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Analysis of RIM11, a Yeast Protein Kinase That Phosphorylates the Meiotic Activator IME1

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Many yeast genes that are essential for meiosis are expressed only in meiotic cells. Known regulators of early meiotic genes include IME1, which is required for their expression, and SIN3 and UME6, which prevent their expression in nonmeiotic cells. We report here the molecular characterization of the *RIM11* gene, which we find is required for expression of several early meiotic genes. A close functional relationship between RIM11 and IME1 is supported by two observations. First, *sin3* and *ume6* mutations are epistatic to *rim11* mutations; prior studies have demonstrated their epistasis to *ime1* mutations. Second, overexpression of *RIM11* can suppress an *ime1* missense mutation (*ime1-L321F*) but not an *ime1* deletion. Sequence analysis indicates that *RIM11* specifies a protein kinase related to rat glycogen synthase kinase 3 and the *Drosophila shaggy/zw3* gene product. Three partially defective *rim11* mutations alter residues involved in ATP binding or catalysis, and a completely defective *rim11* mutation alters a tyrosine residue that corresponds to the site of an essential phosphorylation for glycogen synthase kinase 3. Immune complexes containing a hemagglutinin (HA) epitope-tagged RIM11 derivative, HA-RIM11, phosphorylate two proteins, p58 and p60, whose biological function is undetermined. In addition, HA-RIM11 immune complexes phosphorylate a functional IME1 derivative but not the corresponding *ime1-L321F* derivative. We propose that RIM11 stimulates meiotic gene expression through phosphorylation of IME1.

Cells of the yeast *Saccharomyces cerevisiae* can go through meiosis to produce spores. The decision to enter meiosis is governed by both environmental and genetic signals. The environmental signal, nutrient limitation in the absence of a fermentable carbon source, leads to either G_1-phase arrest or meiosis. The genetic signal, which comes from the mating-type locus, determines how cells respond to nutrient limitation: **a** and α cells arrest in G_1 phase, and **a**/α cells enter meiosis (reviewed in references 6 and 13).

Many genes are expressed at much higher levels in meiotic cells than under other circumstances (reviewed in references 15, 24, 25, and 26). These meiotic genes or sporulation-specific genes are grouped in classes based on their time of expression. Early meiotic genes have functions in recombination (such as *HOP1* [14]), reductional division (such as *SPO13* [53]), and regulation (such as *IME2* and *RIM4* [38, 45]). Expression of early genes depends on *IME1* (28, 38), which is also expressed at highest levels early in meiosis (18).

Expression of *IME1* is coupled to environmental and genetic signals through several regulatory pathways (17–19, 29, 34, 43, 44, 47; reviewed in reference 26). Regulation of most early genes is coupled to these signals, in part, through regulation of *IME1*. For example, overexpression of *IME1* permits sporulation and early gene expression in cells of any type (18, 39). Expression of *IME1* in unstarved cells also permits early gene expression; however, starvation causes a further increase in early meiotic transcript levels (19, 39). Therefore, cell type signals may govern early gene expression only through *IME1* expression levels. On the other hand, nutritional signals act through *IME1* expression levels and at one or more points that are independent or downstream of *IME1* expression (19, 39).

Several studies have identified genes required in addition to IME1 for meiotic gene regulation. These genes, identified largely through effects on reporter fusion genes, include *RIM11*, *RIM15*, *UME6/RIM16*, *RPD3*, and *SIN3/UME4/RPD1*. *RIM11* and *RIM15* are thought to be positive regulators of the early gene *IME2*, based on the finding that recessive *rim11* and *rim15* mutations block *ime2-lacZ* expression (2, 27, 43). *SIN3*, *RPD3*, and *UME6* are negative regulators of many early meiotic genes (2, 41, 50, 51); *UME6* also has a positive role in *IME2* expression (2, 3). The *UME6* gene product is homologous to zinc cluster-DNA-binding proteins and can bind specifically to the URS1 site (42), a *cis* element required for regulation of early meiotic genes (2, 5, 49; reviewed in reference 26). In addition, the BUF protein, which is identical to the heterotrimer RF-A (RP-A), binds to the URS1 site and is thus thought to have both positive and negative roles in URS1 site activity (23). Any of these sites or regulatory proteins may transmit the *IME1*-independent/downstream starvation signal.

IME1 is a nuclear protein with no structural features that provide clues concerning its activity (37, 39). Studies of LexA-IME1 fusions indicate that IME1 can provide a strong transcriptional activation domain (37). These observations and the finding that UME6 and the URS1 site have dual positive and negative roles have led to a simple model for activation of *IME2* expression. The model is that IME1 binds to a UME6-dependent repression complex at URS1 sites upstream of *IME2*; exposure of the IME1 transcriptional activation domain then stimulates *IME2* transcription (2, 37).

Two observations initially indicated a close functional relationship between IME1 and RIM11. First, recessive mutations in either gene reduce *ime2-lacZ* expression and sporulation (27, 43). Second, the *ime1* or *rim11* sporulation defects are suppressed by expression of a *GAL1-IME2* hybrid gene (27, 28). We have suggested that IME1 and RIM11 might act in parallel pathways leading to *IME2* expression, that IME1 and RIM11 might act in a cascade, or that the two proteins might

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TABLE 1. Yeast strains used in this study

Strain	Genotype[a]
AMP108	α
AMP109	**a**/α
AMP115	**a**/α *ime1-12/ime1-12*
AMP918	**a** *ime1-12 ime2-4-lacZ::LEU2 gal80::LEU2 met4-44S*
AMP1004	α P_{GAL1}-*IME1-14::TRP1 gal80::LEU2 his3ΔSK rad52::LEU2*
AMP1145	**a** P_{GAL1}-*IME1 ime2-4-lacZ::LEU2 gal80::LEU2 met4-44S his4G*
AMP1178	α *ime1-12 his3ΔSK gal80::LEU2*
AMP1180	α P_{GAL1}-*ime1-L321-F gal80::LEU2 his3ΔSK*
KB200	α *sin3::LEU2 IME2-5-lacZ::URA3 his3ΔSK arg6*
KB209	**a** *rim11-6* P_{GAL1}-*IME1-14::TRP1 ime2-4-lacZ::LEU2 his4*
KB225	α *rim11-6* P_{GAL1}-*IME1-14::TRP1 gal80::LEU2 arg6-2032*
KB260	**a** *ume6::LEU2 IME2-5-lacZ::URA3 his3ΔSK arg6*
KB263	**a** *rim11::LEU2 ime2-4-lacZ::LEU2 his4*
KB268	**a**/α *rim11::LEU2/rim11::LEU2 IME2/ime2-4-lacZ::LEU2 met4/MET4 his4/HIS4*
KB303	**a** *rim11::LEU2 ime1-12 ime2-7-HIS3 gal80::LEU2 his3ΔSK met4*
KB384	α *rim11::LEU2 ime1-12 ime2-7-HIS3 gal80::LEU2 his3ΔSK met4 arg6*
KB557	**a**/α *rim11::LEU2/rim11::LEU2 met4/MET4*
KB578	**a**/α *HA-RIM11/RIM11 ime2-4-lacZ::LEU2/IME2 his4/HIS4*
KB600	**a**/α *HA-RIM11/RIM11 ime2-4-lacZ::LEU2/IME2 his4/HIS4*

[a] All strains carry the additional markers *ura3 leu2::hisG trp1::hisG lys2 ho::LYS2.*

TABLE 2. Oligonucleotides used in this study

Name	Sequence
RIM11-P1	5′-CCGTCTGGTGGAATC-3′
RIM11-P2	5′-GGAAGACCAGCAGAT-3′
RIM11-P3	5′-CTCAGTAATAACATAGT-3′
RIM11-P4	5′-AGAACAGAGAGCTGG-3′
RIM11-P5	5′-TTTATTAGTAGATCCTG-3′
RIM11-P6	5′-GGTACTCCATCAAAG-3′
RIM11-H3A	5′-CCGAAGCTTAGGATGAATATTCAAAGCAA TAATTC-3′
RIM11-H3B	5′-CCGAAGCTTCTATCATTACTTAGACTTCGG ATATAG-3′
RIM11 K-R	5′-GCAGGACTTTCCTAATAGCAAC-3′
RIM11 K-A	5′-GCAGGACTTTTGCAATAGCAAC-3′
RIM11-N	5′-ATTGCTTTGAATATTGCGGCCGCCCATCCT GCGCGAGTT-3′
RIM11 Y-F	5′-CGTGAACAAATAAAGGAAACGTTAG-3′

associate with or otherwise modify one another (27). We report here a molecular characterization of *RIM11*. Our results have bearing on the function of RIM11, its biochemical role, and its relationship to IME1.

