Phosphorylation of Ime2 Regulates Meiotic Progression in *Saccharomyces cerevisiae*<sup>1,5</sup>

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Ime2p is a meiosis-specific protein kinase in *Saccharomyces cerevisiae* that controls multiple steps in meiosis. Although Ime2p is functionally related to the Cdc28p cyclin-dependent kinase (CDK), no cyclin binding partners that regulate its activities have been identified. The sequence of the Ime2p catalytic domain is similar to CDKs and mitogen-activated protein kinases (MAPks). Ime2p is activated by phosphorylation of its activation loop in a Cak1p-dependent fashion and is subsequently phosphorylated on multiple residues as cells progress through meiosis. In this study, we show that Ime2p purified from meiotic cells is phosphorylated on Thr<sup>242</sup> and Tyr<sup>244</sup> in its activation loop and on Ser<sup>520</sup> and Ser<sup>625</sup> in its C terminus. Ime2p autophosphorylates on threonine in its activation loop in vitro consistent with autophosphorylation of Thr<sup>242</sup> playing a role in its activation. Moreover, autophosphorylation in cis is required for Ime2p to become hyperphosphorylated. Phosphorylation of the C-terminal serines is not essential to sporulation. However, Ime2p C-terminal phosphorylation site mutants genetically interact with components of the FEAR network that controls exit from meiosis I. These data suggest that Ime2p plays a role in controlling the exit from meiosis I and demonstrate that a phospho-modification pathway regulates Ime2p during the different phases of meiotic development.

Meiosis is the program by which diploid cells generate haploids. It shares several key processes with mitosis including DNA replication and chromosome segregation; however, its successful completion requires a series of processes unique to meiosis. These meiosis-specific processes include homolog pairing, synaptonemal-complex formation, genetic recombination, and a reductional division (MI). Subsequently, an equational (mitosis-like) division (MII) occurs without an intervening round of DNA replication. Completion of MII is linked to specialized differentiation programs that produce gametes capable of sexual fusion. Meiosis-specific processes are induced in part, through transcriptional programs that express meiotic genes as they are needed. These programs also express signaling molecules that are required to coordinate meiotic processes and superimpose meiotic regulation on the mitotic cell cycle machinery.

In *Saccharomyces cerevisiae* multiple meiotic processes are regulated by the Ime2p meiosis-specific protein kinase. Ime2p is functionally related to Cdc28p, the sole essential cyclin-dependent kinase (CDK)<sup>2</sup> required for mitosis and meiosis. IME2 transcription begins shortly after meiotic induction and increases further around the time of the chromosomal divisions. This biphasic accumulation of IME2 mRNA first requires Ime1p, the transcription factor specific for the early class of meiosis-specific promoters (1). Its second phase of transcription requires Ndt80p, the transcription factor specific for middle sporulation gene promoters (2).

After induction, Ime2p first positively regulates early gene expression through Ime1p. During this time, Ime2p controls meiotic S phase by functionally replacing some, but not all, of the Cdc28p mitotic S-phase-promoting roles (3–5). For example, whereas Cdc28p is required to target the Sic1p CDK inhibitor for ubiquitin-mediated proteolysis in mitosis, Ime2p is required for this role in meiosis. During the early phase of sporulation, Ime2p also phosphorylates replication protein A (6). Ime2p subsequently promotes the meiotic divisions at least in part by positively regulating Ndt80p through direct phosphorylation (4, 7). In addition, genetic evidence suggests that Ime2p negatively regulates Sum1p, a repressor protein that functions in opposition to Ndt80p (8).

Ime2p also plays a role in terminating early gene expression by promoting the destruction of Ime1p (9). Later in the program, Ime2p has been proposed to negatively regulate Cdh1p, a targeting subunit of the anaphase-promoting complex/cyclosome and may thus regulate chromosome segregation by modulating the activity of this ubiquitin ligase (10). A subset of Ime2p function may be carried out (albeit inefficiently) by Cdc28p in *ime2* mutants (5).

These collective observations demonstrate that Ime2p regulates multiple meiotic processes. Although Ime2p is functionally related to Cdc28p, no cyclin binding partners have been identified that regulate its activity, and relatively little is known about how its multiple functions in meiosis are controlled. Ime2p is degraded shortly after MII is completed (4). Ubiquitin-mediated destruction of Ime2p occurs in a Grr1p-dependent manner when glucose, which inhibits meiosis, is added to sporulating cells (11). In addition, it has been proposed that glucose can inhibit the Ime2p activity through the Gpa2p heterotrimeric GTP-binding protein (12).

In previous studies it was shown that Ime2p is phosphorylated early in sporulation and hyperphosphorylated during middle sporulation (4, 13). Ime2p contains a TXY motif in its activation loop that is similar to the activation loops found in mitogen-activated protein kinases (MAPks). Mutations of either the Thr or Tyr lead to a decrease in Ime2p activity suggesting that these might be sites of activating phosphorylation (13). Interestingly, phosphorylation and activation of Ime2p requires Cak1p, which also activates Cdc28p by phosphorylation of its activation loop. Aspects of this mechanism for activating Ime2p may be evolutionarily conserved in higher eukaryotes since ICK, a mammalian protein kinase with sequence similarity to Ime2p, can be directly phosphorylated by yeast Cak1p (14). Full modification of Ime2p is dependent on its own catalytic activity and Ime2p autophosphorylates in vitro (13).

Cdc28p activity is controlled by multiple mechanisms including phosphorylation and the binding of activators and inhibitors.
Ime2p Phosphomodification Pathway

vegetative growth, Cdc28p activity is down-regulated to allow cells to exit from the mitotic division (15). There are two pathways that work in conjunction to control exit from mitosis: the cdc twelve early anaphase release (FEAR) and the mitotic exit network (MEN) (15, 16). These pathways negatively regulate Cdc28p by controlling release of the Cdc14p dual-specificity phosphatase from the nucleus. Cdc28p must be active for execution of both meiotic divisions but it must also be down-regulated between meiosis I (MI) and meiosis II (MII) (4, 17). Data from several groups indicate that FEAR, and not MEN, is central to regulating the exit from MI (18–20). Cells harboring mutations in the FEAR network (SLK19, SPO12, and CDC14) are defective in down-regulating Cdc28p and disassembling the MI spindle. These cells complete a mixed MI/MII division on the persistent MI spindle and generate dyad rather than tetrad spores. Because cells lacking IME2 block early in the program more is known about its earlier roles than its later roles and it is unclear whether it participates in FEAR network regulation. Ime2p appears to be required for meiotic M phase; however, it is unclear how its activity is controlled during the meiotic divisions.

