D25A-Rad50 complex may impair the 5’ to 3’ resection ability by affecting the unknown function of the Rad32-Rad50 complex, perhaps binding to unidentified proteins. As suggested for the roles of the Rad32-Rad50 complex at telomere ends in taz1-d cells, the main roles of the Rad50 complex might be the recruitment of unidentified nuclease or other factors to DSB ends. These factors are also suggested to exist in S. cerevisiae (28).

Our data suggest that the Rad50 complex is required for the processing of DSB ends and telomere ends in the presence of Ku heterodimer. However, Ku heterodimer inhibits processing of DSB ends and telomere ends by alternative nucleases in the absence of the Rad50-Rad32 protein complex. While we have identified Exo1 as the alternative nuclease targeting DNA break sites, the identity of the nuclease acting on the telomere ends remains elusive. The nuclease function of the Rad50-Rad32 protein complex seems not to be important for degra-
vation of the C-rich strand at telomeres. Moreover, our data allow the speculation that cells regulate the resection of DNA ends in the absence of Rad50 complex through controlled binding of the Ku heterodimer. A similar regulation might underlie the cell-cycle-specific appearance of the G-rich overhang at telomeres.

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