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Nse2, a Component of the Smc5-6 Complex, Is a SUMO Ligase Required for the Response to DNA Damage

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The *Schizosaccharomyces pombe* SMC proteins Rad18 (Smc6) and Spr18 (Smc5) exist in a high-M_r complex which also contains the non-SMC proteins Nse1, Nse2, Nse3, and Rad62. The Smc5-6 complex, which is essential for viability, is required for several aspects of DNA metabolism, including recombinational repair and maintenance of the DNA damage checkpoint. We have characterized Nse2 and show here that it is a SUMO ligase. Smc6 (Rad18) and Nse3, but not Smc5 (Spr18) or Nse1, are sumoylated in vitro in an Nse2-dependent manner, and Nse2 is itself autosumoylated, predominantly on the C-terminal part of the protein. Mutations of C195 and H197 in the Nse2 RING-finger-like motif abolish Nse2-dependent sumoylation. *nse2.SA* mutant cells, in which *nse2.C195S-H197A* is integrated as the sole copy of *nse2*, are viable, whereas the deletion of *nse2* is lethal. Smc6 (Rad18) is sumoylated in vivo: the sumoylation level is increased upon exposure to DNA damage and is drastically reduced in the *nse2.SA* strain. Since *nse2.SA* cells are sensitive to DNA-damaging agents and to exposure to hydroxyurea, this implicates the Nse2-dependent sumoylation activity in DNA damage responses but not in the essential function of the Smc5-6 complex.

SUMO is a small ubiquitin-like protein that is covalently attached to target proteins. In yeasts and lower eukaryotes, SUMO is encoded by a single gene, while in higher eukaryotes there are three isoforms, SUMO-1, SUMO-2, and SUMO-3. The attachment of SUMO to target proteins is similar to the process of ubiquitination: SUMO is produced as a precursor protein which is processed to the mature form by SUMO proteases, revealing a diglycine motif. SUMO is subsequently activated by the formation of a thioester bond with a cysteine residue on the SUMO E1-like activator enzyme, a heterodimer known as SAE. SUMO is then passed to an E2-like SUMO conjugator, with which it also forms a thioester bond at a cysteine residue. SUMO ligases have been identified in several organisms. However, whereas E3 ligases are required for the attachment of ubiquitin to targets both in vitro and in vivo, the requirement for SUMO ligases for the attachment of SUMO to targets appears to be less stringent in vitro, and possibly also in vivo. This would be consistent with reports that several SUMO target proteins interact directly with the E2-like SUMO conjugator (e.g., see reference 4).

Two classes of SUMO ligases have been identified. Proteins in the first category contain C3HC4-like RING domains, while proteins in the second category do not. Members of the first category include the *Saccharomyces cerevisiae* proteins Siz1 and Siz2 (16) and the mammalian PIAS family of proteins (20, 32, 38). Members of the second category include the RanBP2 and Pc proteins (18, 33). In *S. cerevisiae* (budding yeast), the *SIZ1* and *SIZ2* genes are not essential for viability, and null mutants do not show the severe cell and nuclear morphologies (16) that are observed with mutants that are defective in other components of the sumoylation system (17, 39). It remains unclear if there are additional SUMO ligases in *S. cerevisiae* or if the SUMO ligases serve to facilitate only a subset of SUMO conjugation reactions, with the remainder being driven by direct interactions with the E2-like conjugator.

In *Schizosaccharomyces pombe* (fission yeast), SUMO is encoded by the *pmt3* gene (44), while the SAE heterodimer and the SUMO conjugator are encoded by the *fub2*, *rad31*, and *hus5* genes, respectively (1, 12, 40, 44). Early analyses of *rad31* and *hus5* mutants indicated that cells defective in SUMO conjugation were sensitive to DNA-damaging agents, such as UV and ionizing radiation (IR), and to the DNA synthesis inhibitor hydroxyurea (HU) (1, 40). These results imply that sumoylation is required for the DNA damage response in fission yeast. Several DNA replication and repair proteins have recently been shown to be sumoylated. These include PCNA in *S. cerevisiae* (14, 41), topoisomerase I and thymine DNA glycosylase in humans (9, 25, 27), topoisomerase II in both humans and *S. cerevisiae* (2, 24), and Rad22 (the homologue of Rad52) in *S. pombe* (12). In the case of *S. cerevisiae* PCNA, sumoylation occurs on two sites in the protein, one of which is also targeted by ubiquitin. Sumoylation of PCNA occurs during normal S phase, while mono- and polyubiquitination are required for different modes of replication past DNA damage (14, 41).

The precise function of SUMO modification remains unknown. Unlike ubiquitination, sumoylation does not target proteins for proteasome-mediated destruction. SUMO modification has been reported to have a range of effects on protein function, but there is no unifying theme underlying how these effects are mediated. In some cases, SUMO and ubiquitin compete for the same lysine residue, e.g., in the cases of IκBα and PCNA (7, 14, 41). Sumoylation has been proposed to antagonize both ubiquitin-dependent degradation (in the case

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of IκBα) and ubiquitin-mediated changes in protein function (in the case of PCNA). SUMO modification has also been shown to affect protein localization and protein-protein interactions. For example, sumoylation of PML is required for the recruitment of Daax and Sp100 to discrete subnuclear bodies termed PODs (15, 22, 46), and sumoylation of RanGAP1 is required for its association with RanBP2 (23, 35).

Smc6 (Rad18) is a member of the SMC family of proteins and interacts with Smc5 (Spr18) to form the core of an Smc5-6 complex in fission yeast (8, 21) that is required for several aspects of DNA metabolism. Smc6 mutants (*rad18.X* and *rad18.74*) have been characterized as defective in recombinational repair processes (21, 31) and in the maintenance of the DNA integrity checkpoint in the presence of persistent unrepaired DNA damage (45). Unlike proteins involved in homologous recombination and checkpoint functions in fission yeast, the Smc5-6 complex also has an essential function (21). The nature of this defect is unclear, but it results in spontaneous checkpoint activation (10) and may be related to chromosomal fragmentation over several generations of growth (28). Smc6 (Rad18) and Smc5 (Spr18) have been shown to form a high-M_r complex (8) which contains several non-SMC proteins (26; see also the accompanying paper [39a]), namely, Nse1, Nse2, Nse3, and Rad62.

We demonstrate here that Nse2 is an autosumoylating SUMO ligase and confirm that it is part of the Smc5-6 complex. Using an in vitro sumoylation assay to analyze constituents of the Smc5-6 complex, we found that Smc6 (Rad18) and Nse3 are sumoylated in an Nse2-dependent manner but that Smc5 (Spr18) and Nse1 are not. Mutations of two residues in the C3HC4 RING-like domain of Nse2 resulted in a loss of in vitro SUMO ligase activity, and a corresponding strain (*nse2.SA*) containing the mutated gene as a single copy was viable but failed to efficiently sumoylate Smc6 (Rad18) in vivo. *nse2* deletion cells were inviable, whereas *nse2.SA* cells were viable but sensitive to DNA-damaging agents and to the DNA synthesis inhibitor HU.

MATERIALS AND METHODS

Strains and plasmids. The wild-type *S. pombe* strain used for experiments was sp0.011 (*ade6-704 ura4-Δ18 leu1-32 h*$^-$) unless otherwise stated. *rad18.X* was described previously (21), *rad18.74* was obtained from M. O'Connell (45), and *rad18.T2* and *rhp51.d* were described elsewhere (29, 39a). The *nse2* open reading frame (ORF) was amplified from cDNA by a PCR using the following primers: E32_EcoRI (5′ GAATTCAAATGAGTGTGAAGCACAATTAAAAAC 3′) and E32_NcoI (5′ CCATGGGACTAAGCTTCTTTTAAATTAC 3′). The *rad18* (*smc6*) and *spr18* (*smc5*) ORFs have been described elsewhere (8, 21). The *nse1* and *nse3* ORFs and partial sequences of *smc6* (*rad18*) and *nse2* were described in the accompanying paper (39a). The *nse2* ORF was deleted by transformation of a diploid strain with a DNA fragment comprising 1 kb of the *nse2* 5′ region, *ura4*, and 1 kb of the *nse2* 3′ region. The 5′ region was amplified by use of the following primers: Nse2_KpnIF (5′ CTGGTACCGAATCAGACGAGCAAGA ATCTCG 3′) and Nse2_XhoIR (5′ GTGCTCGAGTCATTTACGATTCCATT CGAG 3′). The 3′ region was amplified by the use of primers Nse2_EcoRIF (5′ GAATTCTATTTACATTGTAGTACTGATCCCGG 3′) and Nse2_PstIR (5′ CTGCAGCATCAACAGTTGTTCTGCCTCTCAGAC 3′). Truncated forms of Nse2 were created as described by Sergeant et al. (39a). The *nse2.SA* mutant was created by use of the primers Nse2_C195S,H197A.F (5′ CCCAATATTATCG ACAGCCTCTAATGCTTTTTATGAAAAAGATGC 3′) and Nse2_C195S, H197A.R (5′ GCATCTTTTTCATAAAAAGCATTAGAGGCTGTCGATAAT ATTGGG 3′). The *nse2.SA* mutant sequence was integrated into the genome with the *ura4* gene cloned between the 3′ end of the *nse2* ORF and the *nse2* 3′ region amplified as described above. A wild-type control (*nse2.CH*) containing the *ura4* gene adjacent to the wild-type *nse2* sequence was created in parallel in a similar manner.

