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SUMO modification of Rad22, the *Schizosaccharomyces pombe* homologue of the recombination protein Rad52

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ABSTRACT

The *Schizosaccharomyces pombe rad31* and *hus5* genes are required for the DNA damage response, as mutants defective in these genes are sensitive to DNA damaging agents, such as UV and ionising radiation and to the DNA synthesis inhibitor hydroxyurea (HU). Sequence analysis has suggested that *rad31* and *hus5* encode components of the Pmt3 (SUMO) modification process in *S.pombe*. We show here that the *rad31* null and *hus5.62* mutants display reduced levels of Pmt3 modification. We have initiated a search for proteins required for the DNA damage response, which may be modified by Pmt3 and have identified Rad22, the fission yeast homologue of the recombination protein Rad52. Purification of myc + His-tagged Rad22 protein from cells expressing HA-tagged Pmt3 identifies an 83 kDa species which cross-reacts with anti-HA antisera. We show here that Rad22 interacts with Rhp51 and Rpa70 (the fission yeast homologues of Rad51 and the large subunit of RPA, respectively), but that neither of these proteins appears to be responsible for the 83 kDa species. The 83 kDa species is observed when extracts are prepared under both native and denaturing conditions, and is also observed when myc + His-tagged Rad22 and Pmt3 are expressed at wild type levels, suggesting that Rad22 is modified by Pmt3 *in vivo*. We have established an *S.pombe in vitro* Pmt3 modification system and have shown that Rad22 and Rhp51 are modified *in vitro*, but that Rpa70 is not.

INTRODUCTION

The DNA damage response involves the coordination of a number of processes including DNA repair events and checkpoint arrest mechanisms. Defective DNA damage responses are identified as sensitivities to DNA damaging agents, such as UV and ionising radiation, or to the DNA synthesis inhibitor, hydroxyurea (HU). In mammalian cells, defects in the DNA damage response can lead to cancer or chromosome abnormalities. The fission yeast, *Schizosaccharomyces pombe*, has proved to be a good model for the characterisation of DNA damage response processes as analysis of mutants sensitive to radiation and to HU has identified many genes required either for repair of DNA damage or for the DNA integrity checkpoints, which are conserved between *S.pombe* and mammals (1,2). Among the *S.pombe* DNA damage response mutants are two, *hus5* and *rad31*, which are epistatic to each other and which do not clearly define a repair process (3,4). Double mutant analysis indicates that the genes function in a process that also requires genes involved in the DNA integrity checkpoint, but they are not actually required for the activation of the checkpoint. The *hus5.62* allele and the *rad31* null mutant display morphological abnormalities including aberrant cell and nuclear morphology, sensitivity to UV and ionising radiation and a high rate of loss of mini-chromosomes (3,4). Comparison of the sequences of the *rad31* and *hus5* genes (3–5) with sequence databases suggests that *rad31* and *hus5* encode components of the fission yeast SUMO modification system.

SUMO is a small ubiquitin-like modifier of ~100 amino acids (reviewed in 6). It has been found complexed to a range of proteins, most of which are associated with the nucleus, e.g. PML, p53, IκBα (e.g. 7–12). The role of SUMO modification has not been fully identified, but is likely to include roles in facilitating protein–protein interactions and alterations in the nuclear localisation of proteins (e.g. 7,13). In the fission yeast, *S.pombe*, SUMO is encoded by the *pmt3* gene. Deletion of *pmt3* results in a pleiotropic phenotype (14) similar to that observed in the *rad31* null and *hus5.62* strains, consistent with multiple roles for SUMO modification.

SUMO modification of proteins requires initial processing of precursor SUMO to the mature form to reveal a GG motif necessary for conjugation to target proteins (e.g. 15). Mature SUMO is then activated by attachment to a protein heterodimer (described below), before transfer to a conjugator protein, which in turn is responsible for attachment of SUMO to target proteins (reviewed in 6). In *Saccharomyces cerevisiae* the activator is encoded by *AOS1* and *UBA2* (16), while the conjugator is encoded by *UBC9* (5). In fission yeast, sequence comparisons indicate that the likely homologues to the activator genes are *rad31* (4) and *fub2* (14), while the conjugator is encoded by *hus5* (3) (see Fig. 1A). In support of this, the *S.cerevisiae AOS1* gene, which has a high degree of sequence similarity to *rad31* (16), complements the *rad31* null phenotype (4).

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Two highly conserved recombination proteins, Rad51 and Rad52, interact with either SUMO-1 (one of the mammalian SUMO proteins) or components of the SUMO-1 modification machinery. Using the yeast two-hybrid system, mammalian Rad51 was demonstrated to interact with SUMO-1 (17). In separate screens, the mammalian Rad51 and Rad52 proteins were found to interact with Ubc9, the *S.cerevisiae* Smt3 conjugator (18,19). More recently both Rad51 and Rad52 have been shown to co-immunoprecipitate with SUMO-1 and UBC9 (20). Conjugation of SUMO-1 to either of these proteins was not detected.

Rad51 and Rad52 have major roles in genetic recombination and in the repair of double strand DNA breaks. The mammalian and budding yeast Rad51 and Rad52 proteins have been purified and their activities characterised. Rad51 is a structural and functional homologue of RecA and is required for DNA strand exchange (21). *In vitro* studies suggest that Rad52, along with RPA, promotes the strand exchange activity of Rad51 (22). Rad52 has been shown to function as a heptameric ring (23) and to bind resected DNA ends (24). The fission yeast homologues of the *rad51* and *rad52* genes have been cloned [the *rhp51* and *rad22* genes, respectively (25–27)], but little is known about the activities or interactions of the gene products. Deletion of either gene results in a high degree of sensitivity to both UV and ionising radiation and in the case of the *rhp51* null allele, a slow growth phenotype, indicating major roles in normal cell growth as well as in the repair of damage due to exposure to DNA damaging agents.

