Mts4, a Non-ATPase Subunit of the 26 S Protease in Fission Yeast Is Essential for Mitosis and Interacts Directly with the ATPase Subunit Mts2*

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We have isolated a fission yeast gene, mts4*, by complementation of a temperature-sensitive mutation and show that it encodes subunit 2 (S2) of the 19 S regulatory complex of the 26 S protease. mts4* is an essential gene, and we show that loss of this subunit causes cells to arrest in metaphase, illustrating the importance of S2 for mitosis. The Mts4 protein is 48% identical to S2 of the human 26 S protease, and the lethal phenotype of the null mts4 allele can be rescued by the human cDNA encoding S2. We provide genetic and physical evidence to suggest that the Mts4 protein interacts with the product of the mts2* gene, an ATPase which has previously been shown to be subunit 4 of the 26 S protease.

The selective degradation of proteins is an important means of regulating gene expression and is found increasingly to play a role in the control of various cellular events. Ubiquitin-mediated degradation is the main non-lysosomal proteolytic pathway in the cell, and a number of important regulatory proteins are degraded in this way, including mitotic cyclins, p53, c-Mos, c-Myc, and the Mata2 repressor (1–4). Denatured and abnormally folded proteins are also degraded by this pathway. Proteins are targeted for degradation by the addition of ubiquitin, a 76-amino acid protein that is highly conserved among eukaryotes (5). Further ubiquitin moieties are added to form a polyubiquitin protein conjugate that is recognized as a substrate by a proteolytic complex known as the 26 S protease (20–22). This is a large multisubunit complex composed of two distinct particles, the 20 S catalytic core and the 19 S regulatory cap (9–11).

The 20 S sub-complex is a cylindrical particle consisting of 14 different subunits of 21–32 kDa. The subunits are arranged in four seven-membered rings, the inner two contain the proteolytic activities (12–14). The genes encoding all of the 20 S subunits have been cloned from a number of species and are found to comprise a family of homologous proteins that are greatly conserved between eukaryotes (15, 16). Each 26 S protease consists of two 19 S caps positioned at either end of one 20 S core particle (9, 11). The proteolytic activity of the 20 S core is tightly regulated by the 19 S cap such that degradation is dependent upon ATP and largely, but not exclusively, upon the presence of polyubiquitinated substrates (7, 8, 17).

The 19 S regulatory cap, consists of a number of different subunits, possibly as many as 20, with molecular masses ranging between 25 and 112 kDa. These subunits are named according to their position as determined by SDS-PAGE analysis with S1 being the largest (18). Recently a number of subunits of the 19 S cap have been identified and fall into two classes. First, at least six subunits are members of a family of ATPases (18–21). Their exact role is unclear but could involve hydrolyzing ATP to provide energy for the assembly of the 26 S protease and also for the unfolding and translocation of substrates into the 20 S core. The lethality associated with mutations in various ATPases, in both fission and budding yeast, indicates that these subunits are essential for the function of the 26 S protease (20, 21).

The second class of 19 S subunits has been termed the non-ATPases (11, 18, 22). Their functions are also unknown; however, possible roles could include the capture and unfolding of substrates, the release and recycling of ubiquitin chains, activation of the 20 S core, and the general maintenance of the 26 S protease structure. One non-ATPase subunit S5a has been shown to bind polyubiquitin chains in vitro (23–25). However, the finding that MCB1, which encodes S5a in Saccharomyces cerevisiae, is not an essential gene suggests that there are other factors involved in substrate selection. Once again, the importance of this class of subunits is demonstrated by the isolation of mutations in yeast non-ATPases that are lethal (26–29).