Proteins were expressed in *Escherichia coli* as glutathione *S*-transferase (GST) fusions by use of a modified form of pGEXGH (a gift from H. Lindsay, Sussex, United Kingdom) or as His-tagged proteins by the use of pET15b (Novagen). For expression in *S. pombe*, cDNAs were cloned into pREP42MH or pREP41MH under the control of a modified form of the *nmt1* promoter (6).

Protein methods and in vitro sumoylation assay. An in vitro sumoylation assay was used as described previously (12). *pmt3.GG* and *pmt3.GG,K30R* were created by site-directed mutagenesis as described elsewhere (J. Ho F. Z. Watts, submitted for publication). Gel filtration was performed with 200-ml exponentially growing cultures. Cells were harvested, washed, and then resuspended in 1 ml of lysis buffer (45 mM HEPES [pH 6.8], 300 mM KCl, 5 mM EGTA, 12 mM NaF, 10% glycerol, 80 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 3 mM $MgCl_2$, and a protease inhibitor cocktail consisting of 5 μg each of trypsin inhibitor, pepstatin, leupeptin, and aprotinin/ml, 10 μg each of bestatin and E-64/ml, and 50 μg of chymostatin/ml). The cells were then broken in a ribolyser, and cell debris was removed by centrifugation twice at 45,000 × *g* for 10 min. Proteins (1.5 mg) were loaded onto a Superdex 200 column, and 0.5-ml fractions were collected. Fifteen microliters of each fraction was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Total cell extracts were prepared by the use of trichloroacetic acid as described by Caspari et al. (5). Ni^{2+} pull-down experiments were performed as described previously (12). Immunoprecipitation was undertaken with cells in which the genomic *smc6* (*rad18*) gene was N-terminally tagged with the c-*myc* epitope (8) as in the accompanying paper (39a).

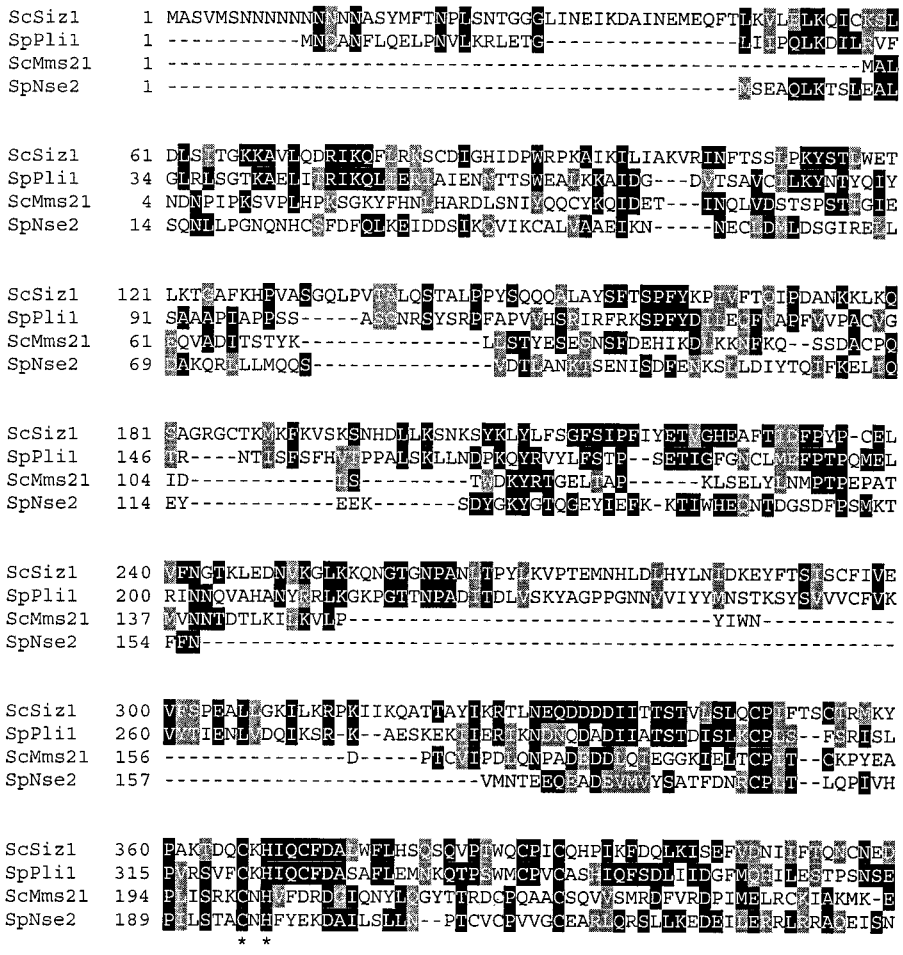
Anti-SUMO antisera were raised as described previously (12), anti-GST antisera were a gift from S. Morley (Sussex, United Kingdom), anti-Smc5 (Spr18) antisera were described previously (8), and anti-Smc6 (Rad18) and anti-Nse1 antisera were described in the accompanying paper (39a). Anti-Nse2 antisera were prepared by the use of a recombinant Nse2 protein, which was prepared by thrombin cleavage of GST-tagged Nse2 to remove the GST, followed by injection into New Zealand White rabbits. The antibodies were affinity purified with glutathione-Sepharose beads to which GST-Nse2 had been cross-linked. Anti-tubulin monoclonal antibodies were purchased from Sigma.

Analysis of DNA damage and HU sensitivity. UV and IR sensitivities were analyzed as described previously (30). HU and methyl methanesulfonate (MMS) sensitivities were analyzed by plating cells on yeast extract-agar plates containing 6 mM HU or 0.005% MMS. Synchronous cultures were prepared with lactose gradients (3). G_2 cells were irradiated with 200 or 400 Gy of IR before incubation at 30°C. Samples were taken at 20-min intervals postirradiation for DAPI (4′,6′-diamidino-2-phenylindole) staining. Pulsed-field gel electrophoresis was performed with 20 A_{595} units of untreated exponentially growing cells or treated cells at various times after exposure to 450 Gy of IR. The cells were washed twice with CSE (20 mM citrate-phosphate [pH 5.6], 40 mM EDTA, 1.2 M sorbitol) and then incubated with 5 ml of CSE containing zymolyase (1.5 mg/ml) at 37°C for 1 h. The cells were then resuspended in 300 μl of TSE (10 mM Tris-HCl [pH 7.5], 45 mM EDTA, 0.9 M sorbitol) and warmed to 37°C. Next, 1.3 volumes of 1% LGT agarose were added, and 100-μl aliquots were dispensed into a plug mold. The cells were lysed by incubating the plugs in a solution containing 50 mM Tris-HCl (pH 7.5), 25 mM EDTA, and 1% SDS for 90 min at 55°C. The plugs were then transferred to a solution containing 1% lauryl sarcosine, 0.5 mM EDTA (pH 9.5), and 0.5 mg of proteinase K/ml and incubated at 55°C for 48 h. Fresh proteinase K was added (to 0.5 mg/ml) after 24 h. The plugs were inserted into wells in 0.8% agarose gels in 1× Tris-acetate-EDTA. The gels were run for 48 h with a pulse time of 1,800 s at 2 V/cm and an angle of 100°. The gels were then stained with ethidium bromide and photographed.

