Mol Gen Genomics (2005) 274: 155–167
DOI 10.1007/s00438-005-0005-8

ORIGINAL PAPER

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Mpg1, a fission yeast protein required for proper septum structure, is involved in cell cycle progression through cell-size checkpoint

Received: 12 November 2004 / Accepted: 29 April 2005 / Published online: 28 July 2005
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Abstract Using a yeast two-hybrid screen we isolated a gene from *Schizosaccharomyces pombe* which corresponds to the previously uncharacterized ORF SPCC1906.01. We have designated this gene as *mpg1*, based on the putative function of its product as a mannose-1-phosphatase guanyltransferase. Mpg1 shows strong similarity to other GDP-mannose-1-phosphate guanyltransferases involved in the maintenance of cell wall integrity and/or glycosylation. This homology, together with the protein's localization pattern demonstrated in this work, strongly suggests that Mpg1 is involved in cell wall and septum synthesis. Moreover, cells lacking Mpg1 present a defect in glycosylation, are more sensitive to *Lyticase*, and show an aberrant septum structure from the start of its deposition, indicating that the Mpg1 function is necessary for the correct assembly of the septum. Interestingly, lack of Mpg1 clearly affects cell cycle progression: *mpg1* null mutants arrest as septated and bi-nucleated 4C cells, without an actomyosin ring. Wee1 is required for the G2/M arrest induced in the absence of Mpg1, since the blockade is circumvented when Wee1 is inactivated. Wee1 is part of a cell-size checkpoint that prevents entry into mitosis before cells reach a critical size. The results presented in this work demonstrate that the G2/M arrest induced in the absence of Mpg1 is mediated by this cell size checkpoint, since oversized mutant cells enter mitosis. The *mpg1* loss-of-function mutant, therefore, provides a good model in which to study how cells coordinate cell growth and cell division.

Keywords Yeast cell cycle · Cell wall/septum structure · GDP-mannose-1-phosphate guanyltransferases · Glycosylation · Cell size checkpoint

Electronic Supplementary Material Supplementary material is available for this article at http://dx.doi.org/10.1007/s00438-005-0005-8

Communicated by E. Bejarano

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Introduction

In order to maintain a specific size, cells must balance growth and division. Yeast cells are thought to use cell-size checkpoints to coordinate these two processes (Nurse 1975; Fantes and Nurse 1977; Hartwell and Unger 1977; Johnston et al. 1977; Rupes 2002). Cell-size checkpoints prevent passage through key cell cycle transitions until cells have reached a critical size. In the fission yeast *Schizosaccharomyces pombe* the critical size requirement is exerted primarily at the G2/M transition, whereas in budding yeast (*Saccharomyces cerevisiae*) it is effected primarily at the G1/S transition (Rupes 2002). Genetic analysis in fission yeast has demonstrated that the kinase Wee1 plays an important role in the checkpoint that coordinates cell growth and cell division (Nurse 1975; Fantes and Nurse 1978; Thuriaux et al. 1978).

During exponential growth *S. pombe* cells spend most of their lives in G2. They grow by extension at one or both ends until they reach a critical size: the G2/M cell size threshold. Growth is accompanied by de novo cell

wall synthesis. Yeast cell wall proteins are highly glycosylated: carbohydrate chains of various lengths are linked via N- or O-glycosidic bonds to asparagine and serine/threonine residues, respectively (Kukuruzinska et al. 1987). The carbohydrate component of *S. pombe* glycoproteins comprises galactose and mannose (Phaff 1971), with mannose being the essential component (Huang and Snider 1995). GDP-mannose acts as the mannose donor in lipid and protein glycosylation. The final reaction in the synthesis of GDP-mannose is the transfer of mannose-1-phosphate onto GTP, a reaction catalyzed by GDP-mannose-1-phosphate guanylyltransferase (EC 2.7.7.13). The relationship between glycosylation, cell wall structure and cell growth has been explored by mutant analysis (Huang and Snider 1995; Takegawa et al. 1996; Cortes et al. 2004). Glycosylation-defective mutants display altered surface properties as a consequence of changes in carbohydrate structure. More specifically, the absence of GDP-mannose pyrophosphorylase can lead to phenotypes such as spontaneous lysis, increased sensitivity to a wide range of antifungal drugs and cell wall inhibitors, defects in cell separation and/or cytokinesis, flocculation, and cell wall fragility (Warit et al. 2000; Agaphonov et al. 2001).

Cell wall synthesis is also required during cytokinesis to construct the septum. *S. pombe* assembles an actomyosin ring at the site of division on entry into mitosis, and divides by binary fission as a consequence of constriction of the ring, in a manner similar to that observed in animal cells (reviewed in Guertin et al. 2002; Rajagopalan et al. 2003). The timing of septum formation coincides with that of ring constriction, which is accompanied by centripetal deposition of the primary division septum. Subsequently, secondary septa are deposited on either side of the primary septum before it is digested by glucanases, which results in the generation of two individual daughter cells (Gould and Simanis 1997; Ishiguro 1998; Martin-Cuadrado et al. 2003). The process of cell separation in fission yeast is mediated by the exocyst, a multiprotein complex which may provide a platform for the docking and fusion of vesicles containing cell-wall degrading enzymes at the division site (Wang et al. 2002, 2003).

As mentioned above, a fundamental element of cell cycle regulation consists of the arrest of the cycle at particular checkpoints to ensure the completion of a previous cell cycle event, or to repair cellular damage (Rhind and Russell 1998). Several different G2/M checkpoints have been described that ensure the genomic integrity of the daughter cells (Al-Khodairy and Carr 1992; Enoch et al. 1992; Murray 1992; Elledge 1996; Rhind et al. 1997; Rhind and Russell 1998; Weinert 1998). A checkpoint that monitors cytokinesis in *S. pombe* has been also demonstrated (Le Goff et al. 1999; Liu et al. 2000). Thus, *S. pombe drc/cps1* mutants are defective in cytokinesis since, although capable of assembling the actomyosin ring, they are unable to assemble the division septum, resulting in a G2/M arrest as bi-nucleated cells with a stable actomyosin ring (Ishiguro 1998; Liu et al. 1999). This cytokinesis checkpoint requires Wee1, F-actin structures, and the Septation Initiation Network (SIN; Le Goff et al. 1999; Liu et al. 1999, 2000, 2002; Balasubramanian et al. 2000), a signalling pathway that regulates the timing of cytokinesis in fission yeast (Simanis 2003). Flp1/Clp1 functions together with Wee1 and the SIN to coordinate the completion of cytokinesis with the initiation of the next cell cycle (Cueille et al. 2001; Trautmann et al. 2001). This cytokinesis checkpoint also appears to be activated in the cytokinesis mutants *cdc3*, *cdc8*, and *cdc12*, which do not assemble their actomyosin rings properly, and are primarily bi-nucleated at the restrictive temperature.

In this study we have isolated and characterized *mpg1*, an essential *S. pombe* gene encoding a putative GDP-mannose-1-phosphate guanylyltransferase. Mpg1 homologues have been proposed to be involved in cell wall maintenance and protein glycosylation in various organisms (Hashimoto et al. 1997; Nickle and Meinke 1998). In addition, the localization pattern of Mpg1, together with the aberrant septum structure and hypersensitivity to Lyticase displayed by Mpg1 null mutants, is consistent with a role for Mpg1 in cell wall synthesis. Mpg1 mutants arrest as bi-nucleated and septated cells with two G2 nuclei. We have found that this arrest is brought about by an inability to reach the required critical size, since *mpg1* mutant cells, which have become highly elongated, are able to enter mitosis and segregate their G2 nuclei.