MATERIALS AND METHODS

Strains and media. All *S. cerevisiae* strains are derivatives of SK-1 and are described in Table 1. Mutations previously described include *ura3*, *leu2::hisG*, *trp1::hisG*, *lys2*, *ho::LYS2*, *ime2-4-lacZ::LEU2*, *IME2-5::lacZ::URA3*, *ime2-7::HIS3*, *ime1Δ12::TRP1*, P_{GAL1}-*IME1-14::TRP1*, P_{GAL1}-*ime1-L321F::TRP1*, *ume6::LEU2*, *sin3::LEU2*, *rim15-1*, *mck1Δ3*, *rad52::LEU2*, *gal80::LEU2*, *his4*, *met4*, and *arg6* (2, 27, 29, 37, 43). Plasmids carrying P_{GAL1}-*IME1* derivatives have been described previously (37). Yeast and bacterial media, including YPD, YPAc, SD, SC, and galactose indicator plates, were prepared according to standard recipes (33). Potassium acetate (KAc) plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) have been described previously (27).

Plasmid constructions. DNA manipulations were carried out by standard methods. The original *rim11-6*-complementing clone, called p2F2, was isolated from a YCp50 library and was described previously (27). Plasmid pKB111 was made by partially digesting plasmid p2F2 with *Bgl*II and religating, resulting in loss of three *Bgl*II fragments totaling 3.6 kb. Plasmid pKB117 was made by digesting p2F2 with *Sal*I and religating. Plasmid pKB120 was made by digesting plasmid pKB117 with *Sal*I and *Xba*I and cloning the 3.5-kb fragment into pRS316 (36), also digested with *Sal*I and *Xba*I. pKB124 was made by digesting pKB120 with *Sac*I and religating.

The high-copy-number *RIM11* plasmid pKB141 was made by digesting pKB124 with *Sac*I and *Sal*I, and the 2.3-kb *RIM11* fragment was ligated to pRS426 (which is similar to pRS326 [36]) that had been digested with *Sac*I and *Sal*I.

To generate RIM11 protein recognized by antibody 12CA5 (BAbCo), pKB141 was subjected to site-specific mutagenesis using oligonucleotide RIM11-N (Table 2) to generate a *Not*I site directly 3′ to the ATG initiation codon. A *Not*I fragment from the GTEP plasmid, encoding a hemagglutinin (HA) triple-epitope tag (48), was inserted to create pKB166, which carries the *HA-RIM11* allele. The entire *Sac*I-to-*Sal*I fragment was subcloned into pRS316 (36) to make a single-copy version, called pKB197. All subsequent mutations were created by site-specific mutagenesis on pKB166, using oligonucleotides described in Table 2. A Lys-to-Arg substitution at amino acid 68 (K68R), was made by using oligonucleotide RIM11 K-R, creating plasmid pKB171. The single-copy version of this plasmid, designated pKB198, was made by subcloning the *Sac*I-to-*Sal*I fragment from pKB171 to pRS316. A Lys-to-Ala substitution at amino acid 68 (K68A) was made by using oligonucleotide RIM11 K-A, creating plasmid pKB199. The single-copy version was made with a *Sac*I-to-*Sal*I subclone into pRS316, creating pKB200. To create a Tyr-to-Phe substitution at amino acid 199 (Y199F), oligonucleotide RIM11 Y-F was used as the mutagenic primer. All mutations were confirmed by sequencing.

***RIM11* sequence determination.** The entire insert in pKB124 was sequenced from nucleotide positions −593 to +1446 by using a set of unidirectional deletions. The opposite strand was sequenced with the complementary oligonucleotides RIM11-P1, -P2, -P3, -P4, -P5, and -P6 (Table 2). Mutant alleles were sequenced either by gap repair (for *rim11-8*) or by cloning of PCR products synthesized by using primers RIM11-H3A and -H3B (Table 2). All mutations were verified by genomic sequencing of the entire coding region, using Amplitaq cycle sequencing (Perkin-Elmer): genomic DNA was amplified with primers RIM11-H3A and -H3B through 30 cycles (94°C for 1 min, 42°C for 2 min, and 65°C for 2 min), and then the purified amplified products were used in cycle sequencing with end-labeled primers (RIM11-H3A, -H3B, -P4, -P5, and -P6) for 20 cycles (95°C for 1 min and 68°C for 1 min).

Sporulation and β-galactosidase assays. Plasmids carrying wild-type and mutant alleles of *RIM11* were transformed into strains indicated in figure legends to uracil prototrophy. Parent vectors pRS316 and pRS426 served as controls. Quantitative β-galactosidase assays were performed on permeabilized cells (39). Saturated cultures in SD containing 1% Casamino Acids (Difco), 0.5% glucose, and 20 mg of tryptophan per liter were filtered and resuspended in sporulation medium (2% KAc plus

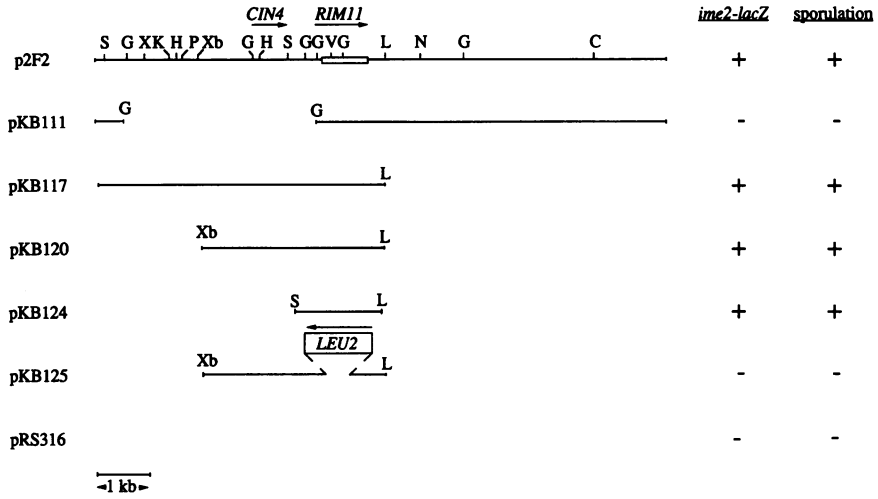

FIG. 1. Map of the *RIM11* locus and plasmids. The *RIM11* coding region is boxed. For each plasmid, the bar represents DNA from the *RIM11* locus contained in the plasmid. Plasmids are described in Materials and Methods. Restriction sites: C, *Cla*I; G, *Bgl*II; H, *Hin*dIII; K, *Kpn*I; L, *Sal*I; N, *Nco*I; P, *Pst*I; S, *Sac*I; V, *Eco*RV; X, *Xho*I; Xb, *Xba*I. Plasmids were transformed into strain KB209 (**a** *rim11-6 ime2-lacZ* P_{GAL1}-*IME1*). *ime2-lacZ* expression was scored on KAc–X-Gal indicator plates. Sporulation was tested by mating transformants to KB225 (α *rim11-6* P_{GAL1}-*IME1*) and transferring resulting diploids to sporulation medium. At least three independent transformants were tested for each plasmid.

required amino acids at 20 mg/liter). After 8 h, 800 μl of cells was collected for assays. The remainder of the culture was used to quantitate sporulation by microscopic examination. Each determination is based upon at least three independent transformants. The range of values was within 15% of the mean for values of 150 Miller units or more, within 30% of the mean for values of 25 to 150 Miller units, and within 50% of the mean for values of 25 Miller units or less.