RESULTS

***nse2* encodes a SUMO ligase.** BLAST searches using the *S. cerevisiae* Siz1 SUMO ligase protein identified a related protein in the fission yeast *S. pombe* encoded by ORF SPAC1687.05. A characterization of this ORF, which we have named Pli1, will be described elsewhere (Ho et al., submitted). Using the Pli1 amino acid sequence to search the database for related *S. pombe* proteins, we identified the protein encoded by SPAC16A10.06c, the closest *S. pombe* homolog of the *S. cerevisiae* Mms21 protein. An alignment of the N-terminal region

A.



B.

	Nse2	Pli1	Mms21	Siz1
Nse2	100 (100)	10 (24)	21 (25)	10 (23)
Pli1		100 (100)	16 (21)	23 (43)
Mms21			100 (100)	12 (23)
Siz1				100 (100)

FIG. 1. Nse2 has homology to SUMO ligases. (A) Sequence alignment of the N-terminal sequences of *S. pombe* Nse2 (aa 1 to 237 or 250) and Pli1 (aa 1 to 375 or 727) with those of *S. cerevisiae* Siz1 (aa 1 to 420 or 750) and Mms21 (1 to 253 or 267), created by use of the ClustalW program. Dark shading, identical amino acids; light shading, conserved residues, *, conserved Cys and His residues (C195 and H197 in Nse2). (B) Percentages of identity between *S. cerevisiae* Mms21 and Siz1 and *S. pombe* Pli1 and Nse2. The first figure in each pair is the percent identity along the full length of the proteins, and the figure in parentheses is the percent identity between RING-finger-like domains.

of these four proteins is shown in Fig. 1A. The SPAC16A10.06c ORF encodes the Nse2 protein, which was recently identified both by McDonald et al. (26) and by ourselves (39a; also see below) as part of the Smc5-6 complex. We amplified the coding sequence of Nse2 from cDNA (see Materials and Methods). A sequence analysis of the cDNA and a comparison with the genomic sequence indicated that the gene contains two introns, I and II, of 247 and 115 nucleotides (nt), respectively. Intron II is larger than that predicted by McDonald et al. (26) and involves a different, upstream 5′ splice site, the sequence of which (5′ GTACGT 3′) closely matches the 5′ splice site consensus sequence. The cDNA thus encodes a protein with a predicted M_r of approximately 29 kDa and with 250 amino acids (aa), which is 17 aa shorter than that previously predicted (26).

Pairwise comparisons (Fig. 1B) of the full sequences of Nse2, Mms21, Pli1, and Siz1 (which comprise 250, 267, 727, and 750 aa, respectively) indicated that the two pairs of sequences with the highest identities are Pli1 and Siz1 (23% identical) and Nse2 and Mms21 (21% identical). A key feature

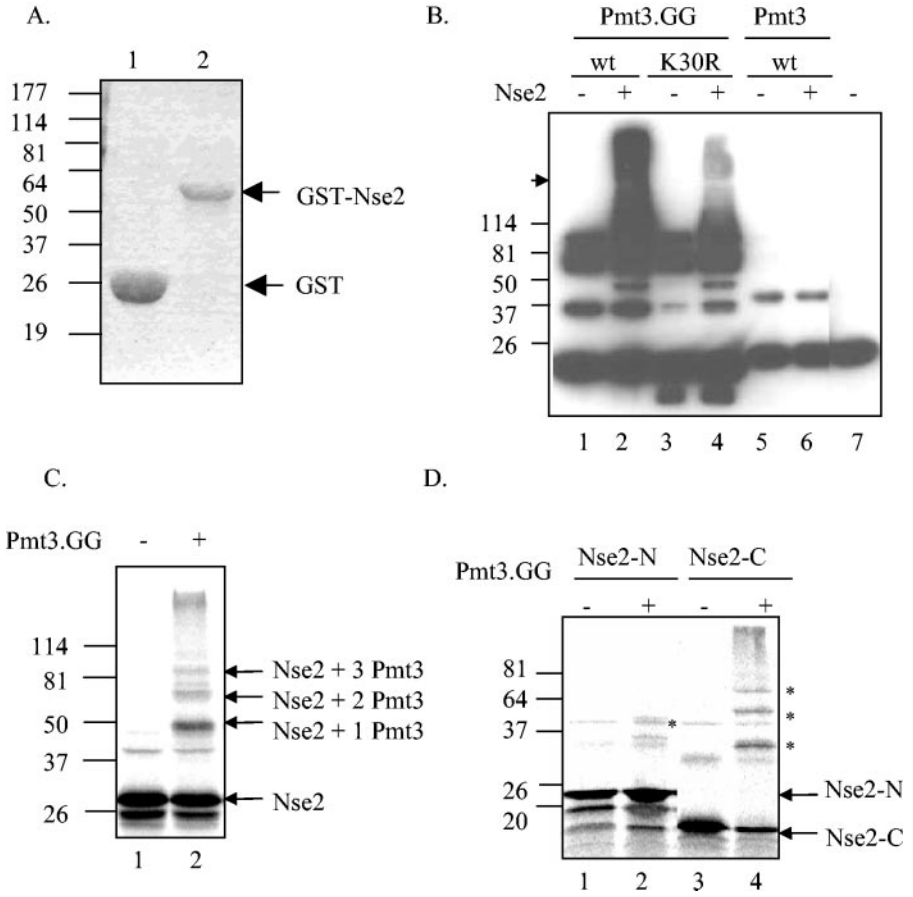


FIG. 2. Nse2 has SUMO ligase activity. (A) Expression of Nse2 in *E. coli*. ORF SPAC16A10.06c, which was amplified by PCR and cloned into pGEX, was expressed in BL21 cells, purified by the use of glutathione-Sepharose (lane 2), and compared to extracts from cells transformed with the empty pGEX vector (lane 1). (B) Pmt3 sequence requirements. The results of an in vitro sumoylation assay to test three forms of Pmt3 (Pmt3.GG, Pmt3.GG,K30R, and Pmt3) are shown. Hus5 (0.05 μg/μl) was used in all assays. The products were analyzed by Western blotting with anti-Pmt3 antisera. The species migrating at about 35 and 50 kDa are likely SUMO chains and reaction intermediates. The arrow indicates the junction of stacking and separating gels. (C) Nse2 is itself sumoylated. An in vitro sumoylation assay was performed with ^{35}S-labeled Nse2 as a substrate and with 0.05 μg of Hus5/μl. The products were separated by SDS-PAGE and detected with a phosphorimager. The species migrating at 37 kDa is a nonspecific band. (D) Sumoylation of Nse2 occurs predominantly on the C-terminal part of the protein in vitro. An in vitro sumoylation assay was performed with ^{35}S-labeled N- and C-terminal fragments of Nse2 as indicated. Nse2-N, aa 1 to 178; Nse2-C, aa 114 to 250. The products were detected as described above. *, modified forms.

of the Siz1 class of SUMO ligases is the presence of a C3HC4 RING-finger-like domain. A related sequence occurs in the Nse2 protein, between aa 179 and 219. Focusing on this region of the proteins, we again observed the highest sequence identity between Pli1 and Siz1 (43%), with all other pairwise comparisons yielding figures of 21 to 25%.

SUMO ligase activity. The cDNA encoding the Nse2 ORF was cloned into the *E. coli* expression vector pGEX and transformed into *E. coli* cells, and the GST-tagged fusion protein was purified by the use of glutathione-Sepharose from induced cell extracts as described in Materials and Methods. The resulting protein migrated close to the predicted size of 55 kDa for GST-tagged Nse2 (Fig. 2A, lane 2). In order to determine whether Nse2 has SUMO ligase activity, we assayed the fusion protein by using a previously established in vitro sumoylation system (12). SUMO has been shown to be capable of forming chains in vitro, as determined by the production of high-M_r SUMO-containing species in vitro in the absence of a target protein (16). The production of these conjugated species is dependent on the addition of the E1-like SAE heterodimer, the E2-like conjugator Hus5, and Pmt3 (SUMO) (data not shown). Under conditions in which the level of the E2-like conjugator Hus5 was kept low (0.05 μg/μl), the level of SUMO conjugates also remained low (Fig. 2B, lane 1). Under these conditions, the addition of Nse2 (0.2 μg/μl) resulted in a substantial increase in the level of SUMO chains (lane 2). This is consistent with Nse2 having SUMO ligase activity when assayed for SUMO chain formation.

