Role of the α -Glucanase Agn1p in Fission-Yeast Cell Separation

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Cell division in the fission yeast *Schizosaccharomyces pombe* yields two equal-sized daughter cells. Medial fission is achieved by deposition of a primary septum flanked by two secondary septa within the dividing cell. During the final step of cell division, cell separation, the primary septum is hydrolyzed by an *endo*-(1,3)- β -glucanase, Eng1p. We reasoned that the cell wall material surrounding the septum, referred to here as the septum edging, also must be hydrolyzed before full separation of the daughter cells can occur. Because the septum edging contains (1,3)- α -glucan, we investigated the cellular functions of the putative (1,3)- α -glucanases Agn1p and Agn2p. Whereas *agn2* deletion results in a defect in endolysis of the ascus wall, deletion of *agn1* leads to clumped cells that remained attached to each other by septum-edging material. Purified Agn1p hydrolyzes (1,3)- α -glucan predominantly into pentasaccharides, indicating an *endo*-catalytic mode of hydrolysis. Furthermore, we show that the transcription factors Sep1p and Ace2p regulate both *eng1* and *agn1* expression in a cell cycle-dependent manner. We propose that Agn1p acts in concert with Eng1p to achieve efficient cell separation, thereby exposing the secondary septa as the new ends of the daughter cells.

INTRODUCTION

Cell separation is the final step of the cell cycle in yeasts and results in an irreversible separation of two independent cells, a mother and daughter cell in budding yeasts or two equal-sized daughter cells in fission yeasts. Cell separation occurs after division of the nucleus (mitosis) and division of the cytoplasm (cytokinesis) have successively been completed. In yeasts, cytokinesis involves formation of a division septum, which is synthesized within the confines of the dividing cell. Subsequent cell separation involves partial dissolution of this division septum. Importantly, the division septum is composed mainly of the same types of polysaccharides that constitute the structural framework of the extracellular matrix (cell wall), which maintains cellular integrity (Hochstenbach *et al.*, 1998; Klis *et al.*, 2002). The molecular mechanisms that are responsible for dissolution of the division septum while maintaining structural integrity are not understood. An attractive model yeast to study this question is the fission yeast *Schizosaccharomyces pombe*, because its complete genome has been sequenced, and mutants with defects in cell separation can be isolated or engineered.

Septum formation requires two separate but interdependent processes, namely, formation of a contractile actomyosin ring in the equatorial plane of the dividing cell and

deposition of the septum (Chang, 2001; McCollum and Gould, 2001). At the end of anaphase, after formation of the actomyosin ring and segregation of the sister chromatids, a signaling cascade called the septation initiation network in *S. pombe* initiates constriction of the actomyosin ring and concomitant synthesis of the primary septum in a centripetal direction (Le Goff *et al.*, 1999; McCollum and Gould, 2001). In *S. pombe*, the primary septum consists of linear (1,3)- β -glucan, and its synthesis involves the (1,3)- β -glucan synthase Cps1p (Liu *et al.*, 1999; Humbel *et al.*, 2001), whereas in the budding yeast *Saccharomyces cerevisiae*, the primary septum consists of chitin, and its synthesis involves the chitin synthase Chs2p (Cabib *et al.*, 2001; Roh *et al.*, 2002). Secondary septa are deposited at both sides of the growing primary septum, starting at the base and becoming progressively thicker as the primary septum develops (Johnson *et al.*, 1973).

The molecular mechanisms of cell separation and their regulation are poorly understood. In *S. cerevisiae*, an *endo*-chitinase, ScCts1p, is involved in hydrolysis of the primary septum, whereas an *endo*-(1,3)- β -glucanase, ScEng1p, also has been shown to be involved in septum hydrolysis (Kuranda and Robbins, 1991; Baladrón *et al.*, 2002). Expression of both hydrolases is regulated by a cascade of transcription factors, including two members of the HNF-3/forkhead family, ScFkh1p, and ScFkh2p (Zhu *et al.*, 2000), in addition to the C2H2 zinc-finger transcription factor ScAce2p (Dohrmann *et al.*, 1992; Spellman *et al.*, 1998; Baladrón *et al.*, 2002). In *S. pombe*, a homolog of ScFkh1p and ScFkh2p, as well as a homolog of ScAce2p, have been identified, denoted Sep1p and Ace2p, respectively (Sipiczki *et al.*, 1993; Ribár *et al.*, 1997; Martín-Cuadrado *et al.*, 2003). Deletion of *sep1*⁺ or *ace2*⁺ caused a severe cell-separation defect with cells displaying a hyphal morphology. Furthermore,

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Martín-Cuadrado *et al.* (2003) showed recently that *S. pombe* expresses a homolog of ScEng1p, Eng1p, which is involved in hydrolysis of the primary septum and is regulated by Ace2p. Whether *eng1*⁺ expression is regulated also by Sep1p is not known.

Cell separation must involve not only dissolution of the primary septum but also dissolution of the cell wall material that surrounds the septum, referred to here as the septum edging to distinguish it from the septum proper. Because the septum edging is cell wall material of the dividing cell, it consists mainly of (1,3)- β -glucan and (1,3)- α -glucan. Although dissolution of the primary septum is known to involve the (1,3)- β -glucanase Eng1p, it remains unclear how the septum edging is dissolved. As (1,3)- α -glucan is present in the septum edging but absent from the primary septum, we studied two genes encoding putative (1,3)- α -glucanases, denoted *agn1*⁺ (systematic gene name, SPAC14C4.09) and *agn2*⁺ (SPBC646.06c). We demonstrate that Agn1p functions as an *endo*-(1,3)- α -glucanase that hydrolyzes septum-edging material, and we conclude that localized hydrolysis of cell wall α -glucan plays an integral part in the cell cycle of *S. pombe*.

MATERIALS AND METHODS

Strains and Culture Media

Mutant strains were derived from *S. pombe* wild type, 972 (Table 1). Cells were grown in YEA medium (Hochstenbach *et al.*, 1998) or in EMM2 medium (Moreno *et al.*, 1991) with addition of 250 mg/l adenine sulfate (EMMA). GEMMA was EMMA with addition of 2% (wt/vol) sodium D-gluconic acid instead of D-glucose, adjusted to pH 4.0 by using 2 M phosphoric acid. In a conjugation and sporulation assay, cells were crossed on agar plates containing ME medium at 25°C for 2 d and treated with iodine vapor to test for ascospore formation. Diploids were selected on EMM2 medium (Moreno *et al.*, 1991).

DNA Sequencing

For *agn1*⁺ and *agn2*⁺ sequencing (GenBank accession nos. AY626901 and AY626902, respectively), total RNA was isolated from vegetatively grown 972 cells or sporulating ND080 cells, respectively (Koerkamp *et al.*, 2002). cDNA of a part of the *agn1*⁺ 5'-untranslated region, the open reading frame (ORF), and 3'-untranslated region was synthesized by polymerase chain reaction (PCR), by using *pfu* polymerase (Stratagene, La Jolla, CA). For *agn2*⁺ sequencing, cDNA of the *agn2*⁺ 5'-untranslated region, ORF, and 3'-untranslated region was synthesized in three separate PCR amplification reactions. These PCR amplification products were used for direct DNA sequencing (BDT sequencing kit; Applied Biosystems, Foster City, CA). The sequence of the 3'-end of the *agn2* ORF also was confirmed by direct DNA sequencing of several PCR amplification products obtained for the region of interest using genomic DNA from strain 972 as template (our unpublished data).