Materials and methods

Genetic techniques and general procedures

All *S. pombe* strains were isogenic to 972 (Leupold 1970). The following strains were used in this study: *leu1-32 ura4-D18 h-* (wt), *cdc25-22* and *wee1-50* (Nurse 1975), and *flp1/clp1::kanMX6* (Cueille et al. 2001). Double mutants were obtained by crossing mutants of opposite mating types and selecting recombinant strains by tetrad dissection. Techniques and media (YES and synthetic EMM media, supplemented as required) used for growth, analysis and transformation of *S. pombe* strains have been described previously (Moreno et al. 1991). DNA sequencing, cloning, and subcloning were carried out according to standard procedures (Sambrook and Russell 2001). Synchronous cultures of *cdc25-22* mutants were prepared by growing the cells to mid-log phase (0.5×10^7 cells/ml) at 25°C in liquid medium, followed by transfer to medium pre-warmed at 35°C. After incubation at 35°C for 4.5 h, G2/M-arrested cells were downshifted by re-suspension in medium pre-warmed to 25°C. Cells were then collected at the desired intervals.

Northern analysis was performed with total RNA prepared as previously described (Moreno et al. 1991) and hybridized with specific DNA probes for *mpg1* (1.4-kb fragment), *cig2* (0.36-kb fragment), and *ura3* (1.2-kb

fragment), containing the complete ORFs of the corresponding genes.

Yeast two-hybrid assay

The two-hybrid plasmid pAS2 (Clontech) was used for the fusion of the bait protein to the GAL4 DNA binding domain (DB). The region encoding the Rep protein of the geminivirus *Tomato yellow leaf curl Sardinia virus* (TYLCSV-ES) was obtained by the digestion of pTYA50 (a plasmid containing the full-length TYLCSV-ES[2] genome—GenBank Accession No. L27708—cloned into the *Eco*RI site of pBluescript IIKS$^+$) with *Kpn*I + *Bgl*II. The fragment was blunt-end cloned into the blunt-ended *Nde*I site of pAS2 to yield pTYC12. The *S. pombe* cDNA library used in this work was constructed in pACT (AD-Gal4; *LEU2*; a gift from S. Moreno, Instituto de Microbiología Bioquímica, CSIC/Universidad de Salamanca, Salamanca, Spain).

For the yeast two-hybrid screen we used *S. cerevisiae* strain Y190 as the host [*MAT***a** *gal4 gal80 his3 trp-901 ade2-101 ura3-52 leu2-3, 112 URA3::GAL1::lacZ LYS2::GAL4(UAS)::HIS3 cyhR*] (Harper et al. 1993). Cells were first transformed with pTYC12 as previously described by Gietz (1995), and then transformed again with the *S. pombe* cDNA library in pACT. For moderate-stringency selection, the transformation mixture was plated on SD/-Ade-Leu-Trp-His medium (Yeast Protocols Handbook, Clontech Laboratories, 2001; http://www.clontech.com) supplemented with 2 mM 3-amino-1,2,4, triazole (3-AT) to reduce the incidence of false positive colonies. To corroborate the interaction between the two fusion proteins, galactosidase activity was assayed as previously described (Fromont-Racine et al. 1997). Library plasmid DNA was recovered from positive colonies by transformation into the *Escherichia coli* leuB mutant strain JA226 and subsequent selection for Leu$^+$ colonies (complemented by the LEU2 gene present in the pACT plasmid).

Cloning of the mpg1 gene

The mpg1 gene was cloned following the detection of an interaction of Mpg1 with TYLCSV-ES Rep. Two independent positive yeast colonies were selected and the library plasmids present were named pACTG11 and pACTG13. Both clones were fully sequenced, and their inserts were subcloned as 1.4-kb and 1.8-kb *Bam*HI fragments into the *Bam*HI site of pBSKII+, to yield pBG11 and pBG13, respectively.

A *S. pombe* genomic DNA library constructed in pUR19 was used to isolate a clone containing the genomic version of *mpg1* by colony hybridization. Using the 1.4-kb *Bam*HI fragment of pBG11 as a probe, we isolated a plasmid (pPIG4) containing a 3.2-kb genomic DNA fragment. This fragment was fully sequenced to confirm that it contained the full-length *mpg1* ORF.

Disruption of *mpg1*

A 2.9-kb *Pst*I genomic DNA fragment containing the entire *mpg1* ORF was ligated into the *Pst*I site of pBSKII+ to generate pBIG41. pBIG45 (*mpg1::ura4*) was constructed by replacement of the 1.2-kb *Eco*RV fragment of *mpg1* (all of the ORF except for the first 140 bp) in pBIG41, with a blunt-ended 1.7-kb *Cla*I fragment containing *ura4*. To replace the wild-type *mpg1* gene with *mpg1::ura4*, pBIG45 was digested with *Pst*I and the insert was isolated and transformed into a diploid strain with the genotype *h+/h− ade6-M210/ade6-M216 ura4-d18/ura4-d18 leu1-32/leu1-32*. Multiple diploids heterozygous for *mpg1::ura4* were confirmed by Southern analysis. Random-spore and tetrad analysis of heterozygous diploids was performed by growing the spores in minimal media with leucine and adenine.

Construction of a conditional *mpg1* mutant

The strain *nmtmpg1* was constructed by the insertion of a *Bam*HI -*Pst*I fragment containing the entire *mpg1* ORF into the *Bam*HI/ *Pst*I sites of pREP3X, to generate pRG11. A *Pst*I -*Sac*I fragment from pRG11 was cloned into the *Pst*I/ *Sac*I sites of the integrative plasmid pJK148 to obtain pJK11. Diploid *mpg1::ura4* heterozygotes were transformed with linearized pJK11 (digested with *Spl*I). Several stable *mnt-mpg1* transformants were analysed by Southern hybridization. A diploid transformant bearing a single integrated copy of the plasmid at the *leu1* locus was selected (Δ*mpg1-nmtmpg1*). Leu$^+$ Ura$^+$ haploid spores derived from this diploid transformant had the endogenous *mpg1* gene disrupted and expressed the *mpg1* cDNA under the control of the inducible *nmt1* promoter (Maundrell 1993). Cells were analysed at different times after the addition of thiamine (which represses this promoter) to EMM liquid cultures incubated at 30°C.

Generation of anti-Mpg1 antibodies

A His_6-tagged Mpg1 protein expressed in and purified from *E. coli* was used for the generation of polyclonal antibodies. The tagged protein was obtained in the following manner. A 1.09-kb *mpg1* fragment containing the entire ORF was amplified by PCR with the primers 5-BamHIGipSP (5′-TCGGGATCCATGAAGGCTCTGATTC-3′) and 3-BamHIGipSP (5′-AGAGGATCCTTACATGACAATAGTACC-3′), digested with *Bam*HI, and ligated into the *Bam*HI site of the His-tag expression vector pQE30 (Qiagen) to generate pQG11. pQG11 was transformed into *E. coli* XL1Blue. His_6-tagged protein was affinity purified from the insoluble fraction with Ni agarose under denaturing conditions, as recommended in the QIAexpress manual (Qiagen). Rabbits were immunized by injection of 200 mg of purified protein.