Previous studies of Pli1 suggested that K30 is the major acceptor lysine for chain formation by SUMO (Ho et al., submitted). A comparison of lanes 2 and 4 in Fig. 2B indicates that Pmt3.GG (K30R) is a less efficient substrate for chain formation by Nse2 than is Pmt3.GG. This result indicates that K30 is a major acceptor site for Pmt3 modification but that it may not be the only lysine capable of accepting Pmt3 linkage in this assay. Previous reports indicated that the mature form of SUMO (Pmt3.GG) is required for both chain formation and sumoylation of target proteins (17; Ho et al., submitted). We

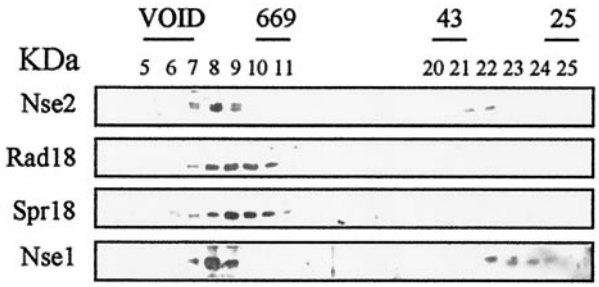


FIG. 3. Nse2 is present in high-molecular-mass complexes. A wild-type cell extract was analyzed by gel filtration on a Superdex 200 column and Western blotted with antisera as indicated.

therefore determined whether this is also the case for Nse2-mediated sumoylation. Figure 2B, lanes 5 and 6, indicates that SUMO chains are not formed when the sole source of SUMO is the precursor form (Pmt3), even in the presence of Nse2 (lane 6). This is in contrast to what was observed with the mature form (Pmt3.GG, lanes 1 and 2). Together, these data demonstrate that the activity associated with Nse2 behaves like a bona fide SUMO ligase activity in vitro.

Nse2 is itself sumoylated. Many of the previously characterized SUMO ligases have been demonstrated to be sumoylated themselves (18, 36, 43). To determine whether Nse2 can also be modified by SUMO, we tested Nse2 labeled with [^{35}S]methionine (by in vitro translation) as a potential substrate in the in vitro sumoylation assay described above. Figure 2C indicates that the incubation of ^{35}S-labeled Nse2 with the SAE heterodimer, Hus5, and SUMO (Pmt3.GG) resulted in the appearance of slower migrating forms with sizes consistent with their being sumoylated species of Nse2 (lane 2). The presence of these species was dependent on SAE, Hus5, and Pmt3 (data not shown), further confirming that they are sumoylated forms of Nse2.

At least three modified forms of Nse2 were observed (Fig. 2C, lane 2). These may have arisen due to the use of more than one SUMO acceptor site or to the production of SUMO chains. Sumoylation occurs on lysine residues that are generally in the context of a consensus sequence, ψKxE (where ψ is a hydrophobic amino acid). Nse2 has 17 lysine residues. We separately tested the N-terminal 178-aa (1–178) region of Nse2 and a C-terminal fragment of 127 aa (114–250) which contains the C3HC4 RING-finger-like domain for the ability to be sumoylated in vitro. Figure 2D, lane 2, indicates that there was a weak modification of the N-terminal fragment when it was incubated in the presence of all of the assay components (compare lane 2 to the situation in the absence of added Pmt3.GG [lane 1]). In contrast, when the C-terminal fragment was used in the in vitro assay, we found a more profound modification in the presence of all of the assay components (compare lane 4 to the situation in the absence of added Pmt3.GG [lane 3]).

Nse2 complexes. Nse2 was recently identified as a component of the Smc5-6 complex in fission yeast (26; see also the accompanying paper [39a]). The SMC proteins Smc6 (Rad18) and Smc5 (Spr18) form the core of a multiprotein complex in *S. pombe*. We investigated whether Nse2 is present in *S. pombe* extracts solely in a Smc6 (Rad18)-containing complex or whether it is also present in other complexes or in a monomer form. Total soluble cell extracts prepared from wild-type *S. pombe* were analyzed by gel filtration as described in Materials and Methods, and the resulting fractions were analyzed by Western blotting. Figure 3 indicates that the majority of the Nse2 protein comigrated with Smc6 (Rad18). Nse2 was present in fractions 7 to 9, which corresponded to molecular masses of >670 kDa. In contrast, Smc6 (Rad18) and Smc5 (Spr18) were present in these fractions but were also abundant in fractions 10 and 11, corresponding to molecular masses of approximately 670 kDa. The absence of Nse1 and Nse2 from fractions 10 and 11 suggests that there may be other forms of the Smc6-Smc5 (Rad18-Spr18) complex, one of which contains Nse1 and Nse2 and another that does not. An alternative explanation is that Nse1 and Nse2 are less tightly associated with the complex and dissociate during purification. Nse1 and Nse2 were also observed in fractions 23 to 26 and in fractions 21 and 22, respectively, consistent with the sizes expected for the monomeric Nse1 and Nse2 proteins.

Nse2 is a SUMO ligase for Smc6 (Rad18) and Nse3. We wished to determine whether other members of the Smc5-6 (Spr18-Rad18) complex are sumoylated and, if so, whether these modifications are dependent on Nse2. Figure 4A to D show the results of an in vitro sumoylation assay using Smc6 (Rad18), Smc5 (Spr18), Nse1, and Nse3 as target proteins. For these assays, the level of Hus5 was kept low (0.05 μg/μl). In the absence of Nse2, modified forms of Smc6 (Rad18) were not observed (panel A, lane 2), and only low levels of modified forms of Nse3 were observed (panel D, lane 2). In both of these cases, the addition of Nse2 to the reaction (0.2 μg/μl) resulted in an increase in the levels of the sumoylated forms compared to those observed in the absence of Nse2 (cf. lanes 3 in panels A and D). The addition of the other *S. pombe* SUMO ligase, Pli1 (0.2 μg/μl), resulted in a low level of sumoylation of Rad18 (panel A, lane 4), but this was lower than that observed with Nse2 (lane 3). In contrast to what we observed with Smc6 (Rad18) and Nse3, we did not detect any sumoylated forms of Smc5 (Spr18) or Nse1 in the in vitro assay (Fig. 4B and C), even when we used large amounts (0.15 μg/μl) of Hus5 (data not shown). Consistent with this, we did not observe modification even in the presence of Nse2 (lane 3). These data indicate that Smc6 (Rad18) and Nse3, but not Nse1 or Smc5 (Spr18), are modified by SUMO in vitro and that this modification is stimulated by Nse2-dependent SUMO ligase activity.

Smc6 (Rad18) is sumoylated in vivo. To investigate the possible biological significance of the sumoylation of Smc6 (Rad18) in vitro, we investigated whether Smc6 (Rad18) is modified in vivo. First, we overexpressed Myc-His-tagged Smc6 (Rad18) in wild-type cells and purified the expressed protein by using Ni^{2+}-agarose beads (see Materials and Methods). The purified proteins were analyzed by Western blotting with anti-Myc (Smc6) and anti-Pmt3 (SUMO) antisera. Figure 4E indicates that no species were observed that cross-reacted with the anti-Myc antiserum when an extract was prepared from cells transformed with the empty vector, pREP42MH (lane 1), and that after the purification of Ni^{2+} binding proteins from this control extract, no anti-Myc-reactive species were detected (lane 3). In contrast, species of 135 and 155 kDa were observed in total extracts prepared from cells transformed with pREP42MH-Smc6 (Rad18) (lane 2). After puri-