Here, we used mass spectrometry of Ime2p purified from meiotic cells and phosphopeptide mapping of in vitro autophosphorylation reactions to identify phosphoacceptor sites in Ime2p and address their significance in Ime2p regulation. We demonstrate that Thr^{242} and Tyr^{244} are phosphorylated in a Cak1p-dependent manner consistent with our previous genetic studies (13). Furthermore, we found that Ime2p autophosphorylates its activation loop. In addition, we found that the C-terminal regulatory domain of Ime2p is phosphorylated on Ser^{250} and Ser^{625} during a time in which cells are completing the meiotic divisions. Mutants lacking these phosphorylation sites show an increase in dyad formation and genetically interact with mutants in the FEAR network. These data provide insight into the mechanism of Ime2p activation and its regulation by phosphorylation during sporulation.

EXPERIMENTAL PROCEDURES

Strain and Plasmid Construction—The yeast strains and plasmids used in this study are described in Tables 1 and 2, respectively. All strains except for those harboring the galactose-inducible IME2 plasmids are in the SK1 genetic background. The IME2 mutant alleles were generated by site-directed mutagenesis (QuikChange, Stratagene) of pAM4, pMRC5, and pCM1. The Ser^{246} codon was mutated from TCC to GCC. These alleles were integrated in the chromosome by transforming the Atp1::URA3 strain KBY443 (a gift from Kirsten Benjamin) with Sac1I/Smal-digested pAM4-derived DNA and with Sac1I/Kpn1-digested pCM1-derived DNA followed by selection of FOA-resistant colonies. Plasmid pCM1 was generated by subcloning the SpeI/Kpn1 fragment from pAM4 into pRS316 cut with SpeI and Kpn1 and pMRC5 was generated by subcloning a SpeI/Kpn1-digested pCM1-derived DNA followed by selection of FOA-resistant colonies.

Media and Growth Conditions—Vegetative growth was maintained in YEPD (1% yeast extract, 2% peptone, 2% glucose), S.D. (0.67% yeast nitrogen base without amino acids, 2% glucose, plus nutrients essential for auxotrophic strains) or YEPA (1% yeast extract, 2% peptone, 2% potassium acetate). For synchronous sporulation, cells were grown in YEPA to an A_{600} of 0.3–0.5. Prior to sporulation, cells were harvested, washed in SM (2% potassium acetate plus 10 μg of adenine, 5 μg of histidine, 30 μg of leucine, 7.5 μg of lysine, 10 μg of tryptophan, and 5 μg of uracil per ml) and resuspended in SM at 2 OD/ml.

DNA Staining and Analysis—To monitor the meiotic divisions, 1 OD of cells was taken at various times, fixed in 90% EtOH, and stained with 1 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI). The completion of MI and MII were scored by counting the proportion of cells containing two DAPI-staining bodies and more than two DAPI-staining bodies observed by fluorescence microscopy. One hundred cells in 3–7 independent isolates per mutant were analyzed.

Protein Isolation and Analysis—Whole cell lysates were prepared as described previously (13). Samples were loaded on 7.5% polyacrylamide gels, run at 12 mA, transferred to Immobilon-P (Millipore), and blocked overnight at 4 °C in phosphate-buffered saline, 0.1% Tween-20 plus 1% (Tropix). Antibody staining was performed for 1 h at room temperature with a polyclonal antibody against Ime2p (1:10,000) (21). After washing, the membrane was incubated for 30 min at room temperature with a secondary anti-rabbit antibody (Promega, 1:7,500). The Myc epitope on Ime2p was detected with a 9E10 monoclonal antibody (Santa Cruz Biotechnology; 1:1,000) and the HA epitope was detected with a HA.11 monoclonal antibody (Babco; 1:5,000) and a secondary anti-mouse (Promega; 1:5,000). The secondary antibodies were conjugated to alkaline phosphatase and were detected by chemiluminescence (CDP-Star, Tropix).

Kinase Assays—Ime2p-myc kinase assays were conducted as described previously (13). Briefly, 20 ODs of sporulating cells were harvested and frozen in liquid nitrogen at 2 h post-induction. Next, the cells were lysed by 3 cycles of 1-min bursts of bead beating followed by 2 min on ice (500 μl of 0.5 μm glass beads, Sigma) in 500 μl of lysis buffer (50 mM HEPES, pH 7.4, 75 mM KCl, 1 mM EGTA, 1 mM MgCl_{2}, 0.1% Nonidet P-40, 50 mM NaF, 50 mM β-glycerolphosphate, 1 mM Na_{2}VO_{4}, 1 mM phenylmethylsulfonyl fluoride, 8.8 μg/ml aprotinin, 4 mg/ml antipain, 0.1 μg/ml pefabloc SC, 2 μg/ml pepstatin A, 1 μg/ml chymostatin, 1 mM benzamidine, and 2 μg/ml leupeptin). Protein from clarified supernatants were used for immunoprecipitation of Ime2p-myc with 20 μl of 1:1 slurry of protein A-agarose beads (Roche Applied Science) and 60 μl of anti-Myc antibody (A-14, Santa Cruz Biotechnology; 200 μg/ml) or anti-HA antibody (Y-11, Santa Cruz Biotechnology; 200 μg/ml) by gentle mixing at 4 °C for 8–24 h. Immunoprecipitates were collected and washed three times in lysis buffer and once in kinase buffer (20 mM HEPES, pH 7.4, 100 mM KCl, 10 mM MgCl_{2}). The immunoprecipitates were stored in a 1:1 mixture of kinase buffer and 100% glycerol at −20 °C until processing. Desired amounts of sample were removed from the stock, washed in kinase buffer, and resuspended in 25 μl of kinase buffer containing 5 μCi of [γ-^{32}P]ATP (PerkinElmer Life Sciences) and 1 μM cold ATP. The reactions were terminated after a 30-min incubation at 30 °C with the addition of SDS gel loading buffer followed by boiling. Samples were resolved on 7.5% polyacrylamide gels, dried, and processed by autoradiography or phosphorimaging (Molecular Dynamics).

Phosphoamino Acid Analysis—Ime2p-myc was isolated from sporulating cells by immunoprecipitation and autophosphorylated as described above. Protein isolation and hydrolysis was conducted as described by Boyle et al. (22). The autophosphorylated material was eluted from a dried 7.5% acrylamide gel by shaking overnight at 37 °C in 50 mM NH_{4}HCO_{3}, 0.1% SDS, and 0.5% 2-mercaptoethanol. The eluted protein was concentrated by precipitation with 20% trichloroacetic acid and washed in cold acetone prior to hydrolysis in 6 N HCl at 110 °C for 1 h. The hydrolyzed material was lyophilized and resuspended in 5 mM Tris-Cl, pH 8 containing 60 μg/ml of cold phosphoamino acid standards (Sigma). 5–8 μl of each sample were spotted onto plastic-backed, cellulose TLC plates (Kodak). Ascending chromatography was performed in a brick TLC tank pre-equilibrated with isobutyric acid-0.5 M NH_{4}OH (5:3, v/v). After drying, the cold standards were visualized with ninhydrin (0.25% in acetone; Sigma), baked at 65 °C for 15 min, and developed by either autoradiography or phosphorimaging.
Phosphopeptides were detected by phosphorimaging, and exposure was spotted onto glass-backed 100% trypsinic acid, was conducted in a glass-brick TLC tank pre-equilibrated with isobutymin at 1.0 kV (10–15 mA). After drying, ascending chromatography was subjected to electrophoresis in pH 1.9 buffer using a Hunter trophenyl-lysine and 1 mg/ml xylene cyanol FF as a standard. First, the sample was lyophilized and washed several times in pH 1.9 oxidized in ice-cold performic acid at 0 °C for 1 h and lyophilized. Next, the sample was lyophilized and washed several times in pH 1.9 —Autophosphorylated Ime2p was isolated and alkylated with 0.5M iodoacetamide in 0.1M NH4HCO3 prior to elution from the beads by boiling in a SDS-containing loading buffer and resolving on a 7.5% polyacrylamide gel.