In this report we first confirm that *rad31* and *hus5* are required for Pmt3 modification in *S.pombe*. We also describe some results of a search for DNA damage response proteins likely to be modified by Pmt3, the fission yeast homologue of SUMO-1. One of the proteins we have identified is Rad22. We have analysed the interactions between Rad22 and Pmt3, as well as between Rad22 and other DNA metabolism proteins, specifically Rhp51 (the *S.pombe* homologue of Rad51) and Rpa70, the large subunit of RPA. To facilitate the investigation into whether Rad22 is modified by Pmt3 we have established an *in vitro* SUMO modification system from *S.pombe* protein components and used this to determine whether Rad22 or the proteins with which it interacts are modified by Pmt3.

MATERIALS AND METHODS

Strains and plasmids

The *S.pombe* strains used in this study were sp.011 (*ade6.704*, *ura4.D18*, *leu1.32*, h^-), sp.333 (*rad31::ura4*, *ade6.704*, *leu1.32*, *ura4.D18*, h^-), sp.376 (*hus5.62*, *ade6.704*, *leu1.32*, *ura4.D18*, h^+), sp.424 [*rad22*::*ura4*, *leu1.32*, *ade6.704* h^+ (26)] and sp.568 (int *mycHis-rad22*/pSTA18, *ade6.704*, *ura4.D18*, *leu1.32*, h^-). sp.568 was created by first amplifying the *rad22* coding sequence by PCR from genomic DNA using the following primers: 5′-AAGCTAGAACATATGTCTTTT-GAGCAA and 5′-TTTGCCTGGATCCTTTTATCCTTTTTT-GG. The *rad22* coding sequence was checked for ability to complement the *rad22* disruption strain (26). The 5′ upstream region of the *rad22* gene was amplified using primers 5′-AAG-TATGGACCATGGGCTT and 5′-CAAAAGACATATGT-TCTAGCT, and cloned upstream of the *rad22* coding sequence. A 100 bp *Nde*I fragment containing 2× myc + 6× His (28) was inserted between the promoter fragment and the *rad22* coding sequence. The promoter-containing tagged *rad22* sequence was then subcloned into the integrating vector pSTA18 (29) and used to transform *S.pombe* sp.011 cells to adenine prototrophy. The integrated tagged *rad22* strain was analysed to ensure that there was no phenotype such as altered cell or nuclear morphology, rate of cell growth or cell viability, or alterations in the DNA damage response, due to the presence of the myc + His tag. For expression studies in *Escherichia coli* the *rad22* ORF was subcloned into pET15b (Novagen). The full-length *pmt3* coding sequence was amplified by PCR using the following primers: SEN4, CTGTGAA-CATATGTCTCATG; and Pmt3-4, TGGAGAGGATC-CACGTATTGG. The *rhp51* coding sequence was amplified by PCR using the following primers: r51a, TAACAAGTTAC-CATGGCAGATA; and r51b, TAGGATCCTTTAGACAG-GTGCGAT, and checked for function by its ability to complement the *rhp51* null mutation. The *rpa70* coding sequence was a gift from A. M. Carr (Sussex) (30). The *rad22*, *rhp51* and *rpa70* ORFs were all amplified as *Nde*I–*Bam*HI fragments to allow subsequent cloning into the appropriate expression vectors. The PML wild type and mutant ORFs cloned into pSG5 were gifts from F. Lehembre (8,9). The *S.pombe* expression vectors used were pREP41 (31), pREP41HA and pREP42MH (28); pREP41HA and pREP42MH contain a haemagglutinin tag and 2× myc + 6× His tags, respectively, and were used to produce in-frame protein fusions by insertion of coding sequences between the *Nde*1 and *Bam*HI sites of the two vectors.

Protein extraction and immunological methods

Whole cell TCA extracts were prepared as described by Caspari *et al*. (32). Ni^{2+} affinity chromatography was undertaken under either non-denaturing or denaturing conditions. The non-denaturing conditions were those described by Novagen with the inclusion of *N*-ethylmaleimide (10 mM), iodoacetamide (10 mM), NaF (50 mM), NaN_3 (10 mM) and mammalian protease inhibitor cocktail (Sigma) (0.2%). Ni^{2+} affinity purification under denaturing conditions was carried out as follows: cells were lysed into buffer A (6 M guanidine–HCl, 0.1 M $NaPO_4$ buffer, 0.01 M Tris pH 8.0, 5 mM imidazole, 10 mM β-mercaptoethanol) and insoluble material removed by centrifugation. The supernatant was incubated with Ni^{2+} agarose for 2 h at room temperature. The Ni^{2+} agarose was then washed in buffer A, followed by buffer B (as buffer A with 8 M urea instead of guanidine–HCl), then in Novagen wash buffer plus 6 M urea, 20 mM imidazole and protein was eluted in 6 M urea, 300 mM imidazole.

The Rad22–Hus5 interaction was investigated by mixing two bacterial cell lysates of 50 ml cultures expressing His-Rad22 (from pET15b) and GST-Hus5 (from pGEX) and purification of the GST-Hus5 protein using glutathione–Sepharose beads (Pharmacia) as described by the manufacturers, followed by SDS–PAGE.

Anti-Pmt3 antisera were produced using a His-tagged fusion protein of an N-terminally truncated version of Pmt3 (NΔPmt3) created by PCR using oligonucleotides Pmt3-1 (CTGAGAACATATGTCTGAATCACC) and Pmt3-4. Anti-ubiquitin antisera were obtained from DAKO, anti-myc antisera were from RDI Inc., anti-HA antisera were from Babco and anti-His antisera were from Pharmacia.