Other non-ATPase subunits recently cloned include S1 encoded by the SEN3 gene in S. cerevisiae (29) and the gene encoding p112 in humans (30), the genes encoding S2 from both humans and budding yeast (30), and S14 encoded by NIN1 and mts3 in budding and fission yeast, respectively (26, 27). Genetic interactions have been identified between some of the yeast 19 S cap subunits implying that these proteins might interact in vivo. For example, a mutation in fission yeast S4, an ATPase, can be rescued by overexpression of another ATPase, S7 (29). Mutations in the S. cerevisiae genes encoding S1 and S14, both non-ATPases, were found to be synthetically lethal, and furthermore, overexpression of S1 was found to suppress the ts allele in the gene encoding S14 (28). To date, however, there have been no published reports of interactions between an

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1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; GST, glutathione S-transferase; DAPI, 4′,6-diamidino-2-phenylindole; ts, temperature-sensitive; TNF, tumor necrosis factor; MBC, methyl benzimidazol-2-yl carbamate; APC, anaphase promoting complex; ts, temperature-sensitive.
ATPase and a non-ATPase in the 26 S protease. Previously, we have isolated two conditional lethal mutants mts2-1 and mts3-1 in the fission yeast Schizosaccharomyces pombe (20, 27). The corresponding genes mts2+ and mts3+ were found to encode subunit 4 and subunit 14, respectively, of the 19 S cap of the 26 S protease. At the restrictive temperature, both mutants were found to arrest in mitosis, at metaphase, implying that the 26 S protease is essential for the metaphase to anaphase transition. Mts2 is a protein that belongs to the ATPase family of regulatory subunits, whereas Mts3 is one of the non-ATPase components. In this paper, we describe another conditional mutant, mts4-1, which was isolated in the same screen. The mts4+ gene encodes a protein with homology to S2 of the human and S. cerevisiae 26 S protease. In contrast to mts2-1 and mts3-1, the mts4-1 mutant does not have a clear cell cycle phenotype. A null allele of mts4, however, is lethal and results in a clear cell cycle defect with cells arrested in metaphase. This lethality can be rescued by the gene encoding human S2. Consistent with the sequence and functional similarities between Mts4 and human S2, we have purified the 26 S protease from fission yeast and find that Mts4 co-purifies with known components of this complex. Therefore we conclude that Mts4 is S2 of the 26 S protease in S. pombe and that this subunit is essential for mitosis. Furthermore, we provide both genetic and physical evidence to suggest that Mts4 and Mts2 interact. This is the first report of interactions between an ATPase and a non-ATPase subunit in the 19 S regulatory cap of the 26 S protease.

**EXPERIMENTAL PROCEDURES**

*S. pombe* Strains and General Techniques—All *S. pombe* strains used were derived from the wild type heterothallic strains 972 h and 975 h+. Standard genetic manipulations and media were as described (31). Yeast transformations were performed by the lithium acetate procedure for library screens (31) or by electroporation for transformation of single genomic DNA from *S. cerevisiae* for library screens (31) or by electroporation for transformation of single plasmids (32).

Cloning and Sequencing—The *mts4* cDNA was isolated from an S. pombe cDNA library in the vector pREPI (a gift from B. Edgar and C. Norbury). The *mts4-leu1-32* h strain was transformed with 20 µg of the library and transformants left to grow at the restrictive temperature of 36 °C. Two colonies were obtained. Plasmids were removed and cDNAs were sequenced using primers located in the nmt1 promoter and terminators from regions in addition to custom-synthesized oligonucleotides. The *mts4* genomic clone was isolated by complementation of the *mts4-leu1-32* h mutant from a library containing a *SacI* digest of genomic DNA from *S. pombe* in the vector pHW5 (a gift from R. Allshire) using oligonucleotides designed according to the CDNA sequence. To generate a linear construct for integration of *mts4* at the mutant locus, the cDNA, under the control of the nmt1 promoter, was ligated into the pREPI vector containing the *ura4* gene but lacking the *ars1* sequence. This plasmid was then linearized within the *mts4* sequence by digestion with *SspI*, and the resulting DNA was used to transform *mts4-leu1-32* h to remove 1.8 kilobase ends, respectively, the sequences are as follows: 5'-CATCGCCATGGGCACTGCTCTTT-3' and 5'-GCTGGATCCATGTCATGAGTTTCAATGTCCT-3'. The resulting fragment was subcloned into the pREPI vector that had been digested with *SauI* and *BamHI* to give the plasmid pREPIHs2 (p97).