Disruption and Tagging

Using a PCR-mediated strategy (Bähler *et al.*, 1998), ORFs of *agn1*⁺ (systematic gene name, SPAC14C4.09), *agn2*⁺ (SPBC646.06c), *eng1*⁺ (SPAC821.09), *ace2*⁺ (SPAC6G10.12C), and *sep1*⁺ (SPBC4C3.12), were replaced by a *loxP*-*kanMX4*-*loxP* cassette (pUG6; Güldener *et al.*, 1996). *agn1*⁺ and *eng1*⁺ ORFs were tagged at their 3' ends with the *13myc-kanMX6* cassette (Bähler *et al.*, 1998). Hot-start PCR amplification products were obtained in 28 cycles by using *Taq* polymerase (TaKaRa). Transformations were performed using a lithium-acetate method (Bähler *et al.*, 1998), and cells were allowed to recover for 30 h at 28°C, followed by replica plating onto YEA plates containing 200 μ g/ml Geneticin (Invitrogen, Carlsbad, CA). PCR analyses were used to verify correct integration, and tetrad analyses were used to confirm single integration events. Double and triple mutants were generated using tetrad dissection, and genotypes were rechecked by PCR analyses.

Cloning and Overexpression of *agn1*⁺

For overexpression of *agn1*⁺ from its chromosomal locus, a *kanMX6-3nmt1* cassette was integrated in front of the ORF by homologous recombination (Bähler *et al.*, 1998). For *agn1p-his* overexpression from a plasmid, the *agn1* ORF was amplified from genomic DNA of strain 972 and cloned into plasmid pSE01, a pREP4-based plasmid (Maundrell, 1993) containing sequences encoding a protease X cleavage site followed by six histidines. After verifying the *agn1* ORF sequence, the resulting plasmid, pND02, was transformed into

Table 1. *S. pombe* strains used in this study

Strain	Genotype
972	<i>h</i> ⁻
cdc10 ^{ts}	<i>h</i> ⁻ <i>cdc10-129</i>
cdc25 ^{ts}	<i>h</i> ⁻ <i>cdc25-22</i>
FYC11	<i>h</i> ⁻ <i>ade6-M210</i>
FYC15	<i>h</i> ⁺ <i>ade6-M216</i>
MBY580	<i>h</i> ⁻ <i>cps1-191 ade6-M210 leu1-32 ura4-D18 lys1-131</i>
ND001	<i>h</i> ⁻ <i>agn1Δ::kanMX4</i>
ND003	<i>h</i> ⁻ <i>agn1::13myc-kanMX6</i>
ND005	<i>h</i> ⁻ <i>eng1Δ::kanMX4</i>
ND007	<i>h</i> ⁻ <i>eng1::13myc-kanMX6</i>
ND019	<i>h</i> ⁻ <i>agn1Δ::kanMX4 eng1Δ::kanMX4</i>
ND028	<i>h</i> ⁻ <i>agn2Δ::kanMX4</i>
ND049	<i>h</i> ⁻ <i>cdc25-22 agn1::13myc-kanMX6</i>
ND051	<i>h</i> ⁻ <i>cdc25-22 eng1::13myc-kanMX6</i>
ND054	<i>h</i> ⁻ <i>cdc10-129 agn1::13myc-kanMX6</i>
ND061	<i>h</i> ⁻ <i>agn2::13myc-kanMX6</i>
ND064	<i>h</i> ⁻ <i>ace2Δ::kanMX4</i>
ND080	<i>h</i> ⁻ / <i>h</i> ⁺ <i>ade6-M210/ade6-M216</i>
ND084	<i>h</i> ⁻ / <i>h</i> ⁺ <i>agn1Δ::kanMX4/agn1Δ::kanMX4 ade6-M210/ade6-M216</i>
ND088	<i>h</i> ⁻ / <i>h</i> ⁺ <i>agn2Δ::kanMX4/agn2Δ::kanMX4 ade6-M210/ade6-M216</i>
ND102	<i>h</i> ⁻ <i>sep1Δ::kanMX4</i>
ND111	<i>h</i> ⁻ <i>cdc25-22 ace2Δ::kanMX4 agn1::13myc-kanMX6</i>
ND132	<i>h</i> ⁻ <i>ace2Δ::kanMX4 agn1::13myc-kanMX6</i>
ND136	<i>h</i> ⁻ <i>ace2Δ::kanMX4 eng1::13myc-kanMX6</i>
ND140	<i>h</i> ⁻ <i>sep1Δ::kanMX4 agn1::13myc-kanMX6</i>
ND144	<i>h</i> ⁻ <i>sep1Δ::kanMX4 eng1::13myc-kanMX6</i>
ND148	<i>h</i> ⁻ <i>ace2Δ::kanMX4 sep1Δ::kanMX4</i>
ND154	<i>h</i> ⁻ <i>cps1-191 agn1::13myc-kanMX6</i>
ND172	<i>h</i> ⁻ <i>agn2::GFP^{S65T}-kanMX6 ade6-M210</i>
ND218	<i>h</i> ⁻ <i>ace2Δ::kanMX4 sep1Δ::kanMX4 eng1::13myc-kanMX6</i>
ND234	<i>h</i> ⁻ <i>agn1Δ::kanMX4 ura4-D18 [pND02]</i>
ND235	<i>h</i> ⁻ <i>agn1Δ::kanMX4 ura4-D18 [pSE01]</i>
ND236	<i>h</i> ⁻ <i>ura4-D18 [pND02]</i>

All strains were constructed for this study, except strain 972 (P. Nurse), strains FYC11, FYC15, *cdc10*^{ts}, and *cdc25*^{ts} (R. Dahr), and strain MBY580 (M. Balasubramanian).

a strain with genotype *h*⁻ *ura4-D18* by using a lithium acetate method (Akada *et al.*, 2000).

Synchronization and Immunoblotting

For synchronization, *cdc25-22* strains were grown at 25°C to an optical density at 595-nm wavelength (OD₅₉₅) of 0.3 and shifted to 37°C for 4 h. Then, cells were released into the cell cycle by quick transfer to 25°C. Septation index was determined by calculating the percentage of cells with a septum after calcofluor-white staining (Fluostain I; F-0386; Sigma-Aldrich, St. Louis, MO). Cells were taken up in 100 mM Tris-HCl, pH 7.6, containing 1 mM phenyl-methylsulfonyl fluoride (PMSF), and broken with washed glass beads by using a FastPrep 120 (Bio 101, Vista, CA) at speed 5.5 for two intervals of 10 s. Protein concentrations were measured using the Bradford method with bovine serum albumin as a standard. Total cell lysates were resolved at 6 μ g of protein per lane on a SDS/8% polyacrylamide gel under reducing conditions, blotted onto nitrocellulose membranes (0.45 μ m; Schleicher & Schuell, Keene, NH), and probed with anti-myc monoclonal antibody (mAb) 9E10 (Evan *et al.*, 1985) and an anti- α -tubulin antibody (T-5168; Sigma-Aldrich). Blots were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad, Hercules, CA) and developed by chemiluminescence (ECL kit; Amersham Biosciences, Piscataway, NJ).

Microscopy

For light microscopy, cells were stained with calcofluor white and embedded in 2% (wt/vol) low-melting agarose. Micrographs were obtained using an Axiophot 2 microscope (Carl Zeiss, Thornwood, NY) equipped with a Cool-snap HQ digital camera (Photometrics, Tucson, AZ). For transmission electron microscopy, cells were fixed with potassium permanganate (Hochstenbach *et al.*, 1998). Thin sections were stained with lead citrate (Reynolds, 1963) and uranyl acetate, and micrographs were obtained using a model 420 trans-

mission electron microscope (Philips, Eindhoven, The Netherlands) operating at 80 kV.