Coomassie-stained Ime2p-myc bands were excised from the gel, washed, and destained in 50% methanol overnight, dehydrated in acetone, and rehydrated in the reducing and alkylating agents described above. MS analysis was performed at the W. M. Keck Biomedical Mass

**TABLE 1**

| Strain          | Genotype Source | Source   |
|-----------------|-----------------|----------|
| LNY65           | MATa/matr6::LEU2/imc2::LEU2 ura3/ura3 leu2::his3/leu2::his3 trp1::his3 trp1::his3 lys2/lys2 trp1::his3 trp1::his3 lys2/lys2 | Lenore Neighborne |
| AMY25           | LNY6s+pSM3      | This study |
| KSY443          | MATa ura3/ura3 leu2::his3/leu2::his3 trp1::his3 trp1::his3 faa1::his3 or trp1::his3 trp1::his3 faa1::his3 or trp1::his3 trp1::his3 lys2/lys2 | This study |
| KSY138          | MATa/matr6::LEU2/imc2::LEU2 ura3/ura3 leu2::his3/leu2::his3 trp1::his3 trp1::his3 lys2/lys2 | This study |
| KSY187          | MATa ura3/ura3 leu2::his3/leu2::his3 trp1::faa1::his3 or trp1::his3 trp1::faa1::his3 or trp1::his3 trp1::faa1::his3 lys2/lys2 | This study |
| KSY162          | KSY187 + ime2-K97R::myc | This study |
| KSY190          | KSY138 + ime2-K97R::myc | This study |
| KSY200          | MATa ura3/ura3 leu2::his3/leu2::his3 trp1::faa1::his3 or trp1::his3 trp1::faa1::his3 or trp1::his3 trp1::faa1::his3 lys2/lys2 | This study |
| KSY201          | KSY200 + pMR1   | This study |
| KSY216          | KSY187 + ime2-Y244F::myc | This study |
| KSY233          | MATa/imc2::LEU2/imc2::LEU2 ura3/ura3 leu2::his3/leu2::his3 trp1::faa1::his3 or trp1::his3 trp1::faa1::his3 or trp1::his3 trp1::faa1::his3 lys2/lys2 | This study |
| KSY268          | KSY233 + pMR5   | This study |
| KSY36          | KSY233 + pKS37  | This study |
| KSY318          | KSY187 + ime2-SS20A::myc::TRPI | This study |
| KSY319          | KSY187 + ime2-S625A::myc::TRPI | This study |
| KSY348          | KSY187 + ime2-SS20A/SS25A-myc::TRPI | This study |
| KSY373          | MATa/matr6::KANMX6::R19::KANMX6/imc2::SS20A/S625A-myc::TRPI/imc2::SS20A/S625A-myc::TRPI ura3::lys2/lys2 trp1::his3 trp1::his3 leu2::his3 leu2::his3 ho::LYS2 or ho::hisG | This study |
| KSY374          | MATa IME2-myc::TRPI ura3::lys2/lys2 trp1::his3 trp1::his3 leu2::his3 leu2::his3 ho::LYS2 or ho::hisG | This study |
| KSY377          | MATa IME2-myc::TRPI ura3::lys2/lys2 trp1::his3 trp1::his3 leu2::his3 leu2::his3 ho::LYS2 or ho::hisG | This study |
| KSY378          | MATa IME2-myc::TRPI ura3::lys2/lys2 trp1::his3 trp1::his3 leu2::his3 leu2::his3 ho::LYS2 or ho::hisG | This study |
| CMY31           | MATa/imc2::KANMX6::R19::KANMX6/SMK1-HA::KAN spm1::spm1::KAN ho::LYS2/ho::LYS2 or ho::hisG | This study |
| CMY32           | MATa/imc2::KANMX6::R19::KANMX6/SMK1-HA::KAN spm1::spm1::KAN ho::LYS2/ho::LYS2 or ho::hisG | This study |

**TABLE 2**

| Plasmid     | Markers | Source    |
|-------------|---------|-----------|
| pMR5        | IME2    | This study |
| pKS37       | imc2-SS20A/S625A | This study |
| pAM4        | IME2    | This study |
| pSM3        | pAM4 + ime2-S246A::myc | This study |
| pRK67       | 2µ + HIS3 + pIME2-SCI1 | This study |

**Phosphopeptide Mapping**—Autophosphorylated Ime2p was isolated and concentrated as described above. After washing, the protein was oxidized in ice-cold performic acid at 0 °C for 1 h and lyophilized. Next, the protein was digested overnight at 37 °C in 50 mM NH4HCO3 containing either 10 µg of TPCK-trypsin or chymotrypsin (Worthington). Finally, the sample was lyophilized and washed several times in pH 1.9 buffer (formic acid (88%), glacial acetic acid, and deionized water (25:78:897, v/v/v)) before resuspension in 5 µl of pH 1.9 buffer. The sample was spotted onto glass-backed 100 µm 20 × 20 cm microcrystalline cellulose TLC plates (EM Scientific) along with 5 µl of 5 mg/ml e-dinitrophenyl-lysine and 1 mg/ml xylene cyano FL as a standard. First, the plate was subjected to electrophoresis in pH 1.9 buffer using a Hunter thin-layer electrophoresis unit 7000 (HTLE, CBS Scientific, Inc) for 45 min at 1.0 kV (10–15 mA). After drying, ascending chromatography was conducted in a glass-brick TLC tank pre-equilibrated with isobutyric acid, n-butyl alcohol, pyridine, acetic acid, and water (65:2:5:3:29). Phosphopeptides were detected by phosphorimaging, and exposure times were adjusted so that the spots were of equal intensities to compare wild-type and catalytically weakened ime2p reactions.

