STT4 Is an Essential Phosphatidylinositol 4-Kinase That Is a Target of Wortmannin in Saccharomyces cerevisiae*

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Wortmannin is a natural product that inhibits signal transduction. One target of wortmannin in mammalian cells is the 110-kDa catalytic subunit of phosphatidylinositol 3-kinase (PI 3-kinase). We show that wortmannin is toxic to the yeast Saccharomyces cerevisiae and present genetic and biochemical evidence that a phosphatidylinositol 4-kinase (PI 4-kinase), STT4, is a target of wortmannin in yeast. In a strain background in which stt4 mutants are rescued by osmotic support with sorbitol, the toxic effects of wortmannin are similarly prevented by sorbitol. In contrast, in a different strain background, STT4 is essential under all conditions and wortmannin toxicity is not mitigated by sorbitol. Overexpression of STT4 confers wortmannin resistance, but overexpression of PIK1, a related PI 4-kinase, does not. In vitro, the PI 4-kinase activity of STT4, but not of PIK1, was potently inhibited by wortmannin. Overexpression of the phosphatidylinositol 4-kinase STT4 conferred wortmannin resistance, as did deletion of phospholipase C-1. These observations support a model for a phosphatidylinositol metabolic cascade involving STT4, MSS4, and phospholipase C-1 and provide evidence that an essential product of this pathway is the lipid phosphatidylinositol 4,5-bisphosphate.

Small cell-permeable compounds have proven valuable tools to study signal transduction. For example, studies on the mechanism of action of the immunosuppressive antifungal natural products cyclosporin A and FK506 led to the identification of the protein phosphatase calcineurin as a critical calcium sensor during T-cell activation and physiological responses in yeast cells. Related studies of another natural product, the immunosuppressant rapamycin, revealed a role for the TOR kinase homologs in regulating cell proliferation in both yeast and mammalian cells (reviewed in Refs. 1 and 2).

Wortmannin is a hydrophobic steroid-related natural product of the fungus Talaromyces wortmannii (reviewed in Ref. 3). Wortmannin is an immunosuppressive and anti-inflammatory agent that inhibits signal transduction events in a variety of cell types. For example, wortmannin inhibits neutrophil and platelet activation by a variety of ligands, such as leukotriene B4, platelet-activating factor, N-formyl-Met-Leu-Phe, and thromboxane, which act via G-protein-coupled receptors (4, 5).

In addition, wortmannin also blocks insulin stimulation of glucose uptake in adipocytes (6). Thus, wortmannin can block signal transduction events emanating from either G-protein-coupled or tyrosine kinase receptors, which signal through distinct pathways. Wortmannin does not affect immediate signal transduction events in these pathways, such as tyrosine phosphorylation, production of inositol trisphosphate (IP3),1 or Ca2+ mobilization. That stimulation of protein kinase C by phorbol ester overcomes the inhibitory effects of wortmannin (7, 8) suggests that wortmannin acts upstream of protein kinase C, possibly at a point at which G-protein-coupled and tyrosine kinase signaling pathways converge.

Several observations suggested that a relevant in vivo target of wortmannin might be an enzyme, namely the lipid kinase that phosphorylates the 3-position of phosphatidylinositol: PI 3-kinase. First, wortmannin blocks antigen-dependent stimulation of PI 3-kinase activity in rat basophils (4). Second, wortmannin markedly inhibits phosphatidylinositol 3,4,5-trisphosphate production in neutrophils stimulated with N-formyl-Met-Leu-Phe, consistent with a block in phosphorylation of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) by PI 3-kinase (5). Third, the ability of insulin to stimulate PI 3-kinase activity in adipocytes is inhibited by wortmannin (6). In mammalian cells, PI 3-kinase is a heterodimer composed of a 110-kDa catalytic subunit and an 85-kDa regulatory subunit that interacts with other signal transduction elements via SH2 domains (9). The activity of purified PI 3-kinase is potently inhibited in vivo and in vitro by wortmannin (4, 5, 10). Finally, using anti-wortmannin antibodies and protease digestion, it has been shown that wortmannin forms a covalent complex with an active site residue of the 110-kDa PI 3-kinase catalytic subunit, lysine 802 (4, 11).

Although wortmannin potently inhibits the PI 3-lipid kinase with an IC50 of 5 nM, other potential targets exist. For example, wortmannin inhibits a protein kinase, myosin light chain kinase, with an IC50 of ~20 nM (12). In addition, wortmannin also inhibits DNA-dependent protein kinase, a member of the PI 3/4-kinase superfamily, with an IC50 of 200 nM (13). Deme-thoxysviridin, a structural analog of wortmannin, inhibits a membrane-associated PI 4-kinase from the fission yeast Schizosaccharomyces pombe (IC50 = 100 nM) (14), whose identity has not been established. Recently a wortmannin-sensitive, membrane-associated PI 4-kinase, shown previously to maintain hormone-regulated PI4P pools in mammalian cells (15–17), was cloned from a human cDNA library (18). Thus, wortmannin inhibits several protein and lipid kinases.

