Ady3p Links Spindle Pole Body Function to Spore Wall Synthesis in *Saccharomyces cerevisiae*

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ABSTRACT

Spore formation in *Saccharomyces cerevisiae* requires the *de novo* synthesis of prospore membranes and spore walls. Ady3p has been identified as an interaction partner for Mpc70p/Spo21p, a meiosis-specific component of the outer plaque of the spindle pole body (SPB) that is required for prospore membrane formation, and for Don1p, which forms a ring-like structure at the leading edge of the prospore membrane during meiosis II. *ADY3* expression has been shown to be induced in midsporulation. We report here that Ady3p interacts with additional components of the outer and central plaques of the SPB in the two-hybrid assay. Cells that lack *ADY3* display a decrease in sporulation efficiency, and most *ady3*Δ/*ady3*Δ asci that do form contain fewer than four spores. The sporulation defect in *ady3*Δ/*ady3*Δ cells is due to a failure to synthesize spore wall polymers. Ady3p forms ring-like structures around meiosis II spindles that colocalize with those formed by Don1p, and Don1p rings are absent during meiosis II in *ady3*Δ/*ady3*Δ cells. In *mpc70*Δ/*mpc70*Δ cells, Ady3p remains associated with SPBs during meiosis II. Our results suggest that Ady3p mediates assembly of the Don1p-containing structure at the leading edge of the prospore membrane via interaction with components of the SPB and that this structure is involved in spore wall formation.

*MAT***a**/*MAT*α diploid cells of *Saccharomyces cerevisiae* cultured in the absence of nitrogen and the presence of a nonfermentable carbon source undergo meiosis and form four haploid spores. Both meiotic divisions occur within a single, continuous nuclear envelope, in which the spindle pole bodies (SPBs), the functional equivalents of centrosomes in higher eukaryotes, are embedded. At the onset of meiosis II, the cytoplasmic face of each SPB, termed the outer plaque, expands and becomes a site for docking and fusion of vesicles, which coalesce into a flattened sac called the prospore membrane. As the meiotic spindles extend during anaphase II to create four nuclear lobes, the prospore membranes grow toward the center of the spindles to engulf the adjacent nuclear lobes. As nuclear division occurs at the end of anaphase II, each prospore membrane fuses with itself, resulting in four haploid nuclei that are each surrounded by two continuous membranes. Spore wall material is deposited into the lumen between the two new membranes around each nucleus to produce mature spores.

The spore wall is composed of four layers of macromolecular polymers (Jones *et al.* 1992). The inner two layers consist of polymers, mannan and glucan, that are also components of the vegetative cell wall. Mannan is made up of glycoproteins with extended α-1,6-mannosyl side chains, and glucan consists of β-1,6- and β-1,3-linked chains of glucose (Kathoda *et al.* 1984). The third layer of the spore wall consists of chitosan, a polymer of β-1,4-linked glucosamine (Briza *et al.* 1988). The outer layer of the spore wall contains dityrosine, protein, and other undefined components and confers the unique resistance of spores to adverse environmental conditions (Briza *et al.* 1986, 1990b). Mutations of genes involved in the synthesis of a specific polymer result in the absence of one or more spore wall layers (Briza *et al.* 1990a; Pammer *et al.* 1992), whereas mutations of putative regulators of spore wall assembly cause heterogeneous spore wall defects (Krisak *et al.* 1994; Straight *et al.* 2000).

Regulation of SPB function during meiosis is critical for spore formation. During sporulation, the primary role of the outer plaque changes from the anchoring of cytoplasmic microtubules to the initiation of prospore membrane synthesis. The functional shift of the SPB during sporulation results from a change in the molecular composition of the outer plaque. Spc72p, a component of the mitotic outer plaque that binds to the gamma-tubulin complex of cytoplasmic microtubules, disappears from SPBs during meiosis II and is replaced by the meiosis-specific components Mpc54p and Mpc70p/Spo21p (Knop and Strasser 2000). Deletion of either *MPC54* or *MPC70* results in abnormal outer plaque formation and abolishes formation of prospore membranes (Knop and Strasser 2000; Bajgier *et al.* 2001).

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Environmental conditions or mutations that selectively block outer plaque modification on daughter SPBs result in formation of two-spored asci, termed nonsister dyads, in which one haploid nucleus from each meiosis II spindle has been packaged (Davidow *et al.* 1980; Okamoto and Iino 1981; Bajgier *et al.* 2001; Ishihara *et al.* 2001; Wesp *et al.* 2001).

Genome-wide analyses have been useful for identifying genes involved in sporulation and for revealing potential interactions between their products. Examination of genes that are induced during sporulation led to the identification and characterization of *MPC54* and *MPC70* (Chu *et al.* 1998; Knop and Strasser 2000; Primig *et al.* 2000; Bajgier *et al.* 2001). Proteomic interaction screens have identified binding partners for the products of these and other genes involved in prospore membrane synthesis, and the *in vivo* significance of several of these interactions is beginning to emerge (Uetz *et al.* 2000; Ito *et al.* 2001). Systematic disruption of meiotically induced genes and analysis of the resulting sporulation phenotypes has uncovered many genes involved in various aspects of sporulation (Rabitsch *et al.* 2001).

One gene that is implicated in prospore membrane formation and/or spore wall synthesis by the results of genome-wide analyses is *ADY3*. Ady3p interacts in the two-hybrid assay with Mpc70p, Ssp1p, which is required for formation of spores but not for meiotic segregation of nuclear DNA, and Don1p, which localizes to a ring-like structure at the leading edge of the prospore membrane during the second meiotic division (Nag *et al.* 1997; Knop and Strasser 2000; Uetz *et al.* 2000; Ito *et al.* 2001). Expression of *ADY3* is induced in midsporulation at approximately the same time as expression of *MPC70* (Chu *et al.* 1998; Primig *et al.* 2000). Loss of *ADY3* has no effect on meiotic chromosome segregation and nuclear division but results in the accumulation of asci with only two mature spores (Rabitsch *et al.* 2001).

We have investigated directly the role of Ady3p in sporulation. Ady3p interacts with components of the outer and central plaques of the SPB in the two-hybrid assay and stably associates with the SPB *in vivo* in *mpc70*Δ/*mpc70*Δ cells. Cells that lack *ADY3* sporulate poorly due to a failure to synthesize spore wall polymers. Ady3p colocalizes with Don1p to ring-like structures that surround the spindles during meiosis II and is required for assembly of Don1p into these structures. We propose that Ady3p recruits Don1p to the leading edge of the prospore membrane during meiosis via interactions with SPB components and may facilitate the recruitment of other factors that are required to promote spore wall formation.

MATERIALS AND METHODS

Yeast strains and methods: Standard *S. cerevisiae* genetic methods and media were used (Rose *et al.* 1990). Strains are listed in Table 1. AN120 and YCJ4 have been described previously (Inouye *et al.* 1997; Neiman *et al.* 2000). All strains except YCJ4 were derived from SK1, and all SK1-derived strains except AN295 are isogenic to AN120. NY48 was provided by H. Tachikawa (SUNY, Stony Brook). AN246 was made by disrupting *ADY3* in AN117-4B to create AN1069, crossing AN1069 to AN117-16D, and mating two of the *ady3*Δ haploid segregants. MND24 was made by chromosomal tagging of *ADY3* with *CFP* in AN117-16D to create MNH17, crossing MNH17 to NY48, and mating two of the *ADY3::CFP DON1::YFP* segregants. MNH13 and MNH14 were made by chromosomal tagging of *ADY3* with *GFP* in AN117-4B and AN117-16D, respectively, and mated to create MND16. MNH29 and MNH30 were made by disrupting *MPC70* in MNH13 and MNH14, respectively, and mated to create MND41. AN295 was created by introducing one wild-type copy of *TRP1* into its natural locus in strain 1702 (Rabitsch *et al.* 2001).