In vitro SUMO-1 modification assay

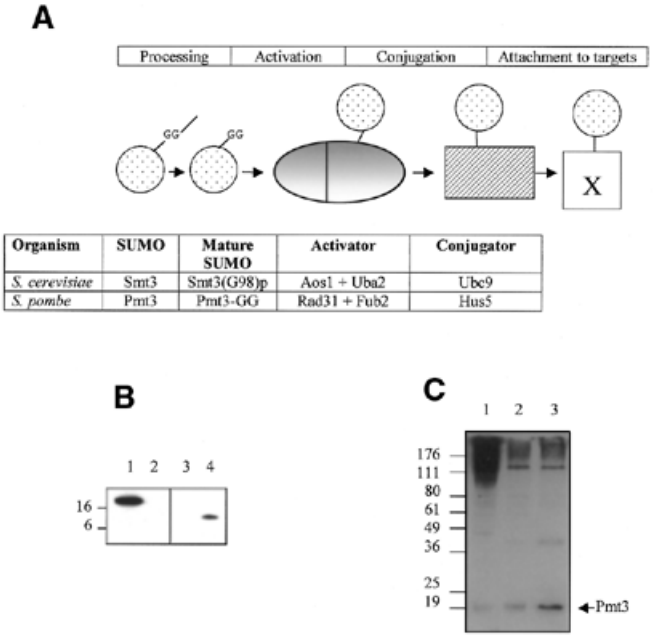
Amplification of the *rad31* cDNA has been described previously (4). The *fub2* ORF was amplified by PCR using the following oligonucleotide primers: Uba2p-N, CATATGCCAAGGCTAATGCAAC; and Uba2p-B, GGATCCAAATGGTTTATTTACATAG. The *hus5* ORF was a gift from A. M. Carr (Sussex). The ORFs were cloned into pGEX and Rad31, Fub2 and Hus5 proteins were purified as GST fusion proteins from *E.coli*. The GST was cleaved off the GST–Hus5 fusion using thrombin (5 U/mg protein) and Hus5 was purified by passage over glutathione–Sepharose. Pmt3-GG, a C-terminally truncated version of Pmt3 equivalent to the mature form was produced with an N-terminal His tag and was constructed by PCR using oligonucleotides SEN4 and Sumo-GG (GGATCCTAAGGAGGTAACTGTTCT). Radio-labelled proteins were produced with [^{35}S]methionine using the TnT T7 coupled reticulocyte lysate system (Promega). The *in vitro* Pmt3 modification assay was carried out in 50 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 5 mM ATP, 10 mM creatine phosphate, 3.5 U/ml creatine kinase, 0.6 U/ml inorganic pyrophosphatase using 3 µg Hus5, 10 µg His-Pmt3, 0.5 µg SAE1 (GST–Rad31 + GST–Fub2) and 2 µl ^{35}S-labelled protein from the TnT reaction at 30°C for 2 h. Reactions were analysed by SDS–PAGE followed by analysis on a phosphorimager.

RESULTS

Pmt3 modification of *S.pombe* proteins

Sequence comparisons suggest that *rad31* and *hus5* encode components of the *S.pombe* Pmt3 (SUMO) modification process. To confirm that the Rad31 and Hus5 proteins are actually required for Pmt3 modification, we first wished to investigate the levels of Pmt3-conjugates in the *rad31* null and *hus5.62* mutant strains. Anti-Pmt3 antisera were prepared as described in the Materials and Methods. Figure 1B shows that they are specific for Pmt3 and do not cross-react with ubiquitin. We next compared the pattern of Pmt3-modified species in wild type, *rad31* null and *hus5.62* mutant cells. Analysis of *rad31* and *hus5* mutant cells indicates a decrease in modified species in both *rad31* null and *hus5.62* strains with a concomitant increase in free Pmt3 (Fig. 1C), confirming that *rad31* and *hus5* have roles in Pmt3 conjugation. The low residual level of Pmt3-conjugates in the *rad31* null mutant most likely results from the activity of Fub2 which contains the conserved cysteine residue that forms a thiolester bond with Pmt3. Deletion of *rad31* may not, therefore, completely abolish the activity of Fub2. In the case of *hus5.62*, this allele has previously been shown to be a partial loss-of-function mutation (3) and presumably allows a low level of Pmt3 conjugation.

We next wished to determine whether exposure of cells to ionising radiation or HU affected the overall pattern of Pmt3-conjugates in wild type cells. We therefore subjected cells to ionising radiation (500 Gy) or HU (20 mM for 3 h), prior to protein extraction and western analysis. We did not detect any major differences after exposure to any of the conditions that we tested (data not shown). We also tested whether over-expression of Pmt3 in *S.pombe* cells affected the resistance of cells to UV or ionising radiation [as over-expression of SUMO-1 has been shown to result in a decrease in the resistance of mammalian cells to ionising radiation (20)]. Cells over-expressing either tagged or untagged Pmt3, using pREP41HA and pREP41, respectively, or an untagged truncated version of Pmt3 (Pmt3-GG), using pREP41, displayed similar sensitivities to UV and ionising radiation as wild type cells transformed with empty vector (data not shown).