Previous studies of antifungal natural products reveal that

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1 The abbreviations used are: IP3, inositol trisphosphate; PI, phosphatidylinositol; PI4,5P2, phosphatidylinositol 4,5-bisphosphate; PI(4,5)P2, phosphatidylinositol 4-phosphate; PI3P, phosphatidylinositol 3-phosphate; PCR, polymerase chain reaction; PLC, phospholipase C; HA, hemagglutinin; GAL, galactose.
both the mechanisms of action as well as drug targets are often highly conserved from unicellular to multicellular eukaryotes. We report here our studies on the mechanism of action and targets of wortmannin in the budding yeast Saccharomyces cerevisiae. Our genetic and biochemical evidence indicates that a target of wortmannin in yeast is the PI 4-kinase STT4 (19). Conditions that rescue cells lacking STT4 also overcome wortmannin toxicity, overexpression of STT4 renders cells wortmannin-resistant, and the PI 4-kinase activity of STT4 is inhibited by wortmannin in vitro. The second yeast PI 4-kinase, PIK1 (20–22), does not appear to be a target of wortmannin in vivo, and the PI 4-kinase activity of PIK1 is not sensitive to wortmannin in vitro. STT4 is tightly associated with a membrane-pellet fraction. In addition, we report that STT4 is essential in some yeast strains. Because wortmannin inhibition causes a nonspecific cell cycle arrest, STT4 function may be required throughout the cell cycle. Mammalian homologs of both yeast STT4 and PIK1 have been recently cloned (15, 18, 23). Curiously, the mammalian PIK1 homolog, and not the mammalian STT4 homolog, is wortmannin-sensitive in vitro. We also find that overexpression of the yeast PI4F 5-kinase homolog MSS4 or deletion of the yeast gene encoding phospholipase C (PLC1) confers wortmannin resistance, supporting a model in which the inhibitory effects of wortmannin may be due to depletion of an essential PI4,5P2 pool. Our studies underscore the utility of yeast as a model system to identify drug targets, suggest caution in the use of wortmannin as a specific inhibitor of mammalian PI 3-kinase, reveal that diverse members of the lipid/protein kinase superfamily are wortmannin-sensitive, and provide evidence for a phosphatidylinositol metabolite cascade in yeast involving sequential action of STT4, MSS4, and PLC1 in which PI4,5P2 is an essential lipid product.

**EXPERIMENTAL PROCEDURES**

**Strains and Media**—Strain YS3–3D (MATa leu2 ura3 his3 ade8 met3) and an isogenic stt4 derivative Δstt4–16C (MATa Δstt4–HIS3 leu2 ura3 his3 ade8) were kindly provided by Yoshi Ohya (19). Strain FM1–5d (MATa leu2–3, 112 ura3–52 trp1 his4 ade2) and FM1–5dGpcF12 (pCF12 contains a ptk1-ts allele) were a kind gift of Mike Hall (22). Strain SEY6210 (MATa leu2–3, 112 ura3–52 his3–200 trp1–ΔSOI lys2–201 suc2–29) and an isogenic vps34 mutant strain PHY102 were from Scott Emr (24). The STT4 gene was disrupted in the JK9–3da/a diploid strain (ura3–52 leu2–3, 112 his4 trp1–ΔSOI HMLa) and in the haploid JK9–3da/a strain obtained by transforming SMY31–1 with the plasmid, sporulating and mating to nonspecific drug binding by yeast extract components as has been observed with other toxins. Consistent with this interpretation, addition of yeast extract to synthetic media prevented wortmannin toxicity (data not shown).

**RESULTS**

**Wortmannin Is Toxic to Yeast**—Wortmannin is a fungal metabolite that potently inhibits mammalian PI 3-kinase (33). We sought to identify wortmannin targets in yeast. Wortmannin inhibited the growth of yeast on synthetic minimal medium with a minimum inhibitory concentration of ~10 μg/ml (Fig. 1A). Wortmannin treatment did not cause cell lysis or a specific cell cycle arrest (data not shown). In addition, wortmannin was not toxic to yeast cells grown on rich YPD media, possibly due to nonspecific drug binding by yeast extract components as has been observed with other toxins. Consistent with this interpretation, addition of yeast extract to synthetic media prevented wortmannin toxicity (data not shown).