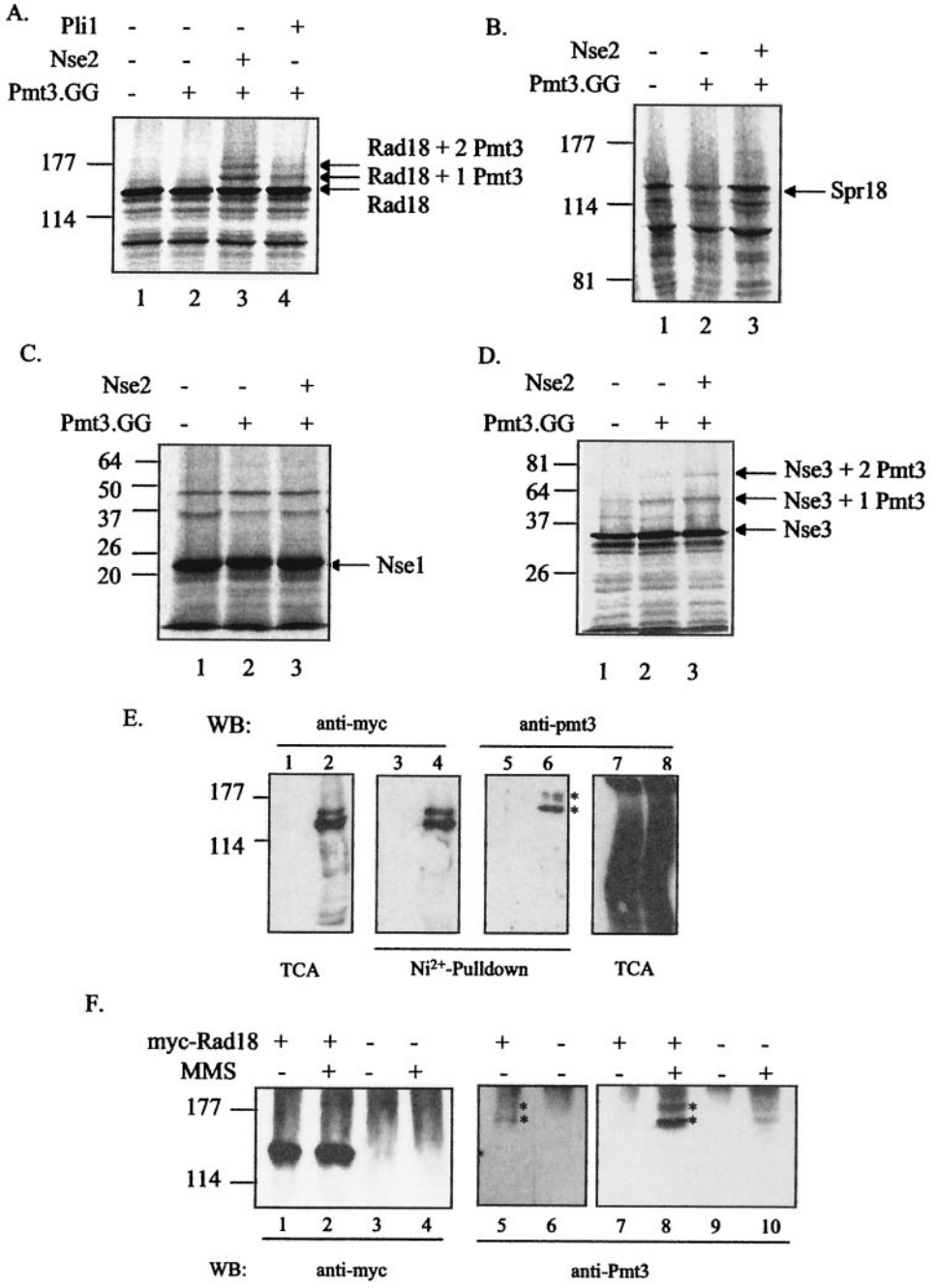


FIG. 4. Smc6 (Rad18) and Nse3 are sumoylated in an Nse2-dependent manner in vitro, and Smc6 (Rad18) is sumoylated in vivo. (A to D) ^{35}S-labeled proteins were tested with in vitro sumoylation assays using 0.05 μg of Hus5/μl and 0 or 0.2 μg of Nse2 or Pli1/μl, as indicated. (A) Smc6 (Rad18); (B) Smc5 (Spr18); (C) Nse1; (D) Nse3. (E) Smc6 (Rad18) is sumoylated in vivo. Ni^{2+} pull-down assays were performed with cell extracts from cells transformed with pREP42MH (empty vector) (lanes 1, 3, 5, and 7) or pREP42MH-Rad18 (Smc6) (lanes 2, 4, 6, and 8). TCA, total cell extract controls. Western analysis was conducted with anti-Myc or anti-Pmt3 antisera as indicated. (F) Sumoylation of Smc6 (Rad18) expressed at wild-type levels increases after exposure to MMS. Lysates (containing 50 mg of total protein) prepared from a Myc-tagged Smc6 (Rad18) strain and an untagged control with or without exposure to MMS (0.01%) were incubated overnight at 4°C with an anti-Myc antibody that had been previously cross-linked to protein G-Sepharose beads. The beads were washed extensively, and bound proteins were eluted by incubation with 100 mM glycine, pH 2.3, separated by SDS-PAGE, and analyzed by Western blotting (WB) with anti-Myc and anti-Pmt3 antibodies, as indicated. Lanes 1, 2, 5, 7, and 8, Myc-tagged Rad18 (Smc6) strain; lanes 3, 4, 6, 9, and 10, wild type (untagged Rad18 [Smc6]). Lanes 5 and 6 are the same as lanes 7 and 9, but with longer exposure times. *, modified forms.

fication of the Ni^{2+} binding proteins by the use of Ni^{2+}-agarose (lane 4), these same species could be detected. Probing a parallel blot of these Ni^{2+}-agarose-purified samples with anti-Pmt3 antisera (lanes 5 and 6) allowed the detection of species of 155 and 175 kDa (lane 6). The faster migrating (155 kDa) band of the anti-Pmt3-reactive species was coincident with the slower migrating anti-Myc-reactive species observed in lanes 2 and 4. The 155- and 175-kDa species likely corresponded to