**Protein Purification and Mass Spectrometry**—Three liters of wild-type and cacl1-Δ cells harboring a chromosomally-integrated allele of IME2-myc were grown overnight at 30 °C in YEPD to an OD of 0.5 prior to sporulation at 2 OD/ml in SM. After 7.5 h cells were harvested, washed, and concentrated in a small volume of ice-cold water plus 25 mM β-glycerophosphate, 1 mM Na3VO4, 100 µg/ml phenylmethylsulfonyl fluoride, and a mixture of protease inhibitors (8.8 µg/ml aprotinin, 4 mg/ml antipain, 0.1 µg/ml pefabloc SC, 2 µg/ml pepstatin A, 1 µg/ml chymostatin, 1 mM benzamidine, and 2 µg/ml leupeptin). The concentrated cells were frozen dropwise in liquid nitrogen prior to grinding into a fine powder with a mortar and pestle in liquid nitrogen and transferring into a 250-ml centrifuge tube (Cornin 430776). The cells were lysed in the centrifuge tube in 50 ml of lysis buffer (50 mM Tris-Cl, pH 7.4, 75 mM KCl, 1 mM EGTA, 1 mM MgCl2, 0.1% Nonidet P-40, 50 mM NaF, 50 mM β-glycerophosphate, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, and the protease inhibitor mixture described above) using a prechilled Polytron homogenizer. Next, the lysate was clarified by centrifugation at 31,000 rpm (110,000 × g) in a Beckman Ultracentrifuge Ti 50.52 for 1.5 h at 4 °C. The clarified lysate was pumped at 1 ml/min over a pre-equilibrated HiTrap Q FF ion exchange column from Amersham Biosciences. After washing with lysis buffer containing 0.2 M KCl, ime2p-myc was eluted in 15 ml of lysis buffer containing 0.4 M KCl. To further purify ime2p-myc, the eluate was immunoprecipitated overnight at 4 °C with 120 µl of protein A-agarose beads and 250 µl of anti-Myc antibody (A-14, Santa Cruz Biotechnology, 200 µg/ml). The immunoprecipitated proteins were reduced with 10 mM dithiothreitol in 0.1 M NH4HCO3 and alkylated with 0.5 mM iodoacetamide in 0.1 M NH4HCO3 prior to elution from the beads by boiling in a SDS-containing loading buffer and resolving on a 7.5% polyacrylamide gel.
Spectrometry Laboratory at the University of Virginia. Gel slices were dehydrated a second time, dried by vacuum centrifugation, rehydrated, and digested overnight at 37 °C in 20 mg/ml trypsin in 50 mM NH₄HCO₃. Peptides were extracted twice using 50% acetonitrile and 5% formic acid, pooled and injected into a Finnigan LCQ DecaXP ion trap mass spectrometer system with a Protagna nanospray ion source (operated at 2.8 kV) interfaced to a Phenomenex Jupiter 10-μm C18 reversed-phase capillary column. Peptides were eluted from the column by an acetonitrile/0.1 M acetic acid gradient at a flow rate of 0.25 μL/min. The digest was analyzed using acquired full mass spectra to determine the molecular weights of the peptides followed by 4 product ion spectra to determine amino acid sequence. The data were analyzed by Sequest search algorithm for matching peptides.

RESULTS

The Ime2p Activation Loop and C-terminal Regulatory Domain Are Phosphorylated in Vivo—To gain insight into how phosphorylation regulates Ime2p, we developed a method to purify phosphorylated Ime2p from sporulating cells suitable for phosphosite identification using mass spectral (MS) analysis. Overexpression of IME2 is lethal to mitotically growing cells and may have negative effects on sporulation (10, 21). Furthermore, we wanted to identify phosphorylation sites that are modified during wild-type sporulation conditions. We therefore chose to purify Ime2p using a strain in which Myc epitope coding information had been integrated at the chromosomal IME2 locus.

Cells of the rapidly sporulating SK1 genetic background were collected at 7.5-h post-induction, when the meiotic divisions are occurring and Ime2p is most abundant and hyperphosphorylated (Refs. 4 and 13 and Fig. 1). Extracts were prepared by mechanical disruption, clarified by ultracentrifugation, and passed over a S-Sepharose anion-exchange resin (see “Experimental Procedures”). Ime2p-myc was subsequently collected by a stepwise 0.2–0.4 M KCl elution and further purified by Myc immunoprecipitation followed by polyacrylamide gel electrophoresis. Coomassie-stainable amounts of Ime2p-myc obtained from wild-type and cak1-Δ cells (non-phosphorylated control) were analyzed by LC/MS. This analysis was conducted twice using three different proteases to maximize sequence coverage. 67% of Ime2p sequence purified from wild-type cells and 68% of Ime2p sequence purified from cak1-Δ cells were analyzed between the three protease-treated samples.

Both Thr²⁴² and Tyr²⁴⁴ were identified as phosphorylated residues in wild-type cells and not in cak1-Δ cells (Table 3 and supplementary Fig. S1). In the tryptic peptide analysis the activation loop was identified as a mixture of two phosphoforms: phosphorylated on Tyr²⁴⁴ alone and doubly phosphorylated on Thr²⁴² and Tyr²⁴⁴. These data are consistent with the Tyr²⁴⁴ being phosphorylated prior to Thr²⁴² phosphorylation (see “Discussion”). Furthermore, Tyr²⁴⁴ was also identified as phosphorylated in the chymotryptic analysis. These phosphorylated peptides were identified in two independent experiments and directly confirm our previous suggestion that these residues are phosphorylated in a Cak1p-dependent manner during sporulation (13).

Two serine residues located in the C terminus of Ime2p were also identified as phosphorylated in wild-type cells and not in the cak1-Δ control (Table 3 and supplementary Fig. S1). Phosphorylated Ser⁵²⁰ was identified in both the trypsin and AspN protease digestes. Both digestes contained a mixture of phosphorylated and non-phosphorylated Ser⁵²⁰-containing peptides (Table 3 and data not shown). Ser⁶²⁵ was identified as being in an exclusively phosphorylated form in the tryptic digest. Both the Ser⁵²⁰- and Ser⁶²⁵-phosphorylated peptides were identified in two independent experiments.

To confirm that Ser⁵²⁰ and Ser⁶²⁵ are sites of phosphorylation we generated single S520A and S625A and double S520A/S625A IME2 mutants and analyzed the electrophoretic migration of the encoded proteins by Western immunoblotting. For these studies we used IME2-containing plasmids that lack the Myc epitope because phosphoforms of untagged Ime2p are more readily resolved from one another than tagged Ime2p (data not shown). As previously reported, when Ime2p is induced in mitotic cells, it is rapidly phosphorylated in a CAK1-dependent fashion. Ime2p therefore migrates slightly slower than unphosphorylated Ime2p when cells are in meiotic phase and prophase (2–5 h post-induction) (38). Hyperphosphorylated forms of Ime2p begin to accumulate around 5–6 h (Fig. 1 and Ref. 13). At this time, the overall level of Ime2p increases as active Ndt80p accumulates, and cells enter MI. Ime2p levels begin to decline (at ~10 h) as cells complete MII and initiate spore formation. Mutation of Ser⁵²⁰ and Ser⁶²⁵ to alanines, both singly and in combination, did not reproducibly affect the timing of Ime2p production, its electrophoretic mobility at early (2–5 h) time points (Fig. 1), or its disappearance after MII has been completed (data not shown). However, the electrophoretic mobilities of these mutant proteins were different than the wild type at the 6–10 h time points, with relative amounts of the slowest migrating forms of Ime2 being reduced in abundance (Fig. 1 and data not shown). More modest electrophoretic migration differences were seen in the single mutant samples; however, the electrophoretic mobility of the double S → A mutant is most similar to the SS20A single mutant (data not shown). The mobilities of the mutant Ime2p proteins were indistinguishable from wild type when expressed in cak1-Δ cells, where Ime2p is not phosphorylated (data not shown, Ref. 13) demonstrating that these mutations do not simply change the electrophoretic migration of Ime2p. The Ime2p mobility differences we observed are consistent with the MS data and confirm that Ser⁵²⁰ and Ser⁶²⁵ are phosphorylated during sporulation. The time when the mobility differences are observed corresponds to the interval when the meiotic divisions occur in this strain background (see Fig. 5). Because the doubly mutant protein is still partially modified

