Phosphatidylinositol 4-Phosphate Is Required for Translation Initiation in Saccharomyces cerevisiae

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The small natural product wortmannin inhibits protein synthesis by modulating several phosphatidylinositol (PI) metabolic pathways. A primary target of wortmannin in yeast is the plasma membrane-associated PI 4-kinase (PI4K) Stt4p, which is required for actin cytoskeleton organization. Here we show that wortmannin treatment or inactivation of Stt4p, but not disorganization of the actin cytoskeleton per se, leads to a rapid attenuation of translation initiation. Interestingly, inactivation of Pik1p, a wortmannin-insensitive, functionally distinct PI4K, implicated in the regulation of Golgi functions and secretion, also results in severe translation initiation defects with a marked increase of the phosphorylation of the translation initiation factor eIF2α. Because wortmannin largely phenocopies the effects of rapamycin (e.g. it triggers nuclear accumulation of Gln3p), it likely also inhibits the PI kinase-related, target of rapamycin (TOR) kinases. Importantly, however, neither inactivation of Stt4p nor Pik1p significantly affects TOR-controlled readouts other than translation initiation, indicating that these PI4Ks do not simply function upstream of TOR. Together, our results reveal the existence of a novel translation initiation control mechanism in yeast that is tightly coupled to the synthesis of distinct PI4P pools.

Under severe environmental conditions, conservation of energy and cellular resources, which is key to cellular survival, is achieved in part by inhibition of translation initiation. The down-regulation of translation initiation depends on multiple signaling pathways that sense internal stresses and subsequently signal downstream effectors that inactivate specific components of the translation machinery. The eIF4E-binding proteins (4E-BPs) are specific translation inhibitors that bind to the translation factor eIF4E and prevent the recruitment of the translation machinery to mRNA (1, 2). In mammalian cells, binding of 4E-BPs to eIF4E is reversible: hypophosphorylated 4E-BPs bind to eIF4E, whereas hyperphosphorylated 4E-BPs do not bind to eIF4E. Growth stimuli including serum, hormones, growth factors, and mitogens induce hyperphosphorylation of 4E-BPs, followed by their dissociation from eIF4E. Conversely, various stresses including nutrient deprivation cause dephosphorylation of 4E-BPs, which results in a rapid association of 4E-BPs with eIF4E and consequent inhibition of translation (1). In the yeast Saccharomyces cerevisiae, two mammalian 4E-BP homologs, Caf20p and Eap1p, have been described (3–5). Although the precise role of these proteins in the regulation of translation initiation is poorly understood, Eap1p may inhibit translation initiation following membrane and/or heat stress (6, 7).

Another mechanism of translation inhibition in response to various environmental stresses is mediated through the phosphorylation of the α subunit of translation initiation factor 2 (eIF2α) (8–10). eIF2α is part of a trimeric GTPase eIF2 complex that, in its GTP-bound form, delivers the charged methionyl initiator tRNA to the small 40S ribosomal subunit. Phosphorylation of eIF2α at serine 51 converts eIF2 to an inhibitor of its guanine nucleotide exchange factor eIF2B. As a result of the inhibition of GDP to GTP exchange, the level of GTP-bound eIF2 decreases, which leads to a reduction of the overall rate of translation initiation. Whereas four eIF2α kinases were identified in mammals (PKR, HRI, GCN2, and PERK), yeast appears to harbor only one eIF2α kinase (Gcn2p), which is mainly activated under amino acid starvation conditions (11, 12). Phosphorylation of eIF2α in yeast specifically stimulates translation of GCN4 mRNA, which encodes a transcriptional activator of genes involved in amino acid biosynthesis.

The elucidation of signaling pathways that regulate translation activity has been largely facilitated by the use of natural cell-permeable compounds, acting as selective enzyme inhibitors. For example, the macrocyclic lactone rapamycin allowed the identification and characterization of the conserved TOR kinases, which, when present in the TOR complex I (TORC1) (13), act as key regulators of eukaryotic cell growth by critically regulating the phosphorylation state and the activity of Gcn2p (14), among other processes (15).

The fungal metabolite wortmannin represents another natural cell-permeable product (16) that acts as a potent cell growth inhibitor, largely by inhibiting the class I phosphatidylinositol (PI) 3-kinase (17). Class I PI 3-kinases are heterodimers composed of a 110-kDa catalytic subunit and an 85-kDa regulatory subunit (reviewed in Ref. 18). In response to insulin and...
other growth factors, the 110-kDa subunit catalyzes the phosphorylation of PI-4,5-P$_2$ to produce phosphoinositide 3,4,5-trisphosphate, which results in recruitment of the serine/threonine kinases PKD1 and PKB/Akt to the plasma membrane. Activation of these effectors ultimately results in the up-regulation of translation initiation through phosphorylation-mediated inactivation of 4E-BP and activation of S6 kinase (56K1) (reviewed in Ref. 19).