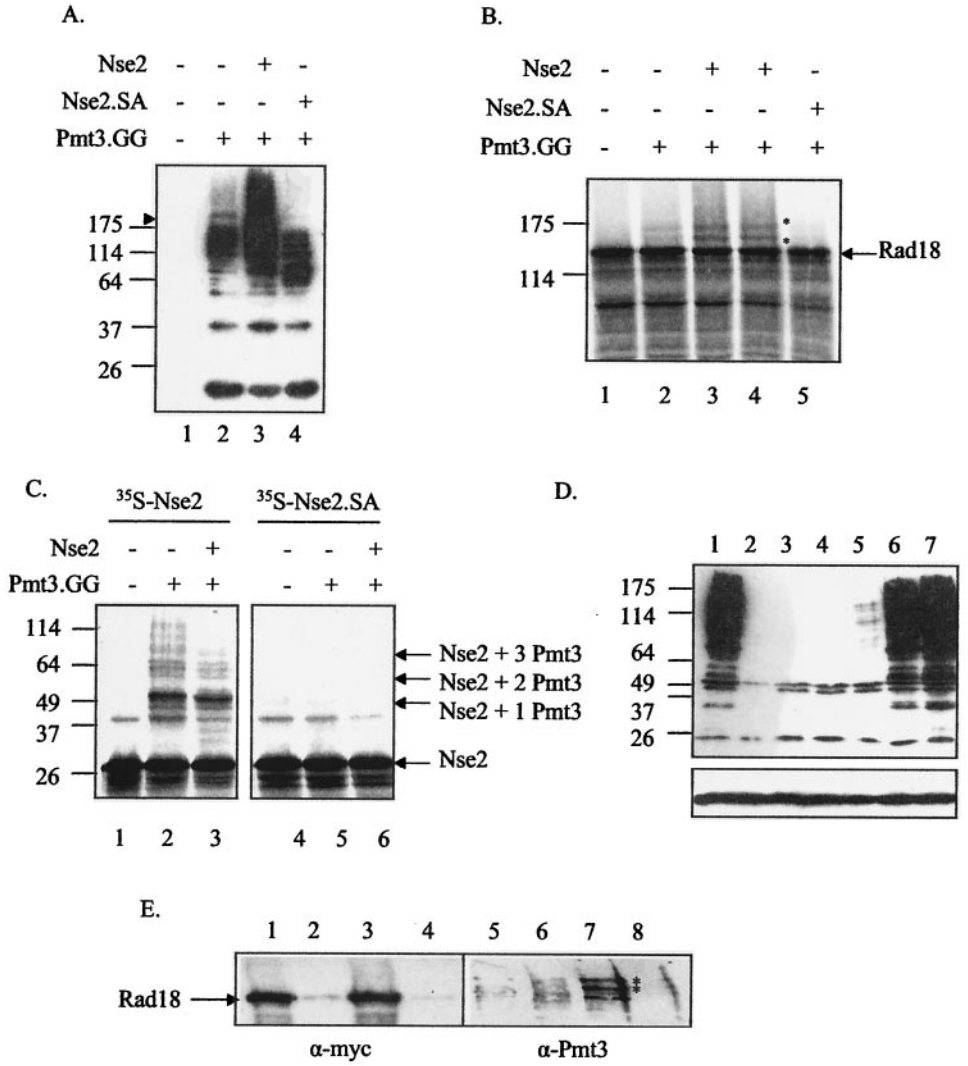


FIG. 5. Mutation of C195 and H197 results in a loss of sumoylating activity in vitro and in vivo. (A) Nse2.SA protein is unable to form SUMO chains in vitro. An in vitro sumoylation assay was performed in the absence of an added target protein, with 0.05 μg of Hus5/μl. The products were detected by Western analysis with anti-Pmt3 antisera. The arrow indicates the junction of stacking and separating gels. (B) Nse2.SA does not facilitate sumoylation of Smc6 (Rad18) in vitro. An in vitro sumoylation assay was performed with ^{35}S-labeled Smc6 (Rad18) and 0.05 μg of Hus5/μl. The products were detected with a phosphorimager. (C) Nse2.SA is not sumoylated in vitro. An in vitro sumoylation assay was performed with an ^{35}S-labeled wild-type or mutant (Nse2.SA) Nse2 protein. The products were detected as described for panel B. (D) Western analysis of total cell extracts probed with anti-Pmt3 antisera (top) or anti-tubulin antisera (bottom). Lane 1, wild type (sp.011); lane2, *rad31.d*; lane 3, *hus5.17*; lane 4, *hus5.62*; lane 5, *pli1.d*; lane 6, *nse2.SA*; lane 7, *nse2.CH*. (E) *nse2.SA* cells have reduced levels of sumoylated Smc6 (Rad18). Extracts of wild-type (*nse2.CH*) (lanes 3 and 4) and mutant (*nse2.SA*) (lanes 1 and 2) strains harboring either pREP41MH-Rad18 (Smc6) (lanes 1 and 3) or the empty pREP41MH vector (lanes 2 and 4) were bound to nickel beads under denaturing conditions, and the bound proteins were analyzed by immunoblotting with either anti-Myc or anti-Pmt3 antibodies as indicated. *, modified forms.

mono- and disumoylated forms of Smc6 (Rad18). Again, these anti-Pmt3-reactive species were not observed in extracts of cells transformed with the empty vector (lane 5). (Lanes 7 and 8 show the results of probing the total cell extract with anti-Pmt3 antisera.) Taken together, these data indicate that Smc6 (Rad18) is sumoylated in vivo.

To demonstrate that Smc6 (Rad18) is sumoylated when expressed at wild-type levels, we performed immunoprecipitation with extracts of cells in which Smc6 (Rad18) was tagged with Myc in the genome (Fig. 4F, lanes 1 and 5), using an untagged strain as a negative control (lanes 3 and 6). Probing the Western blots with anti-Pmt3 antisera indicated two modified forms of Smc6 (Rad18) after a long exposure of the film (lane 5), at 155 and 175 kDa, which were absent from the negative control (lane 6). This demonstrates that Smc6 (Rad18) is sumoylated in vivo when expressed at wild-type levels. Since Smc6 (Rad18) has roles in the DNA damage response, we were interested to determine whether sumoylation of the protein was affected by the exposure of cells to DNA-damaging agents. Even after a short exposure of the film, strong bands of sumoylated Smc6 (Rad18) were observed for cells 3 h after the treatment of cells with MMS (0.01%) (lane 8), in contrast to the weaker levels of sumoylation in untreated cells (lane 7).

The RING domain of Nse2 is required for sumoylation in vitro. To determine whether the C3HC4 RING-finger-like domain of Nse2 is required for its sumoylation activity, we mutated Cys195 and His197 to Ser and Ala, respectively. The mutant protein (Nse2.SA) was expressed in *E. coli*, and its sumoylation activity was assayed in vitro for both the ability to promote SUMO chain formation and the ability to act as a SUMO ligase for Smc6 (Rad18). Figure 5A indicates that, whereas wild-type Nse2 has the ability to form high-M_r SUMO-containing species (lane 3), this ability is lacking in the Nse2.SA mutant protein (lane 4). Figure 5B indicates that, as previously observed in Fig. 4A, the wild-type Nse2 protein increased the level of sumoylated Smc6 (Rad18) forms (lanes 3 and 4) compared to equivalent reactions lacking Nse2 (lane 2). In contrast, the Nse2.SA mutant protein was not able to promote the appearance of modified forms of Smc6 (Rad18) (lane 5).

If the Nse2.SA protein is ligase-dead, we should be able to determine if the SUMO modification we observed for Nse2 itself was a result of in *cis* autosumoylation or of an in *trans* reaction by which one Nse2 molecule promoted the ligation of SUMO onto a second Nse2 molecule. We thus tested the ^{35}S-labeled mutant Nse2 protein for its ability to be sumoylated. Figure 5C indicates that, in contrast to wild-type Nse2 (lanes 1 to 3), the Nse2.SA mutant protein was not modified (lanes 5 and 6). Importantly, this remained the case when the wild-type Nse2 protein (0.2 μg/μl) was added to the reaction (lane 6). This indicates that the Nse2.SA mutant protein is not recognized as a substrate by wild-type Nse2, suggesting that the SUMO modification of Nse2 occurs by autosumoylation. This was confirmed in lane 3, in which the addition of wild-type unlabeled Nse2 to a reaction using ^{35}S-labeled wild-type Nse2 decreased the level of sumoylation of the labeled species. Given the ratio of mutant to wild-type Nse2 in these reactions, we do not favor a model whereby the mutant protein acts as a dominant-negative inhibitor of wild-type Nse2.

The *nse2.SA* mutant has reduced levels of sumoylated Rad18 (Smc6). Smc6 (Rad18), Smc5 (Spr18), and Nse1 are all essential genes (8, 21, 26). We therefore determined whether or not *nse2* is essential for viability. One copy of the *nse2* gene was deleted from a diploid strain (see Materials and Methods), and tetrads were dissected from sporulating heterozygotes. Each tetrad resulted in only two viable colonies, all of which were *ura*$^-$, indicating that *nse2* is essential for cell viability. This confirms the results of MacDonald et al. (26). We were next interested in determining whether cells containing the *nse2.SA* mutation were viable. The *nse2.SA* mutant sequence was therefore integrated into the genome as the sole copy of *nse2* (see Materials and Methods). Colonies containing the *nse2.SA* mutant gene were successfully obtained by use of a haploid strain for transformation, indicating that the mutation does not result in lethality. Backcrossing ensured that suppressor mutations were not required for this viability.

The *nse2.SA* mutant strain grew equally well at a range of temperatures (25 to 36°C), with generation times resembling those of the wild type, indicating that it is not temperature sensitive. By performing Western blotting, we showed that the level of Nse2 protein in *nse2.SA* cells was identical to that observed in wild-type total cell extracts (data not shown). Probing total cell extracts with anti-Pmt3 antisera demonstrated that the total sumoylation levels in *nse2.SA* (Fig. 5D, lane 6) resembled those observed for wild-type cells (lanes 1 and 7). This was in contrast to the situation seen for deletion mutants of the other SUMO ligase, *pli1* (lane 5) (Ho et al., submitted), and for *rad31.d*, *hus5.17*, and *hus5.62* mutants (lanes 2 to 4), in which sumoylation levels were substantially reduced. We have also shown by gel filtration that the Smc5-6 complex was not disrupted in the *nse2.SA* mutant (data not shown).