Gene Disruption—The region of the genomic clone containing mts4 was amplified by PCR using an oligonucleotide introducing a *SauI* site at the 3' end and an oligonucleotide upstream of the start of the gene that incorporated an endogenous *XbaI* site, 5'-TACTGAGCTCATTCTGTTCCGCTATCAGGAGGAGGACGCAAG and 5'-GCTGGATCCATGTCATGAGTTTCAATGTCCT-3'. The resulting PCR product was subcloned into pBluescript that had been digested with *XbaI* and *SauI* to give the plasmid pBSmts4-1. The pBSmts4-1 was digested with *EcoRI* and *Spel* to remove 1.8 kilobase pairs of coding sequence. The remaining fragment was ligated to a 1.8-kilobase pair SpeI-HindIII fragment containing the *ura4* gene to give the construct pBSmts4D-ura4. PCR was used to generate a 2.8-kilobase fragment containing the deleted *mts4* genomic clone. The primers used were 5'-TCATTCAATGGCCTCAGG and 5'-AGGGT-TCTAAAGAGCATT. This deleted copy of *mts4* was used to transform the diploid S. pombe strain *leu1-32/leu1-32ade6-M210/ade6-M216ura4-D18/ura4-D18 h+ h+. Stable uracil prototrophs were isolated and heterozygous deletion mutants identified by Southern blot analysis. Spores were germinated and prepared as described (33).

Flow Cytometry—Flow cytometry was carried out on ethanol-fixed cells stained with propidium iodide using a Becton Dickinson FACScan as described (33).

Fluorescence Microscopy—DAPI staining was as described (34). For immunofluorescence microscopy, cells were grown overnight in yeast extract, and immediately before fixation, sorbitol was added to the culture to give a final concentration of 1.2 M. The cultures were fixed in 38% paraformaldehyde at 32 °C for 30 min. The cells were then prepared as described (35). The anti-tubulin monoclonal antibody TAT1 was used to stain microtubules (36). Stained cells were observed as described (27).

**Purification of Recombinant Mts4 Protein and the Preparation of Antibodies**—For expression in *Escherichia coli*, the *mts4* cDNA was amplified by PCR using primers that incorporated an *NcoI* site at the 5' end and a *SacI* site at the 3' end. The primer is described above, 5'-ACATGCCCATGGGCACTGCTCTTT-3' and 5'-GCTGGATCCATGTCATGAGTTTCAATGTCCT-3'. The resulting fragment was subcloned into pGEX-KG (37) to generate a GST-Mts4 fusion protein. The recombinant protein was expressed in *E. coli* BL21 (DE3) *lysS* for 3 h at 37 °C and purified using glutathione-Sepharose 4B beads (Pharamacia Biotech Inc.) according to the manufacturer's instructions. The mts4 protein was also expressed as a 6-histidine-tagged construct in pET6H. The recombinant protein was expressed in *E. coli* BL21 (DE3) *lysS* for 3 h at 37 °C. Western blots were performed on nitrocellulose (Schleicher & Schuell) using the enhanced chemiluminescence system (Amersham Corp.). Mts2 and Mts3 antisera were used as described (31). For binding in crude extracts, bacterial cells were lysed as described above, and extracts containing equimolar amounts of GST-Mts4 or GST alone were mixed with equimolar amounts of purified his-tagged Mts2 or Mts3 in binding buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride), for 1 h at 4 °C. Following washing, the beads were washed three times with washing buffer, and then GST-Mts4 or GST alone released by elution with glutathione for 10 min at room temperature. Samples were boiled in SDS loading buffer, electrophoresed on 10% SDS-PAGE gels, and analyzed by Western blotting. For binding in crude extracts, bacterial cells were lysed as described above, and extracts containing equimolar amounts of GST, GST-Mts4, or GST-Mts2 were mixed at 4 °C for 1 h. Glutathione-Sepharose 4B beads were then added, and the beads were treated as described above. The purified fractions were separated on a 10% acrylamide gel and stained with Coomassie Blue.

**Purification of the 26 S Protease from *S. pombe***—The purification of the 26 S protease from *S. pombe* was essentially as described (41). 5 liters of wild type *S. pombe* cells were grown in yeast extract media to an *A*620 of approximately 1.0. Cell extracts were fractionated by DEAE-cellulose chromatography (Whatman) and by glycerol gradient centrifugation. The peptide cleavage assays were performed as described (41). 7.5 µg (or 1 µg in the anti-Mts4 blot) of protein from the glycerol gradient fractions were separated by SDS-PAGE on a 10% gel and blotted onto nitrocellulose.