**Yeast Lacking PI 3-Kinase Are Wortmannin-sensitive**—Wortmannin can inhibit the yeast PI 3-kinase VPS34p in vitro (24) at concentrations higher (IC50 = 3 μM) than those that inhibit mammalian PI 3-kinase (IC50 ~ 5 nM) (33). However, yeast cells lacking VPS34 are both viable and remain wortmannin-sensitive, with a minimum inhibitory concentration of ~1 μg/ml (Fig. 1A). Thus, VPS34 is not the only target of wortmannin in yeast. The 10-fold increased sensitivity of Δvps34 mutant cells to wortmannin compared with the isogenic VPS34 mutant strain could result from an increase in the free drug concentration due to the loss of one drug binding target. Alternately, this effect may be nonspecific because Δvps34 mutant cells have perturbed vacuolar function, grow slowly, and are temperature-sensitive, and thus might be inherently more drug-sensitive. The latter explanation is supported by the finding that end1 and vps34 mutations, which also result in vacuolar

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Sorbitol (1m) rescued cells from the toxic effects of 10μg/ml wortmannin in yeast. Tested whether the PIK1 or STT4 PI 4-kinases are targets for wortmannin in different yeast strains, strain FM1–5d expressing the wild-type PIK1 (WT PIK1) or the temperature-sensitive pik1–12 mutant enzyme (pik1-ts), and strain SEY6210 expressing wild-type VPS34 PI 3-kinase (VPS34) or an isogenic derivative lacking VPS34 (Δvps34, strain PHY102) were grown on minimal media with 0, 1, or 10μg/ml wortmannin. Growth was at 25°C for 96 h. Sorbitol rescues cells from wortmannin toxicity. Isogenic derivatives in two different strain backgrounds: strain Y3–6d expressing wild-type STT4 (WT STT4) or lacking STT4 (Δstt4), and strain FM1–5d expressing wild-type PIK1 (WT PIK1) or the temperature-sensitive pik1–12 allele (pik1-ts) were grown on complete synthetic media without (+) or with 10μg/ml wortmannin (+ Sorbitol, lower plates), and without (−) or with 1m sorbitol (+ Sorbitol, lower plates). Growth was at 25°C for 96 h.

defects and temperature-sensitive slow growth, are similarly wortmannin-hypersensitive (data not shown).

Yeast PI 4-Kinase STT4 Is a Wortmannin Target in Vivo—Because yeast cells lacking VPS34 have no detectable PI 3-kinase activity or PI3P, yet retain sensitivity to wortmannin, this drug must inhibit an additional target other than PI 3-kinase in yeast. Recent studies demonstrate that wortmannin or its analog demethoxyviridin also inhibit PI 4-kinases from mammals and fission yeast (14, 16, 18). Two PI 4-kinases, PIK1 or its analog demethoxyviridin also inhibit PI 4-kinases from mammals and fission yeast (14, 16, 18). Two PI 4-kinases, PIK1 and STT4, have been identified in S. cerevisiae (19–22). We tested whether the PIK1 or STT4 PI 4-kinases are targets for wortmannin in yeast.

PIK1 is an essential enzyme (21, 22). A yeast strain expressing a temperature-sensitive PIK1 mutant (pik1-ts) was inhibited by wortmannin at permissive and semi-permissive temperatures with the same minimum inhibitory concentration as the isogenic wild-type PIK1 strain (Fig. 1A). These findings suggest that PIK1 is not a target of wortmannin.

STT4 is also essential for growth on standard yeast growth media, but the inviability of Δstt4 mutant cells can be remediated on rich media, such as YPD, by 1m sorbitol (19) and more poorly on a synthetic medium with 1m sorbitol (Fig. 1B, lower left quadrant). To test if wortmannin inhibits STT4 in vivo, we asked whether 1m sorbitol would rescue cells from wortmannin toxicity. Remarkably, in synthetic complete media, 1m sorbitol rescued cells from the toxic effects of 10μg/ml wortmannin (Fig. 1B). Thus, conditions that allow growth of cells lacking STT4 (Δstt4) also allow growth in the presence of wortmannin.

In several other yeast strain backgrounds, we and others2 have found that Δstt4 mutant cells are inviable and are not rescued by 1m sorbitol (Fig. 2A and data not shown). Thus, in these other strain backgrounds, STT4 is essential under all conditions. Correspondingly, in strain backgrounds where STT4 is essential, 1m sorbitol failed to rescue cells from wortmannin toxicity (Fig. 1B). In summary, in synthetic media lacking sorbitol, STT4 is essential and wortmannin is toxic in all strains. In one unusual strain, both the lethality of an Δstt4 mutation and wortmannin toxicity can be overcome by osmotic support with 1m sorbitol. These findings provide genetic evidence that the PI 4-kinase STT4 is a target of wortmannin in vivo. STT4 is probably not the only wortmannin-sensitive target in yeast, because sorbitol did not rescue cells lacking STT4 from wortmannin toxicity (Fig. 1B, lower left quadrants).