Western analysis

S. pombe cells from a 50-ml culture were collected and resuspended in 200 μl of extraction buffer (EB; 30 mM NaF, 100 mM NaCl, 20 mM Tris–HCl pH 8.0, 10% glycerol, 0.5% Triton X-100) supplemented with 5 μg/ml aprotinin, 5 μg/ml pepstatin A, 5 μg/ml leupeptin and 1 mM PMSF. The cells were then disrupted with 500 μl of acid-washed glass beads (425–600 μm; Sigma) by agitation in a FastPrep FP120 at power setting 5.5 for two 15-s intervals separated by 1 min on ice. Then 300 μl of EB with protease inhibitors was added, and the sample was vortexed. Lysates were subjected to centrifugation at 12,000×*g* at 4°C for 30 min to remove insoluble proteins, the supernatants were recovered, and the protein concentration was determined using the Bradford assay (Bio-Rad). Western analysis was carried out after equivalent amounts of protein had been fractionated by electrophoresis on an SDS-PA gel and transferred to nitrocellulose membrane (Hybond ECL - Amersham). Rabbit polyclonal anti-Mpg1 was used as the primary antiserum, and labelled bands were detected with the ECL chemiluminescence system (Amersham Pharmacia Biotech).

Fluorescence and electron microscopy

For immunofluorescence detection of Mpg1, cells were fixed in formaldehyde and processed as previously described (Alfa et al. 1993a). Preparations were incubated with anti-Mpg1 antibody (1:50 dilution) and goat anti-rabbit IgG rhodamine-conjugated secondary antibody (Sigma; 1:1000 dilution), and observed with a Nikon Microphot-FXA fluorescence microscope. Standard techniques were used for F-actin localization (Alfa et al. 1993b) with an anti-actin monoclonal antibody (Amersham), and for cell staining with Calcofluor white or DAPI (Balasubramanian et al. 1997).

Electron microscopy was performed according to a method supplied by S. Sazer (Department of Biochemistry and Molecular Biology, Baylor College of Medicine, Houston, TX, USA). Cells were washed three times with distilled water, rinsed with 1.5% potassium permanganate in distilled water, and then rinsed until the supernatant was transparent. At this step cells were dehydrated by submersion in serial concentrations of acetone. The pellet was finally embedded in resin and sectioned with a Reichert-Jung ultramicrotome. Samples were analysed with a Phillips CM 100 electron microscope.

Cytological methods

FACS analysis was performed as described previously (Sazer and Sherwood 1990) using a Becton Dickinson flow cytometer. Analysis of mutant viability was performed after the addition of phloxin-B (Sigma) to the incubation medium, at a final concentration of 5 μg/ml.

Assay for sensitivity to Lyticase

Sensitivity of cells to Lyticase (Sigma) was measured as described elsewhere (Ishiguro et al. 1997). The log-phase cells were washed with 50 mM citrate–phosphate buffer (pH 5.6) and incubated at 30°C with continuous shaking in the same buffer containing Lyticase (10 U/ml). The residual absorbance of the cell suspensions was monitored at 595 nm every 30 min.

Detection of invertase activity in gels

S. pombe strains were grown to early log-phase in synthetic EMM medium, containing 2% glucose and supplemented as required. After two washes with distilled water, the cells were resuspended in the same medium containing 0.1% glucose and incubated for 2–3 h. After washing with 0.1 M sodium acetate (pH 4.5), containing 10 mM sodium azide, total cell extracts were obtained as described by Moreno et al. (1990), and fractionated by electrophoresis under native conditions on a 5% polyacrylamide gel for 20 h at 40 V. Invertase activity was detected *in situ* as previously described (Moreno et al. 1990).

Results

Isolation of the *mpg1* gene

We isolated the *S. pombe mpg1* gene in a genetic screen that was designed to identify yeast proteins that interact with the Rep protein encoded by the geminivirus *Tomato yellow leaf curl Sardinia virus* (TYLCSV), a plant virus with a single-stranded (ss) DNA genome. Rep is a multifunctional protein that is essential for viral replication (reviewed in Hanley-Bowdoin et al. 1999; Gutierrez 2000) and interacts with itself (Orozco et al. 1997), with another geminivirus protein (Settlage et al. 1996), and also with a large number of cellular proteins involved in cell cycle regulation (Ach et al. 1997; Kong et al. 2000), development (Kong and Hanley-Bowdoin 2002), DNA replication (Luque et al. 2002, Castillo et al. 2003; Bagewadi et al. 2004), and post-translational protein modification (Castillo et al. 2004). To identify host proteins that interact with the Rep protein of TYLCSV, we used the yeast two-hybrid technology (Fields and Song 1989). Since the processes involved in DNA replication and cell cycle control are highly conserved among eukaryotes, from yeast to human and plants, we decided to screen a library of *S. pombe* cDNAs fused to the coding sequence for the activation domain of GAL4 using TYLCSV Rep fused to the GAL4 DNA-binding domain as bait. Yeast cells

harbouring the bait plasmid were transformed with the *S. pombe* cDNA library and plated on histidine selection medium. Two independent yeast clones were isolated, both of which carried the same complete ORF, encoding a protein of 364 amino acids with a predicted molecular mass of 39,713 Da and a calculated pI of 6.22. This protein was designated Mpg1 (for mannose-1-phosphatase guanyltransferase), because it displays homology to several GDP-mannose-1-phosphate guanylyltransferases (EC 2.7.7.13) from fungi and higher eukaryotes, and has been annotated accordingly in the genome sequence of *S. pombe* (SPCC1906.01). Database searches revealed that Mpg1 is highly conserved in most eukaryotic organisms, including fungi, nematodes, insects, mammals, and plants (see Fig. 1). The deduced amino acid sequence of Mpg1 is 71% identical to that of Psa1p/Srb1p/Vig9p of *S. cerevisiae* (Benton et al. 1996). *PSA1* is an essential gene involved in glycosylation and cell-wall maintenance (Benton et al. 1996; Zhang et al. 1999). The gene encoding Mpg1 is located on *S. pombe* chromosome III.

Expression profile of the *mpg1* gene

To extend our knowledge of the function of *mpg1*, we analysed its expression during the cell cycle, at both RNA and protein levels, using synchronized cultures of *S. pombe* (see Electronic Supplementary Material, Fig. S1). A thermosensitive mutant allele of *cdc25* (*cdc25-22*) was used to arrest cells at G2/M at 35°C (Russell and Nurse 1986). The cells were subsequently released synchronously by shifting the temperature to 25°C. Levels of the *mpg1* transcript were determined by Northern analysis, using the *cig2* transcript (which codes for a G1 cyclin) as a standard for comparison. We could not detect any major variation in the level of *mpg1* transcripts during the cell cycle. These results confirm those obtained using microarray technology (http://www.genedb.org). Western analysis of the same cultures was also performed to determine the amount of Mpg1 protein. Although we could not detect any major variation in the level of *mpg1* transcripts, we did register a slight increase in the amount of the protein just prior to the rise in the cell septation index, followed by a decrease just before the drop in the percentage of septated cells (Supplementary Fig. S1d and e).

Mpg1 localizes in the cytoplasm or to one pole during cell growth

Previous results obtained with mutants for *mpg1* homologues in other organisms (Hashimoto et al. 1997; Nickle and Meinke 1998; Warit et al. 1998;

Fig. 1 Comparison of the amino acid sequence of Mpg1 from *Schizosacharomyces pombe* with homologues from *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, *Caenorhabditis elegans*, *Drosophila melanogaster* and *Homo sapiens*. Sequence identities are highlighted in *black*

Zhang et al. 1999; Agaphonov et al. 2001) suggested that their products are involved in the maintenance of cell-wall integrity. To determine if Mpg1 localization in different stages of the cell cycle could be correlated with such a function, we determined the intracellular distribution of Mpg1 using a polyclonal anti-Mpg1 antibody.