Sedimentation Assay

Cells were grown in YEA medium in an orbital shaker at 200 rpm at 28°C to an OD₅₉₅ of 1. Subsequently, 1 ml of culture was transferred into a cuvette (Sarstedt 67.742), gently mixed, and placed without further disturbance in an Ultrospec III spectrophotometer (Pharmacia AB, Uppsala, Sweden). OD₅₉₅ was registered at 2.5-min intervals over a period of 30 min.

Purification of Agn1p-his

Agn1p-his expression was induced in GEMMA for 24 h. Culture medium was concentrated 10-fold under N₂ pressure at 4°C by using a polyethersulfone filter (10 kDa cut-off; Millipore, Billerica, MA), and its pH was changed to 8.0 by addition of 100 mM MOPS buffer. Then, supernatant was incubated at 4°C for 1 h with nickel-nitrilotriacetic acid beads (QIAGEN, Valencia, CA). Beads were washed with 300 mM NaCl, 50 mM NaH₂PO₄, pH 8.0, buffer (buffer A), containing 15 mM imidazole. Agn1p-his was eluted with buffer A containing 250 mM imidazole, and fractions with (1,3)- α -glucanase activity were pooled. After exchanging buffer A for 20 mM sodium acetate, pH 5.0, by using a gel-filtration column (PD-10; Amersham Biosciences), the eluate was loaded onto an anion-exchange column (Mono-Q; Amersham Biosciences). Agn1p-his was eluted using a linear gradient of KCl to 250 mM, and fractions with highest (1,3)- α -glucanase activity were used for further study. Size exclusion chromatography was performed at a flow rate of 0.5 ml/min, by using a Superdex-75 column (Amersham Biosciences) equilibrated with 50 mM sodium acetate, pH 6.0, and calibrated with low-molecular-mass markers (Amersham Biosciences) and blue dextran.

Purification of MutAp

Fifty mg of Novozym (L-2265; Sigma-Aldrich) was dissolved in 50 mM Tris-HCl, pH 7.6, containing 1 mM PMSF and 10 μ l of protease inhibitor cocktail (P-8215; Sigma-Aldrich) and run over an anion-exchange column (DEAE; Amersham Biosciences) equilibrated with 50 mM Tris-HCl, pH 7.6. Flow-through fractions containing (1,3)- α -glucanase activity were pooled. For adsorption chromatography, buffer was exchanged for 5 mM sodium azide, 50 mM sodium acetate, pH 5.6 (buffer B), and 0.85 mM PMSF and 10 μ l of protease inhibitor cocktail were freshly added. Twenty mg of (1,3)- α -glucan (*Pleurotus ostreatus*) was dissolved in 10 M HCl, incubated at 37°C for 15 min, and reprecipitated overnight by addition of ice-cold 1.25 M sodium acetate and neutralization with 10 M NaOH. Then, MutAp was bound to (1,3)- α -glucan in buffer B containing 10% (vol/vol) ethylene glycol and 250 mM NaCl at 4°C for 45 min, washed, and eluted by a 5-min incubation in 80% (vol/vol) ethylene glycol in buffer B at room temperature. Finally, ethylene glycol was removed using a PD-10 column equilibrated with buffer B.

Isolation of (1,3)- α -Glucan

Fruiting bodies of *P. ostreatus* or *Laetiporus sulfureus* were minced in a blender and boiled in 2% (wt/vol) SDS, 40 mM 2-mercaptoethanol for 15 min to extract cytosolic contaminants. To precipitate the alkali-soluble, water-insoluble fraction, cell wall material was dissolved in 2 mM NaBH₄, 2 M KOH for 30 min on ice and reprecipitated overnight at 4°C by adjusting the pH to 6 with acetic acid. Then, the pellet was resuspended in 5 mM sodium azide, 40 mM 2-mercaptoethanol, 50 mM citrate-phosphate buffer, pH 5.3, containing 45 mg of Zymolyase-100T (Seikagaku America, Rockville, MD) and incubated overnight at 37°C. After collection, the insoluble fraction was reextracted as described above and washed in 5 mM sodium azide.

Mass Spectrometry

For matrix-assisted laser desorption/ionization analysis, protein spots were excised from a Coomassie-stained SDS/8% polyacrylamide gel and treated as described previously (Shevchenko *et al.*, 1996). Dried peptides, cleaned with ZipTip_{C18} (Millipore), were resuspended in 5 μ l 60% (vol/vol) acetonitrile, 1% (vol/vol) formic acid. Approximately 4 μ g of protein also was digested directly with 2 μ g of endoproteinase Glu-C (V8 protease) (Roche Diagnostics, Indianapolis, IN) overnight in 50 mM sodium phosphate, pH 7.9, with 5% (vol/vol) acetonitrile at 25°C. Subsequently, the peptide solution was alkylated (Shevchenko *et al.*, 1996), cleaned, and concentrated on a ZipTip_{C18} (Millipore), and eluted in 10 μ l 60% (vol/vol) acetonitrile, 1% (vol/vol) formic acid. Peptide solutions were mixed with an equal volume of 52 mM (aceton-prewashed) α -cyano-4-hydroxycinnamic acid (Sigma-Aldrich) in 50% (vol/vol) ethanol, 48% (vol/vol) acetonitrile, 2% (vol/vol) trifluoroacetic acid, 1 mM ammonium acetate; spotted on a target plate; and dried at room temperature. Mass spectrometry spectra were collected on a MALDI R (Micro-mass, Wythenshawe, United Kingdom). For tandem mass spectrometry analysis, V8-protease digests were analyzed on a quadrupole time-of-flight (Q-TOF) mass spectrometer (Micromass). Low-energy collision-induced dissociation was performed using argon as a collision gas.

Enzyme Reactions

Substrate specificity was determined by an incubation at 37°C for 20 min of ~2 pmol of Agn1p-his with substrates at a final concentration of 4 mg/ml in 5 mM sodium azide, 50 mM sodium acetate, pH 5.6. Reduced and carboxymethylated (1,3)- α -glucan (*L. sulfureus*), (1,4)(1,6)- α -glucan (starch; 85642; Fluka, Buchs, Switzerland), reduced (1,4)(1,6)- α -glucan (glycogen; G-8751; Sigma-Aldrich), reduced (1,4)(1,6)- α -glucan (pullulan; P-4516; Sigma-Aldrich), reduced (1,6)- α -glucan (dextran; D-9260; Sigma-Aldrich), reduced (1,3)- β -glucan (laminarin; 61430; Fluka), carboxymethylated (1,4)- β -glucan (cellulose; C-5678; Sigma-Aldrich), or reduced (1,3)- α -mannan (mannan; M-7504; Sigma-Aldrich) were used as substrates. O-(Carboxy)methylation and polysaccharide reduction were performed as described previously (Takasaki and Kobata, 1978; Kiho *et al.*, 1989). Reaction products were analyzed using a colorimetric assay (Lever, 1972).

For high-performance anion-exchange chromatography (HPAEC), (1,3)- α -glucan (0.5 mg) purified from *S. pombe* cell walls was incubated with Agn1p-his or MutAp in 5 mM sodium azide, 50 mM sodium acetate, pH 5.6, at 37°C for 5 h. As standards, oligosaccharides from *S. pombe* (1,3)- α -glucan were prepared as described previously (Koizumi *et al.*, 1989). HPAEC was performed using a CarboPac PA-1 column on a DX 500 system (Dionex, Sunnyvale, CA) by using a linear gradient from 0 to 500 mM sodium acetate in 0.1 M NaOH at a flow rate of 1.0 ml/min.