Gene insertions and replacements were performed by transformation of strains with PCR-generated DNA cassettes and verified by PCR (Longtine *et al.* 1998). The sequences of oligonucleotide primers used to amplify insertion and disruption cassettes by PCR are listed in Table 2. The cassette used to replace *ADY3* with *kanMX4* was amplified from genomic DNA of strain 33936 (ResGen Invitrogen) with primers MNO110 and MNO111. The cassettes used to insert *GFP-HIS3MX6* and *CFP-HIS3MX6* tags into the 3′ end of *ADY3* were amplified from plasmids pFA6a-yEGFP-HIS3MX6 and pFA6a-CFP-HIS3MX6, respectively, using primers MNO127 and MNO128. The cassette used to replace *MPC70* with *TRP1* was amplified from plasmid pFA6a-TRP1 using primers ANO194 and MNO101. The cassette used to replace *trp1* with *TRP1* was amplified from plasmid pFA6a-TRP1 using primers ANO270 and ANO271.

Plasmids: Plasmids used in this study are listed in Table 3. pFA6a-CFP-HIS3MX6 was made in two series of steps. In the first series, yEGFP was amplified from pYM12 using MNO155 and MNO156, the PCR product was digested with *Pac*I and *Asc*I, and the resulting fragment was used to replace the 0.8-kb *Pac*I-*Asc*I fragment of pFA6a-GFP(S65T)-HIS3MX6 to create pFA6a-yEGFP-HIS3MX6. pFA6a-yEGFP-HIS3MX6 was created to generate PCR-tagging cassettes with linker sequences longer than those amplified from pFA6a-GFP(S65T)-HIS3MX6, and the longer linker sequence was necessary to create a functional Ady3p-GFP fusion. In the second series of steps, pDH3 was digested with *Msc*I and *Asc*I, and the 0.5-kb fragment was used to replace the fragment of similar size in pFA6a-yEGFP-HIS3MX6 to create pFA6a-CFP-HIS3MX6. pEG202-ADY3 was made by amplifying *ADY3* with MNO130 and MNO132, digesting the PCR product with *Bam*HI and *Xho*I, and subcloning the resulting fragment into the *Bam*HI and *Xho*I sites of pEG202. pGADGH-MPC70 was made by amplifying *MPC70* with MNO115 and MNO116, digesting the PCR product with *Bam*HI and *Xho*I, and subcloning the resulting fragment into the *Bam*HI and *Xho*I sites of pGADGH. The following plasmids were used to express fusion proteins for two-hybrid assays: pEG202, LexA (negative control); pEG202-ADY3, LexA-Ady3p$^{1\text{-}790}$; pLexA$_{202}$-GLC7, LexA-Glc7p$^{1\text{-}312}$; pMK184, LexA-Cnm67p$^{386\text{-}580}$; pSM614, LexA-Nud1p$^{405\text{-}852}$; pGADGH, GAD (negative control); pMK183, GAD-Cnm67p$^{386\text{-}580}$; 3-20, GAD-Gip1p$^{161\text{-}573}$; pGADGH-MPC70, GAD-Mpc70p$^{1\text{-}609}$; pSM613, GAD-Nud1p$^{405\text{-}852}$; and pMK169, GAD-Spc42p$^{1\text{-}363}$.

Sporulation assays: Cells were induced to sporulate in liquid medium essentially as described previously (Neiman 1998). Cells were cultured to saturation in either YPD or SC lacking the appropriate nutrient, cultured overnight to midlog phase in YP acetate, and transferred to 2% potassium acetate at a concentration of 3×10^7 cells/ml ($OD_{600} \sim 2.0$).

Fluorescence microscopy: To visualize naturally fluorescent

TABLE 1

***S. cerevisiae* strains used in this study**

Strain	Genotype
AN117-4B	*MAT*α *arg4 his3 ho::LYS2 leu2 lys2 rme1::LEU2 trp1 ura3*
AN117-16D	*MAT***a** *his3 ho::LYS2 leu2 lys2 trp1 ura3*
AN1069	*MAT*α *ady3::kanMX4 arg4 his3 ho::LYS2 leu2 lys2 rme1::LEU2 trp1 ura3*
MNH13	*MAT*α *ADY3::GFP-HIS3MX6 arg4 his3 ho::LYS2 leu2 lys2 rme1::LEU2 trp1 ura3*
MNH14	*MAT***a** *ADY3::GFP-HIS3MX6 his3 ho::LYS2 leu2 lys2 trp1 ura3*
MNH17	*MAT***a** *ADY3::CFP-HIS3MX6 his3 ho::LYS2 leu2 lys2 trp1 ura3*
MNH29	*MAT*α *ADY3::GFP-HIS3MX6 arg4 his3 ho::LYS2 leu2 lys2 mpc70::TRP1 rme1::LEU2 trp1 ura3*
MNH30	*MAT***a** *ADY3::GFP-HIS3MX6 his3 ho::LYS2 leu2 lys2 mpc70::TRP1 trp1 ura3*
NY48	*MAT*α *arg4 DON1::YFP-HIS3MX6 his3 ho::LYS2 leu2 lys2 rme1::LEU2 trp1 ura3*
YCJ4	*MAT***a** *ADE2::p_{GAL}-URA3 canR gal4 gal80 his3 leu2 LYS2::Lex_{op}-lacZ trp1 ura3*
AN120	*MAT***a**/*MAT*α *ARG4/arg4 his3/his3 ho::LYS2/ho::LYS2 leu2/leu2 lys2/lys2 RME1/rme1::LEU2 trp1/trp1 ura3/ura3*
AN246	*MAT***a**/*MAT*α *ady3::kanMX4/ady3::kanMX4 ARG4/arg4 his3/his3 ho::LYS2/ho::LYS2 leu2/leu2 lys2/lys2 RME1/rme1::LEU2 trp1/trp1 ura3/ura3*
AN295	*MAT***a**/*MAT*α *ady3::HIS3MX6/ady3::HIS3MX6 his3/his3 HO/HO LEU2::p_{URA3}-tetR-GFP/ LEU2::p_{URA3}-tetR-GFP REC8::HA3-URA3/REC8::HA3-URA3 TRP1/trp1 URA3::$tetO_{224}$/URA3::$tetO_{224}$*
MND16	*MAT***a**/*MAT*α *ADY3::GFP-HIS3MX6/ADY3::GFP-HIS3MX6 ARG4/arg4 his3/his3 ho::LYS/ho::LYS2 leu2/leu2 lys2/lys2 RME1/rme1::LEU2 trp1/trp1 ura3/ura3*
MND24	*MAT***a**/*MAT*α *ADY3::CFP-HIS3MX6/ADY3::CFP-HIS3MX6 ARG4/arg4 DON1::YFP-HIS3MX6/ DON1::YFP-HIS3MX6 his3/his3 ho::LYS2/ho::LYS2 leu2/leu2 lys2/lys2 RME1/rme1::LEU2 trp1/trp1 ura3/ura3*
MND41	*MAT***a**/*MAT*α *ADY3::GFP-HIS3MX6/ADY3::GFP-HIS3MX6 ARG4/arg4 his3/his3 ho::LYS2/ho::LYS2 leu2/leu2 lys2/lys2 mcp70::TRP1/mpc70::TRP1 RME1/rme1::LEU2 trp1/trp1 ura3/ura3*

proteins, cells were fixed in 4% formaldehyde for 5 min, washed with PBS (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4), and mounted with Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector, Burlingame, CA). For staining with Calcofluor white, FITC-Concanavalin A, and antibodies to β-1,3-glucan, cells were fixed in 4% formaldehyde for 1–16 hr, washed with 1 M sorbitol, 0.1 M HEPES pH 7.5, 5 mM NaN_3 (SHA), converted to spheroplasts for 1 hr at 30° with 12.5 μg/ml zymolyase and 0.2% β-mercaptoethanol in SHA, washed with SHA, and applied to slides. Calcofluor white (0.2–0.4 mg/ml) was added to some samples during zymolyase incubation. Samples on slides were im-