RNA analysis. Procedures for cell growth, preparation of RNA, and Northern (RNA) filters have been described elsewhere (28, 38). Strand-specific probes (38) included *IME1* (mpHS106) and *IME2* (mp19BE200). Random prime probes included *RIM11* (a *Sac*I-*Sal*I fragment from pKB124), *HOP1* (a *Bam*HI-*Sac*I fragment from pNH50-1 [14]), *RIM4* (a 2.3-kb *Xho*I-to-*Eco*RI fragment from plasmid pSS166ΔK [45]), and the loading control probe, PC4/2 (22, 43).

Genetic manipulations. The *rim11*::*LEU2* deletion was constructed by replacing a 345-bp *Bgl*II fragment in plasmid pKB120 with the *LEU2* gene, creating plasmid pKB125 carrying the *rim11*::*LEU2* allele (Fig. 1). This deletion removes the initiation codon and 278 bp of coding sequences. The **a**/α *leu2*/*leu2* diploid (AMP109) was transformed to leucine prototrophy with a fragment from pKB125 that extends from a *Hin*dIII site upstream of *RIM11* to an *Apa*I site in the polylinker. Two Leu^+ diploids in which the disruption was confirmed by Southern analysis were sporulated and dissected. Each of 31 meiotic tetrads contained four viable spores; two were Leu^+, and two were Leu^-. We observed no general growth or germination defects among the Leu^+ segregants. Diploids from matings between all Leu^+ segregants to *rim11-6* mutant haploids were unable to sporulate or express *ime2-lacZ*. Diploids from matings between the same Leu^+ segregants and *RIM11* haploids were able to sporulate and express *ime2-lacZ*. Therefore, the *rim11*::*LEU2* mutation fails to complement the *rim11-6* allele. Diploids homozygous for *rim11*::*LEU2* were unable to sporulate (<0.1%), whereas diploids heterozygous for *rim11*::*LEU2* sporulated efficiently (>95%). Therefore, the *rim11*::*LEU2* mutation causes a recessive sporulation defect. The similarity in sporulation and growth properties associated with *rim11*::*LEU2* and the nonsense alleles *rim11-4* and *rim11-6* confirms that *rim11*::*LEU2* is a null allele.

Integration of the *HA-RIM11* allele was accomplished through cotransformation of strain KB263 (*rim11*::*LEU2 ime2-lacZ*) with a *Sac*I-to-*Sal*I fragment from pKB166 and plasmid pAM516, a YEp24 derivative carrying *IME1* under control of the *ACT1* promoter (43). Transformants were selected on SC-Ura and screened for β-galactosidase activity on KAc–X-Gal plates. Transformants that were Lac^+ were colony purified and analyzed by Southern blotting to confirm the integration. pAM516 was cured from transformants by selection on 5-fluoro-orotic acid (33); mating of one cured *HA-RIM11* transformant to strain AMP108 yielded diploid KB578.

Immunodetection and immunoprecipitation. Crude protein lysates were prepared by washing cells in lysis buffer (25 mM morpholineethanesulfonic acid [MES; pH 6.2], 10% glycerol, 100 mM NaCl, 100 mM KCl, 1 mM EDTA, 7 mM β-mercaptoethanol, 120 μg of phenylmethylsulfonyl fluoride per ml, 0.5 μg of leupeptin per ml, 10 μg of aprotinin per ml, 1 μg of pepstatin per ml, 1 mM $NaVO_4$, 5 mM NaF, 20 mM β-glycerophosphate, 10 mM *p*-nitrophenyl phosphate). Cells were resuspended in 200 μl of lysis buffer and lysed by vortexing with glass beads. Lysates were clarified by two centrifugations at 15,000 × *g* at 4°C. Protein concentrations were determined with a Bio-Rad protein assay kit. Protein samples (500 or 1,000 μg, depending on the experiment) were diluted into extraction buffer to make a final concentration of 2 mg/ml to which 250 μl of cold immunoprecipitation buffer (50 mM Tris-Cl [pH 7.5], 1% Triton X-100, 0.5 mg of ovalbumin per ml, 100 mM NaCl, 100 mM KCl) was added. Mouse monoclonal antibody 12CA5 (2 μl), which was a 50% ammonium sulfate cut from raw ascites fluid, was added to the lysate. After incubation on a rotator for 2 h at 4°C, 40 μl of a 50% suspension of protein A-Sepharose (Sigma) was added and the mixture was incubated for 1 h at 4°C with rotations. Immune complexes were collected by centrifugation in a microcentrifuge, washed twice with immunoprecipitation buffer, once with immunoprecipitation buffer lacking ovalbumin, and once in kinase buffer (50 mM Tris-Cl [pH 7.5], 1% Triton X-100, 10 mM $MgCl_2$, 100

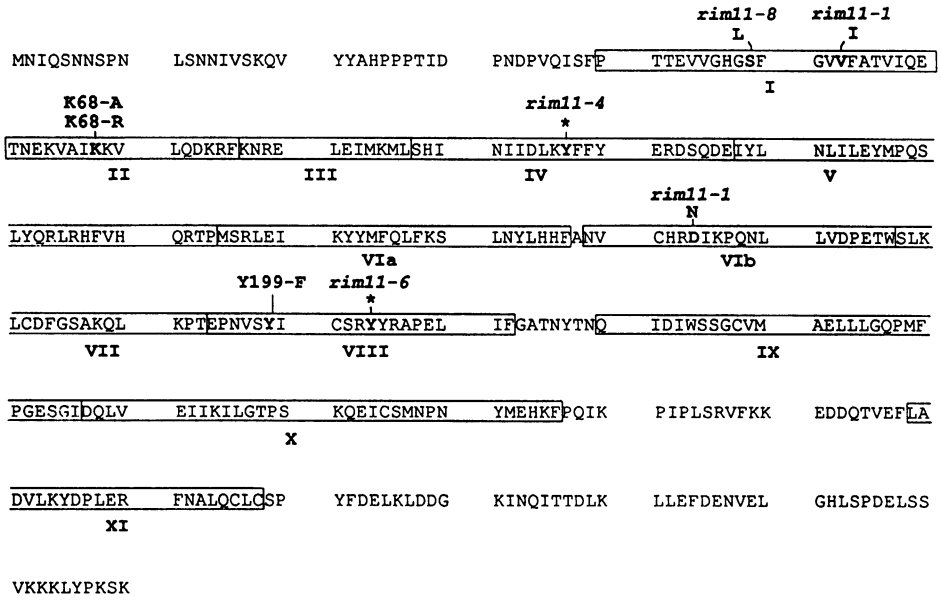

FIG. 2. Deduced amino acid sequence of the *RIM11* product. Mutations in *RIM11* identified by sequence and mutations created by site-specific mutagenesis noted in the text are written above the wild-type *RIM11* sequence. An asterisk above the sequence refers to a nonsense mutation. Conserved kinase subdomains are boxed and designated with roman numerals (11).

mM NaCl, 100 mM KCl), and resuspended in 50 μl of kinase buffer. Kinase reactions were initiated by adding 2.5 μl of [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham), and the reaction proceeded for 20 min at 25°C. Reactions were terminated with 50 μl of 2× Laemmli buffer and boiling. Fifty microliters of the total reaction was loaded per well on a sodium dodecyl sulfate (SDS)-polyacrylamide gel. Gels were treated for autoradiography after staining by five hour-long washes in destaining solution (5% methanol, 10% acetic acid, 10 mM sodium pyrophosphate) and one 30-min wash in 5% glycerol.

Immunoblots of crude extracts or immune complexes were developed with anti-HA monoclonal antibodies and goat anti-mouse conjugated to alkaline phosphatase (Boehringer Mannheim). Immunoblotting with the anti-IME1 antiserum was done as previously described (27).

Nucleotide sequence accession number. The GenBank accession number for the *RIM11* sequence is L29284.