To explore genetic differences between strain backgrounds with respect to the essential function of STT4 a nonisogenic cross was performed between the Δstt4 viable mutant and our wild-type laboratory strain (in the presence of sorbitol). Sporulation and dissection of 24 tetrads revealed 1 tetrad with two wild-type STT4 and two slow growth, sorbitol-dependent Δstt4 segregrants (parental ditotype), 5 tetrads consisting of two wild-type STT4, one slow growth sorbitol-dependent Δstt4 mutant, and one inviable segregrant (tetraparental), and 18 tetrads consisting of two wild-type STT4 spores and two inviable spores (nonparental ditotype). These results are consistent with a minimum of three unlinked extragenic suppressors required for sorbitol rescue of the Δstt4 mutation.

STT4 Is Essential and Overexpression Confers Wortmannin Resistance—As mentioned above, STT4 is essential in our yeast laboratory strain background, and thus an STT4/Δstt4 heterozygous diploid sporulates to yield tetrads containing two viable (STT4) and two inviable (Δstt4) segregrants (Fig. 2A). An HA epitope-tagged version of the STT4 gene was cloned under the transcriptional control of the GAL promoter (pGALHA-STT4). Expression of STT4 complemented the Δstt4 mutation and restored growth even when cells were grown in glucose, which reduces expression from the GAL promoter (Fig. 2B). Rescue of the stt4::G418 mutant cells required the STT4 expression plasmid, as these cells were inviable on 5-fluoroorotic acid medium. These findings provide genetic evidence that the PI 4-kinase STT4 is a target of wortmannin in vivo. STT4 is probably not the only wortmannin-sensitive target in yeast, because sorbitol did not rescue cells lacking STT4 from wortmannin toxicity (Fig. 1B, lower left quadrants).

2 S. Emr, D. Levin, and D. Voelker, personal communications.

FIG. 1. Wortmannin is toxic to yeast. A, isogenic derivatives of two different yeast strains, strain FM1–5d expressing the wild-type PIK1 PI 4-kinase (WT PIK1) or the temperature-sensitive pik1–12 mutant enzyme (pik1-ts), and strain SEY6210 expressing wild-type VPS34 PI 3-kinase (VPS34) or an isogenic derivative lacking VPS34 (Δvps34, strain PHY102) were grown on minimal media with 0, 1, or 10μg/ml wortmannin. Growth was at 25°C for 96 h. B, sorbitol rescues cells from wortmannin toxicity. Isogenic derivatives in two different strain backgrounds: strain Y3–6d expressing wild-type STT4 (WT STT4) or lacking STT4 (Δstt4), and strain FM1–5d expressing wild-type PIK1 (WT PIK1) or the temperature-sensitive pik1–12 allele (pik1-ts) were grown on complete synthetic media without (+) or with 10μg/ml wortmannin (+ Sorbitol, lower plates), and without (−) or with 1m sorbitol (+ Sorbitol, lower plates). Growth was at 25°C for 96 h.

FIG. 2. STT4 is essential. The STT4/Δstt4::G418 heterozygous diploid strain SMY23–3 containing a control plasmid (A) or a plasmid expressing STT4 from the GAL promoter (pHASTT4, B) was sporulated and the four meiotic products from each tetrad were dissected by micromanipulation. Cells containing the control plasmid (A) yielded two viable G418-sensitive and two inviable segregrants on either YPD or YPD plus 1m sorbitol medium, indicating that STT4 is essential. With cells containing the STT4 expression plasmid, four viable segregrants were obtained, of which two were G418-resistant (Δstt4) and two were G418-sensitive (STT4). All of the Δstt4 G418-resistant segregrants were 5-fluoroorotic acid-sensitive (5-FOA, B), indicating that the plasmid-borne STT4 gene complements and is essential in cells bearing the Δstt4 mutation.
acids medium, which is toxic to cells containing the plasmid borne URA3 marker. Interestingly, growth in glucose provided a level of STT4 expression that also conferred resistance to 10 μg/ml wortmannin (Fig. 3). Overexpression of STT4 from the GAL promoter markedly inhibited growth, and thus we could not assess wortmannin toxicity under these conditions (data not shown). That STT4 overexpression is toxic suggests the activity of this enzyme may normally be regulated in vivo. From the GAL promoter of an epitope-tagged version of the PIK1 4-kinase, PIK1, complemented a pika1-ts mutation but did not confer wortmannin resistance and was not toxic when cells were grown with either glucose or galactose (Fig. 3 and data not shown). These findings implicate the STT4 PI 4-kinase as a specific target of wortmannin in yeast.