Importantly, however, the effects of wortmannin on cell growth and proliferation should be interpreted with caution, because this drug may inhibit additional lipid and/or protein kinases (18, 20). For instance, the class III PI-3-kinase hVPS34, which is functionally and structurally distinct from the class I PI-3-kinase, may also be targeted by wortmannin (reviewed in Ref. 18). hVPS34 binds to the Ser/Thr kinase hVP15 on the early and late endosomes to the lysosomes (21–24). In addition, wortmannin also inhibits the type III PI4K$_β$, which is recruited onto Golgi membranes by the small GTPase Arf1p and regulates protein trafficking from the Golgi apparatus to the plasma membranes (25–28). At higher concentrations, wortmannin may inhibit a number of PI3K kinase-related protein kinases (25–28). Finally, at higher concentrations, wortmannin inhibits the primary target of wortmannin in yeast appears to be Stt4p (35), a homolog of the mammalian type III PI4K$_α$. Stt4p is essential, localizes at the plasma membrane, and is required for cell wall integrity and vacuole morphology (36, 37). At the plasma membrane PI4P is metabolized, via the PI 4,5-kinase Mss4p, to PI-4,5-P$_2$, which allows recruitment of the Rho1 GTPase, a key regulator of actin organization and of the cell wall integrity pathway (37). Moreover, Stt4p has a role in the transport of amino-phospholipid phosphatidylinerine (PS) from the ER to the Golgi apparatus (38). Yeast cells also express an essential type III PI4K$_β$, namely Pik1p, which is insensitive to wortmannin (18, 35). Pik1p localizes to both the nucleus and Golgi, regulates Golgi secretory functions, and is required for the integrity of the vacuole and the organization of the actin cytoskeleton (36, 39–41). Finally, the function of a type II PI 4-kinase, Lsb6p, remains unknown at present (42, 43).

In this study, we use wortmannin to modulate PI metabolic pathways and investigate the effects of this drug on translation initiation. Our results demonstrate that decrease of functionally unrelated pools of PI4P in the stt4 and pik1 mutants leads to a rapid attenuation of translation initiation, independently of TORC1 signaling.

**EXPERIMENTAL PROCEDURES**

**Strains, Reagents, and Growth Conditions**—Yeast mutant strains MY128 (sst4::HIS3/p415-sst4–4$^{ts}$), MY63 (pik1::TRP1/p415-pik1–85$^{ts}$), EHY304 (vps34::TRP1), MY30 (vps34::TRP1/vps34$^{ts}$ [URA3, CEN]), MY354 (sac1::TRP1/p416-sac1–23$^{ts}$), ODY371 (mss4::HIS3/p415-mss4–25$^{ts}$) and MY129 (pik1::TRP1 sst4::HIS3/p415-pik1–85$^{ts}$ p415-sst4–4$^{ts}$) are all in the SEY6210 genetic background (Mata leu2–3,112 ura3–52 his3–Δ200 trpl–901 lys2–801 suc2–A9). The insertion of a myc13-kanMX6 tag into the chromosomal GLN3 gene locus of the above strains to create LC49 (SEY6210:GLN3-myc13-kanMX6), LC51 (MY128::GLN3-myc13-kanMX6), LC50 (ODY371::GLN3-myc13-kanMX6), and LC52 (MY63::GLN3-myc13-kanMX6) was performed by PCR-based modification as described (44). Other strains were H1896 (MATa ura3–52 leu2–3 trpl–122 trp1–Δ63::GCN4-LacZ::TRP1 sst4–4::p[SUI2, LEU2]), H1897 (MATa ura3–52 leu2–3 trpl–112 trp1–Δ63::GCN4-LacZ::TRP1 sst4–4::p[sui2-S51A, LEU2]), H1816 (MATa ura3–52 leu2–3 trpl–112 trp1–Δ63::GCN4-LacZ::TRP1 gcn2Δ ssi2Δ/p[SUI2, LEU2]), ODY396 (MATa his4 leu2 lys2 or LYS2 bar1 ura3), ODY397 (MATa hist leu2 lys2 or LYS2 bar1 ura3 acl1–1ts), ODY433 (MATa leu2–3,112 ura3–52 trp1–A901 trpl–Δ63::GCN4-LacZ::TRP1 pik1::TRP1/p415-pik1–85$^{ts}$ ssi2A/pRS316-SUI2), and ODY434 (MATa leu2–3,112 ura3–52 trpl–Δ901 trp1–Δ63::GCN4-LacZ::TRP1 pik1::TRP1/p415-pik1–85$^{ts}$ ssi2A/pRS316-sui2-S51A).