RESULTS

Localization and nucleotide sequence of *RIM11*. *RIM11* was previously cloned by complementation of the *rim11-6* allele (27). We analyzed plasmids containing fragments of the original 10.2-kb *RIM11* clone for *rim11-6*-complementing activity by sporulation and activation of an *ime2-lacZ* reporter gene (Fig. 1). Plasmids pKB117, pKB120, and pKB124 were able to complement a *rim11-6* mutant; plasmid pKB111 and the negative control pRS316 were unable to complement. These results establish that plasmid pKB124, which contains DNA adjacent to the *CIN4* locus on chromosome XIII (27, 40), carries a minimal functional *RIM11* insert.

Sequence analysis of the pKB124 insert revealed a 370-amino-acid open reading frame that we establish below to be the *RIM11* gene (Fig. 2). The deduced 44.4-kDa *RIM11* gene product displays significant homology to serine/threonine protein kinases. It is highly related to rat glycogen synthase kinase 3β (GSK-3β; 50% identity, 69% similarity) and the *Drosophila shaggy/zw3* gene product (51% identity, 70% similarity) when compared over the length of the shorter protein. The *S. cerevisiae* kinase that shares the most identity with RIM11 is MCK1 (37% identity, 59% similarity). MCK1 plays a role in several pathways, including transcriptional control of *IME1*, spore maturation, and chromosome segregation (29, 35).

RIM11 was independently identified in two recent reports. *MDS1* was identified as a dosage-dependent suppressor of an *mck1* mutation (30). *S. cerevisiae GSK-3* was identified by homology to other *GSK-3* family members (1). Our *RIM11* sequence is identical to that of *MDS1* and differs from that of *S. cerevisiae GSK-3* by changes of Val to Ile at amino acid 57 and Glu to Gly at amino acid 343. Silent codon changes and sequence differences in noncoding regions were also noted (listed in GenBank submission; accession number L29284).

Sequence changes in *rim11* mutants. We verified our identification of *RIM11* through sequence determination of *rim11* mutant alleles (Fig. 2). Our studies have been conducted in the SK-1 genetic background, and the cloned gene is from the S288C genetic background (32). We found that the wild-type SK-1 sequence differs from our initial clone by two silent changes: GTT to GTC at codon 65 and GCA to GCG at codon 214. Two *rim11* alleles that abolish sporulation (27) were nonsense mutations. *rim11-4* results in truncation at amino acid residue 97, and *rim11-6* results in truncation at amino acid residue 204. Two *rim11* alleles that impair sporulation (27, 43) were missense mutations. *rim11-1* has two mutations, a change of Val to Ile at amino acid residue 53 (V53I) and a change of Asp to Asn at amino acid residue 164 (D164N). The V53I change, in protein kinase subdomain I (12), is two amino acids C terminal to the consensus GXGXXG domain proposed to form part of the ATP-binding pocket. Valine at this position has been proposed to stabilize the binding pocket (12). The D164N change, in kinase subdomain VI, affects a residue that is invariant among Ser/Thr protein kinases. *rim11-8* has a change of Ser to Leu at amino acid residue 49 (S49L). This change, in protein kinase subdomain I, affects a residue within the GXGXXG consensus. This analysis confirms that the open reading frame identified by sequence corresponds to *RIM11*.

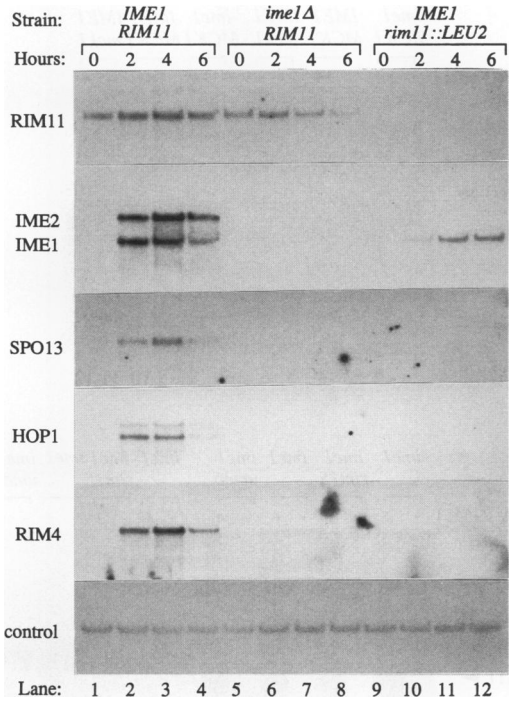

FIG. 3. Northern blot analysis. RNA was prepared from cells grown to mid-log phase in YPAc (lanes 1, 5, and 8) or at the indicated number of hours after transfer to sporulation medium. Strains were AMP109 (**a**/α *IME1/IME1 RIM11/RIM11*; lanes 1 to 4), AMP115 (**a**/α *ime1*Δ/*ime1*Δ *RIM11/RIM11*; lanes 5 to 8), and KB557 (**a**/α *IME1/IME1 rim11*::*LEU2/rim11*::*LEU2*; lanes 9 to 12). A single Northern filter was probed sequentially for the transcripts indicated on the left as described in Materials and Methods.

***RIM11* requirement for accumulation of early meiotic transcripts.** To determine whether *RIM11* is required for expression of meiotic genes, we examined transcript accumulation of several meiotic genes in *RIM11/RIM11* and *rim11*::*LEU2/rim11*::*LEU2* diploids after transfer to sporulation medium. An *ime1*Δ*12/ime1*Δ*12* diploid was included as a negative control. Transcripts of the early meiotic genes *IME2*, *SPO13*, *HOP1*, and *RIM4* accumulated in the wild-type diploid within 2 h after transfer to sporulation medium (Fig. 3, lanes 1 to 4) but were not detectable in *rim11*::*LEU2/rim11*::*LEU2* or *ime1*Δ*12/ime1*Δ*12* diploids (lanes 5 to 12). The transcript of the meiotic activator *IME1* accumulated more slowly in the *rim11*::*LEU2/rim11*::*LEU2* diploid than in the wild-type diploid. Hybridization to a constitutive RNA confirmed that equal amounts of RNA were loaded in each sample. Therefore, *RIM11* is required for expression of several early meiotic genes and stimulates expression of *IME1*.

Relationship between *RIM11* and *UME6*- and *SIN3*-dependent repression. Prior studies have shown that *UME6* and *SIN3* are required to repress several early meiotic genes under vegetative growth conditions (2, 41, 51). We used epistasis analysis to determine the functional relationship between *RIM11*, *UME6*, and *SIN3*. An *ime2-HIS3* fusion gene was used to monitor meiotic promoter activity. *ime2-HIS3* is expressed either in the presence of IME1 or in *sin3* and *ume6* mutants regardless of IME1 function (2). IME1 was expressed from a P_{GAL1}-*IME1* gene, in which the *IME1* coding region is fused to the *GAL1* promoter (39), and His^+ growth was determined on galactose medium. We observed that P_{GAL1}-*IME1* strains were His^+ and P_{GAL1}-*IME1 rim11*::*LEU2* strains were His^-, as expected from studies of *ime2-lacZ* expression (27). In *sin3* or *ume6* mutant backgrounds, both P_{GAL1}-*IME1* and P_{GAL1}-*IME1 rim11*::*LEU2* strains were His^+. In addition, *ime1*Δ *rim11*::*LEU2 sin3* and *ime1*Δ *rim11*::*LEU2 ume6* strains are His^+. Therefore, *sin3* and *ume6* defects are epistatic to the *rim11* defect. We conclude that RIM11 acts upstream of or in parallel to UME6 and SIN3.

Expression of *RIM11*. We used Northern blot analysis to determine if *RIM11* transcript levels change during meiosis (Fig. 3). The *RIM11* probe hybridized to a single 1.3-kb RNA that was abolished by the *rim11*::*LEU2* mutation. In a wild-type diploid, the *RIM11* transcript was detectable during vegetative growth (lane 1) and in sporulation medium (lanes 2 to 4). In an *ime1*Δ/*ime1*Δ diploid, *RIM11* transcript levels were similar to the wild-type levels during vegetative growth (compare lanes 5 and 1) but declined in sporulation medium (lanes 6 to 8). In other experiments, we determined that *RIM11* transcript levels are slightly lower in cells grown in YPD than in YPAc (data not shown), which are rich glucose and acetate media, respectively. We conclude that *RIM11* transcript levels are maintained during sporulation by IME1. Like many genes stimulated by IME1, *RIM11* has an upstream URS1-like site (TAC CCGCCGA, beginning at −526 relative to the ATG codon).