| Protease | Peptide | Mass | Indicated residue |
|---------|---------|------|------------------|
| Trypsin | 234-248 | 1858.84 | Tyr²⁴⁴ |
| Trypsin | 234-248 | 1938.81 | Thr²⁴² and Tyr²⁴⁴ |
| Trypsin | 504-525 | 2630.3 | Ser⁵²⁰ |
| Trypsin | 504-525 | 2644.24 | Ser⁵²⁰ |
| Trypsin | 616-626 | 1396.77 | Ser⁶²⁵ |
| Chymotrypsin | 242-249 | 1063.5 | Tyr²⁴⁴ |
| Chymotrypsin | 242-250 | 1226.5 | Tyr²⁴⁴ |
| AspN | 518-529 | 1477.7 | Ser⁶²⁵ |

Fig. 1. The electrophoretic mobility of Ime2p-S520A/S625A is different from wild type. Protein was isolated from whole cell lysates of sporulating cells at the indicated times from imd2-Δ diploid cells (KSY233) containing plasmids encoding wild-type or S520A/S625A IME2 alleles and probed with a polyclonal antibody against Ime2p. The asterisk indicates a cross-reacting species used as a loading control.

With these results in hand, we proceeded to map the phosphorylation sites in detail. To identify sites of phosphorylation, purified myc epitope tagged Ime2p from wild-type cells was subjected to trypsin, chymotrypsin and AspN protease digestions. The peptides were extracted, reverse phase purified, and analyzed by mass spectrometry. Spectrometry spectra can be found in Fig. S1. Ionization spectra can be found in Fig. S1.

Phosphorylated in Vivo

Myc immunoprecipitation followed by polyacrylamide gelelectrophoresis from wild-type cells and 68% of Ime2p sequence purified from cak1-Δ and sid1/sis. Coomassie-stainable amounts of Ime2p-myc obtained from wild-type cells and not in cak1-Δ.

| Protein was isolated from whole cell lysates of sporulating cells at the indicated times from imd2-Δ diploid cells (KSY233) containing plasmids encoding wild-type or S520A/S625A IME2 alleles and probed with a polyclonal antibody against Ime2p. The asterisk indicates a cross-reacting species used as a loading control. | IME2 | ime2-S520A, S625A |
|---|---|---|
| | 2 hrs | 3 hrs | 4 hrs | 5 hrs | 6 hrs | 7 hrs | 8 hrs |
| | 2 hrs | 3 hrs | 4 hrs | 5 hrs | 6 hrs | 7 hrs | 8 hrs |
| * | Ime2p | Ime2p |

3 K. Schindler, unpublished observations.
Ime2p autophosphorylates its activation loop. Two-dimensional phosphopeptide maps were generated from in vitro autophosphorylated Ime2p. Tryptic (A) and chymotryptic (C) phosphopeptide maps of Ime2p-myc (KSY187). Tryptic (B) and chymotryptic (D) phosphopeptide maps of Ime2p-Y244F-myc (KSY216). The origins are marked with a circle, and the major spot is marked with an arrow.

Ime2p autophosphorylation is relevant to understanding the Ime2p phosphomodification pathway. We used thin-layer electrophoresis followed by chromatography to generate two-dimensional tryptic and chymotryptic phosphopeptide maps of Ime2p autophosphorylation reactions (see “Experimental Procedures”). The tryptic phosphopeptide map of the wild-type autophosphorylated protein showed that Ime2p autophosphorylates primarily on one peptide and several minor peptide species (Fig. 2A). The electrophoretic migration of the major peptide is consistent with the predicted migration of the doubly phosphorylated activation loop peptide (residues 239-NPYTAYVSTR-248). To test whether the major autophosphorylated peptide corresponds to the Ime2p activation loop, we analyzed Ime2p-Y244F autophosphorylation reactions (Fig. 2B and D). This mutant protein autophosphorylates to a decreased level (\(-20\%\) of wild-type) and a homozygous ime2-244F strain shows a decreased ability to promote sporulation consistent with Tyr244 being a site of activating phosphorylation (13). Because the Y244F mutant auto-phosphorylates with a reduced efficiency, we extended the exposure times of these TLC plates to compare the peptide positions with the positions on the wild-type plates. The mutant Y244F activation loop protein also autophosphorylated on one major tryptic peptide; however, the mutant peptide migrated further than the wild-type peptide in the chromatographic dimension (compare Fig. 2A to B). The chromatographic mobilities of the major autophosphorylated Y244F mutant and wild-type peptides were also different in the chymotryptic analysis (compare Fig. 2C to D). These changes in chromatographic migration are consistent with the difference in hydrophobicities between phosphotyrosine and phenylalanine, suggesting that Ime2p autophosphorylates its own activation loop in vitro. These data are also consistent with the MS data demonstrating that Tyr244 is phosphorylated (Table 3). We note that the interpretation of the chymotryptic map in Fig. 2C is most likely complicated by chymotrypsin-inefficient cleavage after phosphotyrosine. Changing Tyr244 to a Phe alleviates this complication making the interpretation of Fig. 2D more straightforward.