Strains were grown at the indicated temperatures in standard rich medium (yeast extract-peptone-dextrose (YPD)) or in synthetic complete medium complemented with the appropriate amino acids for plasmid maintenance and either 2% glucose (SD) or 2% galactose and 1% raffinose (SGal/Raf) (45). Wortmannin, rapamycin, and latrunculin A were purchased from Sigma, LC Laboratories, and Calbiochem, respectively, and were prepared as recommended by the manufacturers.

**Sucrose Gradient Analyses**—Sucrose gradient analyses were performed according to de la Cruz et al. (5). The ratio between the polysome and the 80 S monosome peaks (P/M) was determined using SigmaScan Pro 5.0 program (Systat Software Inc.).

**Northern Blotting**—Total RNA was extracted from 100 $A_{600}$ units of exponentially growing cells and analyzed (5 μg) by Northern blot as described (46). DNA probes were labeled with [γ-32P]CTP using the PRIME-IT II Random Primer labeling kit (Strategene 300385).

**Western Blotting**—Whole cell extracts were prepared as described (47). For the analysis of eIF2α phosphorylation, equal amounts of protein from the different extracts were resolved on 12% SDS-PAGE and subjected to Western blotting using monoclonal antibodies specific for phosphorylated Ser$^{51}$ in eIF2α (BIOSOURCE). The blots were then stripped and reprobed with polyclonal antibodies against total eIF2α. Phosphorylated forms of Gln3p-myc13 were resolved on 7% SDS-PAGE and detected using mouse myc tag monoclonal antibody (9B11, Cell Signaling).

**Sucrose Density Gradients**—For sucrose density gradient fractionation, the isogenic SEY6210 (wild-type) and EHY304 (vps34) strains transformed with pRS316-KEX2-HA (CEN, URA3) and pTOR1-HA (2μ, LEU2) were grown in 1.5 liters of SD-URA/LEU medium to an $A_{600}$ of 1.2 at 30°C. Cells were lysed and sucrose gradients prepared as described (48).

**Indirect Immunofluorescence Microscopy**—Preparation of cells for immunofluorescence was essentially as described (49). Cells were grown to 0.8 $A_{600}$/ml in SD medium at 30°C. Gln3p-myc13 was detected using mouse myc tag antibody (9B11, Cell
eIF2 α wild-type cells prior to and following a 30-min wortmannin (10 μM) treatment. Whole cell extracts were subsequently subjected to immunoblot analysis with antibodies specific for the phosphorylated form of eIF2 α. We found that a short treatment with wortmannin (5 min) already caused a strong induction of eIF2 α-P (Fig. 1C).

Wortmannin Induces the Phosphorylation of eIF2 α by Inactivating TORC1—The phosphorylation of eIF2 α is mainly induced in response to amino acid starvation and inactivation of TORC1 (10, 14). Tor1p and Tor2p (when present in TORC1) function as stress sensors that integrate nutrient quality and abundance to control cell growth (51). Previously, wortmannin was shown to inhibit the intrinsic protein kinase activity of Tor1p in vitro (31). Because the kinase activity is essential for Tor1p function, we asked whether the reduction of translation initiation in wortmannin-treated cells results from a direct inhibition of Tor1p. Cherkasova and Hinnebush (14) previously demonstrated that the activation of Gcn2p depends on Tor1p/2p by showing that overexpression of wild-type Tor2p prevents the increase of eIF2 α phosphorylation observed in rapamycin-treated cells. In a similar approach, we found that overproduction of Tor2p, using an inducible GAL1 promoter, leads to a marked delay in wortmannin-induced eIF2 α phosphorylation (Fig. 2A).

To substantiate the idea that wortmannin may inhibit Tor1p/2p in vivo, we next tested whether wortmannin could phenocopy the effects of the bona fide TORC1 inhibitor rapamycin. For instance, rapamycin treatment (much like nitrogen depletion) results in inactivation of TORC1 and subsequent dephosphorylation and cytoplasmic to nuclear transfer of Gln3p, a transcription activator of nitrogen catabolic genes (52). To compare the effects of wortmannin and rapamycin on both phosphorylation and subcellular localization of Gln3p, we used a wild-type strain containing an integrated myc-tagged GLN3 construct and treated cells with wortmannin or rapamycin for 30 min. Wortmannin treatment, like rapamycin treat-
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**A**

|       | Dextrose | Galactose |
|-------|----------|-----------|
| elf2α-P | 0        | 1        |
| elf2α  | 15       | 0        |
|        | 30       | 5.8      |
|        | 0        | 1        |
|        | 15       | 1.2      |
|        | 30       | 1.5      |

**B**

![Graph showing the effect of wortmannin on translation initiation](image)