Organism	SUMO	Mature SUMO	Activator	Conjugator
S. cerevisiae	Smt3	Smt3(G98)p	Aos1 + Uba2	Ubc9
S. pombe	Pmt3	Pmt3-GG	Rad31 + Fub2	Hus5

Figure 1. Pmt3 modification in *S.pombe*. (**A**) Schematic showing the components of the *S.cerevisiae* and *S.pombe* Pmt3 modification pathway. The *S.cerevisiae* nomenclature is taken from previous studies (5,16). (**B**) Anti-Pmt3 antisera are specific for Pmt3 and do not recognise ubiquitin. NΔPmt3 (lanes 1 and 3) and ubiquitin (0.5 µg) (lanes 2 and 4) were separated by SDS–PAGE (12.5%) and western blotted with anti-Pmt3 antisera (lanes 1 and 2) or anti-ubiquitin antisera (lanes 3 and 4). (**C**) SDS–PAGE and western blotting with anti-Pmt3 antisera of protein extracts from wild type (sp.011) lane 1, *rad31.d* (sp.333) lane 2 and *hus5.62* (sp.376) lane 3.

Search for Pmt3-associated proteins identifies Rad22 as a likely candidate

The fact that the *rad31* and *hus5* mutants are sensitive to UV, ionising radiation and HU suggests that Pmt3 modification is required for some aspect(s) of the DNA damage response. We therefore sought to identify Pmt3-modified targets likely to have a role in the DNA damage response. We screened a number of DNA damage response proteins for interaction with Pmt3 by over-expressing them as myc + His-tagged fusion proteins in cells also over-expressing HA-tagged Pmt3, followed by affinity purification on Ni^{2+} agarose. The characterisation of some of these genes will be described elsewhere. One of the proteins we investigated in more detail was Rad22, the *S.pombe* homologue of Rad52. Figure 2A (lane 3) shows that indeed Rad22 and Pmt3 interact as a new HA-tagged (i.e. Pmt3-modified) 83 kDa species is detected when both proteins are over-expressed. This species is not detected when either HA-Pmt3 (lane 1) or myc + His-Rad22 (lane 2) are expressed on their own. The presence of two bands in lanes 2 and 3 after blotting with anti-myc antisera is likely to be due to proteolysis as only a single species, corresponding to the upper

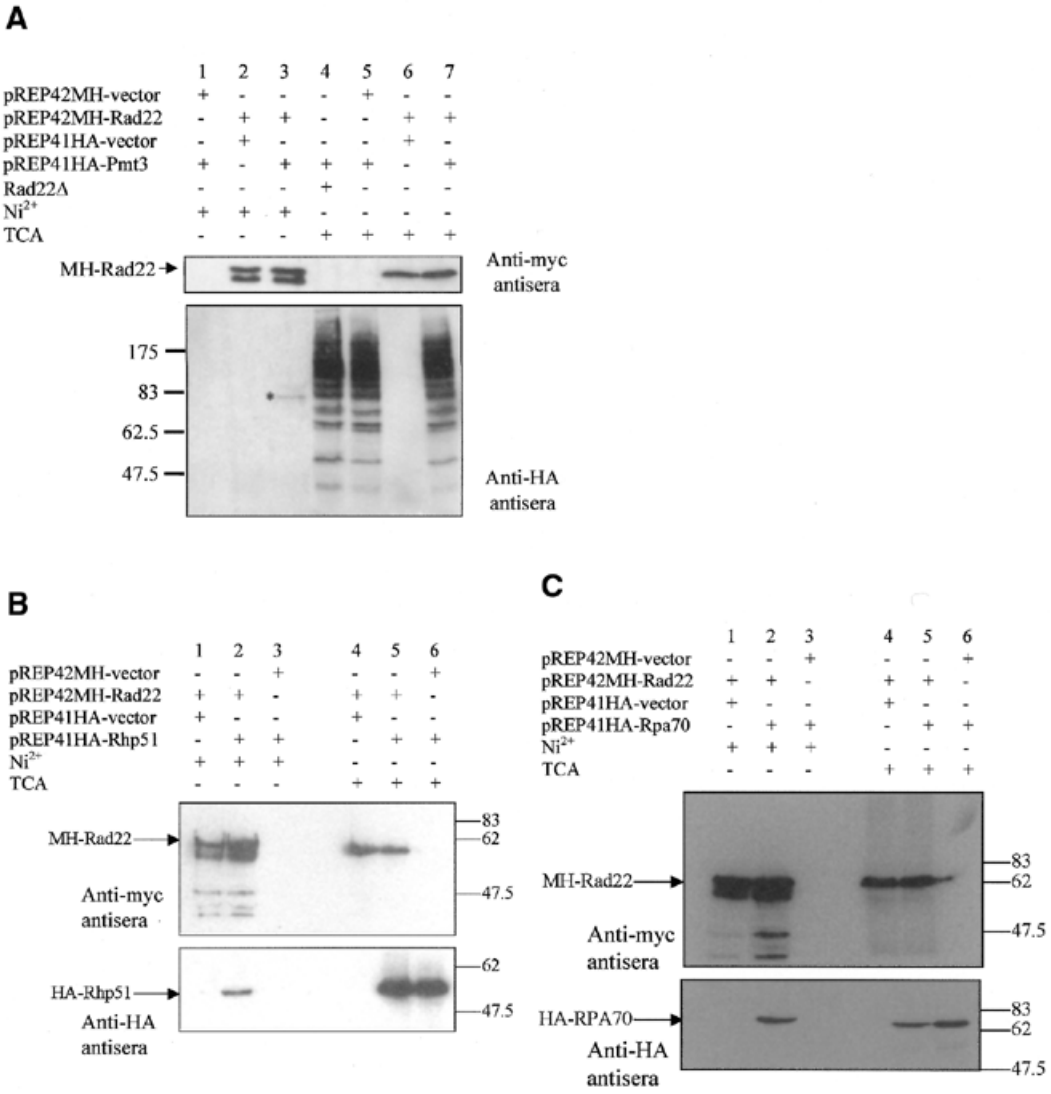


Figure 2. Interaction of Rad22 with Pmt3, Rhp51 and Rpa70. (**A**) Interaction with Pmt3. (**B**) Interaction with Rhp51. (**C**) Interaction with Rpa70. In all cases wild type cells (sp.011) were transformed with plasmids expressing the indicated proteins as tagged fusion proteins under the control of the *nmt1* promoter. Protein interactions were investigated using Ni^{2+} agarose beads, TCA extracts were used as controls to check expression levels. Samples were analysed by SDS–PAGE and western blotted with anti-myc or anti-HA antisera as indicated. *, New HA-cross-reacting species.