**Immunoprecipitations**—*S. pombe* cell extracts were prepared as described (31) in the following buffer: IP1, 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mL of wild type *S. pombe* cells, 50 µl of protein A-Sepharose (Pharamacia) and then covalently coupled using dimethylpimelimidate as described (58). 2 mg of *S. pombe* protein extracts were incubated with the appropriate sera for 1 h at 4 °C. After four washes in the IP1 buffer, the proteins bound to the antibodies were released by boiling in SDS loading buffer.

**REFERENCES**

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The mts4-1 leu1-32 hnmt1 colonies on media lacking thiamine to ensure full expression from the 36 °C (which arrest in metaphase (20, 27). Found to die with heterogeneous phenotypes (data not shown). Study the DNA and microtubules, respectively. The cells were out by staining cells with DAPI and anti-tubulin antibodies to analysis of was found to be recessive to the wild type allele. A cytological insert.

amino acids (also in pREP1), and pREP1 is the vector without a cDNA insert. Mutant was isolated in a screen designed to generate mutations that caused cells to be both resistant to the microtubule destabilizing drug methyl-benzimidazol-2-yl carbamate (MBC) and temperature-sensitive (ts) for growth. Other mutant was isolated by complementation using a genomic library in the pombe genotype leu1-32/ura4D-18, and the ts mutation was found to segregate 2:2 in tetrads. The ts segregants co-segregated with resistance to MBC indicating that a single gene mutation was responsible for conferring resistance to MBC and conditional lethality. A heterozygous diploid strain was generated with the Mts4 Is Subunit 2 of the 26 S Protease in Fission Yeast.

The resulting plasmid was linearized within the nmt1 promoter to left for growth for 6 days at 25 °C (A) or 5 days at 36 °C (B).

RESULTS

Isolation and Characterization of the mts4-1 Mutant—The mts4-1 mutant was isolated in a screen designed to generate mutations that caused cells to be both resistant to the microtubule destabilizing drug methyl-benzimidazol-2-yl carbamate (MBC) and temperature-sensitive (ts) for growth. Other mutants isolated from this screen include mts2-1 (20) and mts3-1 (27). The mts4-1 leu1-32 h- mutant was crossed to the wild type strain leu1-32 h+, and the ts mutation was found to segregate 2:2 in tetrads. The ts segregants co-segregated with resistance to MBC indicating that a single gene mutation was responsible for conferring resistance to MBC and conditional lethality. A heterozygous diploid strain was generated with the genotype mts4+/mts4-1 ade6-M210/ade6-M216 leu1-32/ leu1-32 ura4D-18/ura4D-18 h-/h+, and the mts4-1 mutation was found to be recessive to the wild type allele. A cytological analysis of mts4-1 at the restrictive temperature was carried out by staining cells with DAPI and anti-tubulin antibodies to study the DNA and microtubules, respectively. The cells were found to die with heterogeneous phenotypes (data not shown). The resulting plasmid was linearized within the mts4 sequence and used to transform the mts4-1ura4D-18 h- mutant. Stable uracil prototrophs were selected and crossed to a ura4-D18 h+ strain. Out of 45 full tetrads, no ts mts4-1 alleles were observed indicating that the site of integration was very closely linked to the mts4-1 mutation. Therefore it was concluded that the gene rescuing mts4-1 was in fact the authentic mts4+ gene and not a multicopy suppressor.