Figure 2 shows the intracellular localization of Mpg1 as determined by indirect immunofluorescence with anti-Mpg1 antibodies. Mpg1 clearly accumulates in the cytoplasm. Interestingly, in small uninucleated cells we usually found Mpg1 to be localized at a single pole (see Fig. 2a), while in larger uninucleated or septated cells it was found dispersed throughout the cytoplasm (see Fig. 2b, c), suggesting a relationship between Mpg1 localization and cell wall synthesis. We never detected fluorescence in the nuclei, or in cells probed with pre-immune serum (see Fig. 2d).

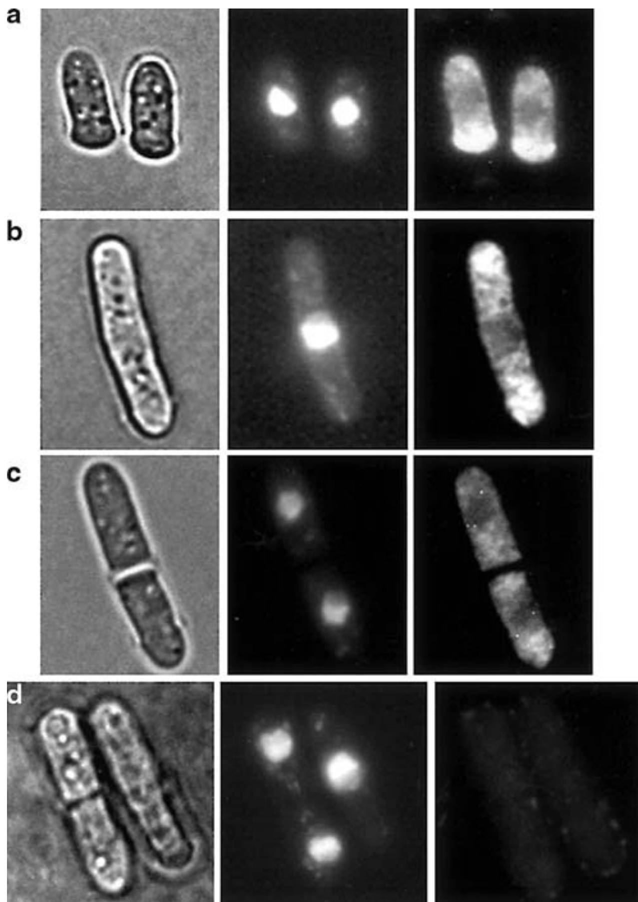

Fig. 2 Mpg1 localizes in the cytoplasm and preferentially to one pole during cell growth. Wild-type cells grown at 30°C were collected, fixed and processed for immunofluorescence microscopy after labelling with an anti-Mpg1 antibody and DAPI. Phase contrast, DAPI and anti-Mpg1 images are shown in each row. Note the accumulation of Mpg1 at one end of small cells (**a**) or throughout the cytoplasm in larger uninucleated (**b**) and septated cells (**c**). Row **d** shows wild-type cells stained with DAPI and processed for immunofluorescence after incubation with pre-immune serum

Loss of the Mpg1 function results in changes in septum/cell wall structure and causes arrest at the G2/M boundary

To determine whether Mpg1 is essential for yeast cell growth, we constructed a diploid yeast strain in which one copy of *mpg1* was disrupted (see Materials and methods). Disruption of the gene was confirmed by Southern analysis (data not shown). After sporulation, 10 asci were dissected on rich medium at 30°C. Tetrad analysis revealed that *mpg1* is an essential gene, since only two spores per tetrad were viable and all resulting colonies were auxotrophic for uracil. The other two spores (*mpg1* nulls) divided once and then ceased to grow (data not shown). To study the *mpg1* loss-of-function phenotype, a conditional mutant was generated. We constructed a *mpg1*-disrupted strain (Δ*mpg1-nmtmpg1*) containing a chromosomally integrated cassette comprising *mpg1* fused to the *nmt1* promoter. Insertion at the *leu1* locus was confirmed by Southern analysis (data not shown). When this conditional mutant is grown in the absence of thiamine, the *nmt1* promoter is active and the strain behaves like the wild type. However, in the presence of thiamine, transcription of the fusion gene is turned off, and after 9 h of incubation under these conditions no Mpg1 is detectable by Western analysis (data not shown), and cell growth ceases (Supplementary Fig. S2).

Wild-type and Δ*mpg1-nmtmpg1* mutant cells growing in the absence of thiamine were shifted to the same medium or to thiamine-containing medium, and cell and nucleus morphologies were scored (see Fig. 3a, c). In a Δ*mpg1-nmtmpg1* culture arrested in the presence of thiamine for 14 h, more than 60% of the cells were bi-nucleate and septated. These cells displayed no obvious changes in nuclei morphology, but had wider septa than normal, as shown by Calcofluor staining (see Fig. 3c). To further characterize the phenotype resulting from the absence of Mpg1, we also determined the cellular DNA content of the samples described above (see Fig. 3b). After incubation for 9–10 h in the presence of thiamine, the proportion of 4C cells was the same as that of septated, bi-nucleated cells. These results indicate that bi-nucleate septated 2C cells can progress through S phase and duplicate their DNA but are unable to proceed into mitosis. These results also show that there is no cell separation even when a new round of DNA replication has taken place, indicating that the primary septum cannot be degraded. Finally, phloxin-B staining revealed that, although loss of the *mpg1*-function causes cell cycle arrest, it is not lethal for the cells (data not shown).

Since septum formation is coupled with the contraction of the actomyosin ring we decided to analyse the distribution of actomysin in arrested Δ*mpg1* cells. Using immunofluorescence techniques with anti-F-actin monoclonal antibodies we confirmed that the ring was indeed absent in the mutant (see Fig. 3d). We found neither a complete actomyosin ring nor disorganized ring components present in arrested mutant cells.