band is observed in the TCA extracts (lanes 6 and 7). The size of the species observed in lane 3 (~83 kDa) is consistent with that expected of an HA-Pmt3-modified form of myc + His-Rad22.

No gross differences in Pmt3-modified species are observed in whole cell TCA extracts of wild type and *rad22* null cells (Fig. 2A, lanes 4 and 5), indicating that the 83 kDa species is not one of the major Pmt3-modified species in *S.pombe*.

It is possible that the 83 kDa species observed in Figure 2A might be an HA-Pmt3-modified form of a protein that interacts with Rad22, rather than Pmt3-modified Rad22. To determine whether this might be the case, we investigated whether Rad22 interacted with two possible candidates, Rhp51 and Rpa70. Rhp51 and Rpa70 are the homologues of Rad51 and the large subunit of the single strand DNA binding protein (RPA) which, in other organisms, have been shown to interact with Rad52, the homologue of Rad22 (33,34). Myc + His-tagged Rad22 was, therefore, over-expressed along with either HA-tagged Rhp51 or HA-tagged Rpa70. Figure 2B (lane 2) shows that Rad22 interacts with Rhp51 as observed by the ~60 kDa species present in lane 2, but which is not present in the negative controls (lanes 1 and 3). Similarly, Rad22 interacts with Rpa70 (Fig. 2C, lane 2) with a species of ~72 kDa pulled down specifically with myc + His-tagged Rad22, and which is not observed in the absence of HA-Rpa70 (lane 1) or myc + His-Rad22 (lane 3). The sizes of the HA-tagged species detected in Figure 2B and C are as expected for tagged, but unmodified, forms of Rhp51 and Rpa70 proteins, respectively, and are distinctly smaller than the 83 kDa Pmt3-associated species identified in Figure 2A, as they migrate at 60 and 72 kDa, respectively. These data suggest that although Rad22 interacts with both Rhp51 and Rpa70, neither of these proteins is likely to be a candidate for an 83 kDa Pmt3-modified Rad22-interacting protein.