**C**

![Image showing the localization of Gln3p and DAPI](image)

**D**

|       | 30     | 60     | 120    |
|-------|--------|--------|--------|
| GLN1  | R      | R      | W      |
| RPS12 | W      | W      | R      |
| SSB1  | R      | W      | W      |

**FIGURE 2.** Wortmannin inhibits TORC1-dependent signaling pathways. A, overexpression of Tor2p partially suppresses the stimulation of elf2α phosphorylation by wortmannin. SEY6210 (wild-type) cells transformed with a plasmid containing TOR2 under the control of an inducible GAL1 promoter were grown to mid-logarithmic phase either in SD medium or SC medium complemented with galactose and raffinose (SGal/Raf). Then, wortmannin (10 μg/ml) was added to both cultures for the times indicated (0, 15, and 30 min). Phosphorylation of elf2α (S51) was measured as described in the legend to Fig. 1C. *Numbers* indicate the relative increase of the elf2α-P/elf2α ratio. B and C, wortmannin induces hypophosphorylation (B) and nuclear localization of Gln3p (C). Wild-type cells expressing genomically tagged Gln3p-myc13 (LC49) were grown to mid-logarithmic phase in SD medium at 30°C and treated with rapamycin (Rap; 1 μg/ml) or wortmannin (Wort; 10 μg/ml) for 30 min, or not treated (–). Whole cell extracts were resolved by SDS-PAGE and the different phosphorylated Gln3p-myc13 forms were detected by anti-myc antibody (B). Gln3p-myc13 was visualized by immunofluorescence using monoclonal anti-myc antibody. DNA was stained with 4’,6-diamidino-2-phenylindole (DAPI) (C). D, wortmannin derepresses GLN1 gene transcription. SEY6210 cells were grown as in B and C and rapamycin (R, 200 ng/ml) or wortmannin (W, 10 μg/ml) were added for the times indicated. Cells were harvested and 5 μg of total RNA from each sample was subjected to Northern analysis.

**Wortmannin Inhibits Translation Initiation via an Additional TORC1-independent Mechanism(s)—**Because wortmannin may not only inhibit Tor1/2p, but also additional protein or lipid kinases, it is formally possible that wortmannin inhibits translation also via TORC1-independent mechanisms. In line with this reasoning, we observed that wortmannin reproducibly caused a much stronger decrease in the P/M ratio than rapamycin (Fig. 3A). In other words, wortmannin inhibits translation initiation more strongly than rapamycin (even when rapamycin was used in significant excess; data not shown). The inhibitory effects of rapamycin on translation initiation are mainly due to enhanced Gcn2p-dependent phosphorylation of elf2α following inactivation of TORC1 (14). To address the relative contribution to the inhibition of translation initiation of phosphorylated elf2α in cells treated with wortmannin and rapamycin, we then analyzed the effects of both drugs in the absence of Gcn2p or in the presence of elf2αS51A, which cannot be phosphorylated by Gcn2p. As expected, the rapamycin-induced redistribution of the polysomes into the 80 S peak was significantly reduced in the absence of Gcn2p (in a gcn2Δ mutant) or in the presence of elf2αS51A (in a sui2-SS1A mutant). Importantly, however, rapamycin treatment still caused a significant decrease in the P/M ratio even in the absence of Gcn2p. Thus, whereas these data substantiate the role of phosphorylated elf2α in the attenuation of translation initiation, they also suggest that TORC1 may control translation initiation via additional Gcn2p-independent mechanisms (see “Discussion”). In comparison to rapamycin treatment, wortmannin treatment consistently caused a much more dramatic inhibition of translation initiation, notably even in gcn2Δ and sui2-SS1A mutant cells (Fig. 3A). Our observation that wortmannin inhibits protein synthesis much more potently than rapamycin is also underlined by the fact that wortmannin inhibited growth of wild-type cells much more rapidly and efficiently than rapamycin (Fig. 3B).

We next investigated the effect of wortmannin on the translational regulation of GCN4 mRNA, which is selectively derepressed due to activation of Gcn2p-mediated elf2α phosphorylation following either nutrient starvation or rapamycin-mediated TORC1 inhibition (12, 53, 54). In line with previous work (14, 55), we found that rapamycin treatment strongly induced GCN4 translation (as assayed using a reporter mRNA, which expresses β-galactosidase under the control of the GCN4 mRNA 5' leader region with the four upstream open reading frames required for translational control; Fig. 3C). Surprisingly, however, even though wortmannin treatment, like rapamycin treatment, caused phosphorylation of elf2α on Ser51 (see Fig. 1C), and hence was expected to induce GCN4 expression, it actually blocked the GCN4 translation under con-
conditions where wortmannin slightly increases the level of GCN4 mRNA (Fig. 3C). Moreover, wortmannin even prevented the translational derepression of GCN4 induced by rapamycin. From these results, we infer that wortmannin targets components of the translational machinery that remain unaffected by rapamycin. Consistent with this idea, wortmannin treatment (10 μg/ml) caused a significantly (4-fold) stronger reduction in translation of GCN4 (as assayed with a reporter construct exhibiting high constitutive expression of Gcn4p due to deletion of the four GCN4 upstream open reading frames) when compared with rapamycin treatment (data not shown).