The activation loop peptide contains 2 threonines (Thr242 and Thr247), 2 tyrosines (Tyr241 and Tyr244), and a single serine (Ser246). Phosphoamino acid analyses demonstrate that only threonine and serine, in a roughly 2:1 ratio, are autophosphorylated (Fig. 3). Thus, the tyrosines are not sites of autophosphorylation. Furthermore, the activation loop serine is not a site of autophosphorylation because both the phosphothreonine:phosphoserine ratio and the total efficiency of autophosphorylation was indistinguishable between the wild type and S246A mutant (Fig. 3 and data not shown). The electrophoretic migration of the major autophosphorylated tryptic peptide in the wild-type Ime2p reaction is inconsistent with it containing more than 2 phosphates. Mutation of Tyr244 to a Phe introduces a chymotryptic recognition site between Thr242 and Thr247. Importantly, the chymotryptic phosphopeptide map of this mutant contains only a single major autophosphorylated peptide (Fig. 2D). Therefore, these data demonstrate that either Thr242 or Thr247 is the major autophosphorylation site. We were not able to unambiguously distinguish the chymotryptic Tp-AF-(242–244) peptide versus a VS-Tp-RW-(245–249) peptide based solely on their predicted mobilities. Both the T242A and T247A mutations almost completely abolish Ime2p autocatalysis in vitro, and therefore we are unable to generate phosphopeptide maps for these mutant alleles (Ref. 13 and data not shown). We have previously demonstrated that premeiotic S and M phases are delayed by \(-3\) h in a homozygous
Ime2p Phosphomodification Pathway

$\text{IME2-myc}$ | WT | K-R | K-R | K-R | WT | K-R
---|---|---|---|---|---|---
$\text{IME2}$ | WT | WT-2μ | WT | WT | WT | Δ

| 1 | 2 | 3 | 4 | 5 | 6 |
---|---|---|---|---|---|

**FIGURE 4.** Ime2p autophosphorylation in cis is required for accumulation of hyperphosphorylated Ime2p. Whole cell lysates were prepared from sporulating cells of the indicated genotype at 4-h post-induction. Ime2p-myc protein was analyzed by immunoblot analysis with an antibody against the Myc epitope.

*ime2-T242A* mutant (13). Importantly, our MS data identified Thr$^{242}$ and not Thr$^{247}$ as being a site of phosphorylation in *in vivo* (Table 3). Taken as a whole, the data indicate that Thr$^{242}$ is the major site of Ime2p autophosphorylation and that this reaction positively regulates Ime2p. Interestingly, this residue aligns with the residue in Cdc28p that is directly phosphorylated by Cak1p (Thr$^{169}$) (Refs. 23 and 24; see "Discussion"; Table 3). The minor species in the phosphopeptide maps represent 1 or more peptides that are autophosphorylated on serine and may also represent improperly cut activation loop peptides or activation loop peptides containing various amounts of incorporated phosphate (Fig. 2). The positions of some of the minor species are different in the wild type and Y244F mutant samples, suggesting that these are autophosphorylated activation loop peptide products. The incorporation of radioactivity in these species was close to background levels; therefore we did not further investigate their identity.

Because the autophosphorylated serine in Ime2p is located outside of the activation loop, we asked whether Ime2p autophosphorylates Ser$^{520}$ or Ser$^{625}$, which were identified in the MS experiments. We found that a double mutant Ime2p-S520A/S625A protein still contained the same 2:1 ratio of phosphothreonine:phosphoserine seen in wild-type Ime2p autophosphorylation reactions (Fig. 3). Furthermore, the tryptic phosphopeptide maps of the single and double serine mutants were indistinguishable from wild type (data not shown). These data suggest that Ime2p does not autophosphorylate Ser$^{520}$ or Ser$^{625}$ in *in vitro*, consistent with another kinase phosphorylating these residues.

**Ime2p Autophosphorylation Controls Ime2p in Vivo and May Occur via an Intramolecular Mechanism**—We previously demonstrated that Ime2p is activated by phosphorylation in a Cak1p-dependent fashion (13). Following activation, Ime2p is sequentially phosphorylated on multiple residues and accumulates in a hyperphosphorylated state around the time when the meiotic divisions are occurring. In this same study we showed that a catalytically inactive form of Ime2p fails to become hyperphosphorylated. However, because active Ime2p is required for meiotic S phase (a process that occurs prior to its hyperphosphorylation), these studies did not establish whether the failure of inactive Ime2p to become hyperphosphorylated reflects a direct requirement of Ime2p catalytic activity or a consequence of the *ime2* mutant blocking early in the program. To gain further insight into the role of Ime2p autophosphorylation in vivo, we compared the phosphorylation of Myc-tagged wild type and catalytically inactive (K97R) Ime2p expressed alone, or in combination with wild-type Ime2p that lacked the Myc epitope. As previously reported, Ime2p-myc from a *IME2-myc/IME2-myc* homozygous diploid strain migrated more slowly than Ime2p-myc from cells lacking $CAK1$ (Fig. 4; compare lanes 1–5). Ime2p-myc from the homozygous control strain migrated slower than Ime2p-K97R-myc (Fig. 4; compare lanes 1, 4, and 6). Importantly, when Ime2p-K97R-myc was made from a heterozygous *ime2-K97R-myc/IME2* strain it was never modified like wild type, despite the fact that these cells completed sporulation (Fig. 4; compare lane 1 to lane 2). Even when wild-type IME2 was produced from a multicopy plasmid, Ime2p-K97R-myc was not hyperphosphorylated (Fig. 4; lane 3). These results suggest that the autophosphorylation of Ime2p plays an essential role in the Ime2p phosphomodification pathway *in vivo*. These data further suggest that Ime2p may be autophosphorylated by an intramolecular mechanism.

**Phosphorylation of Ser$^{520}$ and Ser$^{625}$ Regulate Ime2p**—To study the regulatory role that phosphorylation of Ser$^{520}$ and Ser$^{625}$ has on Ime2p and sporulation we generated strains containing chromosomally integrated serine-to-alanine substitutions at these positions, both singly and in combination. The single and double mutants completed meiosis, formed viable spores, and performed wild-type levels of genetic recombination at the HIS4 recombination hot spot (data not shown). Interestingly, we found a 3-fold increase in the frequency of dyads formed by the single and double mutant cells compared with wild type (Fig. 6A). In addition, we found that 9% ± 3% of the mutants cells formed ascii with greater than four spores whereas <0.1% of wild-type cells formed asci with more than four spores. This supernumerary spore phenotype can occur after extra rounds of premeiotic DNA synthesis and was initially reported in genetic backgrounds where Cdc28p activity was deregulated (25, 26). Because Ime2p controls Cdc28p activity to stimulate meiotic S phase, it is possible that Cdc28p/Cln5p and Cln6p activity is elevated in the *ime2-S520A/S625A* mutant. Using the approach described by Strich et al. (43) we attempted to further up-regulate Cdc28p by overexpressing *CLB1* using the strong *ENO1* promoter in our IME2 mutant background but found no further increase in the frequency of ascis with more than four spores (data not shown). These results indicate that phosphorylation of Ser$^{520}$ and Ser$^{625}$ is not essential for sporulation but may play a role in controlling interactions between Ime2p and Cdc28p.

We also monitored the time at which single and doubly mutant cells completed MI and MII by counting cells stained with DAPI (Fig. 5). Wild-type cells initiated MI between 5 and 6 h post-induction. As the percentage of cells that have completed MI peaks, cells that have completed MI begin to accumulate. In contrast to wild-type cells, both single and double *ime2* mutants show an increased accumulation of cells in MI. The accumulation of MI cells was more severe in the S520A mutant than in the S625A-containing cells (compare Fig. 5, A to B). Notably, the interval separating MI and MII was increased in the phosphorylation site mutants. The increase in the MI peak and delay in MII suggest there may be a defect in regulating exit from MI. This phenotype is characteristic of mutants in the meiotic FEAR network that regulates Cdc28p activity (Refs. 17, 19, and 20 and see below). In addition, we note that the *ime2-S520A/S625A* mutant show a modest but reproducible advancement in the timing of MI (Fig. 5C).