RESULTS

Agn1p Contains a Signal Sequence for Secretion, whereas Agn2p Does Not

Annotation of the *S. pombe* genome sequence suggested that the *agn1*⁺ and *agn2*⁺ genes each contain one continuous ORF (Wood, 2002). This prediction was confirmed by our analysis of the transcripts. *agn1*⁺ cDNA contained a 5'-untranslated region of at least 136 base pairs and a 3'-untranslated region of 422 base pairs, whereas *agn2*⁺ cDNA contained a 5'-untranslated region of 157 base pairs and a 3'-untranslated region of 122 base pairs. Analysis of their ORFs showed that *agn1*⁺ and *agn2*⁺ encode polypeptides of 424 and 433 amino acids, respectively, with calculated molecular masses of 46.9 and 48.0 kDa (Figure 1). These predicted polypeptides contain 22 additional amino acids at the amino terminus of Agn1p and 26 additional amino acids at the carboxy terminus of Agn2p beyond those predicted by the original genome-project annotation. These polypeptides share 39% amino acid sequence identity, suggesting that they are paralogs. Furthermore, Agn1p and Agn2p show 41 and 34% amino acid sequence identity, respectively, to the catalytic domain (residues 38–461) of the known *Trichoderma harzianum* (1,3)- α -glucanase MutAp, the founding member of glycoside hydrolase family 71 (Fuglsang *et al.*, 2000), identifying Agn1p and Agn2p as putative (1,3)- α -glucanases. This prediction is consistent with the absence of Agn homologues in *S. cerevisiae* and *Candida albicans* (our unpublished data), both of which lack cell wall (1,3)- α -glucan. However, Agn1p and Agn2p lack sequences corresponding to the carboxy-terminal Pro-Ser-Thr-rich linker (residues 462–550) and polysaccharide-binding domain (residues 551–634) present in MutAp (Figure 1).

A hydropathy profile of the Agn1 polypeptide reveals a hydrophobic region at its amino terminus (residues 1–20) that is predicted to function as a cleavable signal sequence (Kyte and Doolittle, 1982; Nielsen *et al.*, 1997). However, a signal sequence was found to be absent from the Agn2 polypeptide. To study secretion of Agn1p and Agn2p directly, we tagged these proteins at their carboxy terminus and performed an immunoblot analysis on exponentially growing *agn1-myc* and *agn2-myc* cells. In total cell lysates, Agn1-myc and Agn2-myc protein bands resolved at apparent molecular masses of ~78 and ~76 kDa, respectively (Figure 2, lanes 2 and 3), which are in agreement with the calculated molecular masses of 67.4 and 68.5 kDa. Immunoblot analysis on corresponding culture media revealed the

Figure 1. *S. pombe* Agn1 and Agn2 polypeptides show amino acid sequence identities to the catalytic domain of *T. harzianum* MutAp. cDNA-derived sequences for Agn1p and Agn2p were aligned with that of MutAp (AAF27911). Solid arrowheads indicate cleavage sites in Agn1p and MutAp for signal peptidase predicted by the SIGNALP algorithm, whereas the open arrowhead indicates the start of mature MutA protein observed by Fuglsang *et al.* (2000). Boxes indicate sequence identities and dashes indicate gaps in the PILEUP alignment.

presence of Agn1-myc protein in culture medium of *agn1-myc* cells, whereas no Agn2-myc protein was observed in culture medium of *agn2-myc* cells (Figure 2, compare lanes 5 and 6). Comparison of the cell-bound fraction with the culture-medium fraction revealed that the majority of steady-state Agn1-myc protein was bound to cells (Figure 2, compare lanes 2 and 5). Our data demonstrate that, unlike Agn2p, Agn1p shares a functional signal sequence with characterized MutA proteins of *T. harzianum* and *Penicillium purpurogenum*, both of which also are secreted (Fuglsang *et al.*, 2000).

Deletion of *agn1*⁺ Causes Cells to Clump

To study the cellular function of Agn1p, we deleted the complete *agn1*⁺ ORF from the genome of wild type, 972, and analyzed the phenotype of the resulting deletion mutant. *agn1Δ* cells were viable and displayed a typical cylindrical rod morphology (Figure 3, compare *agn1Δ* with wild type). However, rather than single cells, the *agn1Δ* mutant often formed small clumps of cells attached to each other via an area located near their tips (Figure 3, arrows). These data indicate that *agn1Δ* cells fail to separate effectively at the end of the cell cycle.

Cell clumping has been observed not only with *agn1Δ* cells but also with cells deleted for *eng1⁺* or *ace2⁺* (Martín-Cuadrado *et al.*, 2003). To compare these clumping phenotypes, we deleted the *eng1⁺* and *ace2⁺* ORFs individually and verified the phenotypes of the resulting strains (Figure 3). Although *eng1Δ* and *ace2Δ* cells, like *agn1Δ* cells, formed clumps, the extent of cell clumping seemed to be most severe for the *ace2Δ* mutant in light microscopy. To compare the extents of clumping more quantitatively, we set up a simple sedimentation assay (see MATERIALS AND METHODS). Wild-type cells hardly sedimented during a 30-min time period, whereas *ace2Δ* cells sedimented rapidly, thereby clearing the culture medium to a large extent (Table 2). *agn1Δ* and *eng1Δ* cells, on the other hand, sedimented

Figure 2. Agn1p is secreted into the culture medium, whereas Agn2p is not. Along with wild-type cells (strain 972; lanes 1 and 4), cells of genotypes *agn1-myc* (strain ND003; lanes 2 and 5) or *agn2-myc* (strain ND061; lanes 3 and 6) were grown to midexponential phase in YEA medium. Equivalent amounts of cell-bound fractions were prepared by collecting cells, whereas culture medium fractions were obtained by precipitation on ice with 5% (wt/vol) trichloroacetic acid. Samples were resolved by 8% SDS-PAGE under reducing conditions and visualized by immunoblot analysis using an anti-myc mAb, 9E10. Probing a similar blot with antibodies directed to cytoplasmic protein α -tubulin showed protein in the cell-bound samples, but not in the culture medium samples, from all three strains (our unpublished data).

Figure 3. *agn1Δ* cells clump as a result of inefficient cell separation. Along with wild-type cells (strain 972), cells of genotypes *agn2Δ* (strain ND028), *agn1Δ* (strain ND001), *eng1Δ* (strain ND005), *agn1Δ eng1Δ* (strain ND019), or *ace2Δ* (strain ND064) were grown in EMMA medium to midexponential phase and stained with calcofluor white to visualize septa. Note that *agn1Δ* cells remain attached via a small area near the base of their tips (arrows) and that branching is observed in the *agn1Δ eng1Δ* and *ace2Δ* strains (arrowheads). Bar, 10 μ m.

with intermediate velocities, indicating that they each formed clumps that were generally smaller than those formed by the *ace2Δ* mutant (Table 2). Together, these data indicate that loss of *agn1*⁺ function causes *S. pombe* cells to clump to a similar extent as loss of *eng1*⁺ function.

For a complementation analysis, we expressed the *agn1-his* gene from a multicopy plasmid in the *agn1Δ* strain. Induction of *agn1-his* resulted in a sedimentation index comparable with that of wild-type cells, whereas repression of *agn1-his* gene expression showed a clumping phenotype typical for the *agn1Δ* strain (Table 2, compare induction of pND02 with its repression). The observation that during *agn1-his* repression clumping was not completely identical to that of *agn1Δ* cells is explained by the fact that repression

Table 2. Sedimentation analysis of mutants with a clumping phenotype

Genotype ^a	Strain	Sedimentation time ^b	
		80% of initial OD ₅₉₅	50% of initial OD ₅₉₅
Wild type	972	>30	>30
<i>ace2Δ</i>	ND064	6 ± 1	9 ± 1
<i>sep1Δ</i>	ND102	5 ± 1	10 ± 2
<i>ace2Δsep1Δ</i>	ND148	4 ± 1	6 ± 1
<i>agn1Δ</i>	ND001	17 ± 2	21 ± 2
<i>eng1Δ</i>	ND005	19 ± 1	22 ± 1
<i>agn1Δeng1Δ</i>	ND019	11 ± 1	14 ± 1
<i>agn1Δ</i> [pND02] (ON)	ND234	30 ± 5	>30
<i>agn1Δ</i> [pND02] (OFF)	ND234	19 ± 1	26 ± 1
<i>agn1Δ</i> [pSE01] (OFF)	ND235	16 ± 1	20 ± 1
<i>agn2Δ</i>	ND028	>30	>30

^a Complete genotypes are shown in Table 1.