Both strands of the mts4+ cDNA were sequenced and were found to encode a predicted protein of 887 amino acids with a molecular mass of 97 kDa. The nucleotide sequence has been submitted to the EMBL nucleotide sequence data base with the accession number Y09819. By comparison with the genomic sequence, a small intron of 47 base pairs was found 146 base pairs from the start ATG. One of the two cDNAs was found to be truncated at the N terminus and results in the loss of the first 131 amino acids (also in pREP1), and pREP1 is the vector without a cDNA insert. mts4-1 cells containing these plasmids were streaked to single colonies on media lacking thiamine to ensure full expression from the nmt1 promoter and used to transform the mts4-1 strain in liquid culture. The resulting spores were purified and submitted to the EMBL nucleotide sequence data base with the accession number Y09819. By comparison with the genomic sequence, a small intron of 47 base pairs was found 146 base pairs from the start ATG. One of the two cDNAs was found to be truncated at the N terminus and results in the loss of the first 131 amino acids of the Mts4 protein. This truncated cDNA, however, is still able to rescue the ts defect of mts4-1 (Fig. 1). The predicted Mts4 protein was found to display considerable similarity to human S2 of the 26 S protease which is also known as p97 (30). In addition, Mts4 is also similar to the protein encoded by the S. cerevisiae gene NAS1, believed to be S2 of the 26 S protease in budding yeast (30). Mts4 is 48 and 47% identical to p97 and Nas1, respectively. A comparison of all three sequences is shown (Fig. 2). The identity between these three sequences extends throughout the length of the proteins. Interestingly, there is an insert in the Nas1 sequence of 78 amino acids that is not present in p97 or Mts4, although the significance of this insert is unclear. Both p97 and Nas1 contain a KEKE motif, a hydrophobic region rich in alternating positively and negatively charged amino acids, lysine, glutamate, and aspartate. The exact function of this motif is unknown, but it has been proposed that it may promote protein-protein interactions (38). Despite overall homology to p97 and Nas1 though, a KEKE motif cannot be identified in the Mts4 sequence. There are no other obvious motifs in any of these sequences, but as found with p97 and Nas1 (30), Mts4 shows limited overall identity of 22% with the product of the S. cerevisiae gene SEN3 (data not shown), which encodes S1 of the 26 S protease (29).

mts4+ Is Essential for Cell Viability—The lack of an obvious phenotype associated with mts4-1 at 36 °C could be a result of the ts allele retaining partial function, resulting in the heterogeneous mix of phenotypes. Therefore the consequences of loss of mts4+ function were investigated by constructing a null allele. Most of the coding region of the mts4+ gene was replaced by the ura4+ marker as shown (Fig. 3A). The mts4::ura4+ construct was used to transform a diploid strain, and stable uracil prototrophic transformants were selected. Southern blot analysis was used to identify a transformant in which the mts4::ura4+ construct had integrated at the mts4+ locus (data not shown). The resulting heterozygous diploid was sporulated, and tetrads were dissected. In each of 30 tetrads analyzed, only two spores gave rise to viable colonies. These were always ura- and therefore wild type at the mts4+ locus demonstrating that the mts4+ gene is essential for growth. Microscopic examination of the inviable spores revealed that germination occurred but that the cells subsequently died after a maximum of two rounds of division.

Further examination of the mts4::ura4- cells was carried out by sporulating the heterozygous mts4+/mts4::ura4- diploid strain in liquid culture. The resulting spores were purified and inoculated into minimal medium lacking uracil which allows germination of the mts4::ura4+ spores but not those which are wild type with respect to the mts4+ gene. These wild type spores lack the ura4+ gene. Cells were sampled at various time points after incubation in this media and were analyzed by both fluorescence microscopy and flow cytometric (fluores-
cence-activated cell sorter) analysis. Microscopic examination of cells stained with 4',6-diamidino-2-phenylindole (DAPI) and anti-tubulin antibodies revealed that the mts4 null allele caused cells to arrest in metaphase (Fig. 3B). After 6 h in the medium lacking uracil, 49% of the cells possessed a short mitotic spindle and condensed DNA. This phenotype was transient as upon prolonged incubation, the mitotic spindle disassembled, the DNA decondensed, and septa were formed (Fig. 3C). The germinating spores were also stained with propidium iodide and subjected to flow cytometric analysis to determine DNA content (Fig. 3D). Spores sampled immediately after inoculation into medium lacking uracil (T = 0) were mostly found to have a 1 N DNA content corresponding to cells that had not yet replicated their DNA. After 1–2 h, half of the spores had a