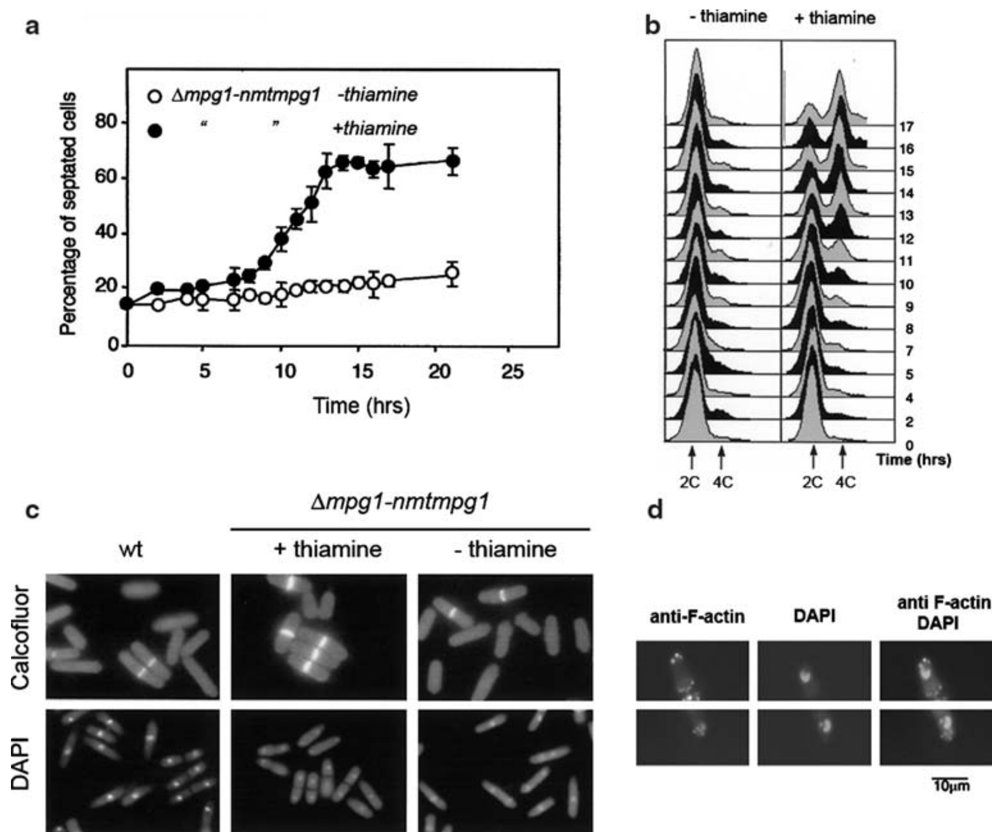

Fig. 3 Lack of expression of *mpg1* blocks cell division at the G2/M transition, which results in an increase in the numbers of bi-nucleated, septated cells. Δ*mpg1 nmt-mpg1* mutant cells were cultured at 30°C until they reached exponential phase, and then shifted to medium containing thiamine to repress *mpg1* expression, or without thiamine as a control. Samples were taken at different times, fixed, and stained with either DAPI or Calcofluor white (**a**), or prepared for flow cytometric analysis (**b**). The percentage of septated cells (**a**) and the distribution of DNA contents (**b**) of Δ*mpg1 nmt-mpg1* cells collected from cultures with or without thiamine are shown. **c** DAPI and Calcofluor staining of Δ*mpg1 nmt-mpg1* cells after growth for 15 h in medium with or without thiamine. Wild-type (wt) cells grown in thiamine-containing medium are also shown. **d** Δ*mpg1 nmt-mpg1* cells were grown at 30°C for 15 h in the presence of thiamine, then samples were removed for fixation and stained with an antibody specific for F-actin antibody, or with DAPI to visualize the nuclei

Interestingly, in septated *mpg1* deficient cells, F-actin patches were mainly found at the division site, which is consistent with a delay in septation, and in many cells the patches were already localized to the tip. The same result was obtained by immunofluorescence analysis using a phalloidin–rhodamine conjugate to localize F-actin (data not shown).

Calcofluor staining of septated bi-nucleated arrested Δ*mpg1* cells indicated that these cells have an abnormally wide medial septum (Fig. 3c). To further investigate this feature, the septum structure was examined by electron microscopy in both mutant and wild-type cells. As shown in Fig. 4a, the septum in *mpg1* mutant cells is clearly wider than that in wild-type cells and displays an abnormal morphology. We also analysed whether the wider septum observed in arrested septated cells was generated as a consequence of sustained deposition onto the medial septum. This alternative was ruled out when we observed that septum structure was aberrant from the earliest stage of deposition (see Fig. 4b, c).

Mpg1 displays homology to several GDP-mannose-1-phosphate guanylyltransferases from different organisms, and is therefore predicted to be involved in glycosylation. As expected, Δ*mpg1-nmtmpg1* mutant cells grown in the presence of thiamine failed to fully glycosylate invertase, indicating that the *mpg1* loss-of-function mutant is indeed defective in glycosylation (Supplementary Fig. S3). To gain further insight into the function of Mpg1 in cell wall structure we investigated whether the absence of Mpg1 affected the sensitivity of mutant cells to a cell-wall-digesting enzyme complex (Lyticase). Both wild type and Δ*mpg1-nmtmpg1* cultures were grown either in the presence or absence of thiamine and then treated with Lyticase and the residual absorbance of the culture was measured at various times (see Fig. 4d). Δ*mpg1-nmtmpg1* cells expressing Mpg1 behaved like wild-type cells, but when Mpg1 expression was repressed the cells became noticeably more sensitive to Lyticase, suggesting that the integrity of the cell wall is affected when Mpg1 is absent.

The G2/M arrest induced by the absence of Mpg1 is mediated by Wee1

As described in the previous section, in the absence of Mpg1 most of the cells arrest as bi-nucleated and

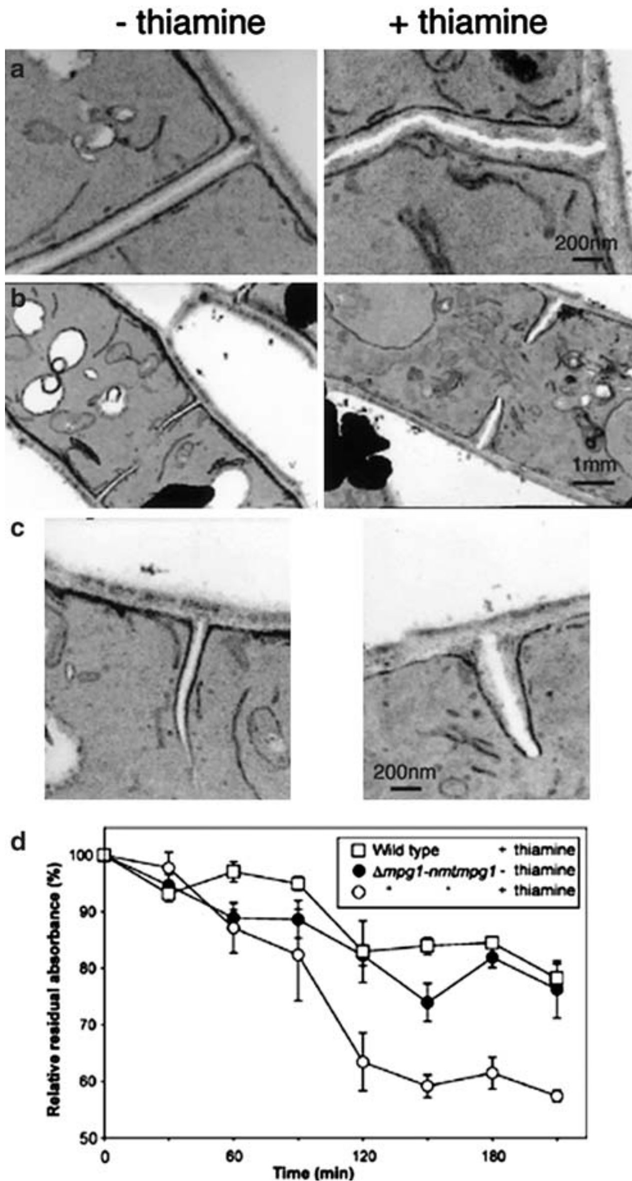

Fig. 4 Arrested cells show an aberrant septum and cell wall. Δ*mpg1 nmt-mpg1* cells grown at 30°C were incubated in medium with or without thiamine for 15 h. Samples were then removed, fixed and prepared for electron microscopy. Cells expressing Mpg1 displayed normal primary and secondary septa. In the absence of Mpg1 the cell wall appeared felt-like, having a spongy-looking outer layer and no obvious changes in the inner layer. Cells also showed an abnormally thickened septum (**a**), either completely or partially formed (**b**, **c**). **d** Δ*mpg1 nmt-mpg1* cells grown at 30°C were placed in medium with or without thiamine for 15 h. Then the culture was treated with Lyticase for the indicated times, and the residual cell absorbance was measured. Wild-type cells grown in the presence of thiamine for 15 h were used as a control