^b Sedimentation times (minutes) are the times required for the OD₅₉₅ of the individual cultures to decrease to 80% or 50% of their initial values and are the mean ± SD of four individual experiments, except those of strains with genotypes wild type (n = 12), *agn1Δ* (n = 8), and *ace2Δ* (n = 8).

of the *nmt1* promoter is not complete. Despite continuous overexpression of the *agn1* ORF under the strong *nmt1* promoter, cells showed a wild-type morphology and did not lyse, indicating that cells are resistant to *agn1*⁺ overexpression (our unpublished data). We confirmed this conclusion by demonstrating that cells overexpressing *agn1*⁺ are as resistant to an incubation with (1,3)- β -glucanase as wild-type cells, providing no evidence for a change in cell wall rigidity (our unpublished data). Together, these data show that Agn1p-his restores Agn1p function in *agn1Δ* cells, but that Agn1p-his, upon overexpression, is not cytolytic.

Agn1p Is an endo-(1,3)- α -Glucanase

To characterize the role of Agn1p in cell separation, we studied its specific enzymatic activity. To obtain recombinant Agn1-his protein, we purified it from culture medium by immobilized metal-ion affinity chromatography and anion-exchange chromatography (Figure 4A, lanes 1 and 2), and assessed its purity as follows. First, SDS-PAGE followed by silver staining resolved only a single band with an apparent molecular mass of ~47 kDa (Figure 4A, lane 2), in close agreement with its calculated molecular mass of 48.4 kDa. In size exclusion chromatography, Agn1p-his eluted as a single peak with an elution volume corresponding to a molecular mass of ~50 kDa, indicating that it is a monomer (Figure 4B). Although mature Agn1p-his possesses one potential N-glycan attachment site at Asn-384, treatment with trifluoromethanesulfonic acid to eliminate glycosyl residues did not change its mobility in SDS-PAGE (our unpublished data), indicating that recombinant Agn1p-his was not glycosylated. Second, the 47-kDa protein band was subjected to peptide mass fingerprinting by matrix-assisted laser desorption/ionization time-of-flight by using trypsin or V8 protease. All major nonprotease peaks in the obtained spectra were accounted for by Agn1p-his, providing no evidence for the presence of contaminating proteins or for posttranslational modification of Agn1-his protein. Electrospray mass spectrometry analysis of Agn1p-his yielded a major peak of

Figure 4. Agn1p-his is an *endo*-(1,3)- α -glucanase producing predominantly (1,3)- α -glucan pentasaccharides. (A) Purification of Agn1p-his and *T. harzianum* MutAp. Agn1p-his was purified from concentrated culture supernatant of strain ND236 by immobilized nickel-nitrilotriacetic acid affinity chromatography (lane 1), followed by anion-exchange chromatography (lane 2). MutAp was purified from a commercial preparation (lane 3) by (1,3)- α -glucan adsorption chromatography (lane 4). Samples were resolved by 8% SDS-PAGE under reducing conditions and visualized by silver staining. (B) Agn1p-his is a monomeric protein. Size-exclusion chromatography of purified Agn1p-his on a Superdex 75 column. Elution volumes of molecular mass markers are indicated. (C) Agn1p-his specifically hydrolyzes (1,3)- α -glucan. Purified Agn1p-his was incubated with indicated substrates at a concentration of 4 mg/ml. To lower backgrounds, substrates were reduced, except for (1,4)- β -glucan (cellulose) and (1,4)(1,6)- α -glucan (starch). To solubilize the (1,3)- α -glucan and (1,4)- β -glucan substrates, they were carboxy-methylated. Data are a percentage of the amount of reducing ends released from reduced and carboxy-methylated (1,3)- α -glucan. (D) Agn1p-his produces mainly (1,3)- α -glucan pentasaccharides. HPAEC analysis with pulsed amperometric detection (PAD) of reaction products released after a 5-h incubation at 37°C from insoluble (1,3)- α -glucan by Agn1p-his (top) or MutAp (bottom). Numbers refer to the degree of polymerization.

molecular mass $45,980.1 \pm 1.2$ Da and a minor peak of mass $46,117.4 \pm 1.8$ Da. The major peak corresponds to an Agn1-his polypeptide without posttranslational modifications that starts, as predicted above, at Asp-21 and terminates with four His residues, indicating that two carboxy-terminal His residues were removed, presumably by proteases. The minor peak corresponds to an Agn1 polypeptide that terminates with five His residues. In conclusion, we purified Agn1-his protein to homogeneity and showed that its signal sequence was cleaved off to generate mature protein.

Substrate specificity of recombinant Agn1p-his was assayed with different glucan or mannan substrates. In the presence of (1,3)- α -glucan, we detected the release of digestion products by using a colorimetric assay (Figure 4C). No activity was detected using (1,3)- β -glucan, (1,3)- α -mannan, (1,4)- α -glucan, or (1,6)- α -glucan as substrates, demonstrating a specificity for (1,3)- α -glucosidic linkages (Figure 4C). Agn1p-his shows a broad pH optimum, with a maximum activity ranging from pH 3.5–5.5 (our unpublished data). Furthermore, 4-nitrophenyl- α -glucose, a synthetic compound that is cleaved only by glucanases with an *exo*-type mechanism of hydrolysis, was not hydrolyzed (our unpublished data). Also, Agn1p-his activity was not inhibited by 1-deoxynojirimycin, *N*-methyl-1-deoxynojirimycin, castanospermin, or D-glucono-1,5-lactone, all inhibitors of *exo*-type enzymes (our unpublished data).

The observation that *S. pombe* Agn1p and *T. harzianum* MutAp share a putative catalytic domain but not a Pro-Ser-Thr-rich linker or polysaccharide-binding domain (see above) prompted us to compare the enzyme characteristics of both (1,3)- α -glucanases. For this purpose, we purified MutA protein by (1,3)- α -glucan adsorption chromatography, yielding a single band in SDS-PAGE analysis that resolved at a molecular mass of ~ 75 kDa (Figure 4A, lane 4), consistent with previous results (Fuglsang *et al.*, 2000). To compare the reaction products of both enzymes, insoluble (1,3)- α -glucan purified from *S. pombe* was incubated with Agn1p-his or MutAp for 5 h. A high-performance anion-exchange chromatography profile of MutAp reaction products showed the presence of mostly glucose, with small amounts of (1,3)- α -glucan trisaccharide (Figure 4D, bottom). Importantly, the reaction product of Agn1p-his lacked glucose, but instead contained (1,3)- α -glucan oligosaccharides with a degree of polymerization of 2–7, with (1,3)- α -glucan pentasaccharide as the predominant reaction product (Figure 4D, top). Together, these results demonstrate that Agn1p is an *endo*-(1,3)- α -glucanase that releases predominantly (1,3)- α -glucan pentasaccharides.