FIG. 2. Comparison of the predicted protein sequence of Mts4 with human p97 and S. cerevisiae Nas1. Sequence data of p97 and Nas1 are taken from Tsurumi et al. (30). Dotted lines represent gaps inserted to achieve the maximum alignment. Identical residues are shown in reverse font. The position within the protein sequence is indicated at the right-hand side. A KEKE motif is found in p97 between residues 623 and 641. An extended KEKE motif is also found in Nas1 between positions 630 and 729. These regions are indicated by a solid black line.
Fig. 3. Phenotype of the mts4 null allele. A, the construction of pBSmts4::ura4'. A restriction map of the mts4' gene is shown. The bar represents 100 base pairs. The EcoRV-SpeI fragment of mts4' was removed and replaced with a HindII-SpeI fragment containing the ura4' gene. Restriction sites are indicated as follows: X, XbaI; H, HindIII; S, SphI; E, EcoRV; Sp, SpeI; B, BamHI; N, NheI; (Sal) indicates a SalI site that was introduced into the sequence for the purpose of subcloning and is not an endogenous site in the gene. The open reading frames (ORFs) of the mts4' gene are also shown as open boxes. Upon insertion into mts4', the EcoRV and HindII sites are lost. B, analysis of the mts4 null phenotype. Following spore germination, mts4::ura4' spores were sampled and stained with an anti-tubulin antibody (TAT-1) and DAPI at various time points. The anti-tubulin antibodies were detected by a secondary antibody conjugated to CY3 and are stained red. The DAPI is indicated by blue staining. The cells shown were sampled after 6 h incubation at 32 °C. The bar represents 10 μm. C, percentage of mts4 null alleles that show the condensed DNA phenotype (squares), short metaphase spindles (circles), and septa (triangles) at various time points. D, flow cytometric analysis of propidium iodide-stained spores. The spores were analyzed at the various time points indicated. The positions of the 1 and 2N peaks are indicated. E, percentage of binucleate cells, and F, percentage of mitotic spindles in mts4 null (filled squares) and wild type alleles (open squares) at various time points during germination.
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1 n DNA content, corresponding to the wild type spores that are unable to germinate without uracil. The remaining spores, which possess the mts4::ura4+ genotype, had a 2 n DNA content due to the replication of the DNA, a finding consistent with the metaphase arrest seen upon cytological examination.

As a control, a wild type diploid strain that was ura4- was sporulated, and the resulting spores treated in an identical manner. Germinating cells were sampled with time and subjected to cytological analysis. As can be seen in Fig. 3, E and F, the wild type and mts4 null spores entered mitosis at an identical time indicating that the mts4 null cells were not defective in cell cycle progression prior to this point. Whereas the wild type spores completed anaphase, as demonstrated by the abundance of binucleate cells, the mts4 null spores became blocked specifically at the metaphase to anaphase transition (as indicated by the low number of binucleate cells).

Human S2 Can Rescue the mts4 Null Allele—To test whether the human S2 (p97) is a functional as well as a sequence homologue of Mts4, the cDNA encoding p97 was subcloned behind the inducible fission yeast promoter, nmt1, in the LEU2-based vector pREP1 (39, 40). mts4-1 cells containing this construct were able to grow at the restrictive temperature of 36 °C (Fig. 4A). In addition, this construct was also transformed into the heterozygous diploid mts4-1ura4-D18leu1-32ade6-M210 h-1 mts4::ura4-1leu1-32ade6-M216 h+. After sporulation of the diploid, segregants that were Ura" Leu" were able to form colonies demonstrating that the cDNA encoding human S2 rescued the mts4 null allele (Fig. 4B). Cells containing the human S2 cDNA formed colonies at the same rate as those containing the mts4-1 cDNA. Furthermore, upon microscopic examination, these cells appeared wild type (data not shown). These results demonstrate that there is a functional similarity between Mts4 and S2 of the human 26 S protease. In addition, the truncated mts4 cDNA was also able to rescue the mts4 null allele. This suggests that the N-terminal 131 amino acids are not essential for the function of the Mts4 protein (Fig. 4B).