**PI 4-Kinase Activity of STT4 Is Wortmannin-sensitive in Vitro**—To further assess the wortmannin sensitivity of STT4 and PIK1 in vitro, these enzymes were immunoprecipitated with an anti-STT4 polyclonal antiserum or with an HA epitope-tagged version of PIK1 and an anti-HA monoclonal antibody. The immunoprecipitates were incubated with PI and [γ-32P]ATP, and PI4P production was detected by borate thin layer chromatography and autoradiography. Immunoprecipitation from cells expressing STT4 yielded detectable PI 4-kinase activity (Fig. 4B, lane 2), whereas cells lacking STT4 (Δstt4) yielded no PI 4-kinase activity (Fig. 4B, lane 4), establishing the specificity of the antiserum for STT4. The PI 4-kinase activity of STT4 was inhibited ~80% and ~95% by 1 and 10 nM wortmannin, respectively (Fig. 4, B–D). In comparison, galactose induction of PIK1-HA led to a marked overexpression of PIK1 as detected by Western blot (Fig. 4A) and to a corresponding increase in HA-precipitable PI 4-kinase activity (Fig. 4B, compare lanes 5 and 7); however, the PI 4-kinase activity of PIK1-HA was completely resistant to wortmannin at a concentration of 1, 10, or 20 μM (Fig. 4A, lane 6, and data not shown). This is in accord with previous observations that the PI 4-kinase activity of PIK1 purified to homogeneity is not sensitive to wortmannin at high concentrations. These findings provide biochemical evidence that the PI 4-kinase activity of STT4 is inhibited by wortmannin, and confirm our genetic evidence that STT4 is a target of wortmannin in yeast.

**STT4 Is Associated with a Membrane-pellet Fraction in Yeast**—To further characterize STT4, cells expressing HA-STT4 were fractionated into soluble (S100) and insoluble (P100) fractions and the amount of STT4 in these fractions was determined by Western blot. STT4 was exclusively detected in the particulate (P100) fraction (Fig. 5). Similar fractionation results were obtained with endogenous STT4 detected with an anti-STT4 polyclonal antisera (data not shown). The strength and nature of the STT4 association to the P100 fraction was tested by treating the cell lysate previous to centrifugation with agents known to disrupt membrane-protein or protein-protein interactions. STT4 was solubilized by 1% SDS; however, treatment with 2% Triton X-100 or with agents that interfere with protein-protein interactions, such as 0.5 M NaCl and 1.6 M urea,
Wortmannin Targets Yeast PI 4-Kinase STT4

The product of STT4, PI4P, can be further metabolized to form other important signaling molecules, including diacetyl glycerol, a proposed model for the effects of wortmannin in yeast. Wortmannin inhibits STT4, decreasing pools of PI4P and thereby regulating parallel pathways with shared and distinct functions. STT4 is essential under all conditions in our strain background. If STT4 does lie upstream of PKC1, it clearly must be relatively resistant to wortmannin. PKC1, the catalytic subunit of PI 3-kinase. Wortmannin inhibits PI 3-kinase, VPS34, and an obvious question is whether the PI 3-kinase activity of VPS34 was found in vitro with an IC50 = 3 μM (14, 24). Our finding that cells lacking VPS34 are still wortmannin-sensitive further supports the view that another protein is a target for wortmannin action in yeast. Why is the VPS34 PI 3-kinase less sensitive to wortmannin compared with the human PI 3-kinase? VPS34 is structurally and functionally distinct from the wortmannin-sensitive heterodimeric mammalian PI 3-kinase. A mammalian PI 3-kinase, distinct from the wortmannin-sensitive heterodimeric mammalian PI 3-kinase, has recently been identified that is insensitive to wortmannin and is likely the mammalian homolog of the yeast VPS34 PI 3-kinase (35). Thus, it appears that yeast lack a homolog of the mammalian p85/p110 PI 3-kinase.

The STT4 gene was previously identified among a collection of mutants resistant to the protein kinase C inhibitor staurosporine (19). Disruption mutants lacking STT4 were inviable but, like pkc1 mutant cells, could be rescued by osmotic remediation with 1 M sorbitol. This finding led to the model that STT4 lies upstream of PKC in the yeast PKC pathway that regulates cell wall biosynthesis (19). In contrast, we and others find that, in our and other strain backgrounds, STT4 is essential and cells lacking STT4 are not rescued by sorbitol (Fig. 2). We have confirmed that, in our strain background, pkc1 mutants are viable in the presence of 1 M sorbitol and inviable in its absence (data not shown). Thus, unlike PKC1, STT4 is essential under all conditions in our strain background. If STT4 does lie upstream of PKC1, it clearly must have additional functions. Alternatively, STT4 and PKC1 could regulate parallel pathways with shared and distinct functions.

The STT4 gene encodes an ~200-kDa protein with a predicted C-terminal region with homology to PI 3-kinases and PI

failed to extract STT4 from the particulate fraction (Fig. 5). These results indicate that STT4 is tightly associated with the pellet fraction, possibly via associations with both proteins and membranes.