TABLE 2

Oligonucleotides used in this study

Name	Sequence
ANO194	TAT GTA TAT ATA GAA TAT TAA GGA TTA TAA AAG AAT TGT TAG CTG TTG AGG TGA ATT CGA GCT CGT TTA AAC
ANO270	CAA TTT GTT AAT ATC CTA ATT CGG TAA AGC TTT GTC GAG ACA TTA ACA AAA CGG ATC CC GGG TTA ATT AA
ANO271	ATG TTT AAG TAA AAG AAC AAA AAG GTA GC CAA TGT AGC GCT CTT ACT TTA GAA TTC GAG CTC GTT TAA AC
MNO101	AGG CAT ATT AAA GAT CTA TTA AAG ATC TAT TAA AGC TTT CTG CTA CCA GTC GGA TCC CCG GGT TAA TTA A
MNO110	GCG GCG GCG GCC GCC ACC GCT GCT TAC TAA TGA AGA C
MNO111	GCG GCG CTC GAG ACA TCT CCA ACA GCA GAT CAG AAG
MNO115	GGG GGG GAT CCC AGT ATG GAT AAT ATT TTA AAG
MNO116	GGG GGG CTC GAG TCA CTT GAT GGA GTG CGG
MNO127	AAG CAT CCT CTT GAG TAT GAT TTG CAA GCG TTG TTT AAA TAT TTC TTT TCT CGG ATC CCC GGG TTA ATT AA
MNO128	TGT GAC TTT ATT TTA GGT GCA CCC CCC ACC AGT AAC TTA TTC TCT AAA TAA GAA TTC GAG CTC GTT TAA AC
MNO130	GCG GCG GGG ATC CGT ATG AAT CAT TGG TTA GCA
MNO132	GCG GCG CTC GAG CTA AGA AAA GAA ATA TTT AAA C
MNO155	GCG GCG TTA ATT AAC CGT ACG CTG CAG GTC GAC GGA
MNO156	GCG GCG GGC GCG CCT GGG CCT CCA TGT CGC TGG

TABLE 3

Plasmids used in this study

Plasmid	Source or reference
3-20 (clone no.)	Tu *et al.* (1996)
pDH3	K. Tatchell, Louisiana State University
pEG202	Gyuris *et al.* (1993)
pEG202-ADY3	This study
pFA6a-CFP-HIS3MX6	This study
pFA6a-GFP(S65T)-HIS3MX6	Longtine *et al.* (1998)
pFA6a-yEGFP-HIS3MX6	This study
pGADGH	Hartley (1993)
pGADGH-MPC70	This study
pLexA$_{202}$-GLC7	Tu and Carlson (1994)
pMK183	Elliott *et al.* (1999)
pMK184	Elliott *et al.* (1999)
pMK169	Elliott *et al.* (1999)
pSB8	Tachikawa *et al.* (2001)
pSM613	Elliott *et al.* (1999)
pSM614	Elliott *et al.* (1999)
pYM12	Knop *et al.* (1999)

TABLE 4

Two-hybrid interactions with LexA-Ady3p

GAD fusion	Interaction
None	−
Cnm67p$^{386\text{-}580}$	++
Gip1p$^{161\text{-}573}$	−
Mpc70p$^{1\text{-}609}$	+++
Nud1p$^{405\text{-}852}$	+++
Spc42p$^{1\text{-}363}$	+

A LexA-Ady3p fusion was tested for interactions with fusions of the Gal4 activation domain (GAD) to residues of indicated proteins using a β-galactosidase filter lift assay as described in materials and methods. Three independent transformants for each combination of constructs were analyzed. None of GAD fusions used here interacted with LexA alone. The following LexA fusions were used as positive controls for interactions with GAD fusions: Glc7p$^{1\text{-}312}$ for Gip1p$^{161\text{-}573}$; Nud1p$^{405\text{-}852}$ for Mpc70p$^{1\text{-}609}$ and Nud1p$^{405\text{-}852}$; and Cnm67p$^{386\text{-}580}$ for Spc42p$^{1\text{-}363}$.

mersed sequentially in −20° methanol for 5 min, −20° acetone for 30 sec, and PBS for 2 sec and then washed three times with PBS containing 1% BSA and 0.1% Triton (P/B/T). A monoclonal antibody to β-1,3-glucan (Biosupplies Australia, Parkville, Australia) was diluted 1:3000 in P/B/T and incubated with samples overnight. Samples were washed three times with P/B/T, incubated 1–2 hr with secondary antibody, washed three times with P/B/T, and mounted with Prolong Antifade (Molecular Probes, Eugene, OR). Alexa Fluor 488 and 546 anti-mouse IgG antibodies (Molecular Probes) were used at 1:200–1:400 dilutions in P/B/T. Totals of 50 μg/ml FITC-Concanavalin A (Sigma, St. Louis) and 0.2 μg/ml DAPI were added to secondary antibody solutions.

Images of naturally fluorescent proteins were collected with a Zeiss (Thornwood, NY) Axioplan 2 microscope and Zeiss AxioCam HRm digital camera using Axiovision 3.0.6 software. Images of cells stained with Calcofluor white, FITC-Concanavalin A, and antibodies to β-1,3-glucan were collected with a Zeiss Axioskop microscope and SPOT camera (Diagnostic Instruments, Sterling Heights, MI) using Adobe Photoshop 5.0 (Adobe Software, San Jose, CA). Figures were prepared using Adobe Photoshop 6.0 (Adobe Software) and Canvas 5.0.2 (Deneba Software).

Electron microscopy: Cells were prepared for electron microscopy as described previously (Bajgier *et al.* 2001). Figures were prepared using Scion Image 1.62 (National Institutes of Health) and Canvas 5.0.2 (Deneba Software) software.

β-Galactosidase assays: Assays for two-hybrid interactions were performed in strain YCJ4. Cells that had been cotransformed with pEG202-ADY3 and each of various GAD plasmids were cultured overnight at 30° on a Whatman 50 filter on the surface of an SD-L,W plate, and the filter was immersed in liquid N_2 for 10 sec and incubated at 30° in Z buffer (Miller 1972) containing 0.1% 5-bromo-4-chloro-3-indolyl-β-d-galactopyrannoside (X-gal) and 0.027% β-mercaptoethanol.

RESULTS

Ady3p interacts with several components of the spindle pole body in the two-hybrid assay: To examine whether Ady3p interacts with proteins of the spindle pole body other than the meiosis-specific outer plaque component Mpc70p, a LexA-Ady3p fusion was tested for its ability to bind various fusions to the Gal4p activation domain in the yeast two-hybrid assay. In addition to Mpc70p, Ady3p interacted specifically with fusions containing sequences from Cnm67p and Nud1p, components of the outer plaque during both mitotic and meiotic growth, and the central plaque protein Spc42p (Table 4; Uetz *et al.* 2000; Ito *et al.* 2001). Cnm67p has been shown to interact with Spc42p and Nud1p in two-hybrid assays (Elliott *et al.* 1999). Thus, the two-hybrid interactions we have identified with Ady3p may be either direct or mediated by one or more additional proteins naturally present in yeast cells. Moreover, Ady3p bound to 31 different partners in genome-wide two-hybrid screens, and the physiological relevance of most of these interactions is uncertain at present (Uetz *et al.* 2000; Ito *et al.* 2001). Nonetheless, the ability of Ady3p to bind to multiple components of the SPB in the two-hybrid assay suggests that it may associate with this structure *in vivo*.

***ady3Δ/ady3Δ* cells sporulate poorly:** To investigate whether Ady3p plays a role in spore formation, an *ady3Δ/ady3Δ* strain was tested for its ability to sporulate. In *ADY3/ADY3* cultures, >90% of the cells sporulated, and most asci contained four spores (Figure 1 and Table 5). In *ady3Δ/ady3Δ* cultures, fewer than one-half of the cells sporulated, and most of the cells that did sporulate formed asci with only one or two spores (Figure 1 and Table 5). The preponderance of dyads (two-spored asci) in sporulated cultures of *ady3Δ/ady3Δ* mutants is consistent with previous findings (Rabitsch *et al.* 2001).