Agn1p Is Involved in Hydrolysis of the Septum Edging but Not the Primary Septum

Given that Agn1p and Eng1p are both glucanases, we investigated whether their functions are redundant. First, we compared the cellular morphologies of mutants with single or double deletions for *agn1*⁺ and *eng1*⁺. Although the morphology of *agn1* Δ *eng1* Δ cells resembled that of *eng1* Δ cells, the double mutant often contained more cells per chain of cells (Figure 3). Frequently, these chains of cells contained branches (Figure 3, arrowheads), a morphology hardly ever observed with either single deletion mutant. Second, we compared the extent of clumping of the single and double mutants in our sedimentation assay. Interestingly, *agn1* Δ *eng1* Δ cells sedimented significantly faster than *agn1* Δ or *eng1* Δ cells (Table 2), indicating that clumps of the double mutant were on average significantly larger than those of either single mutant. Together, these data suggest that *agn1*⁺ and *eng1*⁺ fulfill nonoverlapping functions.

Figure 5. *agn1Δ* cells remain attached via remnants of the septum edging. Electron micrographs of thin sections from cells of genotypes: (A) *agn1Δ* (strain ND001). (B and D) *eng1Δ* (strain ND005). (C and E) *ace2Δ* (strain ND064). Cells were grown to midexponential phase in YEA medium. Solid arrow indicates locations of cell attachment, open arrow indicates remnants of the primary septum, solid arrowheads indicate branched outgrowths, and * indicates fission scars. Bar, 1 μm. (F) Schematic representation of the septum and septum edging immediately before (left) and during (right) cell separation. Adapted from Johnson *et al.* (1973).

To gain insight into the precise roles of Agn1p and Eng1p in cell separation, we analyzed the ultrastructures of *agn1Δ* and *eng1Δ* cells. Electron microscopy analysis of *agn1Δ* cells showed that septum assembly (and thus cytokinesis) and dissolution of the primary septum progressed normally in this mutant (Figure 5A). These results were corroborated by microscopy analysis on *agn1Δ* cells stained with calcofluor white, a dye that specifically stains the primary septum and remnants thereof. Staining of the septa in *agn1Δ* cells was intense during cytokinesis, but diminished in intensity during cell separation (Figure 3). By contrast, a defect in dissolution of the septum edging was evident in *agn1Δ* cells (Figure 5A). The septum edging in *agn1Δ* cells was broken down only partially, causing daughter cells to remain attached via a small stretch of septum-edging material (Figure 5A, solid arrow).

The ultrastructure of the septal region in *agn1Δ* cells is clearly distinct from that in *eng1Δ* cells. In *eng1Δ* cells, we observed that the primary septum often fails to break down

(Figure 5B), exposing primary-septum material to the extracellular space (Figure 5D, open arrow). This defect causes *eng1Δ* cells to remain attached via the primary septum, which is visualized clearly by staining with calcofluor white (Figure 3). The septum edging, on the other hand, seems to be broken down normally in *eng1Δ* cells (Figure 5B), resulting in invaginations at the septa and in exposure of fission scars (Figure 5B, asterisks). We conclude that *eng1⁺* and *agn1⁺* fulfill distinct roles in cell separation: *eng1⁺* is involved in dissolution of the primary septum, whereas *agn1⁺* is involved in dissolution of the septum edging.

Agn1p Protein Expression Oscillates during the Cell Cycle

The observation that Agn1p acts during cell separation prompted us to examine whether Agn1 protein expression was up-regulated during this stage of the cell cycle. To this end, we arrested temperature-sensitive (ts) *cdc25-22 agn1-myc* cells at the G2-M transition and then released them synchronously into the cell cycle, while monitoring cell syn-

Figure 6. (A–C) Periodic expression of Agn1p-myc resembles that of Eng1p-myc and depends on *ace2*⁺. Cells of genotypes *cdc25-22 agn1-myc* (strain ND049) (A), *cdc25-22 eng1-myc* (strain ND051) (B), or *cdc25-22 ace2Δ agn1-myc* (strain ND111) (C) were arrested at G2-M transition and then released synchronously. Cells were collected at indicated times after start of the release. Degrees of synchrony are indicated by septation index. A indicates asynchronous controls. Quantitation of the blots by normalization to the α-tubulin loading control confirmed the apparent peak in Agn1p levels at about the time of septation and cell separation both in the experiment shown and in a second experiment in which the synchronous population was followed into the second cell cycle (our unpublished data). Note that upon *ace2*⁺ deletion Agn1p-myc is not expressed at any stage of the cell cycle. (D) Agn1-myc protein expression is up-regulated during G1 and S phases. Cells of genotypes *cdc25-22 agn1-myc* (strain ND049; lane 2), *cps1-191 agn1-myc* (strain ND154; lane 3), and *cdc10-129 agn1-myc* (strain ND054; lane 4) were grown in YEA medium to midexponential phase at 28°C and then arrested at G2 phase, cytokinesis (C phase), or G1 phase, respectively, by a shift to 36°C for 4 h. For an arrest at S phase, exponentially growing cells of genotype *agn1-myc* (ND003) were incubated with hydroxyurea at a concentration of 11 mM at 28°C for 4 h (lane 5). Along with a total lysate of asynchronous cells (indicated as A, lane 1), cell lysates were resolved by 8% SDS-PAGE under reducing conditions and visualized by immunoblot analysis by using an anti-myc mAb, 9E10. α-Tubulin served as a loading control.

chrony by septation-index analysis and Agn1-myc protein levels by immunoblot analysis. Agn1-myc protein levels oscillated considerably as cells traversed the first cell cycle, peaking concomitantly with septation and cell separation (Figure 6A). During the following cell cycle, the oscillation was less dramatic, because Agn1-myc protein is a stable protein with a half-life of >2 h (our unpublished data), and its levels did not decrease to their initial levels observed at arrest. In an arrest-release experiment on *cdc25-22 eng1-myc* cells, Eng1-myc protein levels oscillated in a manner similar to the oscillation of Agn1-myc protein levels (Figure 6, compare B and A). This oscillation in Eng1 protein levels is in good agreement with the oscillation reported for *eng1*⁺ transcript levels (Martín-Cuadrado *et al.*, 2003).

To compare more precisely Agn1 protein levels at specific stages of the cell cycle, we used, along with the *cdc25*^{ts} mutant to block entry into mitosis, a *cps1*^{ts} mutant to block septum assembly and a *cdc10*^{ts} mutant to block in late G1 phase. Also, we treated *agn1-myc* cells with hydroxyurea for arrest in S phase. Immunoblot analysis showed that during the G2 and C phases Agn1-myc protein levels are low, whereas during the G1 and S phases they increased (Figure 6D). These data not only confirm the results of the *cdc25-22* arrest-release experiment, they also narrow down the broad period of septation and cell separation to the G1 and S phases. In summary, we conclude that Agn1 and Eng1 pro-

tein levels oscillate during the cell cycle and that Agn1 protein levels peak during G1 and S phases.

agn1⁺ and *eng1*⁺ Expression Depend on Transcription Factors *Sep1p* and *Ace2p*

To investigate whether transcription factors Sep1p and Ace2p are involved in regulating Agn1 protein levels, we crossed *agn1-myc* into *ace2Δ* and *sep1Δ* backgrounds. Immunoblot analysis showed that the Agn1-myc protein level had decreased dramatically in both these mutants (Figure 7, compare lanes 2 and 3 to lane 1). To exclude the possibility that the observed lack of Agn1-myc protein expression was the result of a change in the frequency of cells in G1 and S phases, we performed an arrest-release experiment on *cdc25-22 agn1-myc* cells deleted for *ace2*⁺. At no stage of the cell cycle were we able to detect Agn1-myc protein in the *ace2Δ* background (Figure 6, compare A and C). These data show that *ace2*⁺ and *sep1*⁺ each are required for Agn1 protein expression.