**MSS4 Overexpression or PLC1 Mutation Confer Wortmannin Resistance**—The product of STT4, PI4P, can be further metabolized to form other important signaling molecules, including PI4,5P2, diacetyl glycerol, and IP3. Inhibition of STT4 could result in a depletion of all of these molecules. To address whether depletion of these metabolites may be responsible for the growth inhibitory effects of wortmannin, the effect of lipid-metabolizing enzymes on wortmannin sensitivity was investigated. Two genes in *S. cerevisiae*, MSS4 and FABI, show marked identity with the human C isoform of phosphatidylinositol 4-phosphate 5-kinase (34). Because MSS4 is essential (30), we could not test the effects of deleting the gene. Expression of MSS4 from a multicopy 2μ plasmid conferred wortmannin resistance, allowing J9–3da cells to grow on 7.5 μg/ml wortmannin (Fig. 6A). In contrast, we found no evidence that the second yeast PI4P 5-kinase homolog FABI plays any role in wortmannin action. Thus, sfab1 mutant cells are viable, grow poorly on minimal media, and were neither wortmannin-hypersensitive nor resistant (data not shown). Moreover, expression of FABI from a multicopy 2μ plasmid did not alter wortmannin sensitivity (Fig. 6A, data not shown). These findings indicate a specific role for MSS4, but not for FAB1, in a wortmannin-sensitive phosphatidylinositol cascade.

We also tested whether yeast PLC1 participates in the wortmannin-sensitive phosphatidylinositol cascade involving STT4 and MSS4. In fact, strains in which the PLC1 gene was deleted were viable and resistant to growth inhibition by wortmannin at 10 μg/ml (Fig. 6B). Wortmannin resistance co-segregated with the Δplc1 mutation in a genetic cross (data not shown), and reintroduction of the wild-type PLC1 gene complemented the plc1 deletion mutation to restore wortmannin sensitivity (Fig. 6B). These findings indicate that PLC1 plays a role in a wortmannin-sensitive phosphatidylinositol metabolic cascade in yeast, possibly by cleaving an essential product of STT4 and MSS4 such as PI4,5P2.

**DISCUSSION**

Our studies identify the PI 4-kinase STT4 as a target of the natural product wortmannin in the yeast *S. cerevisiae*. First, in a strain background in which the lethal phenotype of an stt4 deletion is remediated by sorbitol, wortmannin toxicity is similarly mitigated by sorbitol. In contrast, in other strain backgrounds STT4 is essential, and wortmannin is toxic, under all conditions. Second, we show that modest overexpression of STT4 confers wortmannin resistance, as does overexpression of MSS4 and deletion of PLC1. Finally, we demonstrate that the PI 4-kinase activity of STT4 is potently inhibited (IC50 = 1 nm) in vitro by wortmannin. Our findings suggest a model in which inhibition of STT4 PI 4-kinase activity by wortmannin leads to a lethal depletion of a PI4,5P2 pool in yeast.

A target of wortmannin in mammalian cells is the 110-kDa catalytic subunit of PI 3-kinase. Wortmannin inhibits PI 3-kinase with an IC50 = 1–5 nM and forms a covalent complex with the active site of the enzyme. Yeast cells express a single PI 3-kinase, VPS34, and an obvious question is whether the VPS34 PI 3-kinase is also a target of wortmannin in yeast. In previous studies, the PI 3-kinase activity of VPS34 was found to be relatively resistant to wortmannin in vitro, with an IC50 = 3 μM (14, 24). Our finding that cells lacking VPS34 are still wortmannin-sensitive further supports the view that another protein is a target for wortmannin action in yeast. Why is the VPS34 PI 3-kinase less sensitive to wortmannin compared with the human PI 3-kinase? VPS34 is structurally and functionally distinct from the wortmannin-sensitive heterodimeric mammalian PI 3-kinase. A mammalian PI 3-kinase, distinct from the wortmannin-sensitive heterodimeric mammalian PI 3-kinase, has recently been identified that is insensitive to wortmannin and is likely the mammalian homolog of the yeast VPS34 PI 3-kinase (35). Thus, it appears that yeast lack a homolog of the mammalian p85/p110 PI 3-kinase.

The STT4 gene was previously identified among a collection of mutants resistant to the protein kinase C inhibitor staurosporine (19). Disruption mutants lacking STT4 were inviable but, like pkc1 mutant cells, could be rescued by osmotic remediation with 1 M sorbitol. This finding led to the model that STT4 lies upstream of PKC in the yeast PKC pathway that regulates cell wall biosynthesis (19). In contrast, we and others find that, in our and other strain backgrounds, STT4 is essential and cells lacking STT4 are not rescued by sorbitol (Fig. 2). We have confirmed that, in our strain background, pkc1 mutants are viable in the presence of 1 M sorbitol and inviable in its absence (data not shown). Thus, unlike PKC1, STT4 is essential under all conditions in our strain background. If STT4 does lie upstream of PKC1, it clearly must have additional functions. Alternatively, STT4 and PKC1 could regulate parallel pathways with shared and distinct functions.