Mts4 Co-purifies with Known Subunits of the 26 S Protease—The sequence and functional similarities between Mts4 and p97 suggested that Mts4 might be S2 of the fission yeast 26 S protease. To determine whether this was the case, the 26 S complex was purified from S. pombe cell extracts and the presence of Mts4 determined by Western blot analysis. Polyclonal antibodies were raised against a GST-Mts4 fusion protein, and their specificity was tested on total protein extracts from wild type S. pombe cells. The antibodies recognized one band corresponding to a protein of approximately 97 kDa. In contrast, no band was seen in protein extracts made from the mts4 null allele containing the human S2 cDNA (Fig. 5A). These results show that the antibodies are specific for the Mts4 protein. In light of the reasonable level of homology between Mts4 and p97, it is interesting to note that the antibodies do not cross-react with the human S2 protein. The 26 S protease was purified as described previously (41). Briefly, cell extracts were passed over an anion exchange column and then fractions containing activity pooled and fractionated further by centrifugation through a glycerol gradient. The fractions containing 26 S activity were identified by their ability to cleave a fluorogenic peptide. Although cleavage of the peptide is due to activity of the 20 S core, ATP-stimulated proteolysis is indicative of the whole 26 S protease. The profile of peptidase activity of alternate fractions from the glycerol gradient centrifugation step is shown (Fig. 5B). Mts4 was found to co-purify with those fractions containing the highest specific activity which corresponded to fractions 4–6 of the glycerol gradient (Fig. 5C).

Furthermore, these are the fractions where known subunits of the complex were previously shown to migrate (41). This was tested using antibodies against Mts2 (S4) and against subunits of the 20 S core. Both Mts2 and the 20 S complex were found predominantly in fractions 4–6 indicating that Mts4 does indeed co-purify with the 26 S protease. Taking these results together with the sequence and functional similarity found between Mts4 and subunit 2 of the human 26 S protease, we conclude that mts4 encodes S2 of the 26 S protease in S. pombe.

mts4 Interacts Genetically with mts2—To try and identify genes whose products interact with Mts4, the mts4-1 mutant strain was transformed with a cDNA library as detailed above to isolate multicopy suppressors that could rescue the temperature-sensitive phenotype at 31 °C. This lower temperature was used because only the authentic mts4+ gene was isolated in this way at 36 °C. One suppressing cDNA encoded the mts2+ gene (20) and its ability to rescue mts4-1 at 31 °C is shown (Fig. 6). In addition, the mts4-1 and mts2-1 alleles were found to be synthetically lethal at the permissive temperature of 25 °C. Tetrad analysis was carried out on crosses between the two
The presence of wild type colonies, after crossing the two strains together, indicated that the tetrad also contained the double mutant. Asci containing four viable, three viable, and two viable spores segregated in a ratio of six, seven, and four, respectively. In the asci containing three viable spores, the missing one was deduced to be the \textit{mts4-1mts2-1} double mutant. The same conclusion was drawn from the four asci containing two viable spores which both gave rise to wild type colonies. The double mutant spores were examined microscopically and were found to divide once or twice to give a maximum of four cells (data not shown). The \textit{mts3} gene encodes S14 of the 26 S protease and was cloned by complementation of the \textit{mts3-1} mutant (27). No synthetic lethality was found between \textit{mts4-1} and \textit{mts3-1}. Furthermore, overexpression of the \textit{mts3} cDNA was unable to rescue \textit{mts4-1} at 31 °C. The most likely explanation of these results is that the Mts4 protein binds to Mts2 \textit{in vivo} but not to Mts3.

\textbf{In Vitro Association of Mts4 and Mts2}—To test whether the Mts4 and Mts2 proteins are capable of binding to each other, an in vitro binding assay was carried out. The GST-Mts4 fusion protein was expressed in \textit{E. coli} and purified using glutathione-Sepharose 4B beads. Constructs containing Mts2 and Mts3 fused to a six-histidine tag were engineered, and the resulting fusion proteins were purified from \textit{E. coli} using a nickel agarose column. The purified 6His-Mts2 or 6His-Mts3 proteins were mixed with the beads bound to GST-Mts4. As a control, 6His-Mts2 and 6His-Mts3 were also mixed with beads bound to GST alone. After mixing, the GST or GST-Mts4 beads were extensively washed, and the GST or GST-Mts4 protein was eluted with glutathione, and this fraction was analyzed by SDS-PAGE and Western blotting. As shown, 6His-Mts2 was able to bind to GST-Mts4 but not to GST alone (Fig. 7A). In contrast, GST-Mts4 was not able to bind to 6His-Mts3 (Fig. 7A), but instead the 6His-Mts3 protein was found in the wash fractions (data not shown). When the crude bacterial lysates containing GST-Mts4 and 6His-Mts2 proteins were mixed together followed by purification using glutathione-Sepharose, Mts2 was found to be the major protein bound to GST-Mts4 (as analyzed by SDS-
Mts4 Is Subunit 2 of the 26 S Protease in Fission Yeast

We have shown that the Mts4 protein co-purifies with other components of the fission yeast 26 S protease. Therefore we conclude that Mts4 is S2 of the 26 S protease in fission yeast.