Not only did Agn1-myc protein levels decrease considerably in both the *sep1Δ* and *ace2Δ* backgrounds but also Eng1-myc protein levels (Figure 7, compare lanes 5 and 6 to lane 4). Simultaneous deletion of *sep1*⁺ and *ace2*⁺ did not further decrease Eng1-myc protein levels, suggesting that their effects are not additive (Figure 7, lane 7). This obser-

Figure 7. Agn1-myc protein expression depends on *ace2*⁺ and *sep1*⁺. Cells of genotypes *agn1-myc* (strain ND003; lane 1), *agn1-myc sep1Δ* (strain ND140; lane 2), *agn1-myc ace2Δ* (strain ND132; lane 3), *eng1-myc* (strain ND007; lane 4), *eng1-myc sep1Δ* (strain ND144; lane 5), *eng1-myc ace2Δ* (strain ND136; lane 6), or *eng1-myc sep1Δ ace2Δ* (strain ND218; lane 7) were grown in YEA medium to midexponential phase. Cell lysates were resolved by 8% SDS-PAGE under reducing conditions and visualized by immunoblot analysis by using an anti-myc mAb, 9E10. α -Tubulin served as a loading control. Ratios indicate normalized Agn1p-myc or Eng1p-myc levels corrected for α -tubulin loading controls.

vation was substantiated by the finding that the *ace2Δ* and *sep1Δ* single and double mutants formed clumps to a similar extent, although the double mutant sedimented a little faster than either single mutant (Table 2).

To study the effect of a simultaneous loss of *agn1*⁺ and *eng1*⁺ expression in the *ace2Δ* mutant (see above), we studied its ultrastructure. We observed that in *ace2Δ* cells both the septum edging and the primary septum remained intact (Figure 5C). Also, in this mutant, apical growth was redirected toward the side of the septum, causing branching (Figure 5E, solid arrowheads). This phenotype was also observed for the *agn1Δ eng1Δ* double mutant (Figure 3, arrowheads). From these experiments we conclude that both Sep1p and Ace2p are required for periodic expression of both Agn1 and Eng1 proteins.

Agn2p Is Essential for Endolysis of the Ascus Wall during Sporulation

To investigate whether the cellular function of *agn2*⁺ is similar to that of *agn1*⁺, we deleted the *agn2*⁺ ORF and analyzed the resulting deletion phenotype. *agn2Δ* cells were viable and grew in YEA culture medium at 28°C with a generation time similar to that of wild-type cells (our unpublished data). *agn2Δ* cells also displayed a cylindrical rod morphology indistinguishable from that of wild-type cells (Figure 3), and they did not form clumps (Table 2). Furthermore, northern blot analysis indicated that *agn2* transcript levels did not fluctuate during the cell cycle (our unpublished data). We conclude that, unlike *agn1*⁺, *agn2*⁺ does not seem to have a role during vegetative growth.

agn2⁺ expression is up-regulated temporarily during sporulation (Mata *et al.*, 2002). To localize Agn2p in sporulating cells, we crossed haploid *agn2-gfp* cells with haploid wild-type cells of opposite mating type and analyzed Agn2p-GFP fluorescence in living mating products. Although Agn2p-GFP fluorescence in the haploid *agn2-gfp* cells was weak and diffuse, it was more intense in sporulating diploid cells. Agn2p-GFP was excluded from ascospores, but instead localized to the residual cytoplasm within the ascus wall (Figure 8A), known as the epiplasm (Yoo *et al.*, 1973). To assess Agn2p function, we analyzed *agn2Δ* cells in a simple mating assay. No differences in conjugation and sporulation efficiency were observed with *agn2Δ* cells compared with wild-type cells (our unpublished data). To determine whether *agn2*⁺ plays a role in the subsequent step of

endolysis of the ascus wall, during which the ascospores are released, we transferred *agn2Δ/agn2Δ* diploid cells to sporulation medium. Like the majority of matching wild-type diploids, the majority of *agn2Δ/agn2Δ* cells developed into asci containing four ascospores, called tetrads, within 48 h after start of induction, confirming that sporulation was not affected by *agn2*⁺ deletion (our unpublished data). However, whereas the ascus walls lysed in a majority of wild-type tetrads, releasing individual ascospores within the following 48 h, the ascus walls remained intact in a majority of *agn2Δ/agn2Δ* tetrads, even after 5 d after tetrad formation (Figure 8B). Small numbers of free ascospores were observed, but they represented only ~10% of the tetrads. We conclude from these experiments that Agn2p is involved in endolysis of the ascus wall.

DISCUSSION

In this report, we have identified Agn1p as an *endo*-(1,3)- α -glucanase that is involved in the dissolution of cell wall material that surrounds the septum, the septum edging (Figure 5F). Deletion of *agn1*⁺ abrogates the ability of *S. pombe* cells to fully separate as free cells and results in mutant cells that remain attached to each other via remnants of the septum edging. However, *agn1*⁺ deletion does not affect hydrolysis of the primary septum, based on the normal dissolution of primary-septum material stained by calcofluor white (Figure 3) and on the ultrastructure of *agn1Δ* cells (Figure 5A). These observations are consistent with the presence of (1,3)- α -glucan in the septum edging and with its absence from the primary septum. By contrast, deletion of *eng1*⁺ does not affect dissolution of the septum edging, based on the observed invaginations at the site of the septum in *eng1Δ* cells, but rather abrogates dissolution of the primary septum (Figure 5B). The roles of Agn1p and Eng1p in cell separation seem functionally distinct in that the *agn1Δ eng1Δ* double mutant clumps more extensively than either single mutant (Table 2). Furthermore, the *ace2Δ* mutant, which expresses decreased levels of both Eng1p and Agn1p (Figure 7), displays a hyphal morphology characterized by intact primary septa and intact septum edgings (Figure 5C). We conclude that the dissolution of both the primary septum and the septum edging are facilitated by hydrolases but that these processes involve dedicated hydrolases Eng1p and Agn1p.

Figure 8. Agn2p localizes to the cytoplasm of the ascus and contributes to hydrolysis of the ascus wall. (A) Haploid cells of genotype *agn2-gfp* (strain ND172) and matching wild-type cells (strain FYC15) were allowed to mate on a sporulation plate and resulting zygotic asci were examined using fluorescence microscopy. Agn2p-GFP fluorescence is excluded from ascospores, but instead localizes to the cytoplasm of the ascus, the epiplasm. Bar, 10 μ m. (B) Along with wild-type diploid cells (strain ND080), diploid cells of genotype *agn1Δ/agn1Δ* (strain ND084) and *agn2Δ/agn2Δ* (strain ND088) were grown to exponential phase in EMM medium containing 2% ammonium sulfate to inhibit sporulation. Subsequently, sporulation was induced synchronously by a shift to EMM medium containing 0.5% sodium glutamate, a poor nitrogen source. At the indicated time intervals, percentages of free ascospores were determined. Note that in the absence of *agn2*⁺ the ascus wall fails to undergo endolysis.