Correlation between in vivo activity and in vitro *trans*-phosphorylation activity. To study the RIM11 protein product, we created an epitope-tagged *RIM11* allele (designated *HA-RIM11*). Plasmid complementation assays described below established that *HA-RIM11* is a functional allele. HA-RIM11 was identified on an immunoblot as a 45-kDa doublet present in an **a**/α *HA-RIM11/RIM11* diploid (Fig. 4C, lane 2) and absent from an **a**/α *rim11*::*LEU2/RIM11* strain (lane 1). A 46-kDa protein that cross-reacts with anti-HA antiserum was evident in extracts lacking HA-RIM11 (lane 1). HA-RIM11 levels were slightly lower in YPD-grown cells (lane 2) than in YPAc-grown cells (lane 3) and declined after 6 h in sporulation medium (lanes 7 and 8). We conclude that HA-RIM11 protein levels vary little under these growth conditions.

We examined HA-RIM11 phosphorylation activity in immune complexes. Immune complexes were incubated with [γ-^{32}P]ATP, and phosphoproteins were visualized by SDS-polyacrylamide gel electrophoresis and autoradiography. We observed three phosphoproteins, of 45, 58, and 60 kDa, only in samples containing HA-RIM11 (Fig. 4A; compare lanes 1 and 2). The 45-kDa phosphoprotein is autophosphorylated HA-RIM11, as judged from its comigration with HA-RIM11 identified on immunoblots. Autophosphorylation activity was fairly constant in growing and sporulating cells (Fig. 4A, lanes 2 to 7) except that HA-RIM11 recovery decreased late in sporulation (as assessed by immunoblotting; Fig. 4B). However, the amounts of phosphorylated 58- and 60-kDa proteins, which we refer to as p58 and p60, were greatest in glucose-grown cells (lane 2) and lower in acetate-grown cells (lane 3), and decreased further during sporulation (lanes 4 to 8). Changes in p58 and p60 phosphorylation activity may reflect changes in the amounts of these proteins or in HA-RIM11-associated protein kinase activity.

p58 and p60 are apparently not derived from HA-RIM11 because they fail to react with anti-HA antibodies in an immunoblot (Fig. 5A, lanes 2 and 8). Immune complexes prepared from *ime1*Δ, *mck1*Δ, *rim15-1*, or *ume6*::*LEU2* mutants contain phosphorylatable p58 and p60 (Fig. 5). We conclude that none of these mutations cause a severe reduction in HA-RIM11-associated protein kinase activity or presence of phosphorylatable p58 and p60.

We examined the relationship between HA-RIM11-associ-

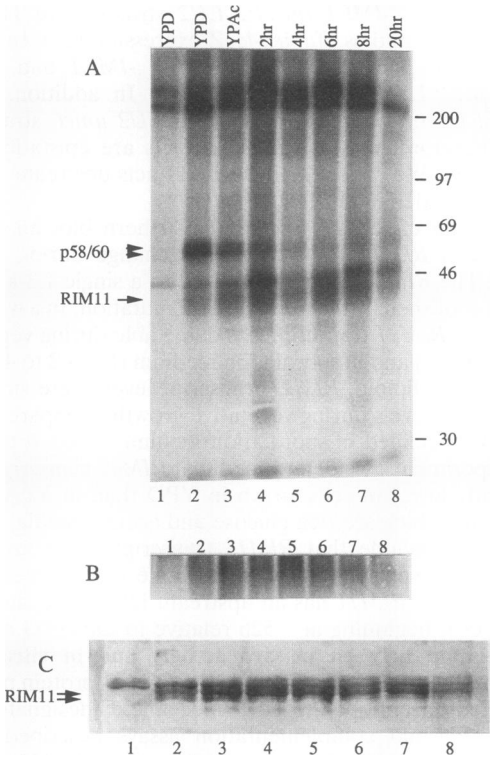

FIG. 4. Immune complex kinase assays of HA-RIM11. Extracts of diploid strains KB263 × AMP108 (*rim11*::*LEU2*/*RIM11*; lane 1) or KB578 (*HA-RIM11*/*RIM11*; lanes 2 to 8) were prepared during growth in YPD (lanes 1 and 2) or YPAc (lane 3) or at 2, 4, 6, 8, and 20 h after transfer to sporulation medium (lanes 4 to 8). Immune complexes were collected with anti-HA antibodies and split; half was used for kinase assays (A), and half was used for an immunoblot developed with anti-HA antibodies (B). In addition, an aliquot of each extract containing 50 μg of protein was used for an immunoblot developed with anti-HA antibodies (C). Migration of molecular size standards is indicated in kilodaltons at the right.

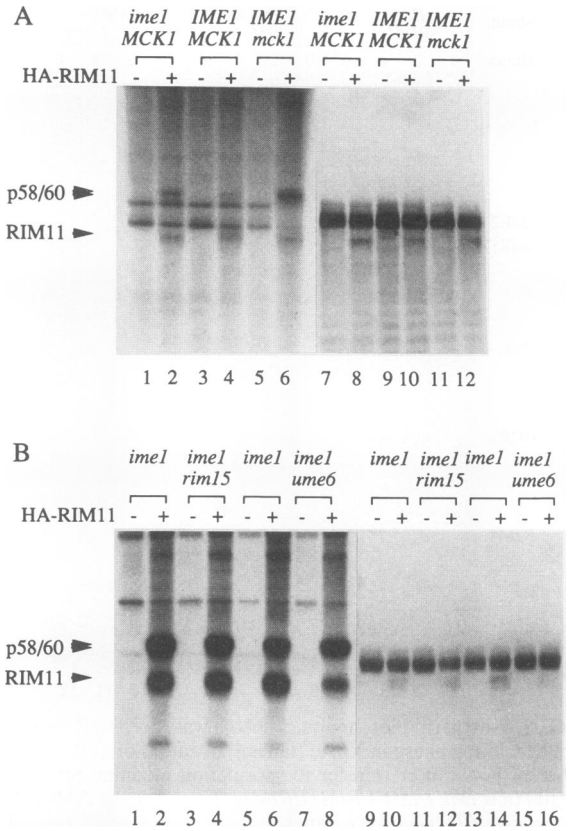

FIG. 5. Immune complex kinase assays in meiotic mutant extracts. Immune complexes were collected with anti-HA antibodies from extracts of YPAc-grown cells and used for kinase assays (A, lanes 1 to 6; B, lanes 1 to 8) and for immunoblots developed with anti-HA antibodies (A, lanes 7 to 12; B, lanes 9 to 16). In panel A, strains had either a wild-type *RIM11* allele (odd-numbered lanes) or an *HA-RIM11* allele (even-numbered lanes) and were otherwise wild type (lanes 3, 4, 9, and 10), *ime1Δ12* (lanes 1, 2, 7, and 8), or *mck1Δ3* (lanes 5, 6, 11, and 12). In panel B, strains carried high-copy-number plasmids with *RIM11* (odd-numbered lanes) or *HA-RIM11* (even-numbered lanes) and the mutation *ime1Δ12* (lanes 1, 2, 5, 6, 9, 10, 13, and 14), *ime1Δ12 rim15-1* (lanes 3, 4, 11, and 12), or *ime1Δ12 ume6*::*LEU2* (lane 7, 8, 15, and 16).

ated phosphorylation activity and biological function for three HA-RIM11 missense derivatives. Two mutants were constructed with substitutions of alanine (*HA-RIM11-K68A*) or arginine (*HA-RIM11-K68R*) for a conserved lysine residue in the ATP-binding site (subdomain II [11, 12, 20]). A third mutant was constructed with a substitution of phenylalanine for a tyrosine (*HA-RIM11-Y199F*) conserved in other kinases related to GSK-3 (subdomain VIII). Phosphorylation of the corresponding tyrosine residues in GSK-3α, GSK-3β, and the *shaggy*/*zw3* gene product is required for protein kinase activity (16).