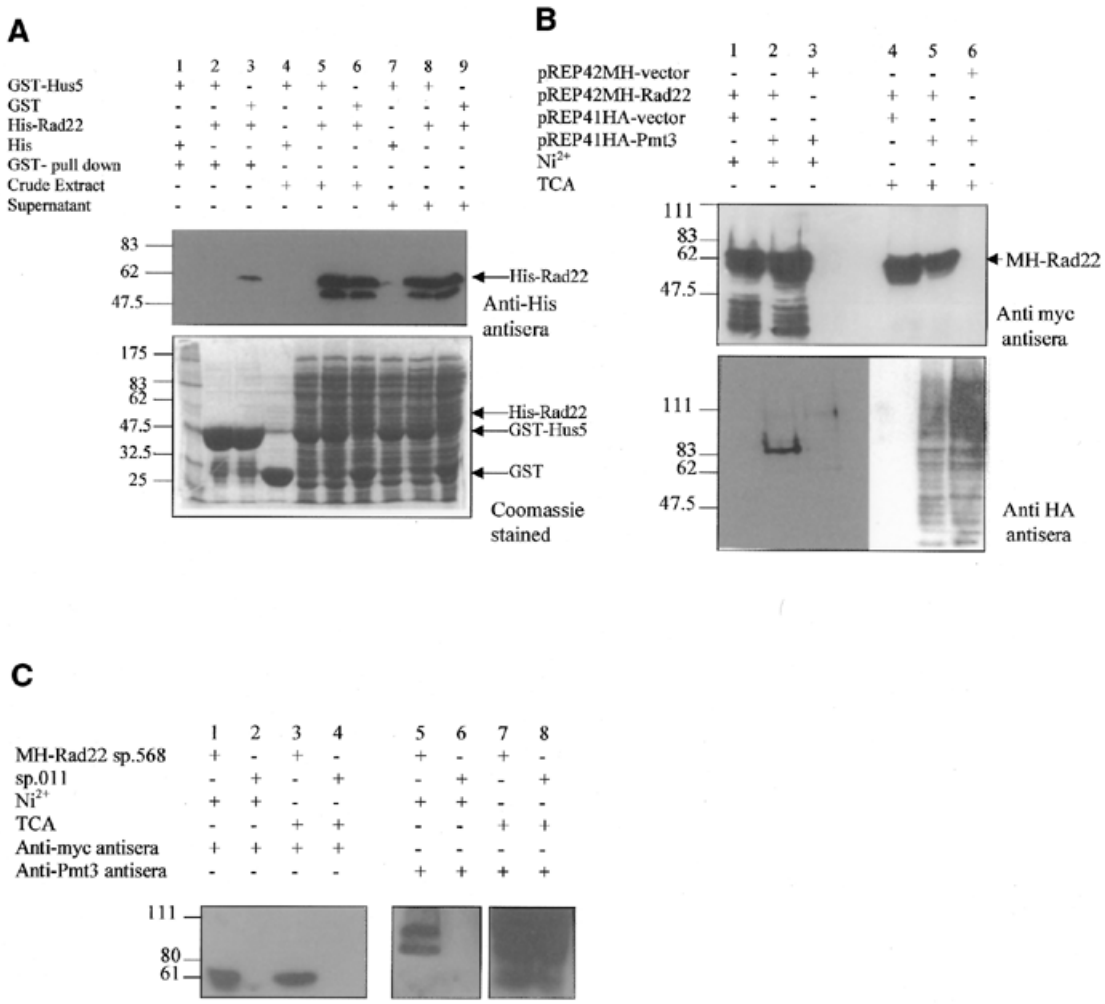


Figure 3. Interaction of Rad22 with Hus5 and Pmt3. (**A**) Bacterial cell lysates were mixed as indicated and proteins were fractionated on glutathione–Sepharose. Proteins binding to glutathione (GST-pull down), crude extract and unbound material (supernatant) were separated by SDS–PAGE and western blotted with anti-His antisera or stained with Coomassie Brilliant Blue. (**B**) Interaction of Rad22 and Pmt3 was analysed using extracts from cells over-expressing both proteins as in Figure 2A, but in the presence of 6 M guanidine–HCl. (**C**) Interaction of Rad22 and Pmt3 expressed at wild type levels. Strains used were an integrated myc + His-tagged Rad22 strain (sp.568) and sp.011 as the negative control. Samples were analysed by SDS–PAGE and western blotted with anti-myc or anti-Pmt3 antisera as indicated. Lanes 1–4, 7 and 8 were exposed to X-ray film for 10 s, while lanes 5 and 6 were exposed for 30 min.

Further analysis of the interaction between Rad22 and components of the Pmt3 modification process

As analysis of the interactions of Rad22 with Rhp51 and Rpa70 suggests that neither Rhp51 nor Rpa70 appears to be responsible for the 83 kDa species detected in Figure 2A, we wished to further analyse the relationship between Rad22 and Pmt3 and with components of the Pmt3 modification process. We first investigated whether Rad22 interacts with the Pmt3 conjugator, Hus5. This was analysed *in vitro* using GST-Hus5 and His-tagged Rad22. Figure 3A shows that GST-Hus5 interacts with Rad22 protein *in vitro*, as Rad22 is pulled down with GST-Hus5 using glutathione–Sepharose (lane 2), but not in the absence of Hus5 (lanes 1 and 2). The relative amount of Rad22 which purifies with Hus5 (lane 2) and which remains in the supernatant is low (lane 8), suggesting that the interaction between Rad22 and Hus5 is transient in nature.

To further investigate whether Rad22 is modified by Pmt3, we wished to establish whether HA-Pmt3 is associated with Rad22 under denaturing conditions. The Ni^{2+} pull down assay described in Figure 2A was, therefore, repeated in the presence of guanidine–HCl which would be expected to disrupt non-covalent interactions. Figure 3B shows that the 83 kDa species is still pulled down with the myc + His-Rad22 protein (lane 2), but not when Rad22 or Pmt3 are over-expressed alone (lanes 1 and 3), implying that Rad22 is modified by HA-Pmt3.

The interactions observed so far between Rad22 and Pmt3 or Hus5 have been either under conditions where Rad22 and Pmt3 are over-expressed, or *in vitro*. To confirm that the 83 kDa species we observe in Figures 2A and 3B is not an artefact due to over-expression of either Rad22 or Pmt3, we created an *S.pombe* strain containing an integrated myc + His-tagged copy of *rad22*, repeated the Ni^{2+} pull down assay and probed with anti-Pmt3 antisera. Figure 3C shows that the 83 kDa species is observed in cells expressing wild type levels of both Rad22 and Pmt3 (lane 5), but not in the control sample (lane 6). The level of the 83 kDa species is low in comparison