***ady3Δ/ady3Δ* dyads result from random packaging of meiotic nuclei:** To determine whether the dyads produced in *ady3Δ/ady3Δ* cells are nonsisters, segregation of the centromere-linked marker *TRP1* was analyzed. An *ady3Δ/ady3Δ TRP1/trp1* strain was induced to sporulate,

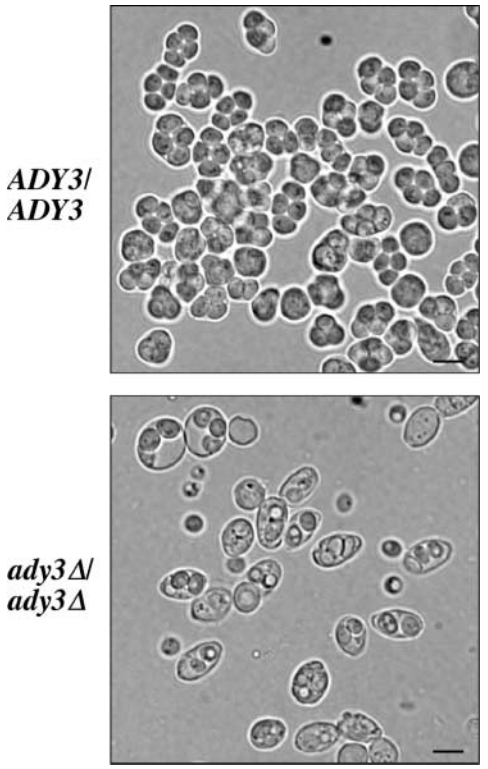

FIGURE 1.—*ADY3/ADY3* and *ady3Δ/ady3Δ* asci. Cells from strains AN120 and AN246 were photographed after 1 day in 2% potassium acetate. Bars, 5 μm.

dyads were dissected, and progeny were tested for the ability to grow on medium lacking tryptophan. Less than 75% of *ady3Δ/ady3Δ* dyads produced one Trp+ and one Trp− segregant (Table 6). The expected frequency of 1:1 segregation of *TRP1* in nonsister dyads is 98%, significantly different ($P < 0.001$) from the value observed in *ady3Δ/ady3Δ* dyads. These data indicate that the dyads formed in *ady3Δ/ady3Δ* cells result from random packaging of meiotic nuclei.

Meiotic progression and prospore membrane formation occur normally in *ady3Δ/ady3Δ* cells: To determine whether poor sporulation of *ady3Δ/ady3Δ* cells is due to a defect in meiosis, segregation of chromatin was analyzed by fluorescence microscopy of DAPI-stained cells. The rate of progression through meiosis and the fraction of cells that completed both divisions were comparable for *ADY3/ADY3* and *ady3Δ/ady3Δ* cells (Figure 2). This result indicates that Ady3p is dispensable for meiotic progression.

TABLE 6

Segregation of *TRP1* in *ady3Δ/ady3Δ* dyads

Dyad type	Expected frequency (%)		Observed frequency (%)
	Random	Nonsister	
+/+	17	1	14
+/−	67	98	74
−/−	17	1	12

Expected frequencies of segregation of *TRP1* in random and nonsister dyads and observed frequencies of segregation of *TRP1* in *ady3Δ/ady3Δ* dyads are shown. A total of 74 dyads were analyzed. The observed distribution of dyad types for *ady3Δ/ady3Δ* was significantly different ($P < 0.001$) from the frequency expected from nonsister packaging.

To test whether the sporulation defect in *ady3Δ/ady3Δ* cells is attributable to aberrant prospore membrane formation, prospore membranes were analyzed by immunofluorescence microscopy using antibodies that recognize Sso1p and Sso2p. No difference in the morphology or number of prospore membranes could be detected between *ADY3/ADY3* and *ady3Δ/ady3Δ* cells (Table 7). Moreover, modification of the outer plaque of the SPB in *ady3Δ/ady3Δ* cells appeared normal by electron microscopy (data not shown). These results suggest that Ady3p is not required during meiosis for SPB modification or prospore membrane biogenesis.

The sporulation defect in *ady3Δ/ady3Δ* mutants is due to a block in spore wall formation: The observation that meiotic progression and prospore membrane formation occur normally in *ady3Δ/ady3Δ* cells led us to investigate whether the sporulation defect in this mutant was due to aberrant spore wall synthesis. To restrict the analysis of spore wall synthesis to cells that have completed meiosis and prospore membrane formation, the stages of sporulation of individual cells in sporulating cultures were determined microscopically as described previously (TACHIKAWA *et al.* 2001). DAPI was used to monitor segregation of chromatin, FITC-Concanavalin A (ConA) was used for formation of prospore membranes, an antibody to β-1,3-glucan was used for glucan synthesis, and Calcofluor white was used for chitosan synthesis. Cells that have completed meiosis and

TABLE 5

Sporulation in *ADY3/ADY3* and *ady3Δ/ady3Δ* cells

Strain	Relevant genotype	Distribution of ascal types (%)				
		Unsporulated	Monad	Dyad	Triad	Tetrad
AN120	*ADY3/ADY3*	8.9 ± 10	0.4 ± 0.4	3.5 ± 1.9	10.6 ± 3.8	76.6 ± 7.9
AN246	*ady3Δ/ady3Δ*	56.9 ± 8.0	21.6 ± 2.9	17.6 ± 5.8	3.6 ± 1.3	0.3 ± 0.3

A total of 200 cells of indicated strains were analyzed by phase contrast microscopy after 1 day in 2% potassium acetate. Data shown are averages of four independent experiments.

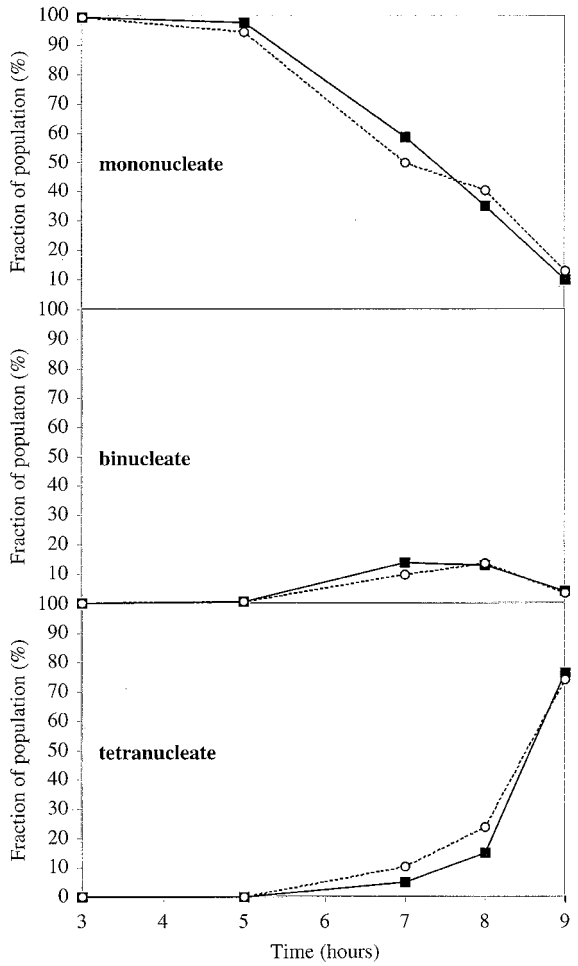

FIGURE 2.—Meiotic progression of *ADY3/ADY3* and *ady3*Δ/*ady3*Δ cells. Cells from strains AN120 and AN246 were cultured in 2% potassium acetate, and aliquots were removed at various times, fixed as for indirect immunofluorescence, and stained with DAPI. Cells were categorized by the configuration of their nuclear DNA. Values for *ADY3/ADY3* cells are indicated by solid squares connected by solid lines, and values for *ady3*Δ/*ady3*Δ cells are indicated by open circles connected by dashed lines.

prospore membrane formation but not spore wall synthesis have four discrete lobes of nuclear DNA by DAPI staining and four spherical prospore membranes by staining with FITC-ConA (TACHIKAWA *et al.* 2001).