**Loss of Vps34p Does Not Significantly Affect TORC1 Activity**—Although wortmannin appears to directly inhibit Tor1p/2p, it is possible that it additionally inhibits lipid kinases that act upstream of TORC1. In this context, two recent studies reported that hVPS34 is required for mTOR and S6 kinase activities (56, 57). Whereas a similar role of Vps34p has not yet been described in yeast, it is intriguing that both Vps34p and at least part of the TORC1 pool were found associated with internal and/or endocytic membraneous structures (58, 59). We therefore tried to assess whether Vps34p may affect TORC1 activity. To this end, we first reexamined Tor1p localization on sucrose density gradients. A whole cell extract from wild-type cells expressing a functional Tor1p-HA was first subjected to differential centrifugation to yield a low-speed pellet fraction (P13; 13,000 × g for 15 min), and a high-speed pellet fraction (P100; 100,000 × g for 60 min). Similar amounts of Tor1p-HA were detected in both pellets (data not shown), which is consistent with previous findings that Tor1/2 proteins associate with different membrane fractions (58, 60, 61). Because Golgi and endocytic elements predominantly sediment in the high-speed pellet (P100), we then loaded the P100 fraction onto a sucrose gradient and centrifuged at 100,000 × g for 18 h. Fractions were collected and analyzed by immunoblotting. Tor1p-HA was detected as a single peak in denser fractions than Mnn1p and Pep12p markers of the medial Golgi and late endosomal compartments, respectively (Fig. 4A). In addition, Tor1p-HA distribution was very similar to those of Kex2p and Mon2p-HA (data not shown), both markers of the late-Golgi/early endosomes. Thus, Tor1p distribution could in principle depend on the PI3P that is synthesized on the late-Golgi by Vps34p. To test this idea, we performed the same sucrose gradient with cells lacking Vps34p and found Tor1p-HA distribution to be unchanged, indicating that Vps34p is not needed for the association of Tor1p-HA with the late-Golgi compartment. Because these data still do not rule out the possibility that Vps34p is required for Tor1p activation, we also determined whether inactivation (in a vps34Δ mutant) and/or loss of Vps34p (in a vps34Δ mutant) affect TORC1-controlled readouts. Inhibition of TORC1 results in a rapid translational repression of ribosomal genes (e.g. RPS12) and induction of stress-responsive genes (e.g. HSP26 and GLN1; Fig. 2D and data not shown), and in synthesis of glycogen (62, 63). Interestingly, inactivation (incubation of the vps34Δ mutant for 4 h at 37 °C; Fig. 4B) or loss of Vps34p (Fig. 4C; and data not shown) did not change significantly any of these readouts when compared with wild-type cells. From these data we conclude that in yeast, unlike in mammalian cells, Vps34p is not required for normal regulation of TORC1 activity.

**The PI 4-Kinase Stt4p Is Required for Normal Translation Initiation**—Whereas mTOR is inhibited in vitro by submicromolar concentrations of wortmannin (64), both in vitro and in vivo studies in yeast have identified the PI4K Stt4p as a primary target of wortmannin (IC_{50} = 1 nM (35)). To test whether inactivation of Stt4p, like wortmannin treatment, causes a rapid drop in translation initiation activity, we used a temperature-
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Inhibition of translation initiation. Our analyses of act1-1ts mutant cells further substantiate this conclusion. Accordingly, even though act1-1ts mutant cells grow slower and have intrinsically lower polysome levels than wild-type cells (at the permissive temperature of 24°C), their polysome profiles remain largely unchanged even after a 45-min incubation at the restrictive temperature (37°C; Fig. 6A), which is otherwise sufficient to cause complete disorganization of actin filaments (data not shown). Despite the slow growth of the act1-1ts mutant at 24°C, basal eIF2α phosphorylation levels are low and, as in wild-type cells, increase only transiently following a heat shock at 37°C (Fig. 6B). Moreover, treatment of wild-type cells with latrunculin A, which blocks actin polymerization, does not induce any significant changes in eIF2α phosphorylation levels (Fig. 6C). Finally, we also found that inactivation of Rho1p, a downstream effector of PI-4,5-P2, required for actin polarization, does not significantly affect translation initiation (data not shown). From these data we infer that the inhibition of translation initiation following Stt4p inactivation (and hence the decrease in PI4P levels) does not result from an actin organization defect.