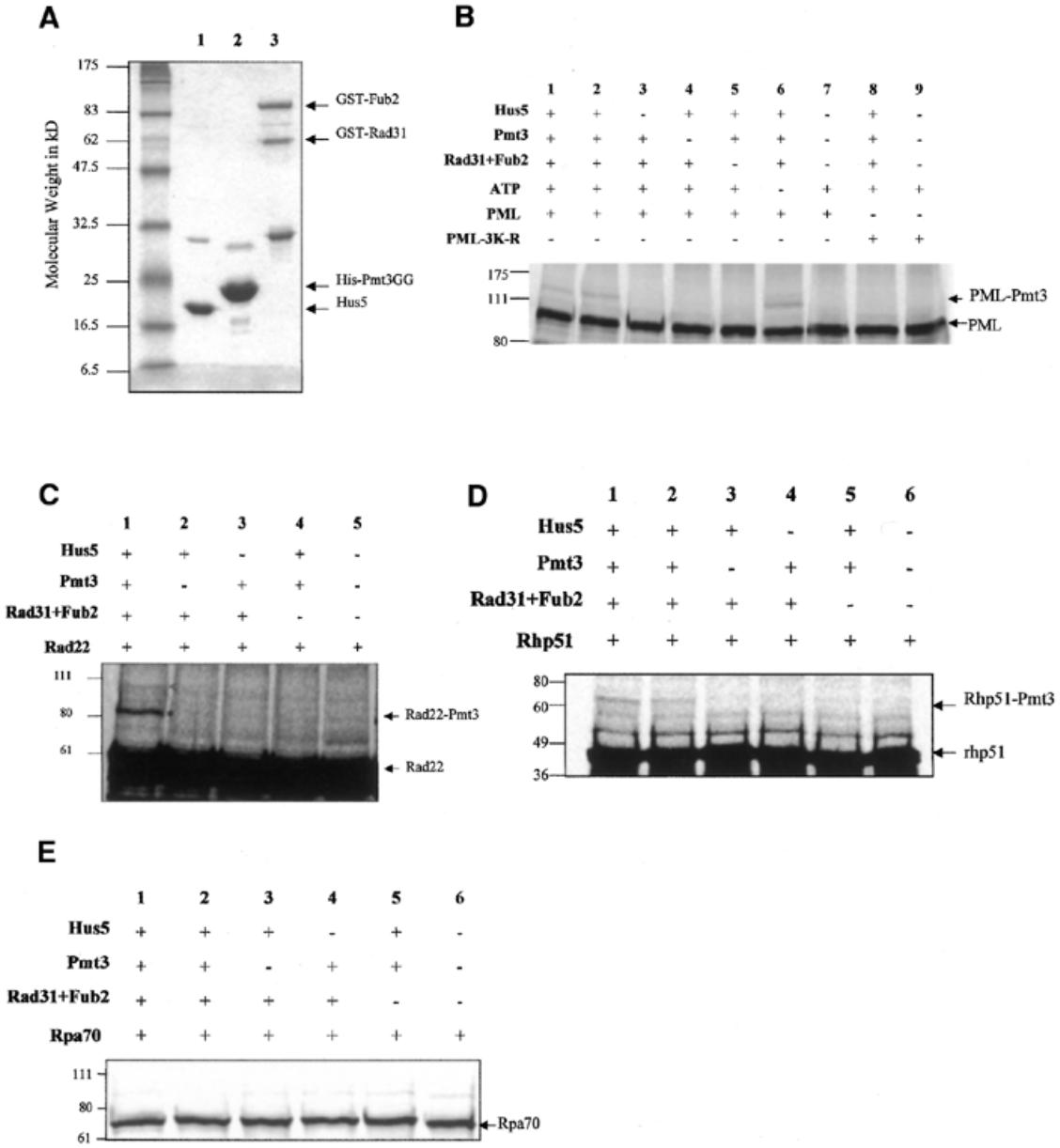


Figure 4. *Schizosaccharomyces pombe in vitro* Pmt3 modification system. (**A**) Protein components used to establish the assay. Coomassie stain of SDS–PAGE. Lane 1, 4 µg Hus5; lane 2, 10 µg His-Pmt3-GG; lane 3, 1 µg GST-Rad31, GST-Fub2. (**B**) Modification of PML. Assays were set up as described in the Materials and Methods; all samples were incubated in assay buffer for 2 h at 30°C. Lanes 1–7, wild type PML; lanes 8 and 9, PML-3K-R. Lanes 1 and 2, all assay components; lane 3, no Hus5; lane 4, no Pmt3; lane 5, no Rad31 + Fub2; lane 6, no added ATP; lanes 7 and 9, no Hus5, Pmt3, Rad31 and Fub2. (**C**–**E**) Testing for modification of Rad22 (C), Rhp51 (D) and Rpa70 (E). Addition of components was as indicated. (C) *, Major Pmt3-modified product; <, minor Pmt3-modified species of 105 kDa.

to the amount of myc + His-tagged Rad22 pulled down in this experiment (note the different exposure times of lanes 5 and 6 compared with that of the other lanes). The bands observed in lane 5 were visible at the exposure time used for lanes 1–4, but were faint. In addition to the 83 kDa species, an additional species at ~110 kDa is also observed. The identity of this is unknown, but its size is consistent with it being Rad22-modified by two Pmt3 molecules. Our inability to detect the 83 kDa species in anti-myc blots may reflect masking of the myc epitope by the Pmt3 modification of the different affinities of the anti-myc and anti-HA antisera.

Rad22 is SUMO-1-modified *in vitro*

The results of the analysis of Rad22 *in vivo* are strongly suggestive that Rad22 is modified by Pmt3. However, as Pmt3 modification is difficult to observe in cells because the modification appears to be labile and Pmt3-modified species are present at a low level, we wished to use a further method for analysing Pmt3 modification of proteins. We therefore established an *in vitro* Pmt3 modification system using *S.pombe* components (as described in the Materials and Methods). Figure 4A shows the protein components that were purified for use in the assay. By analogy with the *S.cerevisiae*

and mammalian homologues it was presumed that Pmt3 needs to be processed to reveal the GG motif for conjugation onto target proteins. Thus, for the *in vitro* system we prepared His-tagged Pmt3-GG as described in the Materials and Methods (Fig. 4, lane 2). Several procedures were tested for the preparation of the activator (Rad31 + Fub2) and the conjugator (Hus5) with the result that we obtained maximum yields of soluble protein by preparing the three proteins as GST fusion proteins. Rad31- and Fub2-containing *E.coli* extracts were mixed together prior to purification on glutathione–Sepharose as this prevented precipitation of the individual proteins. The GST moiety was removed from Hus5, but not from Rad31 and Fub2.

The *S.pombe in vitro* Pmt3 modification system was first tested using the well established mammalian SUMO-1 target protein, PML. Figure 4B shows that the system is capable of modifying wild type PML protein (lanes 1 and 2), but not an unmodifiable version of it (lane 8). The modification is dependent on the presence of Rad31, Fub2 and Hus5 as omission of any one of these components results in loss of the modification (lanes 3–5, 7 and 9). Omission of added ATP did not prevent the reaction. This appears to be in contrast to what has been observed in the mammalian *in vitro* SUMO-1 modification system (12), but is likely to be due to the presence of ATP in the reticulocyte lysate used here to create labelled substrates.