Synthesis of the β-glucan layer of the spore wall in *ady3*Δ/*ady3*Δ cells was analyzed by immunofluorescence microscopy. Cells from sporulating cultures were fixed and stained with anti-β-1,3-glucan, FITC-ConA, and DAPI, and cells that had four discrete lobes of nuclear DNA and four spherical, FITC-ConA$^+$ prospore membranes were scored. In the *ADY3/ADY3* culture, 38% of cells at this stage of sporulation displayed β-1,3-glucan staining, and 89% (34/38) of β-1,3-glucan$^+$ cells contained four β-1,3-glucan$^+$ prospores (Figure 3, A–C, and Table 8). In the *ady3*Δ/*ady3*Δ culture, only 23.5% of cells at the same stage of sporulation displayed β-1,3-glucan staining, and only 11% (2.5/23.5) of β-1,3-glucan$^+$ cells contained four β-1,3-glucan$^+$ prospores (Figure 3, D–L, and Table 8). These results indicate that the majority of prospores in *ady3*Δ/*ady3*Δ cells fail to synthesize the glucan layer of the spore wall and that the number of prospores within an individual ascus that are competent to do so is variable.

Assembly of the chitosan layer of the spore wall in *ady3*Δ/*ady3*Δ cells was examined by fluorescence microscopy using Calcofluor white. Cells from sporulating cultures were fixed, separately stained with either DAPI or Calcofluor white, and analyzed. In the *ADY3/ADY3* culture, 53.5% of cells had completed meiosis, and

TABLE 7

Prospore membrane formation in *ADY3/ADY3* and *ady3*Δ/*ady3*Δ cells

		No. of Sso$^+$ prospore membranes per cell				
Strain	Relevant genotype	0	1	2	3	4
AN120	*ADY3/ADY3*	5	0	2	2	91
AN246	*ady3*Δ/*ady3*Δ	6	0	1	3	90

A total of 100 meiosis II cells of indicated strains were analyzed by indirect immunofluorescence microscopy after 8 hr in 2% potassium acetate.

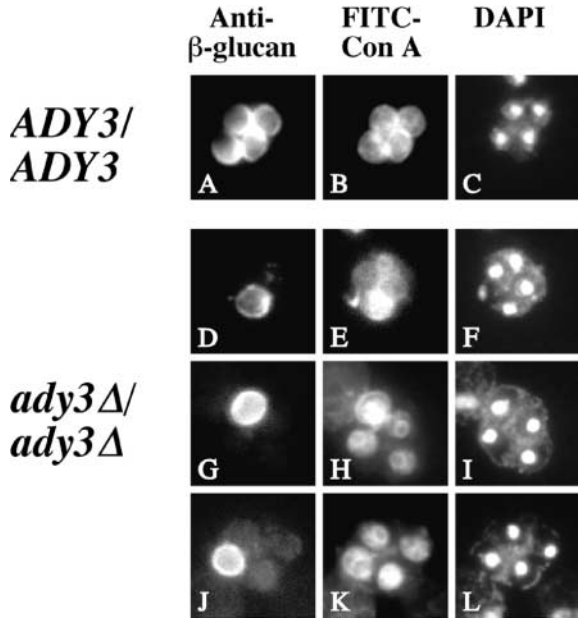

FIGURE 3.—β-1,3-Glucan in sporulating *ADY3/ADY3* and *ady3*Δ/*ady3*Δ cells. Cells from strains AN120 and AN246 were cultured for 6–8 hr in 2% potassium acetate and processed for indirect immunofluorescence as described in MATERIALS AND METHODS. (A, D, G, and J) Indirect immunofluorescence images of cells stained with a monoclonal antibody to β-1,3-glucan. (B, E, H, and K) Fluorescence images of the same cells stained with FITC-Concanavalin A. (C, F, I, and L) Fluorescence images of the same cells stained with DAPI.

TABLE 8

β-1,3-glucan synthesis in *ANY3/ADY3* and *ady3Δ/ady3Δ* cells

			Distribution of cell types (%)				
Strain	Relevant genotype	No. of β-1,3-glucan$^+$ prospores/cell:	0	1	2	3	4
AN120	*ADY3/ADY3*		62	1	1	2	34
AN246	*ady3Δ/ady3Δ*		76.5	15	3.5	2.5	2.5

Cells were cultured for 8 hr in 2% potassium acetate, fixed, and processed for immunofluorescence microscopy. A total of 200 cells that had completed meiosis and contained four prospore membranes as judged by staining with DAPI and FITC-ConA were analyzed.

41.4% of cells were chitosan$^+$ (Table 9). Seventy-six percent (31.3/41.4) of chitosan$^+$ *ADY3/ADY3* cells contained four chitosan$^+$ prospores (Figure 4, A and B, and Table 9). In the *ady3Δ/ady3Δ* culture, 72% of cells had completed meiosis, but only 7.6% were chitosan$^+$ (Table 9). Moreover, <7% (0.5/7.6) of chitosan$^+$ *ady3Δ/ady3Δ* cells contained four chitosan$^+$ prospores (Figure 4, C–H, and Table 9). These results indicate that the majority of postmeiotic nuclei in *ady3Δ/ady3Δ* cells do not become surrounded by a chitosan layer and that the number of postmeiotic nuclei within an individual cell that do become surrounded by chitosan is variable. The numbers of β-glucan$^+$ and chitosan$^+$ prospores per cell during sporulation resemble the ultimate distribution of mature spores per ascus in *ady3Δ/ady3Δ* cultures, suggesting that the block in spore wall synthesis in this mutant occurs uniformly among affected prospores prior to deposition of the glucan layer.

To further characterize the spore wall defect in the *ady3Δ/ady3Δ* mutant, cells were analyzed by transmission electron microscopy. Aliquots from sporulating cultures were fixed and either stained with DAPI or processed for OsO_4 staining and electron microscopy. In sections of *ADY3/ADY3* cells in which multiple spores were visible, all spores had walls composed of three distinct layers characteristic of the mature spore wall (Figure 5, A and B). In contrast, sections of *ady3Δ/ady3Δ* cells that contained multiple meiotic products revealed that either none or only one of the prospores had spore wall material (Figure 5, C–F). These results corroborate the findings by fluorescence microscopy that the sporulation defect in *ady3Δ/ady3Δ* cells is due to a sporadic failure of prospores to initiate spore wall synthesis.

Ady3p localizes to rings around meiosis II spindles: To gain insight into the role of Ady3p in spore wall synthesis, the subcellular localization of Ady3p was analyzed. A strain homozygous for a functional *ADY3-GFP* fusion was induced to sporulate, and fixed cells were stained with DAPI and visualized by fluorescence microscopy. Ady3p-GFP was first visible in cells completing the first meiotic division as two discrete foci at the ends of the spindle (data not shown), although one or two additional spots of Ady3p-GFP were occasionally seen elsewhere in cells at this stage. At the onset of meiosis II, Ady3p-GFP was visible as four spots, two on opposite sides of each mass of chromatin (Figure 6, A–D). As meiosis II progressed, Ady3p-GFP resolved into structures that appeared as bars when viewed from the side (Figure 6, E and F) and as rings when viewed *en face* (Figure 6, G and H). These Ady3p-GFP rings surrounded the segregating chromatin and moved away from the SPBs toward the center of the spindles as meiosis II neared completion (Figure 6, I–L). The Ady3p ring-like structures in meiosis II are similar to those formed by Don1p at the leading edge of the prospore membrane, suggesting that the interaction of these two proteins identified by proteomic screens may be physiologically relevant (Knop and Strasser 2000; Ito *et al.* 2001).