The PI 4-Kinase Pik1p Is Also Required for Normal Translation Initiation—As mentioned above, Pik1p synthesizes a distinct pool of PI4P that is essential for Golgi function and secretion (Fig. 5A). In this context, we previously showed that a secretory block results in a rapid attenuation of translation initiation (6). Based on these observations, we expected Pik1p
function to be also required for normal translation initiation. Indeed, we observed in pik1+ cells, which exhibited nearly wild-type P/M ratio at 24 °C, a dramatic, 6-fold decrease in the P/M ratio following incubation at the non-permissive temperature (Fig. 5B). This observed polysome redistribution was slightly more pronounced in stt4+ pik1+ double mutant cells (Fig. 5B). Together, these data indicate that the function of distinct PI 4-kinases are required for normal translation initiation.

To confirm the role of PI4P in protein synthesis, we also analyzed the effects of the stt4+ and pik1+ mutations with respect to ribosome biosynthesis. To this end, cells were grown in rich medium to mid-logarithmic phase at 22 °C and then shifted to 37 °C. Aliquots were taken at the indicated times, and mRNA levels were quantified by Northern blot analysis. As previously noted, the mRNA levels of RPS12 and RPL42 genes transiently decrease following heat shock in wild-type cells. In the stt4+ and pik1+ mutants, however, the corresponding mRNA levels did not resume to wild-type levels (Fig. 8B). This indicates that proper ribosome biosynthesis requires the function of both Stt4p and Pik1p. In control experiments, we found that in mss4+ mutant cells, like in wild-type cells, RPS12 and RPL42 mRNA levels decreased only transiently (Fig. 8B).

In further support of a role for PI4P in the control of protein synthesis, we found that inactivation of Pik1p or (to a lesser extent) Stt4p is associated with an increase of Ser51 phosphorylation in elf2α (Fig. 7A). Interestingly, both the temperature-sensitive sac1+ mutant, defective in phosphoinositide 4-phosphatase activity (66), and the vps34+ mutant exhibited very low elf2α Ser51 phosphorylation levels at the permissive temperature of 22 °C (Fig. 7A). These results suggest that subtle changes in the intracellular levels of PI4P and PI3P affect the phosphorylation state of elf2α. To determine whether the observed increase in elf2α phosphorylation following inactivation of Pik1p is also paralleled by a reduction in translation initiation, we then studied the polysome profiles in pik1+ and pik1+ su2i2-S51A double mutant cells. Surprisingly, both the pik1+ single and pik1+ su2i2-S51A double mutant cells exhibited a strong reduction in the P/M ratio following their transfer to the restrictive temperature (Fig. 7B). Therefore, elf2α-P appears to contribute only marginally to the inhibition of translation initiation that results from Pik1p inactivation. Consistent with this notion, we also observed that stt4+ pik1+ double mutant cells, even though dramatically compromised with respect to translation initiation (Fig. 5B), exhibited only a moderate (1.4-fold) increase in elf2α phosphorylation following their exposure to the restrictive temperature (Fig. 7A). Taken together, even though PI4P levels affect the phosphorylation status of elf2α, they appear to control translation initiation mainly via an elf2α-independent mechanism(s).

Pik1p and Stt4p Are Not Required for Normal Regulation of TORC1-controlled Readouts—The results shown above indicated that the protein synthesis defect in the stt4+ mutant is likely not related to a decrease of the PI-4,5-P$_2$ levels or an actin organization anomaly. We therefore next asked whether the Stt4p-dependent regulation of protein synthesis is linked to its function in amino-phospholipid PS transport from the ER to the Golgi (38). We reasoned that a decrease of the PS level on the Golgi apparatus in the stt4+ mutant, similar to a block of the secretory pathway in the pik1+ mutant, might cause a membrane stress on the late-Golgi that could reduce protein synthesis indirectly by inhibition of TORC1. To address this possibility, we determined the cellular localization of Gln3p (a TORC1-controlled process) as described above. Wild-type, stt4+, mss4+, and
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![Figure 8](image)

**FIGURE 8.** TORC1-dependent readouts are not affected in the *stt4* and *pik1* cell strains. A, the isogenic LC49 (wild-type), LC51 (*stt4*–4*ts*), LC50 (*mss4*–25*ts*), and LC52 (*pik1*–85*ts*) strains expressing genomically tagged Gln3p-myc13 were grown in YPD at 22 °C to mid-logarithmic phase of growth and were shifted to 37 °C for 60 min. Rapamycin (Rap) was added for an additional 30 min in the LC51 (*stt4*–4*ts*) strain. Gln3p-myc13 was visualized by immunofluorescence as described in the legend to Fig. 2C. B, the isogenic SEY6210 (wild-type), MYF128 (*stt4*–4*ts*), ODY371 (*mss4*–25*ts*), and MYF63 (*pik1*–85*ts*) were grown as in A. At the indicated times, cells were harvested and Northern analysis of the indicated genes was performed as described in the legend to Fig. 2D.