We next investigated whether Rad22 was modified in this *in vitro* system. Figure 4C (lane 1) indicates that Rad22 is indeed modified *in vitro*. The size of the major modified species corresponds well with the size of the Pmt3-associated species observed *in vivo* (e.g. Fig. 3C). (The species detected *in vivo* would be expected to migrate slightly slower than the forms detected *in vitro* as the Rad22 species expressed in yeast contains two additional myc epitopes.) There is also a very minor species at 105 kDa which migrates with similar relative molecular mass to the larger species observed in Figure 3C, lane 5. As with PML, the modification of Rad22 is dependent on the presence of added SAE (Rad31 + Fub2), Hus5 and Pmt3 (lanes 2–4).

We also investigated whether proteins which interact with Rad22 are modified by Pmt3 *in vitro*. Figure 4D, lanes 1 and 2, shows that Rhp51 is modified by Pmt3 *in vitro*, while Rpa70 does not appear to be (Fig. 4E, lanes 1 and 2). Modification of Rhp51 is dependent on the presence of added Hus5 and SAE (Rad31 + Fub2) (Fig. 4D, lanes 3–5).

DISCUSSION

We have confirmed here our speculation, derived from sequence comparisons, that the *rad31* and *hus5* genes encode components of the *S.pombe* SUMO-1 (Pmt3) conjugation machinery. The *S.pombe in vitro* Pmt3 modification system, which we describe and which uses purified Rad31, Fub2 and Hus5 enzymes, is capable of modifying the mammalian protein PML, but not a mutant version of it that has been shown in the mammalian SUMO-1 modification system to be unmodifiable (8). The levels of Pmt3 modification in the *S.pombe* system, while low, resemble the levels of SUMO-1 modification observed using the mammalian system (e.g. 12). The Pmt3 modification that we detect requires the presence of added Pmt3-GG (the mature form of Pmt3), the activator (Rad31 + Fub2) and the conjugator, Hus5. As with the mammalian *in vitro* SUMO-1 modification system, the *S.pombe* system does not appear to require a Pmt3 ligase, although it is possible that such an activity might be contributed by the rabbit reticulocyte lysate system used to prepare ^{35}S-labelled substrates. We have obtained similar results using substrates labelled in the TnT wheatgerm system (data not shown), indicating that if a Pmt3 ligase is required it is also being provided by the wheatgerm extract. A Pmt3 ligase is unlikely to have co-purified with any of the modification components, as they were all prepared from *E.coli*, which does not have ubiquitin or ubiquitin-like proteins.

The UV, ionising radiation and HU sensitivities of the *rad31* null and *hus5.62* mutants imply that Pmt3 modification has a role in one or more aspects of the DNA damage response. Previous work on SUMO-1 has shown that the majority of SUMO-1-modified proteins are located within the nucleus (35), although there are exceptions to this (e.g. 36). The range of defects observed in *rad31*, *hus5* and *pmt3* mutant strains (3,4,14) suggest that there are likely to be several, or possibly many, proteins involved in aspects of DNA metabolism or the DNA damage response which have their function modulated by Pmt3 modification. Among a number of DNA damage response proteins we have tested, we identified Rad22 as a potential Pmt3-modified target. Taken together, the data described here, in particular the interaction of Rad22 and Pmt3 under denaturing conditions (Fig. 3B) and when both proteins are expressed at wild type levels (Fig. 3C), strongly suggest that Rad22 is modified by Pmt3. From these experiments we estimate that the level of the modified species is ~2–5% of the level of the unmodified Rad22 protein. The presence of two Pmt3-modified species observed in Figure 3C may indicate that Rad22 is modified by two Pmt3 molecules. The majority of the cases of multiple sumoylation have been shown to be due to single SUMO-1 molecules attached to multiple sites. However, there is a recent report of SUMO-2 and SUMO-3 forming polymeric chains (37). Whether Pmt3 is able to form chains remains to be determined.

In addition to Rad22, the *in vitro* Pmt3 modification system is also capable of modifying Rhp51. However, in contrast to what we observe with Rad22 *in vivo*, we have not detected any species which might correspond to Pmt3-modified Rhp51 when, for example, we over-express myc + His-Rhp51 and HA-Pmt3 under similar conditions to those described in Figure 2. The reason for this is not known but may be due to the presence of very low levels of Pmt3-modified Rhp51 or that Pmt3-modified Rhp51 is highly unstable *in vivo*. The fact that we do not detect modification of Rpa70 indicates that not all substrates tested in the *in vitro* system give positive results. During these and other *in vivo* studies that we have undertaken to investigate Pmt3 modification of *S.pombe* proteins, we have found Pmt3-modified species to be present at low levels and difficult to purify from cell extracts. This makes the task of identifying novel Pmt3-modified targets *in vivo* a laborious process. The *S.pombe in vitro* Pmt3 modification system will thus provide a useful and relatively straightforward first step in the identification of potential Pmt3 targets, which can then be tested *in vivo*.

Multiple possible roles have been proposed for SUMO-1 modification. One of these is demonstrated in the case of IκBα, where SUMO-1 competes with ubiquitin for the same lysine

residue (12). SUMO-1 modification of IκBα prevents ubiquitination and hence protects the protein from degradation by the proteasome. We have no direct evidence that Rad22 is ubiquitinated or is subject to ubiquitin-dependent proteolysis. However, Rad22 is somewhat unstable in cell extracts, even in the presence of the mammalian protease inhibitor cocktail as can be observed by the doublet of bands in Figures 2 and 3. The upper band corresponds to the band seen in TCA extracts, with the lower species likely to be a degradation product. Alternatively Pmt3 modification of Rad22 might affect the location of Rad22 within cells or within distinct compartments within the nucleus or might alter its ability to interact with different proteins.

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