Ady3p colocalizes with Don1p: To test the possibility that Ady3p and Don1p form a single complex at the

TABLE 9

Chitosan synthesis in *ADY3/ADY3* and *ady3Δ/ady3Δ* cells

			Distribution of cell types (%)				
Strain	Relevant genotype	No. of chitosan$^+$ prospores/cell:	0	1	2	3	4
AN120	*ADY3/ADY3*		58.6	1.4	2.3	6.4	34.3
AN246	*ady3Δ/ady3Δ*		92.4	5.1	1.7	0.3	0.5

Cells were cultured for 8.5 hr in 2% potassium acetate and fixed. Aliquots from each sample were stained separately with either DAPI or Calcofluor white. A total of 200 cells were analyzed to assay meiosis, and 1000 Calcofluor white-stained cells were analyzed to assay chitosan synthesis. Totals of 53.5% of AN120 cells and 72% of AN246 cells in these samples had completed meiosis.

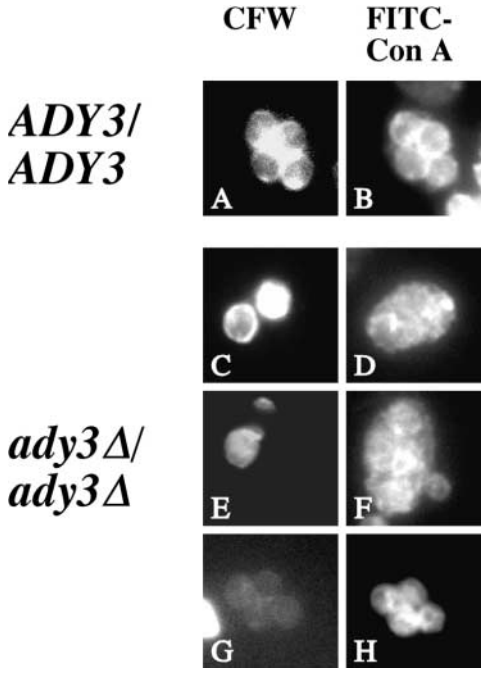

FIGURE 4.—Chitosan in sporulating *ADY3/ADY3* and *ady3*Δ/*ady3*Δ cells. Cells from strains AN120 and AN246 were cultured for 6–8 hr in 2% potassium acetate and processed as for indirect immunofluorescence as described in MATERIALS AND METHODS. (A, C, E, and G) Fluorescence images of cells stained with Calcofluor white. (B, D, F, and H) Fluorescence images of the same cells stained with FITC-Concanavalin A.

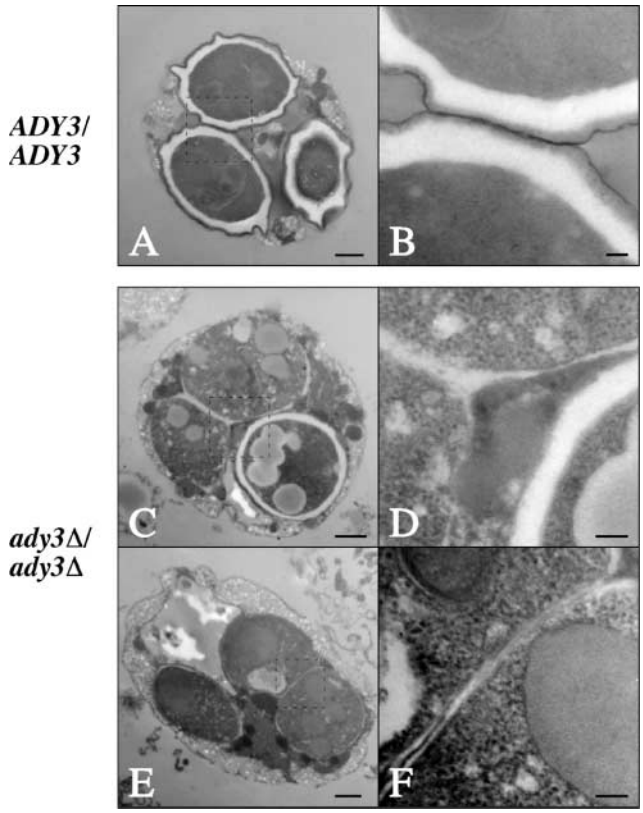

FIGURE 5.—Cross sections of sporulating *ADY3/ADY3* and *ady3*Δ/*ady3*Δ cells. Cells from strains AN120 and AN246 were cultured in 2% potassium acetate for 10 hr and processed for transmission electron microscopy as described in MATERIALS AND METHODS. Bars: A, C, and E, 500 nm; B, D, and F, 100 nm.

leading edge of the prospore membrane, we examined whether Ady3p colocalizes with Don1p. A diploid strain homozygous for *DON1-YFP* and *ADY3-CFP* fusions was induced to sporulate and examined by fluorescence microscopy. Both Ady3p-CFP and Don1p-YFP formed rings around the spindles during meiosis II, and these structures were coincident throughout the second meiotic division (Figure 7). These data suggest that Ady3p and Don1p associate in a complex at the leading edge of the prospore membrane.

Ady3p is required for formation of Don1p ring-like structures: To further investigate the possibility that Ady3p and Don1p form a complex *in vivo*, each protein was tested for the ability to assemble into rings *in vivo* in the absence of the other. To examine the localization of Don1p in cells that lack Ady3p, *ADY3/ADY3* and *ady3*Δ/*ady3*Δ cells carrying a centromeric plasmid with *DON1-GFP* were induced to sporulate and examined by fluorescence microscopy. In *ADY3/ADY3* cells Don1p-GFP assembled into characteristic rings around the spindles during meiosis II (Figure 8, A–D). In contrast, in *ady3*Δ/*ady3*Δ cells Don1p-GFP did not form recognizable structures at any stage of meiosis (Figure 8, E–H). These results indicate that Ady3p is required to assemble and/or stabilize Don1p ring-like structures during formation of prospore membranes.

To examine the localization of Ady3p in cells that lack Don1p, *DON1/DON1* and *don1*Δ/*don1*Δ cell strains homozygous for *ADY3-GFP* were induced to sporulate and analyzed by fluorescence microscopy. Ady3p-GFP assembled into rings around meiosis II spindles in both *DON1/DON1* and *don1*Δ/*don1*Δ cells, and these structures were indistinguishable between the two strains (data not shown). These results indicate that Don1p is dispensable for the formation of Ady3p ring-like structures during formation of prospore membranes.

Ady3p remains associated with SPBs during meiosis II in *mpc70*Δ/*mpc70*Δ cells: To test the idea that Ady3p transiently associates with the SPB before assembly into a ring at the leading edge of the prospore membrane, localization of Ady3p was analyzed in *mpc70*Δ/*mpc70*Δ cells, in which prospore membrane formation is blocked. *MPC70/MPC70* and *mpc70*Δ/*mpc70*Δ strains homozygous for *ADY3-GFP* were induced to sporulate and analyzed by fluorescence microscopy. Ady3p-GFP was localized to the SPBs in 78% of *mpc70*Δ/*mpc70*Δ cells in meiosis II, whereas this was never observed in *MPC70/MPC70* cells in meiosis II (Figure 9). This result indicates that Ady3p stably associates with the SPB *in vivo* in an Mpc70p-independent manner when formation of prospore membranes is blocked, consistent with the idea