pik1*ts* cell strains bearing a genomically tagged version of GLN3 (GLN3-myc) were grown exponentially and temperature shifted to 37 °C for 60 min. In contrast to rapamycin-treated cells (Fig. 2C), none of the mutants showed nuclear accumulation of Gln3p after 30 (data not shown) or 60 min incubation at 37 °C (Fig. 8A). In control experiments, all mutants showed nuclear accumulation of Gln3p following an additional 30-min treatment with rapamycin (200 ng/ml; data only shown for the *stt4*–4*ts* mutant). We also measured the mRNA levels of HSP26 and GLN1 (Fig. 8B), which are normally derepressed following TORC1 inactivation (data not shown). The transient induction of HSP26 mRNAs (a normal response to the imposed heat shock), and the expression levels of GLN1 were largely unaffected by the *stt4*–4*ts*, *mss4*–25*ts*, and *pik1*–85*ts* mutations (Fig. 8B). Finally, GLN1 expression remained also unaffected by the *sec7*–*ts* and *sec14*–*ts* mutations, both of which block secretion at the Golgi stage at the restrictive temperature (data not shown). Together, these results indicate (i) that the attenuation of translation initiation in both *stt4*–4*ts* and *pik1*–85*ts* mutants does not result from TORC1 inhibition and (ii) that a defective transport through the Golgi network does not perturb the activity of TORC1.

**DISCUSSION**

The yeast TOR kinases regulate protein synthesis in response to cellular nutrient availability in part by controlling the activity of the Gcn2p kinase, which directly phosphorylates eIF2α (14). The data presented here support the idea that wortmannin directly inhibits the PIK-related TOR kinases. Accordingly, treatment with wortmannin, like treatment with rapamycin ((52), results in nuclear accumulation of Gln3p and expression of Gln3p-dependent genes. In addition, wortmannin treatment rapidly reduces translation initiation and increases the level of eIF2α-P, an effect that can be significantly reduced/delayed by overproduction of Tor2p. A previous work reported that phosphorylation of eIF2α by Gcn2p accounts for 50% of the observed translation initiation inhibition in rapamycin-treated cells (14). Surprisingly, in our strain background (S288c), phosphorylation of eIF2α by Gcn2p accounts for less than 25% of the inhibition of translation initiation in rapamycin-treated cells (Fig. 3; notably both *sui2*-S51A and *gcn2Δ* mutants were similarly rapamycin-sensitive as their isogenic wild-type parent; data not shown). In yet another strain background (MC1278b) translation initiation was previously found to remain largely unaffected by rapamycin treatment, despite the fact that eIF2α phosphorylation and GCN4 translation both increased significantly following application of the drug (67). Collectively, these findings not only show that the contribution of eIF2α phosphorylation to the inhibition of translation initiation varies among different strain backgrounds, but also suggest the presence of additional eIF2α-independent regulatory mechanisms that inhibit general mRNA translation initiation following inactivation of TORC1. These mechanisms may include (among others) the previously reported control of the stability of eIF4G, a component of the eIF4F complex that is needed to recruit the 40 S ribosome to capped mRNAs, by TORC1 (68) (see also Fig. 9).

We noted that wortmannin treatment affects the polysome profiles much stronger than rapamycin treatment (both drugs were used at saturating concentrations), and that wortmannin, in contrast to rapamycin, prevents the activation of GCN4 translation. A possible explanation for these findings could be...
that wortmannin blocks the activity of PI kinases, which may be indirectly involved in control of translation. In support of this model, we found that the PI4K Stt4p, a known wortmannin target, is indeed required for normal translation initiation (Fig. 9). Notably, Stt4p generates a pool of PI4P required for the production of PI-4,5-P₂, which regulates cell wall integrity and actin polarization at the cell surface (37). An actin organization defect per se, however, did not affect translation initiation, indicating that Stt4p controls translation initiation by another yet unidentified mechanism(s). An interesting possibility is that this mechanism(s) is related to the function of Stt4p in the transport of PS from the ER to the Golgi (38). Accordingly, PS is normally enriched in the Golgi membrane leaflet facing the cytoplasm where it recruits specific proteins involved in the formation of transport vesicles and/or in maintenance of Golgi functions. The asymmetric localization of PS is generated and maintained by ATP-driven lipid transporters or translocases such as, for instance, Drs2p, which functions with clathrin molecules to form a specific class of secretory vesicles from the late-Golgi (69, 70). Interestingly, we found that drs2–31 (for deficient ribosome subunit) mutants, initially identified in a genetic screen for cells defective in ribosome synthesis (71), exhibit a drastically decreased P/M ratio as assayed in the temperature-sensitive drs2–31ts mutant (data not shown). Future studies that focus on the possible functional relationship between the Golgi network and protein synthesis are therefore likely to provide further insight into the role of Stt4p in translation control.