We have shown that in vitro, the Nse2.SA protein has a reduced ability to direct the sumoylation of Smc6 (Rad18). To determine whether the sumoylation of Smc6 was affected in the *nse2.SA* strain in vivo, we performed Ni^{2+}-agarose affinity purification as described for Fig. 4E. Extracts were prepared from *nse2.SA* and wild-type cells that had been transformed with either the pREP41MH empty vector control or pREP41MH-Smc6 (Rad18). *nse2.SA* and wild-type cells transformed with pREP41MH-Smc6 (Rad18) expressed similar levels of Myc-His-Smc6 (Rad18) (Fig. 5E, lanes 1 and 3, respectively), indicating that the stability of the expressed Smc6 (Rad18) protein was not affected in the mutant strain. After the purification of Myc-His-Smc6 (Rad18) by Ni^{2+}-agarose affinity chromatography from extracts prepared from wild-type cells that had been transformed with pREP41MH-Smc6 (Rad18), bands corresponding to SUMO-containing species of 155 and 175 kDa were observed (Fig. 5E, lane 7). These species were absent from equivalent purification reactions from extracts of wild-type cells that had been transformed with the empty vector (Fig. 5E, lane 8). In contrast, the high-M_r SUMO-containing species were barely detectable in extracts prepared from *nse2.SA* cells that had been transformed with pREP41MH-Smc6 (Rad18) (Fig. 5E, lane 5). These data indicate that the sumoylation of Smc6 (Rad18) is substantially reduced in vivo in *nse2.SA* cells.

The *nse2.SA* mutant is sensitive to DNA-damaging agents and to HU. Since the Smc5-6 complex is required for a range of functions associated with the response to DNA damage in addition to its essential function, we tested the response of the *nse2.SA* mutant to DNA-damaging agents and to exposure to the replication inhibitor HU. Figure 6A and B indicate that the *nse2.SA* mutant was significantly, but not dramatically, sensitive to both UV and ionizing radiation compared to wild-type cells. It was markedly less sensitive to UV and IR than is the *nse2-1* allele (26). Strikingly, *nse2.SA* colonies appeared more slowly than wild-type cells after exposure to UV (data not shown). This is reminiscent of the response we observed for *rad18.X* cells when they were exposed to UV, for which colony formation was retarded substantially (data not shown). *nse2.SA* cells were also sensitive to both HU (6 mM) and MMS (0.005%), a similar profile to that seen for *rad18.X* cells (Fig. 6C).

A previous report demonstrated a direct DNA repair defect associated with *rad18.X* (45). We thus investigated whether *nse2.SA* cells are able to repair DNA double strand breaks by using pulsed-field gel electrophoresis (PFGE). Figure 6D, lane 1, shows the three undamaged chromosomes in wild-type cells. After exposure to 450 Gy of ionizing radiation (lane 2), the chromosomes were damaged, as evidenced by the low-M_r smear of DNA (lane 2). There was an almost complete repair of the damage, as judged by the reappearance of intact chromosomes. The chromosomes in *rad18.X* appeared somewhat damaged even before exposure to IR (lane 11), which was

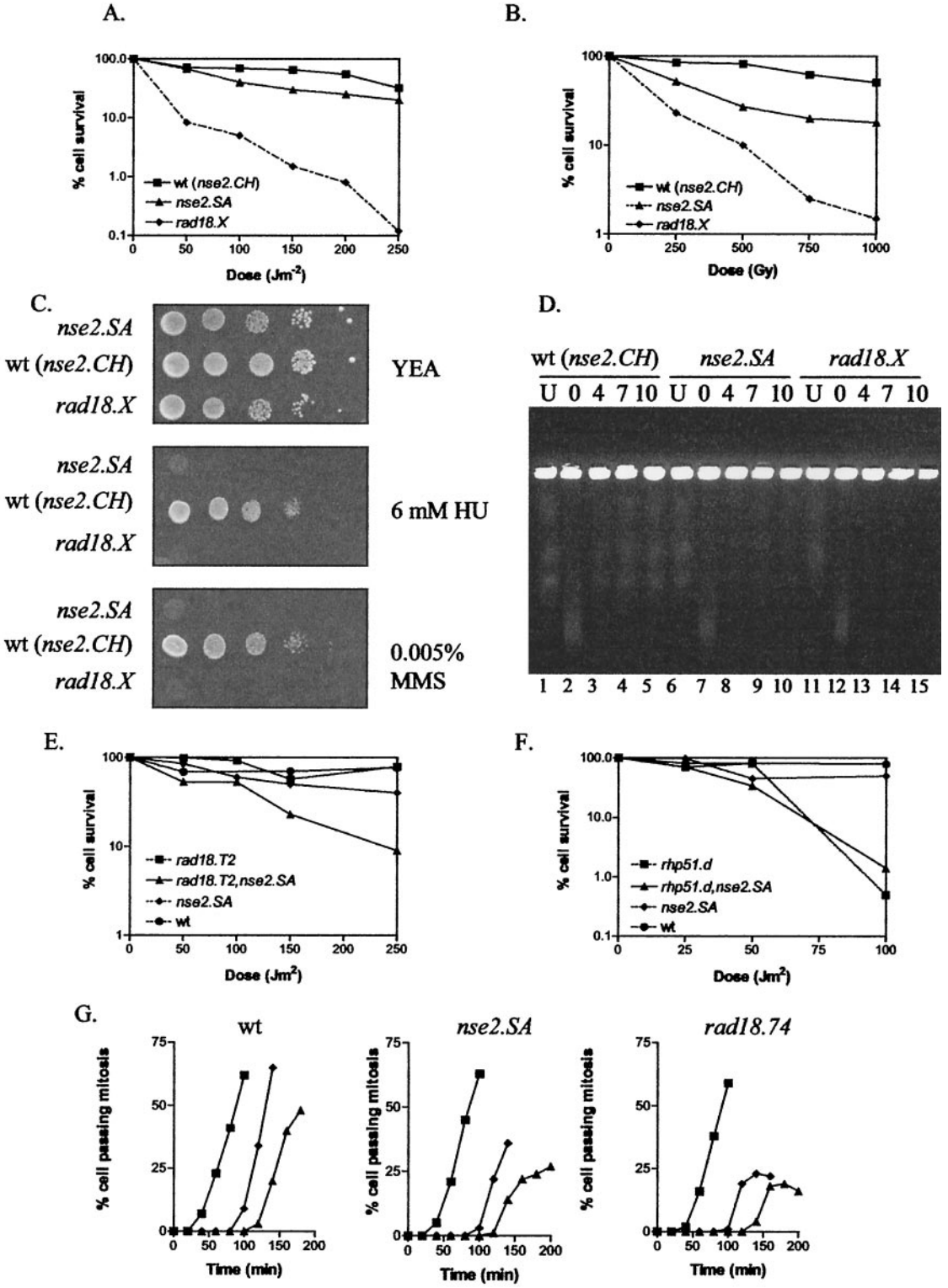


FIG. 6. *nse2.SA* cells are sensitive to DNA-damaging agents. (A) UV survival analysis. (B) IR survival analysis. (C) HU and MMS sensitivities. Five-microliter samples of 10-fold dilutions of exponentially growing cultures (5×10^6/ml) were plated onto yeast extract-agarose plates with supplements as indicated. (D) PFGE. DNAs from wild-type, *nse2.SA*, and *rad18.X* cells before and after exposure to 450 Gy of IR were analyzed by PFGE. (E and F) Epistasis analysis with *rad18.T2* (E) and *rhp51.d* (F) cells. (G) Analysis of DNA damage checkpoint. Cells that were synchronized by lactose gradients were incubated at 30°C after no treatment (■), 200 Gy of IR (◆), or 400 Gy of IR (▲). Samples were taken at 20-min intervals and fixed in methanol. They were stained with DAPI and calcofluor, and the percentages of cells that passed mitosis were assessed.

possibly a sign of a DNA replication defect. Consistent with previous reports (45), intact chromosomes were not seen even 10 h after the exposure of *rad18.X* cells to IR, indicating that they cannot repair double strand breaks. Prior to irradiation, *nse2.SA* cells displayed intact chromosomes (lane 6), implying that DNA replication is not affected in these cells (as a replication defect would result in retention of the DNA in the wells, as seen for the *rad4ts* mutant [37]). The restoration of intact chromosomes in *nse2.SA* cells after IR exposure (lanes 7 to 10) was intermediate between that observed for wild-type (lanes 2 to 5) and for *rad18.X* (lanes 12 to 15) cells, with a fraction of whole chromosomes observed after 7 to 10 h (lanes 9 and 10). These data indicate that *nse2.SA* cells are partially defective in double-strand-break repair after exposure to IR.