septated cells with a 4C DNA content, indicating that each nucleus has duplicated its DNA (2C/nucleus) but cannot progress through mitosis. Wee1 is an inhibitor of the G2/M transition in fission yeast, and indeed Wee1 homologues inhibit the G2/M transition in all eukaryotes examined to date (Russell and Nurse 1986). To determine whether the cell cycle block at G2/M in the absence of Mpg1 can be relieved when Wee1 is inactivated, a double mutant strain was constructed harbouring the thermosensitive *wee1-50* mutation and the conditionally repressible Δ*mpg1-nmtmpg1*. *wee1-50*, Δ*mpg1-nmtmpg1* and Δ*mpg1-nmtmpg1 wee1-50* strains were grown without thiamine at 25°C, and then shifted to thiamine-containing medium at the same temperature, to block cell cycle by turning off Mpg1 expression. Under these conditions, both Δ*mpg1-nmtmpg1* and Δ*mpg1-nmtmpg1 wee1-50* mutant cells arrested as 4C binucleated and septated cells, as expected (see Fig. 5b). Arrested cells were then shifted to 35°C to inactivate Wee1 in the double mutant, samples were taken at 1-h intervals for 5 h, and both cell morphology and DNA content were analysed. Interestingly, the number of nuclei per cell increased in the Δ*mpg1-nmtmpg1 wee1-50*

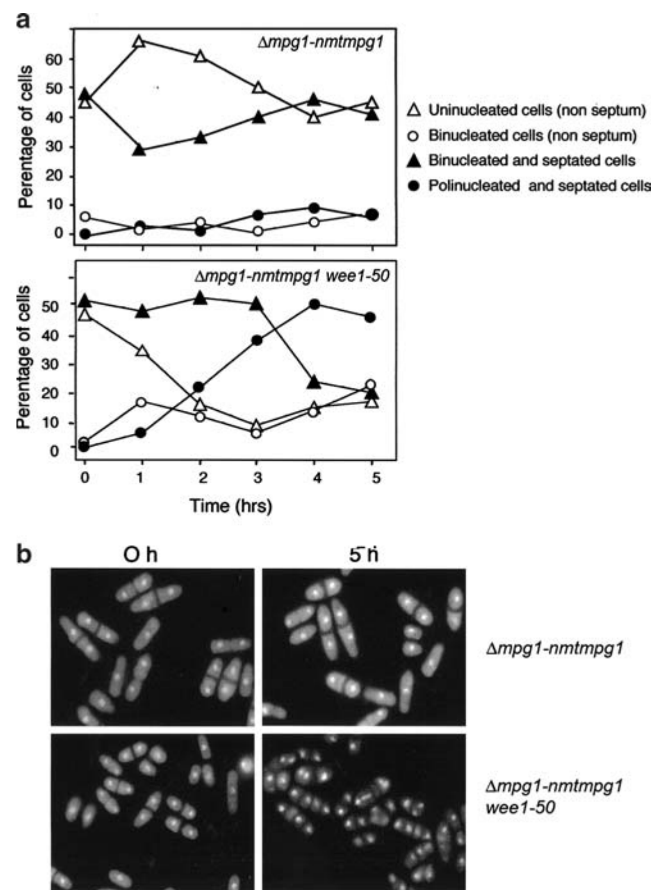

Fig. 5 Arrest at G2/M in Δ*mpg1 nmt-mpg1* cells is mediated by *wee1*. Δ*mpg1 nmt-mpg1* and Δ*mpg1 nmt-mpg1 wee1-50* cells were cultured to exponential growth phase at 25°C, shifted to medium containing thiamine for 15 h, and then shifted to 35°C. Samples were taken at 1-h intervals, fixed and stained with DAPI and Calcofluor white. Cells were classified into four groups, according to the number of nuclei and the presence or absence of a septum: (1) uninucleated and non-septated, (2) bi-nucleated and non-septated, (3) bi-nucleated and septated, and (4) multi-nucleated (more than two nuclei) and septated. **a** The percentage of cells in each class plotted against time after the temperature shift; 200–300 cells were analysed per sample. **b** Samples were collected at 0 and 5 h after the temperature shift, fixed, and stained with DAPI

double mutant (more than 45% of cells contained four nuclei after 4 h at 35°C). In the Δ*mpg1*-*nmtmpg1* single mutant the number of nuclei per cell remained unaltered (see Fig. 5a). These results clearly show that inactivation of Wee1 releases the block imposed by the absence of Mpg1p, and allows the cells to enter mitosis. However, although septation is allowed, cell separation does not take place, perhaps due to the defects previously observed in the septum. These results also clearly show that Mpg1 is necessary for cell growth as nuclear segregation and septation takes place in the Δ*mpg1*-*nmtmpg1 wee1*-*50* double mutant in the absence of cell growth.

Flp1/Clp1 is not required for the G2/M arrest observed in the absence of Mpg1

A checkpoint that monitors cytokinesis has been shown to operate in fission yeast (Le Goff et al. 1999; Liu et al. 2000). This cytokinesis checkpoint, which requires Wee1, Flp1/Clp1, F-actin structures, and the SIN (Le Goff et al. 1999; Liu et al. 1999, 2000, 2002; Balasubramanian et al. 2000), blocks the G2/M transition until the preceding cytokinesis has been completed.

The phosphatase Flp1/Clp1 is essential for the cytokinesis checkpoint activated by the loss of the *cps1* function (Cueille et al. 2001; Trautmann et al. 2001). Since there are some similarities between the terminal phenotypes of *cps1* and *mpg1* mutants, we decided to investigate whether the G2/M arrest imposed by the absence of Mpg1 could also be bypassed when Flp1 is absent. We compared the phenotype of the Δ*mpg1*Δ*flp1* double mutant with that of the Δ*mpg1* single mutant when both strains were shifted to a thiamine-containing medium, scoring both cell and nuclear morphology, as well as DNA content (see Fig. 6). Δ*mpg1*-*nmtmpg1* and Δ*mpg1*-*nmtmpg1*Δ*flp1* cells arrested in the presence of thiamine displayed the same number of 4C septated, binucleated cells (see Fig. 6a, b), and no increase in the number of nuclei per cell was observed. This result clearly shows that the pathway activated in the absence of Mpg1 is independent of Flp1. Therefore, the cytokinesis checkpoint activated in the *cps1* mutant is not required for the G2/M arrest observed in the absence of Mpg1.