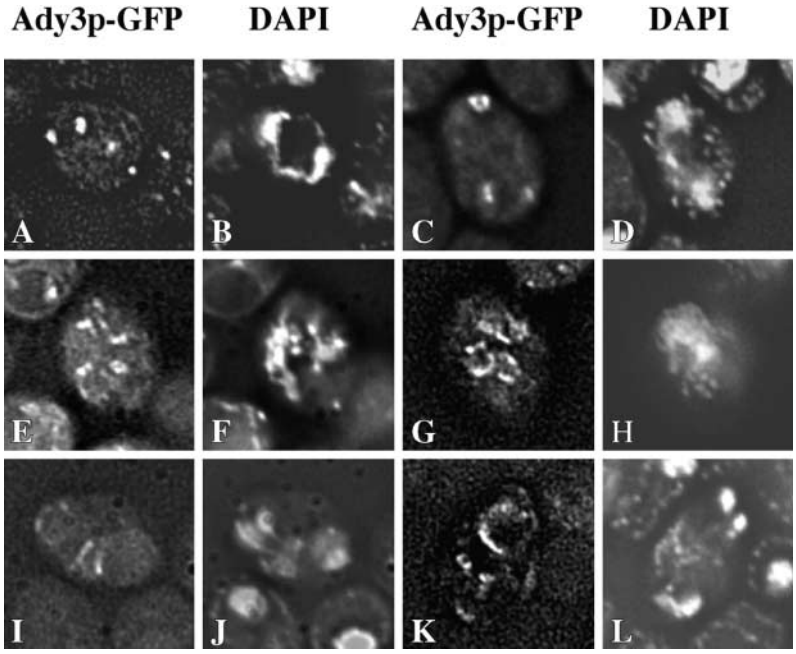

FIGURE 6.—Fluorescence from Ady3p-GFP and DAPI in sporulating cells. Cells from strain MND16 were cultured for 6–8 hr in 2% potassium acetate and processed for visualization of fluorescent proteins as described in MATERIALS AND METHODS. (A, C, E, G, I, and K) Fluorescence from Ady3p-GFP. (B, D, F, H, J, and L) Fluorescence images from same cells stained with DAPI.

that assembly of the Ady3p-Don1p structure is initiated by a transient interaction between Ady3p and the SPB.

DISCUSSION

We report here the identification of a role for Ady3p in synthesis of the spore wall in *S. cerevisiae*. Ady3p interacts with components of the SPB in the two-hybrid assay and associates with SPBs *in vivo*. Cells that lack *ADY3* display normal modification of SPB outer plaques and formation of prospore membranes during meiosis but fail to synthesize spore walls around the majority of prospores. Ady3p colocalizes with Don1p to a ring-like structure at the leading edge of the nascent prospore membrane and is required for recruitment of Don1p into this structure.

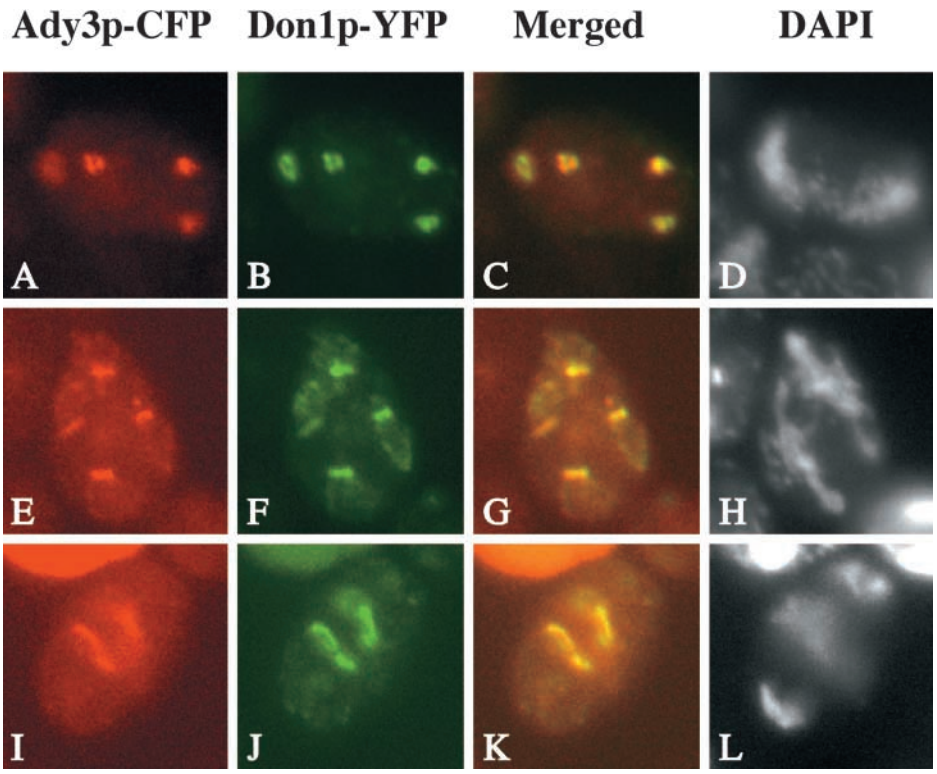

FIGURE 7.—Fluorescence from Ady3p-CFP, Don1p-YFP, and DAPI during meiosis II. Cells from strain MND24 were cultured for 6–8 hr in 2% potassium acetate and processed for visualization of fluorescent proteins as described in MATERIALS AND METHODS. (A, E, and I) Fluorescence from Ady3p-CFP. (B, F, and J) Fluorescence in same cells from Don1p-YFP. (C, G, and K) Overlaid images of fluorescence from Ady3p-CFP and Don1p-YFP. (D, H, and L) Fluorescence images from same cells stained with DAPI. Colors from emission of fluorescent proteins at their natural wavelengths have been changed to facilitate visualization.

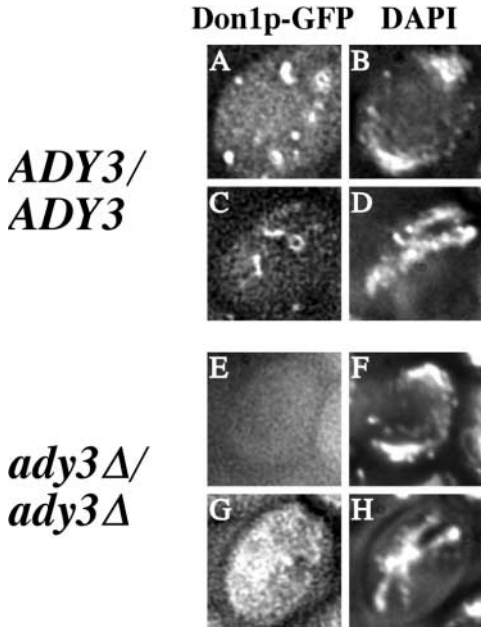

FIGURE 8.—Fluorescence from Don1-GFP and DAPI staining during meiosis II in *ADY3/ADY3* and *ady3*Δ*/ady3*Δ cells. Cells from strains AN120 and AN246 that had been transformed with pRS314-DON1-GFP were cultured for 6–8 hr in 2% potassium acetate and processed for visualization of fluorescent proteins as described in MATERIALS AND METHODS. (A, C, E, and G) Fluorescence from Don1p-GFP. (B, D, F, and H) Fluorescence images of same cells stained with DAPI.

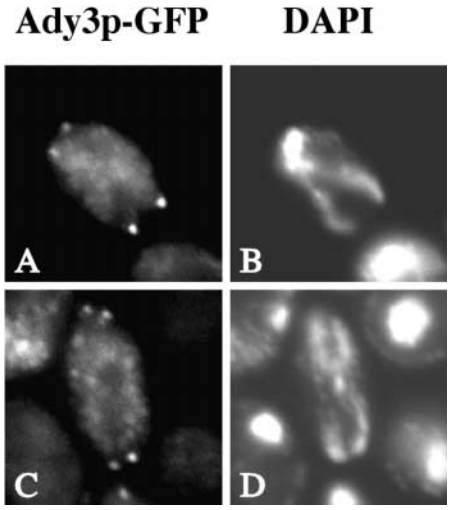

FIGURE 9.—Fluorescence from Ady3p-GFP and DAPI during meiosis II in *mpc70*Δ*/mpc70*Δ cells. Cells from strain MND41 were cultured for 6–8 hr in 2% potassium acetate and processed for visualization of fluorescent proteins as described in MATERIALS AND METHODS. (A and C) Fluorescence from Ady3p-GFP. (B and D) Fluorescence images of same cells stained with DAPI.