**The Ime2p C-terminal Phosphorylation Mutants Genetically Interact with Mutants in the FEAR Network**—The IME2 C-terminal phosphorylation site mutant alleles were introduced into three FEAR-network mutants, and the percentage of cells completing one or two meiotic divisions was analyzed. Consistent with previously published data, *slk19-Δ* sporulation cultures contained a mixed population of cells completing one and two divisions (Fig. 6B and Refs. 17 and 20). The *ime2-S520A/S625A* allele substantially increased the frequency of cells completing a single division in the *slk19* genetic background (from 26–69%). The temperature-sensitive *cdc14-1* mutant sporulated at the semi-permissive temperature of 30 °C, also contained a mixed popula-

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tion of cells completing 1 and 2 divisions. The ime2-SS20A/S625A allele further increased the frequency of single division cells (from 48–72%), similar to results with the slk19 mutant. Deletion of SPO12 caused the tightest FEAR phenotype with almost all cells completing a single division. The double ime2-S520/S625A spo12-/H9004 mutant had the same percentage of cells that completed a single division as the spo12-/H9004 single mutant. It is likely that the severe MI-exit defect of the spo12 mutant makes it difficult to observe any further difference when IME2 is mutated in this genetic background. Interestingly, both of the single IME2 serine mutant alleles in the slk19-/H9004 and cdc14-1 genetic backgrounds also increased the frequency of cells only able to complete 1 division. This data suggests that phosphorylation at both Ser520 and Ser625 function to regulate Ime2p during meiotic M phase and is consistent with the time at which we purified Ime2p for MS analysis. Taken together, these results suggest that Ime2p may function to regulate exit from MI. Because MI exit is, in part, controlled by down-regulating Cdc28p activity, these data also imply that Ime2p modulates Cdc28p activity during meiotic M phase.

A recent report from Kamieniecki et al. (19) suggested that the Cdc14p phosphatase controls Cdc28p activity between MI and MII through stabilizing the CDK inhibitor, Sic1p. This group found that expression of SIC1 driven by the sporulation-specific IME2 promoter suppressed the “FEAR phenotype” of the slk19-/H9004 mutant. This mutant expressing SIC1 contained fewer cells that blocked after a single division than slk19-/H9004 strains without the Sic1p plasmid. Ime2p regulates the destruction of Sic1p during meiotic S phase; however, it is unknown if it continues to regulate Sic1p later in the program (4, 27). We asked whether a multicopy plasmid expressing SIC1 from the IME2 promoter could suppress the dyad phenotype observed in the ime2 phosphorylation site mutant but found no difference in the frequency of cells that

**FIGURE 5.** ime2-SS20A and S625A mutants show defects in meiotic progression. Completion of meiosis I (MI) and meiosis II (MII) were monitored by fixing and DAPI staining cells at the indicted times during sporulation in IME2 (KSY187) (A–C), ime2-SS20A (KSY318) (A), ime2-S625A (KSY319) (B), and ime2-SS20A/S625A (KSY348) (C). Completion of MI (2 DAPI-staining bodies) and MII (more than 2 DAPI-staining bodies) was scored by fluorescence microscopy (n = 7).
formed dyads (data not shown). This result suggests that Ime2p may be regulating meiotic M phase and Cdc28p in a Sic1p-independent mechanism or through multiple substrates.

**DISCUSSION**

Ime2p is a key meiosis-specific protein kinase that controls multiple steps in meiotic development. It is produced early in meiosis and is subsequently activated by phosphorylation in a Cak1p-dependent fashion (13). Later, it is phosphorylated on multiple residues as cells progress through the program. Ime2p accumulates in a hyperphosphorylated state during meiotic M phase and is subsequently degraded. It is likely that the phosphorylation of Ime2p not only activates its enzymatic activity, but also regulates multiple meiotic functions of the activated kinase. In this study we analyzed autophosphorylation reactions and used mass spectrometry of Ime2p purified from meiotic cells to identify residues in Ime2p that are phosphorylated. The data show that the activation loop of Ime2p is phosphorylated at Thr242 and Tyr244 in a Cak1p-dependent manner, consistent with our previously published genetic findings (Ref. 13 and Table 3). In addition, we show that Ime2p autophosphorylates its activation loop (Fig. 2). Finally, we found that Ser520 and Ser625 located in the C-terminal regulatory domain of Ime2p are phosphorylated when cells are undergoing the meiotic divisions and that mutants lacking phosphorylatable residues at these positions display modest defects in the timing of the meiotic divisions and genetically interact with FEAR network mutants (Fig. 5). These results are relevant to understanding how phosphorylation controls Ime2p during meiosis and demonstrate a previously unrecognized role for Ime2p in regulating the transition between MI and MII.

**Ime2p Activation**—Many protein kinases are activated by phosphorylation of activation loop residues that cause local and global structural changes that promote catalytic activity and substrate interactions (28). Thr242 and Tyr244 in Ime2p align with the sites of activating phosphorylation (TXY motifs) in MAPKs. Furthermore, Ime2p and MAPKs share similar catalytic cores (5 of the 10 most similar yeast proteins identified by BLAST comparisons are MAPKs). Structural studies have demonstrated that phosphorylation of the TXY motif in the mammalian ERK2 MAPK changes the conformation of the activation loop and activates the enzyme by causing changes to the active site and substrate.
specificity pocket (29). The sequence similarity between Ime2p and MAPKs and the demonstration that the TXY motif in Ime2p is phosphorylated suggest that Ime2p is activated by structural changes that are comparable to the changes seen in ERK2. Despite these similarities, the pathway that activates Ime2p does not appear to require a dual specificity MAPKK-like enzyme (see below).

Although phosphorylation by upstream kinases is generally thought to activate many kinases, several recent reports demonstrate that auto-phosphorylation plays an important role in activation among a diverse group of kinases (30–33). This study indicates that Ime2p autophosphorylates its activation loop at Thr\(^{242}\). In a previous study, we demonstrated that Ime2p-T242A purified from meiotic cells showed dramatically reduced autophosphorylation in vitro (less than 5% of the level seen in the wild-type enzyme) (13). The ability of the Ime2p-Y244F protein to autophosphorylate was more modestly reduced (20% of the level seen in the wild-type). In contrast to the in vitro results, ime2-T242A mutant cells were still capable of forming spores (albeit with reduced efficiency and kinetics compared with wild-type cells) whereas the ime2-Y244F mutant failed to form spores. Thus, there was not a correlation between the autocatalytic readout and in vivo function in this previous study. Our demonstration that the major site of Ime2p autophosphorylation is in its activation loop provides an explanation for this apparent paradox. These data indicate that the T242A substitution dramatically reduced autophosphorylation not because it was catalytically inactive but because it lacked the major phosphoacceptor site. Our results suggest that even in the absence of Tyr\(^{244}\) phosphorylation that Thr\(^{242}\) can be phosphorylated via an autocatalytic mechanism. We infer that autophosphorylation of Thr\(^{242}\) promotes the Ime2p activity based on the phenotypes of the T242A mutant but suggest that this reaction is insufficient to efficiently promote sporulation in the absence of Tyr\(^{244}\) phosphorylation.