The STT4 gene encodes an ~200-kDa protein with a predicted C-terminal region with homology to PI 3-kinases and PI 3-kinase activity by wortmannin leads to a lethal depletion of a PI4,5P2 pool in yeast.

**FIG. 6. Lipid metabolizing enzymes effect wortmannin sensitivity.** A, yeast strain JK9–3da containing a 2μ plasmid lacking (vector) or expressing the PI4P 5-kinase homolog MSS4 (2μ MSS4) or FAB1 (2μ FABI) was grown on synthetic media lacking (0) or containing wortmannin (7.5 μg/ml) for 5 days at 30 °C B, isogenic yeast strains lacking (Δplc1) or expressing phospholipase C (PLC1) and containing a 2μ plasmid lacking (vector) or expressing PLC1 (2μ PLC1) were grown on synthetic media lacking (0) or containing wortmannin (10 μg/ml) for 10 days at 24 °C. C, a proposed model for the effects of wortmannin in yeast. Wortmannin inhibits STT4, decreasing pools of PI4P and thereby decreasing the availability of substrate for MSS4, which results in depletion of a PI4,5P2 pool necessary for cell proliferation. Overexpression of MSS4 increases PI4,5P2 and confers wortmannin resistance. Deletion of PLC1 prevents the cleavage of PI4,5P2 into diacetyl glycerol and IP3, increasing resistance to wortmannin.
Wortmannin Targets Yeast PI 4-Kinase STT4

4-kinases. In previous studies, extracts from Δstt4 mutant strains had a marked reduction in PI 4-kinase activity, providing evidence that STT4 was indeed a PI 4-kinase (19). We have immunoprecipitated STT4 and have found that the protein indeed has PI 4-kinase activity that is readily inhibited by wortmannin. In addition, we find that wortmannin is toxic to yeast and causes a nonspecific cell cycle arrest. These findings suggest that the PI 4-kinase activity of STT4 is essential. The essential product of STT4 could be PI4P itself, or a further metabolite such as PI4,5P2. Two genes in yeast, MSS4 and FAB1, have been shown to share marked identity with the mammalian C isoform of PI4P 5-kinase (36), which phosphorylates PI4P to produce PI4,5P2. MSS4 was isolated as a multifunctional kinase that is essential in yeast (30). MSS4 does not fully suppress the cell cycle defect of an Δstt4 deletion and does not suppress the staurosporine sensitivity of an Δstt4 mutant. We find that overexpression of MSS4 confers wortmannin resistance, providing further evidence that MSS4 acts downstream of STT4 (Fig. 6A). The homology of MSS4 to mammalian PI4P 5-kinase and its genetic interactions with STT4 suggest a model in which the product of STT4 lipid kinase activity, PI4P, is phosphorylated by MSS4 to produce PI4,5P2 (Fig. 6C). Deletion of the gene encoding yeast PLC1, which breaks down PI4,5P2 to produce diacylglycerol and IP3 (31, 37, 38) also confers wortmannin resistance. Both overexpression of MSS4 and deletion of PLC1 would be predicted to increase PI4,5P2 levels. That these mutations also confer wortmannin resistance implies that wortmannin may act to decrease essential pools of PI4,5P2 by inhibiting STT4 and decreasing production of its precursor, PI4P (Fig. 6C).

PI4,5P2 has been shown to play important signaling roles in mammalian cells, involved in phospholipase D activation (39), providing evidence that STT4 was indeed a PI 4-kinase (19). We have immunoprecipitated STT4 and have found that the protein indeed has PI 4-kinase activity that is readily inhibited by wortmannin. In addition, we find that wortmannin is toxic to yeast and causes a nonspecific cell cycle arrest. These findings suggest that the PI 4-kinase activity of STT4 is essential. The essential product of STT4 could be PI4P itself, or a further metabolite such as PI4,5P2. Two genes in yeast, MSS4 and FAB1, have been shown to share marked identity with the mammalian C isoform of PI4P 5-kinase (36), which phosphorylates PI4P to produce PI4,5P2. MSS4 was isolated as a multifunctional kinase that is essential in yeast (30). MSS4 does not fully suppress the cell cycle defect of an Δstt4 deletion and does not suppress the staurosporine sensitivity of an Δstt4 mutant. We find that overexpression of MSS4 confers wortmannin resistance, providing further evidence that MSS4 acts downstream of STT4 (Fig. 6A). The homology of MSS4 to mammalian PI4P 5-kinase and its genetic interactions with STT4 suggest a model in which the product of STT4 lipid kinase activity, PI4P, is phosphorylated by MSS4 to produce PI4,5P2 (Fig. 6C). Deletion of the gene encoding yeast PLC1, which breaks down PI4,5P2 to produce diacylglycerol and IP3 (31, 37, 38) also confers wortmannin resistance. Both overexpression of MSS4 and deletion of PLC1 would be predicted to increase PI4,5P2 levels. That these mutations also confer wortmannin resistance implies that wortmannin may act to decrease essential pools of PI4,5P2 by inhibiting STT4 and decreasing production of its precursor, PI4P (Fig. 6C).