A series of distinct steps is required for successful delivery of cell-separation hydrolases to their target destination at the right stage of the cell cycle, including cell cycle-regulated synthesis, targeted secretion, and localized hydrolysis. For the first step in cell separation, transcription factors are involved. We show that expression of Agn1p and Eng1p in *S. pombe* requires transcription factors Sep1p and Ace2p. In *S. cerevisiae*, two homologues of Sep1p (ScFkh1p and ScFkh2p) regulate the cell cycle oscillation of an entire gene cluster that includes ScAce2p, the *S. cerevisiae* homologue of Ace2p (Zhu *et al.*, 2000). ScAce2p, in turn, directly regulates cell cycle-dependent expression of cell-separation hydrolases ScEng1p and ScCts1p (Dohrmann *et al.*, 1992; Baladrón

et al., 2002). The peaks in ScENG1 and ScCTS1 transcript levels during G1 phase in *S. cerevisiae* (Dohrmann *et al.*, 1992; Spellman *et al.*, 1998) overlap with the peak in Agn1 protein levels during G1 and S phases in *S. pombe* (Figure 6D), suggesting that *agn1*⁺ expression may be directly controlled by Ace2p. During targeted secretion, the second step in cell separation, hydrolases are transported to the septal region via the secretory pathway. We hypothesize that secretory vesicles containing cell-separation hydrolases, such as Agn1p and Eng1p, may be targeted to the septum via the exocyst complex, a multiprotein complex present in yeasts (but also in humans) (Hsu *et al.*, 1998). The exocyst complex might be involved in recruiting secretion vesicles to specific regions of the plasma membrane (Hsu *et al.*, 1999), and the cell-separation defect observed in exocyst mutants in *S. pombe* (Wang *et al.*, 2002) is consistent with this hypothesis. Also certain septins, filament-forming proteins that form a ring structure near the septal region, together with the septin-organizing protein Mid2p, may be involved in localizing secretion of cell-separation hydrolases to the septal region, because their gene deletion phenotypes show similar cell-separation defects (Berlin *et al.*, 2003; Tasto *et al.*, 2003). Localized hydrolysis, the third and final step in cell separation, follows targeted secretion of cell-separation hydrolases. Oulevey *et al.* (1970) observed a triangular region of dense material in the extracellular space between the septum and the septum edging opposite the intracellular exocyst ring (Figure 5F). It seems not unreasonable to suggest that this region may contain secreted cell-separation hydrolases that diffuse toward the septum and the septum edging. Indeed, one of these hydrolases, Eng1p, localizes to a ring structure overlaying the primary septum (Martín-Cuadrado *et al.*, 2003).

Cell separation was not inhibited completely in any of the mutants studied here, not in the *agn1Δ* and *eng1Δ* single and double mutants, nor in the *ace2Δ* and *sep1Δ* single and double mutants. Our observation that cells of the *agn1Δ eng1Δ* double mutant can separate (Figure 3 and Table 2) suggests that additional factors may contribute to cell separation in *S. pombe*. One possibility is physical breakage of septum or septum edging by mechanical forces caused by agitation. More likely, however, is the presence of additional cell-separation hydrolases besides Agn1p and Eng1p. Perhaps one (or more) of the three putative *exo*-(1,3)- β -glucanases encoded by the *S. pombe* genome are involved (Martín-Cuadrado *et al.*, 2003). Furthermore, our finding that cells of the *ace2Δ sep1Δ* double mutant can separate, albeit to the lowest extent of all mutants studied here (Table 2), may indicate that one (or more) additional transcription factors are involved in regulating expression of cell-separation hydrolases. Indeed, even after deletion of *ace2*⁺ and *sep1*⁺, low levels of Eng1p remain detectable (Figure 7).

Overexpression of *endo*-(1,3)- α -glucanase Agn1p did not compromise the structural integrity of *S. pombe* cells and was not cytolytic, even though we overexpressed this hydrolase continuously during the entire cell cycle. This observation may be explained as follows. First, it is possible that Agn1p did not reach concentrations high enough to weaken the cell wall effectively. Second, effective impairment of cell wall (1,3)- α -glucan may be avoided by the enzyme's *endo*-catalytic mode of action, cleaving intrachain glycosidic linkages, rather than an *exo*-catalytic mode, which would have cleaved glycosidic linkages at one of the ends of the polysaccharide. Perhaps, accumulation of internal breaks may be less disruptive to the three-dimensional structure, and thus to the rigidity, of (1,3)- α -glucan than accumulation of successive cleavages from one end. Third, it is conceivable that

its simple domain structure prevents Agn1p from compromising structural integrity. Sequence comparisons with *T. harzianum* MutAp (Figure 1) and *Penicillium purpurogenum* MutAp (Fuglsang *et al.*, 2000) show that Agn1p lacks a carboxy-terminal polysaccharide-binding domain together with a Pro-Ser-Thr-rich linker. The presence of a polysaccharide-binding domain is common among hydrolases, such as cellulases, xylanases, chitinases, and amylases (Gilkes *et al.*, 1991). Because *T. harzianum* and *P. purpurogenum* are parasitic fungi, they secrete (1,3)- α -glucanases to degrade (1,3)- α -glucan of host fungi, producing glucose, which can be taken up as a carbon source. Our analyses on Agn1p show (Figure 4C) that a polysaccharide-binding domain is not essential for hydrolysis of (1,3)- α -glucan. Nonetheless, the absence of a polysaccharide-binding domain may diminish the efficiency of hydrolysis by Agn1p or may limit its access to (1,3)- α -glucan, thereby preventing cytolysis.

The paralog of Agn1p in *S. pombe*, Agn2p, lacks a signal sequence for entry into the secretory pathway (Figure 1), fails to be secreted into the culture medium (Figure 2), and localizes to the cytoplasm of the ascus, the epiplasm (Figure 8A). To our knowledge, Agn2p is the first member of glycoside hydrolase family 71 that localizes, at least initially, to the cytoplasm. DNA-microarray analysis showed that *agn2*⁺ is up-regulated 180-fold during sporulation (Mata *et al.*, 2002). *agn2Δ/agn2Δ* diploid cells induced to sporulate were able to generate four ascospores per ascus, which is typical for *S. pombe*, but then failed to undergo effective endolysis of the ascus wall. As a consequence, few ascospores were released as free entities and most remained enclosed within the ascus wall, even after a prolonged incubation (Figure 8B). We speculate that in wild-type asci the ascus membrane, which encloses the four ascospores, may disintegrate after formation of the ascospores, bringing proteins of the epiplasm including Agn2p into direct contact with the ascus wall. Given that the ascus wall is derived from the cell wall of the diploid cell that underwent sporulation, it contains (1,3)- α -glucan, which, after disintegration of the ascus membrane, may be broken down from within by hydrolases, such as Agn2p. The *S. pombe* genome encodes not only *endo*-(1,3)- α -glucanases in two forms, one with and the other without a signal sequence, but also *endo*-(1,3)- β -glucanases. Whereas Eng1p contains a signal sequence, its paralog in *S. pombe*, Eng2p, lacks such a sequence. Like Agn2p, Eng2p is temporally up-regulated during sporulation (Mata *et al.*, 2002), suggesting that it may also be involved in endolysis of the ascus wall. Homologues of these two forms of *endo*-(1,3)- β -glucanases also have been identified in *S. cerevisiae* and in *C. albicans* (Baladrón *et al.*, 2002).

Characterization of the roles of the Agn proteins in *S. pombe* has provided new insights into our understanding of the cellular functions of (1,3)- α -glucanases. Not only *S. pombe* but also medically important dimorphic fungi, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Blastomyces dermatitidis*, and *Coccidioides immitis*, contain substantial amounts of cell wall α -glucan in their pathogenic yeast form (Hogan *et al.*, 1996), which must be hydrolyzed locally during cell separation to allow efficient dispersal in the infected organism. Furthermore, cell wall α -glucan is abundantly present in the human fungal pathogen *Cryptococcus neoformans* (James *et al.*, 1990), for which not only immunocompromised patients but also healthy individuals are at risk due to its ubiquitous environmental presence (Stephen *et al.*, 2002). Interestingly, the *C. neoformans* genome encodes four homologues of the *S. pombe* *endo*-(1,3)- α -glucanases, allowing future investigations into potential roles of these enzymes for virulence.

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