An independent study on the composition of the leading edge of the prospore membrane recently reported findings similar to those presented here (MORENO-BORCHART *et al.* 2001). The authors demonstrate that Ady3p is a component of the leading-edge structure that interacts with SPBs and is required for assembly of Don1p rings. Additionally, Moreno-Borchart *et al.* identify Ssp1p as a component of the leading-edge structure and show that Ssp1p is required for proper shaping of the prospore membrane and for assembly of both Ady3p and Don1p rings.

The use of simple genetic criteria to categorize mutants like *ady3*Δ*/ady3*Δ that form dyads may provide a rapid way to identify new components involved in specific processes during sporulation. The type of dyads formed by mutants that have been characterized can be categorized by the genetic endowment of their spores: diploid, nonsister haploid, and random haploid. Mutations in *SPO12* and *SPO13* cause formation of dyads that have diploid spores, and the products of these genes are involved in progression through the meiotic division (KLAPHOLZ and ESPOSITO 1980). Mutations in *ADY1*, *MPC70*, and *SPC42* lead to formation of nonsister dyads, and these genes encode proteins required for meiosis-specific modification of the outer plaque of the SPB (BAJGIER *et al.* 2001; DENG and SAUNDERS 2001; ISHIHARA *et al.* 2001; WESP *et al.* 2001). Mutations in *ADY3* and *SSP1/SPO3* result in formation of random dyads, and the products of these genes are components of the leading-edge structure involved in regulation of prospore membrane shape and spore wall synthesis (Table 6, Figures 6 and 7; ESPOSITO *et al.* 1974; MORENO-BORCHART *et al.* 2001). Analysis of dyads formed by cells that lack *ADY2* or *ADY4* (RABITSCH *et al.* 2001) may expedite the assignment of roles for their products in specific processes of spore formation, and a similar approach can be used for other dyad-forming mutants.

The analysis of Ady3p function and localization reveals novel roles in regulation of spore wall synthesis for two protein complexes: the SPB and the ring-like structure at the leading edge of the prospore membrane. Ady3p interacts with the SPB and is the first such protein to have been identified that is dispensable for prospore membrane biogenesis yet required for normal spore wall synthesis. Ady3p is also a newly identified component of the ring at the leading edge of the nascent prospore membrane. Loss of Don1p, the first component of this structure to have been described, has no effect on sporulation, and the absence of Ssp1p leads to aberrantly shaped prospore membranes (KNOP and STRASSER 2000; MORENO-BORCHART *et al.* 2001). The observation that *ady3*Δ*/ady3*Δ cells have apparently normal prospore membranes but defects in spore wall synthesis indicates that the role of the leading-edge structure in regulating spore wall synthesis is distinct from its role in shaping the prospore membrane.

The spore wall defect in *ady3*Δ*/ady3*Δ cells is different from the phenotypes caused by mutations of genes involved in spore wall assembly. Several mutants have been described in which spore walls are not properly assembled but polymeric precursors are present at the pro-

spore membrane (Briza *et al.* 1990a; Pammer *et al.* 1992; Krisak *et al.* 1994; Christodoulidou *et al.* 1996). For example, *cda1*Δ/*cda1*Δ *cda*Δ*2*/*cda2*Δ cells lack the chitin deacetylase activity required to convert chitin to chitosan, and this double mutant produces viable spores that lack the chitosan and dityrosine layers of the spore wall but show normal staining with Calcofluor white (Christodoulidou *et al.* 1996). In contrast, prospores in *ady3*Δ/*ady3*Δ cells either develop into mature, UV-fluorescent spores (M. Nickas and A. Neiman, unpublished data) or fail to deposit spore wall polymers altogether.

The complete absence of spore wall material around the majority of prospores in *ady3*Δ/*ady3*Δ cells (Figures 3–5 and Tables 8 and 9) suggests that initiation of spore wall synthesis is impaired in this mutant. Although not completely penetrant, the spore wall defect in *ady3*Δ/*ady3*Δ cells is qualitatively similar to the phenotype of *gip1*Δ/*gip1*Δ cells (Tachikawa *et al.* 2001). Spore wall synthesis is blocked in *gip1*Δ/*gip1*Δ cells due to a failure in either closure of prospore membranes or signaling that this event has occurred (Tachikawa *et al.* 2001). The localization of Ady3p to the leading edge of the prospore membrane is consistent with a role for this protein in mediating or monitoring the closure of this organelle. Moreover, Ady3p fails to redistribute to the prospore cytoplasm upon completion of meiosis in *gip1*Δ/*gip1*Δ cells (M. Nickas and A. Neiman, unpublished data), indicating that the function of Gip1p influences the localization and, potentially, activity of Ady3p.

Our findings support a model in which Ady3p mediates assembly of the Don1p-containing structure at the leading edge of the prospore membrane through its interaction with the SPB at the onset of the second meiotic division. Recruitment of Don1p into this ring-like structure is dependent on Ady3p, whereas Ady3p assembles into a ring in the absence of Don1p. Ady3p binds to both Don1p and components of the SPB, the site of prospore membrane biogenesis. Thus, Ady3p may serve as a bridge that draws Don1p into position during initiation of prospore membrane synthesis to form a complex at the growing edge.

How are the functions of Ady3p in assembly of the structure at the leading edge of the prospore membrane and regulation of spore wall synthesis coordinated? One possibility is that Ady3p directly facilitates closure of the prospore membrane from the leading-edge structure or initiation of spore wall synthesis upon its release to the prospore cytoplasm. Alternatively, Ady3p may help to assemble another protein that performs these functions into the leading-edge structure. Don1p is dispensable for spore wall synthesis and therefore not a candidate for such a protein, but Ssp1p may play such a role (Knop and Strasser 2000). Ady3p is not required for assembly of Ssp1p into the leading-edge structure but does facilitate the recruitment of Ssp1p-containing prospore membrane precursors to the SPB (Moreno-Borchart *et al.* 2001). Thus, the leading-edge structure in *ady3*Δ/*ady3*Δ cells may contain a decreased and/or variable amount of Ssp1p that renders some spores unable to trigger spore wall synthesis. A third possibility is that the functions of Ady3p in assembly of the leading-edge structure and regulation of spore wall synthesis are independent. For example, Ady3p may abet the recruitment of a factor to the outer plaque of the SPB at the onset of meiosis II, and the release of this factor upon disassembly of the outer plaque may trigger spore wall synthesis.

Our findings highlight the value of using an integrative approach to apply data available from genomic technologies toward the study of a biological system. A role for Ady3p in sporulation was implicated by the results of three different types of large-scale analysis: proteomic interaction screens, genome-wide transcriptional analyses, and systematic disruption and characterization of meiotically induced genes (Chu *et al.* 1998; Primig *et al.* 2000; Uetz *et al.* 2000; Ito *et al.* 2001; Rabitsch *et al.* 2001). While these approaches have provided a plethora of information, the biological relevance of data from a single type of genomic data set can be difficult to assess. For example, 31 binding partners for Ady3p have been identified in genome-wide two-hybrid screens (Uetz *et al.* 2000; Ito *et al.* 2001), suggesting that its structure may promote promiscuous binding of other proteins in this assay and casting doubt on the *in vivo* significance of interactions identified by this method alone. Of the 31 gene products identified as binding partners for Ady3p by proteomic screening, only three genes displayed transcriptional profiles similar to that of *ADY3*: *DON1*, *MPC70*, and *SSP1*. Despite the fact that individual deletions of *ADY3*, *DON1*, *MPC70*, and *SSP1* all confer distinct sporulation phenotypes, our results and those of others demonstrate the *in vivo* relevance of these two-hybrid interactions (Nag *et al.* 1997; Knop and Strasser 2000; Bajgier *et al.* 2001; Moreno-Borchart *et al.* 2001). Thus, cross-referencing the results from different types of genomic data sets may provide an effective means of identifying *in vivo* functional relationships.

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