The gene encoding human S2 was previously identified in screens to isolate proteins that bind to the cytoplasmic domain of type 1 TNF receptor (42, 43). The significance of this finding is currently unclear, but perhaps the 26 S protease regulates the TNF signaling pathway in mammalian cell and this regulation is mediated through S2. TNF is a cytokine associated with a variety of functions including the induction of cell death, the modulation of differentiation patterns, and the activation of certain immune processes (44–46). The signaling of apoptosis is mediated by a specific region in the C terminus of the TNF receptor, namely the death domain. The human S2 protein was found to associate with a region of the TNF receptor that is distinct from this death domain (42), raising the possibility that S2 is involved in mediating TNF signaling pathways other than apoptosis.

One possible function of the non-ATPase subunits is that they each recognize different subsets of substrates. Previously, S5a has been shown to bind polyubiquitin chains in vitro and is thought to play a role in substrate selection (23–25). However, Saccharomyces cerevisiae cells deleted for the MCB1 gene, which encodes S5a, are viable and only partially defective in degrading polyubiquitated proteins indicating that other factors are involved in ubiquitin-dependent substrate recognition by the 26 S protease (25). Consistent with this view is genetic evidence from studies on a number of mutations in other non-ATPases in yeast. For example, a mutation in the S. cerevisiae gene SEN3 (S1) causes an increase in the levels of a protein involved in tRNA splicing (29). A mutation in the S. cerevisiae gene NIN1 (S14) results in the failure to activate Cdc28 kinase activity (26), and a mutation in the fission yeast gene mts3 (S14) causes cells to re-plicate their DNA at the restrictive temperature (27). Recently, the S. cerevisiae gene encoding S2 was identified in a screen to select for mutants that are deficient in the degradation of an isoyme of 3-hydroxy-3-methylglutaryl-CoA reductase (47). Such a finding could indicate that S2 is involved in selecting 3-hydroxy-3-methylglutaryl-CoA reductase for degradation. Further analysis of such 19 S cap mutants should help elucidate the role of the non-ATPases within the 26 S protease and their possible role in substrate selection.

Previous analyses of the S. cerevisiae S2 gene have shown

\[ \text{C. R. M. Wilkinson and C. Gordon, unpublished data.} \]
that it is an essential gene (30, 47). In our study, however, we have demonstrated not only that S. pombe S2 is essential but also that it is required for the metaphase to anaphase transition in the mitotic cell cycle. It was not possible to identify a cell cycle phenotype associated with the ts allele of mts4-1 at the restrictive temperature, probably due to the mutation being somewhat leaky. In contrast, the null allele of mts4 was found to arrest in metaphase with condensed DNA and a short mitotic spindle. This phenotype is also found with disruptions of the fission yeast genes mts2 and mts3, which encode S4 and S14 of the 26 S protease, respectively (20, 27). Although the mts4 null spores do eventually disassemble their spindle, this is also seen in the other mts alleles and with other mutations where the corresponding genes are required for the metaphase to anaphase transition, namely mts2, mts3, nuc2, cut4, and cut9. The nuc2, cut4, and cut9 mutants (50), like the mts2-1, mts3-1, and mts4-1 alleles, arrest at the metaphase to anaphase transition; the corresponding Nuc2, Cut4, and Cut9 proteins are known to be part of the anaphase promoting complex (APC, 59). A number of other proteins have also been identified in yeast and frogs that form the APC. This multisubunit particle catalyzes the addition of polyubiquitin chains identified in yeast and frogs that form the APC. This multi-
plex (APC, 59). A number of other proteins have also been identified in yeast and frogs that form the APC. This multi-
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