The in vitro reactions indicate that autophosphorylation of Thr\(^{242}\) is reduced by ~80% in the Y244F mutant (13).\(^3\) This mutant shows a similar decrease in phosphorylation of Ndt80p, a known Ime2p substrate, indicating that this substitution impairs autophosphorylation and phosphorylation of substrates to similar extents. These results suggest that Tyr\(^{244}\) phosphorylation promotes Thr\(^{242}\) autophosphorylation. Based on these data we propose that phosphorylation of Tyr\(^{244}\) by an upstream kinase is a key initiating step that must occur in order to activate Ime2p, and that Ime2p autophosphorylates its activation loop on Thr\(^{242}\) to further enhance its activity. The finding that the Ime2p activation loop is phosphorylated on Tyr\(^{244}\) alone, on both Thr\(^{244}\) and Thr\(^{242}\), but not on Thr\(^{242}\) alone is consistent with a 2-step activation model (34). Interestingly, we find that a catalytically inactive form of Ime2p fails to be fully modified by phosphorylation in sporulation-competent cells that contain wild-type Ime2p even when Ime2p is overproduced (Fig. 4). This result suggests that Ime2p phosphorylates itself through an obligatory intramolecular mechanism and that autophosphorylation is required for it to become fully hyperphosphorylated. These data do not necessarily indicate that the majority of the phosphorylated residues in Ime2p are the consequence of intramolecular autophosphorylation. It is possible that an early autocatalytic event, or the acquisition of catalytic activity is required for Ime2p to become associated with additional proteins that are necessary for it to become further modified by other protein kinases. Additional studies will be necessary to elucidate the underlying mechanisms that couple catalytic activity of Ime2p to its hyperphosphorylation.

Which kinase phosphorylates Tyr\(^{244}\)? Both genetic and biochemical studies indicate that Ime2p produced in mitotic cells can become activated suggesting that the activating kinase is not meiosis-specific (10, 35). RIM11 encodes a dual-specificity protein kinase found in both mitotic and meiotic cells that is required for meiosis (36). RIM11 genetically interacts with IME2 and is an attractive candidate for an activating kinase. However, Ime2p ectopically expressed in mitosis is equally active in rim11Δ and wild-type cells as assayed using autokatalysis (see supplementary data). These data indicate that Rim11p is not required for Ime2p activity during sporulation. In addition, mutants lacking the Ste7p, Mkk1p, Mkk2p, or Pbs2p MAPKKs are able to sporulate indicating that they are not uniquely required for Ime2p activation.\(^4\) Whereas Cak1p has thus far been shown to activate protein kinases via threonine phosphorylation, it has also been shown to phosphorylate a synthetic substrate on tyrosine (37). One possibility is that Cak1p, which is present in mitotic cells but transcriptionally up-regulated in meiosis, directly phosphorylates Ime2p on Tyr\(^{244}\). Ime2p is most similar to a family of mammalian protein kinases that include male-germ cell associated kinase (MAK) and intestinal cell kinase (ICK), which show significant similarity to both CDKs and MAPKs throughout their catalytic cores (14). These enzymes also contain a TXY motif in their activation loops. Recent work by Fu et al. (14) demonstrates that ICK autophosphorylates on tyrosine in its TXY motif and that the threonine is phosphorylated by an upstream kinase. The yeast Cak1p enzyme is able to phosphorylate the ICK activating threonine residue. Thus, while Cak1p-mediated phosphorylation and autophosphorylation can activate both mammalian ICK and yeast Ime2p, the Cak1p target site and secondary autophosphorylated residue appear to be reversed in the 2 kinases. The phosphoamino acid analysis showed that Ime2p autophosphorylates on both threonine and serine (Fig. 3). Our results show that Ime2p does not phosphorylate itself at Ser\(^{246}\) in its activation loop or at Ser\(^{220}\) or Ser\(^{625}\), the residues in the C terminus of Ime2p identified as phosphorylated in our MS analysis. Truncation mutants lacking the first 18 or the last 190 amino acids are still able to autophosphorylate on serine in vitro (data not shown). This suggests that the autophosphorylated serine(s) is located within the catalytic core or a loop region in the kinase. These data also suggest that another kinase(s) functions in the Ime2p phosphomodification pathway that phosphorylates Ime2p at Ser\(^{220}\) and Ser\(^{625}\) (see below).

\(^3\) E. Winter, unpublished data.
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wild-type Ime2p and the Ime2p-S520A/S625 isolated from sporulating cells at different times post-induction using Ndt80p as a substrate (data not shown). Phosphorylation at these sites may therefore alter the activity of Ime2p by influencing its subcellular localization and/or substrate specificity instead of controlling gross catalytic activity.

Because it appears that Ser520 and Ser625 are not autophosphorylated in vitro, we suggest that another kinase(s) phosphorylates the C terminus of Ime2p at these positions. Ser520 lies within a potential Polo kinase consensus sequence ((D/E)X(S/T)DX(D/E)) (X14DHSLSN25) where the acidic residue at position +2 and hydrophobic residue at −1 are the most critical for recognition (38). CDC5, the budding yeast Polo homolog, is transcriptionally induced as the nuclear divisions are occurring. Cdc5p has also been shown to positively regulate the FEAR network in mitosis (39, 40). Furthermore, defects in the accumulation of higher molecular weight forms of Ime2p in cdc5 mutants have been described (40). One of the many functions of Cdc5 may therefore be to phosphorylate Ime2p at Ser520. Ser625 is located within a highly basic region of the protein (623KKSRE628) and, to our knowledge, is not located within an obvious phosphorylation consensus motif.

Ime2p and Cdc28p are key interacting members of the protein kinase network that governs meiotic development (3). Cdc28p is controlled by activating and inactivating phosphorylation and by the binding of activator and inhibitor molecules. Ime2p is required for multiple meiotic events but little is known about its regulation. We propose that a phosphomodification pathway exists that is central to the activation and regulation of this enzyme. Ime2p activation is the first step in this pathway and requires Cak1p and a TXY motif in its activation loop. The phenotypes that we observed in our C-terminal phosphosite mutants show that specific regulatory modifications occur in this region of Ime2p. Because Ime2p is central to meiotic progression deciphering this pathway will be key to understanding how meiosis is controlled.

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