PI4,5P2 has been shown to play important signaling roles in mammalian cells, involved in phospholipase D activation (39), cytoskeletal dynamics (40), and integrin-mediated cell adhesion (41). PI4,5P2 has been found to bind to the pleckstrin homology domain of several signaling molecules (42–44). Intriguingly, STT4 has a pleckstrin homology domain, opening the possibility that the pathway proposed in Fig. 6C may be autoregulated, either positively or negatively.

It has previously been reported that Δstt4 mutation results in a G2/M cell-cycle arrest at nonpermissive temperatures (19, 30). Wortmannin treatment, however, results in a nonspecific cell cycle arrest. One possible explanation is that STT4 serves additional functions, both at G2/M and at other points in the cell cycle. This is consistent with the interpretation, depriving Δstt4 deletion cells of sorbitol resulted in a nonspecific cell cycle arrest (data not shown). Thus, one possible explanation is that while wortmannin inhibits all STT4 functions, the temperature-sensitive allele may be defective in only one function. Another possibility is that targets of wortmannin other than STT4 are required at different points during the cell cycle. Indeed, that an Δstt4 deleted strain is still wortmannin-sensitive and that STT4 overexpression confers relative but not absolute wortmannin resistance indicate that there are additional targets of wortmannin in yeast.

We find that the STT4 PI 4-kinase is associated with an insoluble membrane fraction in yeast. STT4 may serve to produce PI4P in a distinct membranous compartment of the cell. Yeast cells also express another essential PI 4-kinase, PIK1 (20–22). Why do yeast cells express two different essential PI 4-kinases? One model is that these enzymes are localized to different intracellular compartments and serve to generate two different pools of PI metabolites, both of which are essential for cell function. Consistent with this view, we find that overexpression of PIK1 does not complement a Δstt4 mutation, and overexpression of STT4 does not complement conditional Δpik1 mutations. MSS4 overexpression also fails to rescue Δpik1 mutations (data not shown). Thus, STT4 and PIK1 play distinct roles and cannot substitute for each other, even when overexpressed.

Mammalian homologs of both the yeast STT4 and PIK1 PI 4-kinases have recently been identified. The mammalian homolog of STT4 is PI4Kα and the two share 50% identity in the kinase domain (15, 23). The human homolog of PIK1 is PI4Kβ, and the two share 42% identity in the kinase domain (18). Interestingly, PI4Kα, the STT4 homolog, is insensitive to micromolar concentrations of wortmannin, while PI4Kβ, the PIK1 homolog, is inhibited by wortmannin (IC50 = ~50 nM) (18). This is in marked contrast to our findings that the yeast PIK1 PI 4-kinase is not inhibited by wortmannin, whereas yeast STT4 is potently inhibited by wortmannin. The autophosphorylation activity of the mammalian target of rapamycin (mTOR), a PI kinase homolog, has also recently been shown to be inhibited by submicromolar concentrations of wortmannin, suggesting another possible target in mammalian cells (45). Alignment of amino acid sequence of wortmannin-sensitive and wortmannin-insensitive enzymes fails to reveal any residues unique to either group. All have a high degree of identity in the kinase domain, and all contain an invariant lysine residue that is the site of cross-linking to wortmannin in the mammalian PI 3-kinase. These observations suggest that subtle secondary structural differences dictate sensitivity to wortmannin inhibition.

The STT4 PI 4-kinase is a member of a larger family of proteins, the PI 3-kinase-related lipid/protein kinase superfamily. This family of proteins includes both PI 3-kinases and PI 4-kinases, receptor tyrosine kinases, and their mammalian homolog RAFT1/FRAP/mTOR, the catalytic subunit of DNA-dependent protein kinase, and checkpoint control proteins, including TEL1 and MEC1 in yeast, and the ataxia telangiectasia protein (ATM) and its related protein ATR/FRP in mammals (see Ref. 46 for review). Several members of this family of unusual lipid and protein kinases are now known to be inhibited by wortmannin, including the mammalian PI 3-kinase, DNA-dependent protein kinase, mTOR, human PI4Kβ, and the yeast PI 4-kinase STT4. Because of the growing number of members of this protein family, and their medical and pharmacological importance, wortmannin represents a valuable lead compound in the analysis of both their intracellular functions and in the rational design of other inhibitors that might more specifically target different family members.

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