To assess biological function, an **a**/α *rim11*::*LEU2*/*rim11*::*LEU2 ime2-lacZ*/*IME2* diploid transformed with *HA-RIM11* plasmids was tested for *ime2-lacZ* reporter gene expression and sporulation (Table 3). Transformants carrying low- or high-copy-number vectors failed to express *ime2-lacZ* or sporulate. Transformants carrying *RIM11* or *HA-RIM11* plasmids expressed *ime2-lacZ* and sporulated with 25 to 50% efficiency. Transformants carrying *HA-RIM11-K68A* or *HA-RIM11-Y199F* plasmids did not express *ime2-lacZ* or sporulate. Transformants carrying a low-copy-number *HA-RIM11-K68R* plasmid expressed *ime2-lacZ* at low levels and sporulated weakly; transformants carrying the same allele on a high-copy-number plasmid displayed further elevated *ime2-lacZ* expression and sporulation. We conclude that the K68R substitution reduces but does not abolish RIM11 function; the K68A and Y199F substitutions reduce RIM11 function below the detection limits of these assays.

Protein kinase activity associated with these *HA-RIM11* products was determined in immune complexes prepared from the transformants described above (Fig. 6A). The low-copy-number *HA-RIM11* transformant yielded phosphorylated HA-RIM11, p58, and p60 (lane 2). Low-copy-number *HA-RIM11-K68R* and *-K68A* transformants yielded none of these phosphoproteins at detectable levels (lanes 3 and 4). This result establishes that HA-RIM11 itself is responsible for protein kinase activity in these complexes. The high-copy-number *HA-RIM11* transformant yielded elevated levels of the three phosphoproteins (lane 6) compared with the low-copy-number *HA-RIM11* transformant. The high-copy-number *HA-RIM11-K68R* transformant had no detectable autophosphorylation product but had low levels of phosphorylated p58 and p60 (lane 7); these phosphoproteins were more apparent in a longer exposure (lane 10). The high-copy-number *HA-RIM11-K68A* transformant had no detectable autophosphorylation

TABLE 3. Functional assays of mutant *RIM11* alleles

Plasmid[a]	*RIM11* allele	*ime2-lacZ* expression[b] (U)	Sporulation (%)[c]		
			1 day	2 days	3 days
Low copy number					
pRS316		2	<0.2	<0.2	<0.2
pKB124	*RIM11*	229	23	24	24
pKB197	*HA-RIM11*	217	29	33	36
pKB198	*HA-RIM11-K68R*	22	1	12	12
pKB200	*HA-RIM11-K68A*	2	<0.2	<0.2	<0.2
High copy number					
pRS426		3	<0.2	<0.2	<0.2
pKB141	*RIM11*	159	45	46	53
pKB166	*HA-RIM11*	167	33	44	41
pKB171	*HA-RIM11-K68R*	83	9	24	21
pKB199	*HA-RIM11-K68A*	3	<0.2	<0.2	<0.2
pKB201	*HA-RIM11-Y199F*	2	<0.2	<0.2	<0.2

[a] Plasmids were transformed into strain KB268 (a/α *rim11*::*LEU2*/*rim11*::*LEU2 ime2-lacZ*/*IME2*).
[b] β-Galactosidase levels were determined after 8 h in sporulation medium for three independent transformants.
[c] Determined after 1, 2, and 3 days in sporulation medium for three independent transformants.

product, p58, or p60 (lanes 8 and 11). The high-copy-number *HA-RIM11-Y199F* transformant had high levels of autophosphorylation product but no detectable phosphorylated p58 and p60. Thus, there is little correlation between HA-RIM11 biological function and autophosphorylation activity. For example, the *HA-RIM11-Y199F* mutation abolishes biological function but does not abolish autophosphorylation ability; the *HA-RIM11-K68R* mutation does not abolish biological function but does abolish autophosphorylation ability. On the other hand, there is a correlation between HA-RIM11 biological function and p58/p60 phosphorylation. The *HA-RIM11-Y199F* and *-K68A* mutations abolish both function and p58/p60 phosphorylation; the *HA-RIM11-K68R* mutation reduces both function and p58/p60 phosphorylation.

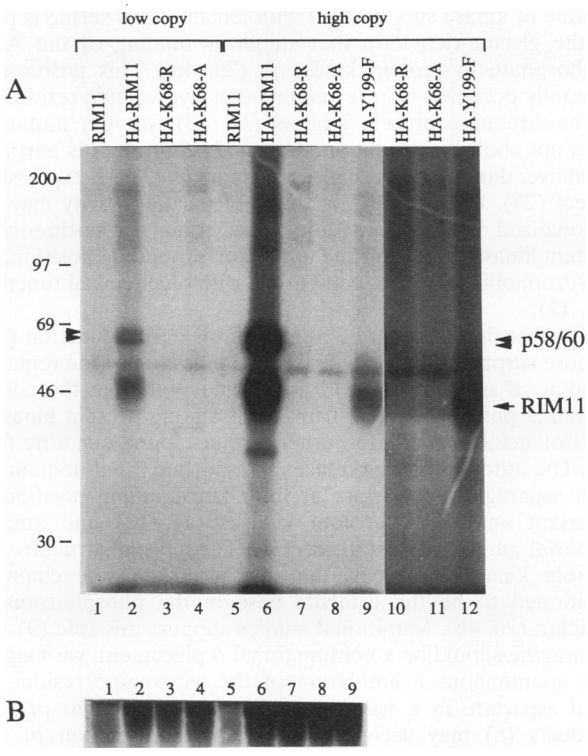

FIG. 6. Immune complex kinase assays of mutant HA-RIM11 derivatives. The *RIM11* alleles indicated above the lanes were carried in low-copy-number plasmids (lanes 1 to 4) or high-copy-number plasmids (lanes 5 to 12) in strain KB268 (a/α *rim11*::*LEU2*/*rim11*::*LEU2*). Immune complexes were collected from extracts of YPAc-grown cells and used for both kinase assays (A) and an immunoblot (B) as described for the corresponding panels of Fig. 4. Exposure times were 16 h (lanes 1 to 9) and 9 days (lanes 10 to 12). Sizes are indicated in kilodaltons.

Phosphorylation of IME1 by HA-RIM11. *IME1* and *RIM11* display a close functional relationship. They are among the few known genes that are absolutely essential for early meiotic gene expression. The sporulation defect of both *ime1* and *rim11* mutants can be suppressed by overexpression of *IME2*. In addition, *sin3* and *ume6* mutations are epistatic to both *ime1* and *rim11* mutations. Previous studies have shown that expression of *IME1* from the *ACT1* or *GAL1* promoters does not bypass the need for *RIM11* (27, 43). We also observed that presence of a high-copy-number *RIM11* plasmid does not bypass the need for *IME1*, as indicated by assays of *ime2-lacZ* expression and sporulation in an *ime1*Δ/*ime1*Δ diploid (Table 4). These observations are consistent with the idea that one protein may be required for activity of the other. An *ime1*Δ mutation had not reduced HA-RIM11 protein kinase activity, so IME1 does not apparently stimulate RIM11. We considered

TABLE 4. Suppression of an *ime1* missense mutation by overexpression of *RIM11*

Relevant genotype[a]	High-copy-no. *RIM11* allele	*ime2-lacZ* expression[b] (U)	Sporulation (%)[c]
*ime1*Δ	None	3	<0.2
*ime1*Δ	*HA-RIM11*	11	<0.2
P_{GAL1}-*ime1-L321F*	None	4	0.3
P_{GAL1}-*ime1-L321F*	*HA-RIM11*	196	19
P_{GAL1}-*ime1-L321F*	*HA-RIM11-K68A*	9	0.2
P_{GAL1}-*IME1*	None	372	24
P_{GAL1}-*IME1*	*HA-RIM11*	442	42

[a] Strains AMP1178 × AMP918 (a/α *ime1*Δ/*ime1*Δ *ime2-lacZ*/*IME2*), AMP1180 × AMP918 (a/α P_{GAL1}-*ime1-L321F*/*ime1*Δ *ime2-lacZ*/*IME2*), and AMP1004 × AMP918 (a/α P_{GAL1}-*IME1*/*ime1*Δ *ime2-lacZ*/*IME2*) were transformed with plasmids pRS426, pKB166, and pKB171.
[b] β-Galactosidase levels were determined after 8 h in sporulation medium.
[c] Determined after 24 h in sporulation medium.