We next investigated interactions between the *nse2.SA* mutant and some of the *rad18* alleles. Double mutants of *nse2.SA* with *rad18.X* and *rad18.74* were not viable, and a *nse2.SA rad18.T2* double mutant grew more slowly than a *rad18.T2* mutant. Epistasis analysis indicated that *nse2.SA* and *rad18.T2* are epistatic in their response to IR (data not shown), but not in their response to UV (Fig. 6E). To confirm that the *nse2.SA* repair defect is epistatic with *rhp51*-dependent homologous recombination repair, as previously demonstrated for the *rad18.X* mutant (21) and the *nse2-1* allele (26), we performed epistasis analysis for *nse2.SA* and *rhp51.d*. *nse2.SA* was epistatic with *rhp51.d* for its response to both UV and IR (Fig. 6F and data not shown). Furthermore, like the *rad18.X* mutant, *nse2.SA* was not epistatic with mutations in the nucleotide excision repair pathway (*swi10.d* and *rad16.d* [data not shown]).

We compared the cell cycle arrest kinetics of wild-type, *nse2.SA*, and *rad18.74* cells. Interestingly, asynchronous cultures of *nse2.SA* and *rad18.74* cells both contained a small proportion of elongated cells and cells with aberrant chromosomes (data not shown), suggesting that there is a mitotic defect in these cells. We analyzed checkpoint profiles of irradiated synchronized cultures after irradiation by DAPI staining to determine whether the cells were able to arrest the cell cycle in response to DNA damage. G_2-phase cultures were exposed to 200 or 400 Gy of ionizing radiation, and the percentages of cells that passed mitosis were monitored. As observed with wild-type cells, *nse2.SA* and *rad18.74* cells arrested the cell cycle after exposure to IR in a dose-dependent manner (Fig. 6G) and returned to the cell cycle with similar kinetics to the wild type.

DISCUSSION

The role of sumoylation is beginning to be elucidated for many proteins. For example, sumoylation has roles in enhancing protein-protein interaction capabilities and in conferring specific subcellular localization properties. However, a coherent molecular explanation for the influence of SUMO modification on the change in function for individual proteins is not yet forthcoming. It is possible either that a single physical change in properties underpins the diverse functions assigned to SUMO modification or that the modification can cause specific proteins to acquire one or more of a diverse range of properties. Equally poorly understood is the precise role of the SUMO ligases in promoting SUMO modification. In *S. cerevisiae*, deletion of the genes encoding the Siz1 and Siz2 proteins results in only a weak phenotype (16), whereas the complete loss of SUMO conjugation activity has a more dramatic effect (e.g., see reference 39). This is consistent with the fact that the SUMO conjugators have often been reported to be direct binding partners for SUMO target proteins and that SUMO modification can occur in vitro without the presence of a ligase in a manner that is enhanced by increased concentrations of the conjugator. Thus, it is possible that in vivo, as seen in vitro, SUMO ligases act as efficiency factors for sumoylation rather than being absolutely required for modification. This contrasts with the role of the majority of ubiquitin ligases (11).

In *S. pombe*, a complete loss of SUMO conjugation results in very sick cells that cannot be propagated beyond microcolonies (1, 44). Here we demonstrated that the essential *nse2* gene has SUMO ligase activity. We also demonstrated that the highly conserved Cys and His amino acids in the C3HC4 RING-finger-like domain are required for SUMO ligase activity in vitro. The requirement for this RING-finger-like domain has also been observed for Siz1 and some of the PIAS proteins (19, 32, 42). Our data show, however, that the inviability of the *nse2* null mutant is not a consequence of a lack of Nse2-dependent SUMO ligase activity.

We and others (26, 39a) have shown that Nse2 is a component of the *S. pombe* Smc5-6 complex. Previous reports of C3HC4-like domain-containing SUMO ligases have not identified these proteins as existing as part of larger protein complexes, although RanBP2, a member of a separate class of SUMO ligases, is a component of the nuclear pore (33). Gel filtration of *S. pombe* cell extracts indicated that the majority of the Nse2 protein in these extracts was in the Smc5-6 complex, with only a small amount present in the monomer form. Nse2 was not observed in any other high-M_r complexes. We cannot determine directly whether there is free functional Nse2 in intact cells or whether the small quantity of monomeric Nse2 seen in our fractionation studies was a result of the dissociation of Nse2 from the Smc5-6 complex during the preparation of extracts. However, our data strongly suggest that the major role of Nse2 is performed as part of the Smc5-6 complex.

Of the six components of the Smc5-6 complex, we have shown that three (Smc6 [Rad18], Nse3, and Nse2 itself) are sumoylated in an Nse2-dependent manner in vitro, whereas Smc5 (Spr18) and Nse1 do not appear to be modified. Furthermore, Smc6 (Rad18) was sumoylated in vivo and the levels of the modified form increased substantially after exposure to MMS, implicating that the sumoylation of Smc6 (Rad18) has a role in the DNA damage response. The modification was dramatically reduced in the *nse2.SA* mutant. In vitro, the Nse2.SA mutant protein had lost its SUMO ligase activity, and it is reasonable to predict on the basis of the function of the RING domain in ubiquitin ligases that this will also be the case in vivo. This would imply that one function of Nse2 is to promote the sumoylation of Smc6 (Rad18). It is intriguing that Nse2 appears to bind via its N terminus to Smc5 (Spr18) (39a) and that it sumoylates the Smc5 partner protein Smc6 (Rad18) via the RING motif in the C terminus. The fact that the ligase-dead *nse2.SA* RING domain mutant was viable whereas a deletion mutant was inviable may indicate that, in addition to its SUMO ligase activity, Nse2 plays an essential structural role in the Smc5-6 complex. Whatever the essential function of Nse2 is, our data show the following two separate functions of

Nse2: an essential role (possibly in the integrity of the Smc5-6 complex) and a nonessential role promoting the sumoylation of targets (one of which is Smc6 [Rad18]).

An analysis of the phenotype of the *nse2.SA* mutant showed that it was similar to, but somewhat less severe than, those of *rad18.X* and *rad18.74.* Specifically, there were similarities in the responses of the different mutants to UV, ionizing radiation, HU, and MMS. *nse2.SA* mutants were also deficient in the repair of double strand breaks (as judged by PFGE), and the slow growth of *nse2.SA* colonies after exposure to UV may also suggest that the mutant has a reduced ability to repair UV-induced damage. Mutants with defects in the related protein in *S. cerevisiae* (Mms21) are also sensitive to DNA-damaging agents, including UV, ionizing radiation, and MMS. The *mms21-1* mutant displays increased spontaneous mitotic segregation, which is consistent with a deficiency in the repair of single strand DNA breaks, possibly arising during DNA replication (34).

The UV- and IR-sensitive phenotypes of the *nse2.SA* mutant also resembled, but were slightly less severe than, those of the *rad31.d* mutant and the *hus5* mutants, *hus5.17* and *hus5.62*, which are defective in one-half of the SUMO activator and the SUMO conjugator, respectively (1, 13, 40). In contrast, the *rad31.d* and *hus5* mutants were more sensitive to MMS and displayed a slow growth phenotype. The similar sensitivities to UV and IR of the *nse2.SA*, *rad31.d*, and *hus5* mutants suggest that while, by analogy with the situation in other organisms, many proteins may be sumoylated in *S. pombe*, the loss of Nse2-dependent sumoylating activity (which may be limited to a small number of targets) may account for a significant proportion of the *rad31.d* and *hus5* mutant UV- and IR-sensitive phenotypes.

Taken together, our data suggest that the Nse2-dependent SUMO modification of several target proteins is an important aspect of the DNA damage response that involves the Smc5-6 complex. Indeed, we have identified Smc6 (Rad18) as a target of SUMO modification in vivo and have shown that this largely depends on the integrity of the ligase domain of Nse2. Several other components of the Smc5-6 complex, including Nse2 itself, are targets of Nse2 ligase in vitro, although this remains to be verified in vivo. The presence of the majority of soluble cellular Nse2 in the Smc5-6 complex further suggests that the major role of Nse2 is its function as part of this complex. The synthetic lethality of *nse2.SA* with the *rad18.X* and *rad18.74* mutants suggests that sumoylation of Rad18 may be required for its stability. One intriguing possibility is that the Smc5-6 complex localizes Nse2 to additional target proteins, perhaps in response to DNA damage or during DNA replication. Further biochemical and genetic analyses will determine whether this is the case.

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