Yeast cells possess two elf4E-BPs, namely Csf20p and Eap1p, that inhibit translation initiation of mRNAs (3–5). The activity of Eap1p may be regulated by TORC1, as inferred from the observation that loss of Eap1p causes resistance to low concentrations of rapamycin (20 ng/ml) (4). We found that in response to rapamycin treatment, isogenic wild-type and eap1Δ cells both displayed a similar decrease in the P/M ratio (data not shown), which argues in fact against a major role of TORC1 in regulating translation initiation via Eap1p. Interestingly, however, Eap1p is predominantly associated with membranes and interacts genetically and biochemically with the ER-associated polyribosomal protein Scp160p (72). Moreover, it is known that Eap1p inhibits translation initiation following a membrane stress imposed by either application of the amphiphilic drug chlorpromazine or a block (in sec mutant cells) of the membrane transport to the plasma membrane (4, 6). Finally, the essential lipid sphingoid base, which is involved in many cellular functions including membrane trafficking, also regulates mRNA translation via Eap1p in response to a heat shock (7). Together, these data may be explained by a currently speculative model in which a membrane trafficking defect induced by a decrease of the intracellular PI4P levels in stt4Δ and piki1 mutant cells (see below) causes activation of Eap1p and subsequent (TORC1-independent) inhibition of translation initiation (Fig. 9).

Interestingly, the PI4K Pik1p, which is functionally unrelated to Stt4p and which regulates the formation of secretory vesicles at the late-Golgi, is also required for normal translation initiation. Because a part of TORC1 resides on the endosomal/Golgi compartment in a complex with Lst8p (1, 51), a protein that regulates the transport of amino acid permeases from the late-Golgi to the plasma membrane (13, 58, 59, 73), we envisaged the possibility that a decrease of PI4P levels in the Golgi and the associated transport defect to the cell surface may cause a reduction in TORC1 activity and therefore indirectly inhibit translation initiation. Our finding that piki1ts mutant cells (as well as sec14ts and sec7ts mutants cells; data not shown) were not significantly affected in the control of known TORC1 read-outs, however, argues against this possibility. Thus, our present results not only support our previous finding that translation initiation requires a functional secretory pathway (6), but also newly implicate the function of Pik1p in this process (Fig. 9).

Remarkably, whereas a block of the secretory pathway in the piki1ts mutant results in a 4-fold increase of the elf2α phosphorylation, a membrane transport defect to the vacuole in the vps34ts mutant leads to a hypophosphorylation of elf2α. These results suggest that PIs on late-Golgi and endosomal compartments regulate the phosphorylation status of elf2α. It is possible for instance that PI4P-dependent membrane transport processes are sensed by an elf2α-P-targeting phosphatase(s). The type 1 phosphatase Glc7p, for instance, which plays a critical function in several cellular trafficking events, including homotypic vacuole fusion, ER to Golgi transport, and endocytic transport (74), has previously been reported to (directly or indirectly) affect elf2α phosphorylation levels (75). A secretory transport defect may also more indirectly affect elf2α phosphorylation presumably via an effect on amino acid metabolism. Accordingly, inactivation of Pik1p may result in mis-sorting of amino acid transporters thereby causing a drop in intracellular amino acid levels, activation of Gcn2p, and consequent hyperphosphorylation of elf2α (Fig. 9).

Finally, it is interesting to note that loss of Pik1p function, like any other secretory block, leads to the activation of the transcription factor Hac1p, which requires the function of both Gcn2p and Gcn4p to control the expression of UPR genes (data not shown) (76, 77). Thus, elf2α phosphorylation following inactivation of Pik1p may also serve to activate a specific transcriptional program, whose gene products are necessary to alleviate ER stress and facilitate protein trafficking (76). Notably, in mammalian cells the UPR involves activation of a PKR-like ER kinase (PERK, which is absent in yeast) that directly phosphorylates elf2α and thereby inhibits translation initiation (78). In view of our results, it will therefore be interesting to determine whether a secretory stress may, via activation of the UPR pathway and subsequent PERK-dependent phosphorylation of elf2α, also cause a reduction in mammalian translation initiation. Because the role of PI4P in the regulation of protein secretion appears to be conserved (28, 79), it will, furthermore, be interesting to test whether the control over protein translation initiation is also intimately linked to the PI4P-dependent secretory pathway in higher eukaryotes.

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