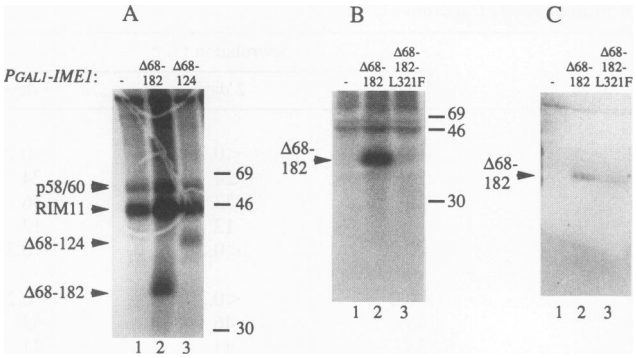

FIG. 7. Phosphorylation of IME1 derivatives in HA-RIM11 immune complexes. (A) Extracts of YPAc-grown strain KB600 (*HA-RIM11/RIM11*) carrying no plasmid (lane 1), YCpP_{GAL1}-*IME1Δ68-182* (lane 2), or YCpP_{GAL1}-*IME1Δ123-182* were used for immune complex kinase assays as described for Fig. 4A. (B and C) Extracts of YPAc-grown strain KB600 carrying plasmids YCpP_{GAL1} (lane 1), YCpP_{GAL1}-*IME1Δ68-182* (lane 2), or YCpP_{GAL1}-*IME1Δ68-182-L321F* were used for immune complex kinase assays (B) and an immunoblot with anti-IME1 antiserum (C). Sizes are indicated in kilodaltons.

the possibility that RIM11 phosphorylates IME1 and thus stimulates IME1 activity.

To detect phosphorylation of IME1, we used two functional deletion derivatives, *IME1Δ68-182* and *IME1Δ123-182* (37). Full-length IME1 has an apparent molecular mass of 44 to 54 kDa, which might be indistinguishable from that of HA-RIM11, whereas these deletion derivatives have apparent molecular masses of 36 and 40 kDa, respectively (37). HA-RIM11 immune complexes were collected from strains overexpressing these IME1 derivatives from the *GAL1* promoter and used for immune complex protein kinase assays (Fig. 7A). Phosphoproteins detected from an extract containing no IME1 derivative included HA-RIM11, p58, and p60 (lane 1). Phosphoproteins detected from an extract containing IME1Δ68-182 included a novel 36-kDa protein (lane 2); phosphoproteins detected from an extract containing IME1Δ123-182 included a novel 40-kDa protein (lane 3). The *HA-RIM11-K68A* mutation blocked phosphorylation of IME1Δ68-182 (data not shown). We conclude that HA-RIM11 can phosphorylate these IME1 derivatives.

If the ability of IME1 to serve as a RIM11 substrate is important for its function, then some nonfunctional *IME1* alleles may cause defects in IME1 phosphorylation. We examined the *ime1-L321F* missense allele, which results in defects in *ime2-lacZ* expression and sporulation (37). This allele had been isolated in a selection that also yielded *rim11* mutants. If the L321F substitution reduces ability of IME1 to serve as a substrate for RIM11, the *ime1-L321F* mutation may be suppressed by overexpression of RIM11. Suppression was tested in diploid AMP1180 × AMP918 (**a**/α P_{GAL1}-*ime1-L321F*/*ime1Δ ime2-lacZ*/*IME2*) carrying either the high-copy-number *HA-RIM11* plasmid or vector alone, through assays of *ime2-lacZ* expression and sporulation (Table 4). The *HA-RIM11* plasmid stimulated both *ime2-lacZ* expression and sporulation. A high-copy-number *HA-RIM11-K68A* plasmid did not stimulate either response in this strain, so it is RIM11 kinase activity that is required for suppression. These results are consistent with the proposal that the L321F substitution impairs a functional IME1-RIM11 interaction in vivo.

To determine whether the missense mutation prevents phosphorylation of IME1, we constructed an *IME1Δ68-182-L321F* deletion derivative that carries the L321F substitution (and is expressed from the *GAL1* promoter). An immunoblot confirmed that IME1Δ68-182 and IME1Δ68-182-L321F accumulated to detectable levels (Fig. 7C, lanes 2 and 3). In HA-RIM11 immune complex kinase assays, we could detect phosphorylation only of IME1Δ68-182 and not of IME1Δ68-182-L321F (Fig. 7B, lanes 2 and 3). We were unable to detect either IME1 derivative in immunoblots of the immune complexes (data not shown), so it is unclear whether the L321F substitution impairs binding of IME1 to RIM11 or its phosphorylation. However, these results indicate that IME1-L321F is a defective substrate in vitro.

DISCUSSION

Mutations in *RIM11* were first identified among mutants defective in *ime2-lacZ* expression and sporulation (27, 43). *RIM11* was thought to specify a positive regulator of *IME2* expression because these *rim11* mutations were recessive. Our characterization of the *RIM11* gene in this report has established that RIM11 functions as a protein kinase. We have provided strong correlative evidence that the biological role of RIM11 is to phosphorylate the meiotic activator IME1.

RIM11 protein kinase activity. *RIM11* specifies a polypeptide that includes regions conserved among serine-threonine protein kinases and little else. Thus, there seemed no doubt that RIM11 functions as a protein kinase. In that context, the sequences and properties of *rim11* missense alleles were surprising. For example, the *rim11-8* allele replaces a serine residue of kinase subdomain I with leucine. This serine is part of the glycine-rich loop that stabilizes binding of the ATP β-phosphate in protein kinase A (20, 46). This position is generally occupied by a serine or other hydrophilic residue in serine-threonine protein kinases (11, 12), yet the mutation does not abolish sporulation. The *rim11-8* product is partially defective, though, because it results in an *ime2-lacZ* expression defect (27). For *rim11-8*, presence of partial activity may be rationalized by the observation that some serine-threonine protein kinases have a valine at the corresponding position, so a hydrophobic residue is compatible with biochemical function (11, 12).

The fact that the *rim11-1* allele retains partial function (43) is more surprising. This allele has two mutations. One replaces a valine of subdomain I with isoleucine. Valine at the corresponding position is almost invariant among protein kinases, but isoleucine is one of few other residues found in nature (11, 12). The other mutation replaces an aspartate in subdomain VI with asparagine. Aspartate at the corresponding position is invariant among all protein kinases (11, 12) and among bacterial phosphotransferases (4). The crystal structure of protein kinase A reveals that the aspartate side chain is positioned to be the catalytic base in the phosphotransfer reaction (20, 46). Mutational studies support this role (9). As asparagine should be a nonfunctional replacement, we suggest that spontaneous deamidation of the asparagine residue to yield aspartate in a fraction of the *rim11-1* mutant protein products (8) may account for the partial function of the *rim11-1* allele.

Among the mutations that we constructed, *HA-RIM11-K68R* also retains partial function. This mutation replaces an invariant lysine in subdomain II (11, 12) with arginine. The corresponding lysine in protein kinase A stabilizes the α and β phosphates of ATP (20, 46). Substitutions at this position in other protein kinases do not invariably abolish activity (57). Substitution of alanine at this position (*HA-RIM11-K68A*) does abolish biological function and both substitutions abolish HA-

RIM11 autophosphorylation activity, thus confirming the importance of this site.