The G2/M arrest caused by the absence of Mpg1 is a manifestation of the cell size checkpoint

The Wee1 kinase is part of the cell-size checkpoint that prevents entry into mitosis before cells have reached a critical size (Nurse 1975; Fantes and Nurse 1978; Thuriaux et al. 1978; Sveiczer et al. 1996). It therefore seemed possible that the *mpg1* loss-of-function mutant, which is defective in cell elongation (see Figs. 3, 5) and displays a *wee1*-dependent G2/M arrest phenotype, does not accumulate sufficient cell mass to allow the two G2 nuclei to proceed into M phase. In order to investigate

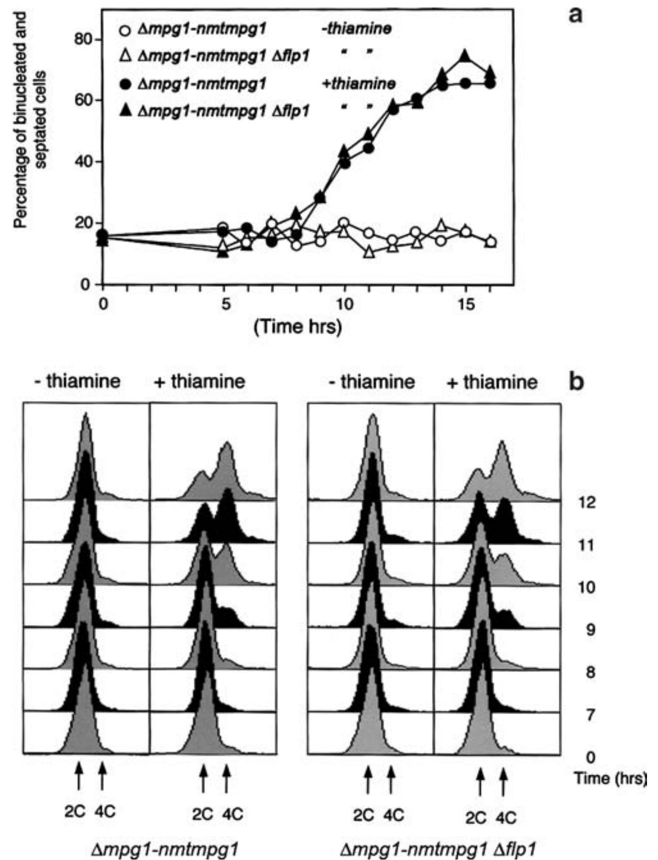

Fig. 6 *flp1*/*clp1* is not involved in the G2/M arrest observed in the absence of *mpg1*. Δ*mpg1 nmt-mpg1* and Δ*mpg1 nmt-mpg1* Δ*flp1* cells were cultured to exponential phase at 30°C and shifted to thiamine-containing medium to repress *mpg1* expression, or without thiamine as a control. Samples were taken at different times, fixed and stained with either DAPI or Calcofluor white (**a**), or prepared for flow cytometric analysis (**b**). **a** Percentage of binucleated and septated cells plotted against time. **b** DNA content of Δ*mpg1 nmt-mpg1* and Δ*mpg1 nmt-mpg1* Δ*flp1* cells collected from cultures with or without thiamine

this possibility, a double mutant strain was constructed that harboured the thermosensitive *cdc25-22* mutation and was also capable of conditional expression of *mpg1*. Δ*mpg1*-*nmtmpg1 cdc25-22* double mutant cells were first grown without thiamine at 25°C, and then shifted to medium with or without thiamine at the same temperature for 14 h (after this period in the presence of thiamine the *mpg1* mutant phenotype has not yet become manifest, so cell growth is still possible). Next, each culture (+/− thiamine) was divided into two halves: one was shifted to 35°C for 5 h to inactivate Cdc25 (allowing cells to elongate) and the other was kept at 25°C as a control. Finally, all cultures were transferred again to 25°C.

As expected, the elongated Δ*mpg1*-*nmtmpg1 cdc25-22* cells that expressed Mpg1 (i.e. those grown at 35°C in the absence of thiamine) were released synchronously when shifted to 25°C. On the other hand, in the thiamine-containing medium at 25°C, the Δ*mpg1*-*nmtmpg1 cdc25-22* double mutant arrested as binucleated septated

cells as a consequence of the repression of *mpg1* and the depletion of its product (Fig. 7b). Interestingly, however, when Cdc25 function was restored in highly elongated *Δmpg1-nmtmpg1 cdc25-22* double mutant cells lacking Mpg1 (i.e., those shifted to 25°C in the presence of thiamine), the number of nuclei per cell clearly increased: after 4 h upto 36% of septated cells had more than two nuclei (3 or 4), and this fraction reached 50% after 5 h (Fig. 7a, b). These results clearly show that the G2/M arrest in the absence of Mpg1 is circumvented when cells are highly elongated, indicating that the cell cycle arrest is mainly a manifestation of the cell-size checkpoint. Interestingly, the G2/M arrest is bypassed and a new round of septation is allowed in oversized cells, but cell separation does not take place, as in the case of the double mutant *Δmpg1-nmtmpg1 wee1-50*.

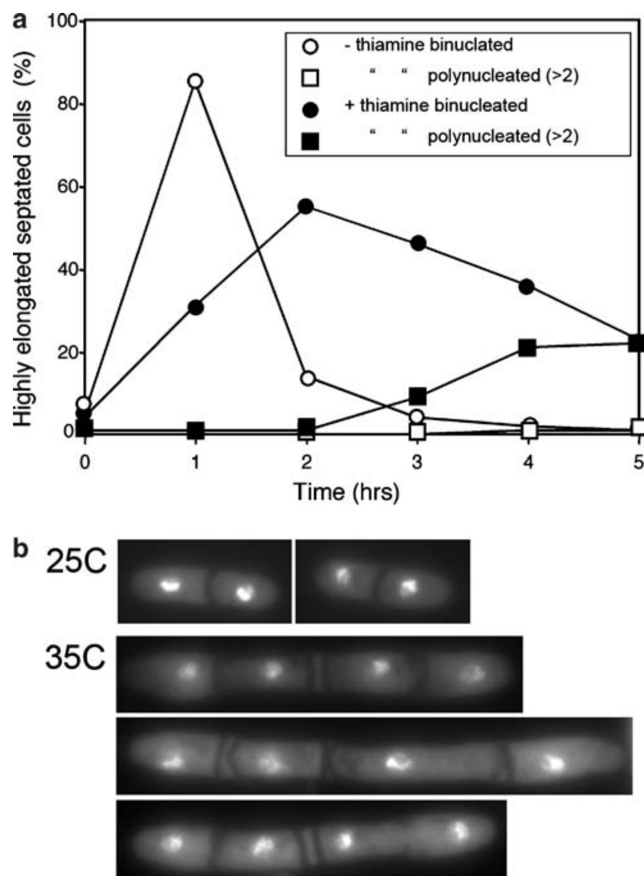

Fig. 7 Cell cycle arrest in *mpg1* mutant cells is brought about by an inability to reach the critical size and activate the size checkpoint. *Δmpg1-nmtmpg1 cdc25-22* double mutant cells were grown without thiamine at 25°C, and then shifted to medium containing (or not) thiamine at the same temperature for 14 h. Cultures were then shifted to 35°C for 5 h to inactivate Cdc25, allowing cells to elongate. Then cells were transferred back to 25°C. **a** Highly elongated *Δmpg1-nmtmpg1 cdc25-22* double mutant cells containing two nuclei (binucleated cells) or more (polynucleated) were then quantified at various times up to 5 h after the final shift to 25°C (0 h). The data are the means of two independent experiments. **b** Representative DAPI images of arrested *Δmpg1-nmtmpg1 cdc25-22* double mutant cells incubated at 25°C (binucleated septated cells) or at 35°C (polynucleated cells) in thiamine-containing medium

Discussion

In this paper, we report the isolation and characterization of an essential *S. pombe* gene, which we have named *mpg1*. This gene was isolated in a two-hybrid screen designed to identify yeast proteins capable of interacting with Rep, a geminivirus protein that is essential for viral DNA replication. Although homologues of *mpg1* have been identified in plants such as *A. thaliana* (Nickle and Meinke 1998) and *Lycopersicon esculentum* (Donoso et al., unpublished data), the work presented here has focused on the characterization of this gene in fission yeast. The significance of the interaction between Rep and Mpg1 during viral infection in plants is currently being investigated.