The *HA-RIM11-Y199F* mutation was unusual in that it abolished biological function and p58/p60 phosphorylation but did not abolish autophosphorylation activity. The corresponding tyrosine, in subdomain VIII, is universally conserved among GSK-3 homologs and its replacement with phenylalanine abolishes GSK-3β activity (16). One explanation of this mutant defect is that the kinase is specifically defective in substrate recognition or binding. The corresponding region of protein kinase A is in close proximity to the peptide substrate (20, 46), and a mutation of the nearby universal glutamate in protein kinase A causes a specific defect in peptide substrate binding (9). A second explanation comes from the observation that the corresponding tyrosine in GSK-3 is the site of a stimulatory phosphorylation (16). Thus, active mammalian GSK-3 is recognized by antiphosphotyrosine antibodies; GSK-3 preparations with reduced phosphotyrosine content have reduced activity. We have been unable to detect tyrosine phosphorylation of HA-RIM11 with antiphosphotyrosine antibodies (3). Therefore, the possibility that RIM11 is stimulated by phosphorylation of this tyrosine remains speculative.

Role of RIM11 in IME1 activity. We found that a deletion of *RIM11* causes an absolute sporulation defect. We consider this *rim11*::*LEU2* allele a null allele because it makes no detectable *RIM11* transcript and because it causes the same phenotype as two *rim11* nonsense alleles. Recently, Puziss et al. (30) reported that a deletion of the entire *RIM11* coding region also causes an absolute sporulation defect. We found no obvious growth defects associated with *rim11* mutations, as did Puziss et al. in their more extensive phenotypic survey. Thus, the major role unique to RIM11 is to stimulate meiosis.

Our study indicates that RIM11 is formally a positive regulator of meiotic gene expression. The *rim11*::*LEU2* mutation causes a severe defect in transcript accumulation of the early meiotic genes *IME2*, *RIM4*, *SPO13*, and *HOP1*. These genes are thought to respond to a common regulatory pathway: all possess 5′ URS1 sites, all depend on IME1 for expression, and at least two (*IME2* and *SPO13*) are negatively regulated by SIN3 and UME6 (reviewed in reference 26). Our epistasis analysis indicated that RIM11, like IME1, does not act downstream of these negative regulators in stimulating *IME2* expression.

Three observations suggest a close functional relationship between RIM11 and IME1. First, *rim11* mutations suppress the toxicity of *IME1* overexpression to haploid cells (27); we showed here that two such *rim11* mutations are nonsense alleles that must result in loss of function. Second, epistasis experiments indicate that IME1 and RIM11 both act upstream of or in parallel to SIN3 and UME6. Third, we found that overexpression of RIM11 can suppress an *ime1* missense defect. This *ime1* mutation does not cause a defect in IME1 accumulation (37). The finding that LexA-IME1 is a RIM11-dependent transcriptional activator (37) argues that RIM11 is required for IME1 activity.

The simplest model is that RIM11 stimulates IME1 through phosphorylation. We have detected phosphorylation of two IME1 derivatives in HA-RIM11 immune complexes. These two IME1 derivatives are functional in vivo (37). Moreover, the L321F substitution in IME1, which impairs IME1 activity, prevents IME1 from serving as a RIM11 substrate in vitro. The fact that *ime1-L321F* is suppressed by increased RIM11 dosage is consistent with a mutation that causes a defective substrate in vivo. We are uncertain whether IME1-L321F is defective in binding to RIM11 or is defective only in phosphorylation. Current efforts to purify suitable in vitro substrates will permit us to address this issue directly. However, we have provided biochemical support for the model that RIM11 phosphorylates IME1 and that phosphorylation is functionally relevant (Fig. 8).

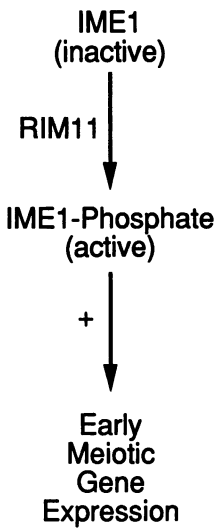

FIG. 8. Model for the relationship between IME1 and RIM11. We propose that IME1 is synthesized in an inactive form. Phosphorylation by RIM11 activates IME1. Active IME1 stimulates early meiotic gene expression.

We note a simple alternative to our model: IME1 might be a stimulatory subunit of a RIM11 protein kinase complex; RIM11-IME1 would phosphorylate some other protein to activate meiotic genes. The best candidate for this other protein is UME6. Our observation that HA-RIM11 immune complex kinase activity is unaffected by absence of IME1 lends no support to this second model. Nonetheless, current evidence does not rule this model out.

We are uncertain about the site of RIM11 phosphorylation in IME1. Studies of LexA-IME1 activity suggest that a region including IME1 residues 295 to 360 transmits a RIM11-dependent signal (37). This region includes a segment that resembles a GSK-3 target site ($S\text{-}X_3\text{-}S\text{-}X_3\text{-}S$, in which S represents phosphorylated serine residues [31, 54]). One simple model is that RIM11 phosphorylates this region of IME1 directly to permit exposure of a transcriptional activation domain, much as phosphorylation by protein kinase A of CREB exposes a functional transcriptional activation domain (10, 55). Our inference that this region of IME1 is also the specificity determinant (37) suggests that RIM11-dependent phosphorylation might also influence the presumed interaction of IME1 with UME6.

Role of RIM11 in *IME1* expression. The *rim11*::*LEU2* mutation caused a slight defect in *IME1* transcript accumulation. This observation is consistent with the study of Puziss et al. indicating shared substrate specificity of RIM11 and the related protein kinase MCK1 (30). They found that RIM11 and MCK1 can phosphorylate the same peptide substrate and that overexpression of *RIM11* can suppress some *mck1* growth defects. One role of MCK1 is to stimulate *IME1* transcript accumulation (29). Thus, it seems reasonable that RIM11 and MCK1 might both stimulate *IME1* expression through phosphorylation of a common substrate. However, the mild *IME1* expression defect is unlikely to be the cause of the severe *rim11* sporulation defect for two reasons. First, other mutants with comparable *IME1* expression defects (*mck1* [29] and *rim1/8/*

9/13 [43]) have decreased sporulation efficiency, not a complete defect like *rim11* mutants. Second, expression of *IME1* from the *GAL1* promoter does not permit sporulation of *rim11-4* or *-6* nonsense mutants, despite presence of IME1 protein (27), whereas P_{GAL1}-*IME1* does permit efficient sporulation in *mck1* mutants (29). Therefore, stimulation of *IME1* expression appears to be a second role of RIM11, distinct from its vital role in phosphorylation of IME1.

Phosphorylation of p58 and p60. HA-RIM11 immune complexes contain two proteins, p58 and p60, that serve as in vitro phosphorylation substrates. These proteins are not modified forms of HA-RIM11 because they do not react with anti-HA antibodies in immunoblots of crude extracts. Phosphorylation of p58 and p60 by mutant HA-RIM11 kinases correlates with biological activity. Their phosphorylation by HA-RIM11 is greater in growing cells than in starved cells that have entered meiosis. These proteins may have some role in meiotic gene expression, such as to stimulate or inhibit phosphorylation of IME1, or may simply associate with HA-RIM11 immune complexes adventitiously. We have detected the proteins in extracts of *ume6*, *mck1*, and *ime1* deletion/disruption mutants, so these genes do not encode p58 or p60. In addition, we detected p58 and p60 in a *rim15-1* mutant, but the nature of this mutation is unclear (43). The proteins are unlikely to be encoded by *SIN3* because the deduced SIN3 polypeptide migrates at 170 kDa (52). Association of p58 and p60 with HA-RIM11 may permit interaction-based cloning, which has successfully identified components of the SNF1 and CDK2 protein kinase complexes (7, 21, 56).

Regulatory role of RIM11. We had initially speculated that RIM11 might transmit a starvation signal (27). Support for that idea came from Smith et al. (37), who found that the C terminus of IME1 confers both RIM11 dependence and starvation responsiveness to transcriptional activation by a LexA-IME1 fusion. Further support for a relationship between starvation and RIM11 comes from two new observations. First, RIM11 stimulates *IME1* RNA accumulation, which is stimulated by starvation. Second, p58/p60 phosphorylation by HA-RIM11 is greater in immune complexes prepared from growing cells than sporulating cells. Thus, it appears that these immune complexes are different under different growth conditions. Biochemical analysis of immune complex components may reveal a starvation-dependent subunit or modification.

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