The protein encoded by *mpg1* is likely to be a GDP-mannose-1-phosphate guanylyltransferase, considering its strong homology with similar proteins from fungi and higher eukaryotes. Mpg1 homologues have been proposed to be involved in the glycosylation of cell wall proteins (Phaff 1971; Kukuruzinska et al. 1987) and consequently in the establishment and/or maintenance of cell wall structure (Huang and Snider 1995; Takegawa et al. 1996; Hashimoto et al. 1997; Nickle and Meinke 1998; Warit et al. 2000; Agaphonov et al. 2001). Taken together, our results indicate that *mpg1* has a similar function. Like its homologues, *mpg1* is involved in glycosylation and it is required for correct cell wall formation, as inferred from the analysis of glycosylation activity and the sensitivity of *mpg1* mutant cells to Lyticase. Cell wall structure is altered in cells lacking Mpg1, since they are clearly more sensitive to Lyticase than cells that express the protein.

The role of *mpg1* in de novo cell wall synthesis, and therefore in cell growth, is compatible with the localization of the protein at the growing end of small uninucleated cells. The idea that Mpg1 could also be involved in septum formation is supported by the increase in Mpg1 levels that takes place just before the start of septation, and also by electron microscopy of cells lacking *mpg1* expression, where an abnormal septum structure is observed from the beginning of material deposition (see Fig. 4). Interestingly, when G2 nuclear segregation and new septation are allowed to occur in the absence of Mpg1 (by inactivation of Wee1 in the *Δmpg1-nmtmpg1 wee1-50* double mutant) or elongated, Mpg1-deficient cells are released into the cell cycle (by shifting oversized *Δmpg1-nmtmpg1 cdc25-22* cells to the permissive temperature for Cdc25 expression), cell separation does not occur. These results suggest that, although a new septum can be formed when Mpg1 is absent, its abnormal structure prevents cell separation. Moreover, as *Δmpg1-nmtmpg1* is a glycosylation-defective mutant, this work demonstrates that glycosylation is important for cell separation.

The absence of Mpg1 also affects cell cycle progression, as *mpg1* mutants arrest in the form of bi-nucleated septated cells (Fig. 3). The DNA content of bi-nucleated

and septated Δ*mpg1-nmtmpg1* cells kept under restrictive conditions was 4C, indicating that each cell has two G2 (2C) nuclei. This result clearly shows that, in the absence of Mpg1, cells are able to replicate their DNA after mitosis and produce a septum, but they can neither segregate their 2C nuclei nor separate, generating a final population of 4C, bi-nucleated and septated cells arrested at the G2/M transition. As mentioned above, septum structure in these arrested cells becomes aberrant from the beginning of septum deposition. A high percentage of 4C binucleated cells with a single, uncleaved septum is also produced following the addition of cycloheximide to wild-type cells late in the cell cycle (Fantes 1982; Sohrmann et al. 1996), in a DMSO-sensitive *sec13-ds293* mutant affected in the secretory pathway (Poloni and Simanis 2002), or in the absence of Pmr1, an essential protein involved in glycosylation (Cortes et al. 2004). How does loss-of-function of *mpg1* lead to a defect in cell cycle progression? Mpg1 absence might function, directly or indirectly, as an activation signal for a regulatory mechanism that normally allows cell cycle progression only if a step that requires the Mpg1 function has been performed successfully. The G2/M arrest induced in cells lacking expression of Mpg1 is bypassed when Wee1 is inactivated (see Fig. 5) indicating that it is triggered through a *wee1*-dependent pathway.

The checkpoint mechanism described in *S. pombe* (Le Goff et al. 1999; Liu et al. 1999; 2000; Balasubramanian et al. 2000) that blocks entry into mitosis until cytokinesis has been completed seems to be activated by the actomyosin ring present in arrested *cps1-191* cells. This checkpoint depends on Wee1, SIN, and the phosphatase Flp1 to maintain the block to nuclear division (Cueille et al. 2001; Trautmann et al. 2001). Though the Δ*mpg1-nmtmpg1* and *cps1* mutant phenotypes present some similarities, in Δ*mpg1*-arrested cells an aberrant septum is formed, but neither an actomyosin ring nor disorganized ring components are present (see Figs. 3d, 4). Moreover, the absence of Flp1 does not allow segregation of the G2 nuclei in arrested Δ*mpg1* cells, as it occurs in arrested *cps1* mutant cells (see Fig. 6) indicating that the cytokinesis checkpoint is not activated in the *mpg1* mutant.

Entry into mitosis requires that the cells reach a critical size in *S. pombe* (Nurse 1975). This size-related checkpoint is also mediated by *wee1*. It was therefore possible that the Δ*mpg1* arrest occurred because the cells do not accumulate sufficient mass to allow the two G2 nuclei to proceed into M phase. Indeed, when highly elongated cells were allowed to re-enter the cell cycle in the absence of Mpg1, within 5 h approximately 50% of septated cells were found to have an increased number of nuclei, indicating that they had segregated their G2 nuclei (Fig. 7). This clearly demonstrates that the arrest in *mpg1* mutant cells is brought about by an inability to reach the critical size, resulting in the activation of the size-related checkpoint mediated by *wee1*. It is important to emphasize that as consecutive cycles partially overlap in *S. pombe*, the absence of *mpg1* produces a defect in septum formation that prevents cell separation. However, nuclei are able to enter G1, complete the S phase and go into G2 phase. Due to the absence of Mpg1, and most probably due to a defect in growth, neither of the linked daughter cells can reach the critical size to enter mitosis and the cell-size checkpoint is activated during the second cycle.

In summary, *mpg1* is an essential *S. pombe* gene which is involved in glycosylation, and is required for the formation of a correct septum structure and cell growth. In the absence of Mpg1, cells arrest as 4C bi-nucleated septated cells as a result of activation of the cell size checkpoint. This *mpg1* mutant provides a convenient tool to further our understanding of how the fission yeast coordinates cell growth and cell division.

Acknowledgements We thank Juan Jiménez and his laboratory for their interest and collaboration in this project. We also thank Manuel Cifuentes for his help with the generation of anti-Mpg1 antibodies. We are grateful to Antonio Jiménez and Manuel Cifuentes for their help with electron microscopy, to Manuel Muñoz for discussions and helpful suggestions, and Lucía Cruzado for technical support. We thank Sergio Moreno for the *S. pombe* library, Avelino Bueno for the Δ*flp1* strain and Juan Jiménez for the *wee1-50* and *cdc25-22* strains. We thank Dr. Javier Ruiz-Albert and Carmen R. Beuzón for critical reading of the manuscript and many helpful suggestions. I. Donoso was supported by a Predoctoral Research Fellowship from the Ministerio Español de Educación. M. A. Sánchez-Durán was supported by a Predoctoral Research Fellowship from the Junta de Andalucía. This work was supported by the Comisión Interministerial de Ciencia y Tecnología, Grants AGL2001-1857-C04-01, Plan Andaluz de Investigación, Group CVI 264, Universidad de Málaga and Gentech

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