Yeast phosphatidylinositol 4-kinase, Pik1, has essential roles at the Golgi and in the nucleus

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Phosphatidylinositol 4-kinase, Pik1, is essential for viability. GFP-Pik1 localized to cytoplasmic puncta and the nucleus. The puncta colocalized with Sec7-DsRed, a marker of trans-Golgi cisternae. Kap95 (importin-β) was necessary for nuclear entry, but not Kap60 (importin-α), and exportin Msn5 was required for nuclear exit. Frq1 (frequenin orthologue) also is essential for viability and binds near the NH_2 terminus of Pik1. Frq1-GFP localized to Golgi puncta, and Pik1 lacking its Frq1-binding site (or Pik1 overexpressed in $frq1\Delta$ cells) did not decorate the Golgi, but nuclear localization was unperturbed. Pik1(Δ10-192), which lacks its nuclear export sequence, displayed prominent nuclear accumulation and did not rescue inviability of $pik1\Delta$ cells. A Pik1-CCAAX chimera was excluded from the nucleus and also did not rescue inviability of $pik1\Delta$ cells. However, coexpression of Pik1(Δ10-192) and Pik1-CCAAX in $pik1\Delta$ cells restored viability. Catalytically inactive derivatives of these compartment-restricted Pik1 constructs indicated that PtdIns4P must be generated both in the nucleus and at the Golgi for normal cell function.

Introduction

Phosphatidylinositol (PtdIns) and derived molecules have crucial functions in eukaryotes, including yeast (Michell et al., 2003). PtdIns can be phosphorylated on certain hydroxyls in its head group by specific lipid kinases; phosphoinositides (PIPs) so generated can be hydrolyzed by various lipid phosphatases. PIPs, especially $PtdIns4,5P_2$, can be cleaved by PLC, producing DAG and water-soluble inositol-1,4,5-P_3 (IP_3). IP_3 can be acted on by other kinases and phosphatases to generate other inositol-phosphate species (IP_xs). Thus, different membrane PIPs and soluble IP_xs are well suited to serve as spatial and temporal regulators of diverse cellular processes.

The *Saccharomyces cerevisiae* genome encodes three PtdIns 4-kinase (PI4K) isoforms that generate PtdIns4P: Pik1 (Flanagan et al., 1993), Stt4 (Yoshida et al., 1994), and, Lsb6 (Han et al., 2002; Shelton et al., 2003). All are conserved from yeast to humans (for review see Heilmeyer et al., 2003). The mammalian Pik1 orthologue is PI4KIIIβ (Kapp-Barnea et al., 2003). Pik1 (120 kD) is soluble in cell extracts (Flanagan and

Thorner, 1992) and Stt4 (215 kD) is localized in the plasma membrane (PM; Audhya and Emr, 2002). Each is essential for cell viability (Flanagan et al., 1993; Yoshida et al., 1994), indicating they serve nonoverlapping roles. Lsb6 (70 kD) is membrane associated, but dispensable, and is purported to function in endosome motility (Chang et al., 2005).

The first *sst4* mutants were identified because they were hypersensitive to staurosporine, which is an inhibitor somewhat specific for PKC-related protein kinases (Yoshida et al., 1994). This phenotype is now understood. Pkc1 is essential for cell viability (Levin et al., 1990) and activated by the small GTPase, Rho1 (Kamada et al., 1996). Rho1 activation depends on the recruitment of its cognate guanine nucleotide exchange factor (GEF) Rom2, which contains a $PtdIns4,5P_2$-specific pleckstrin homology (PH) domain (Audhya and Emr, 2002). Stt4 generates the pool of PtdIns4P at the PM that is converted to $PtdIns4,5P_2$ by the PtdIns4P 5-kinase, Mss4 (Desrivieres et al., 1998; Audhya and Emr, 2003). Thus, PtdIns4P serves as an intermediate in the generation of $PtdIns4,5P_2$ and, in many eukaryotes (but not *S. cerevisiae*), $PtdIns3,4,5P_3$, which each have been ascribed roles in actin dynamics, receptor-tyrosine kinase signaling, and endocytosis (Martin, 1998). However, compelling cumulative evidence, obtained first in yeast, shows that PtdIns4P itself has an important function in the Golgi (Hama et al., 1999; Walch-Solimena and Novick, 1999; Levine and Munro, 2002; Wang et al., 2003).

A GFP-Pik1 DIC

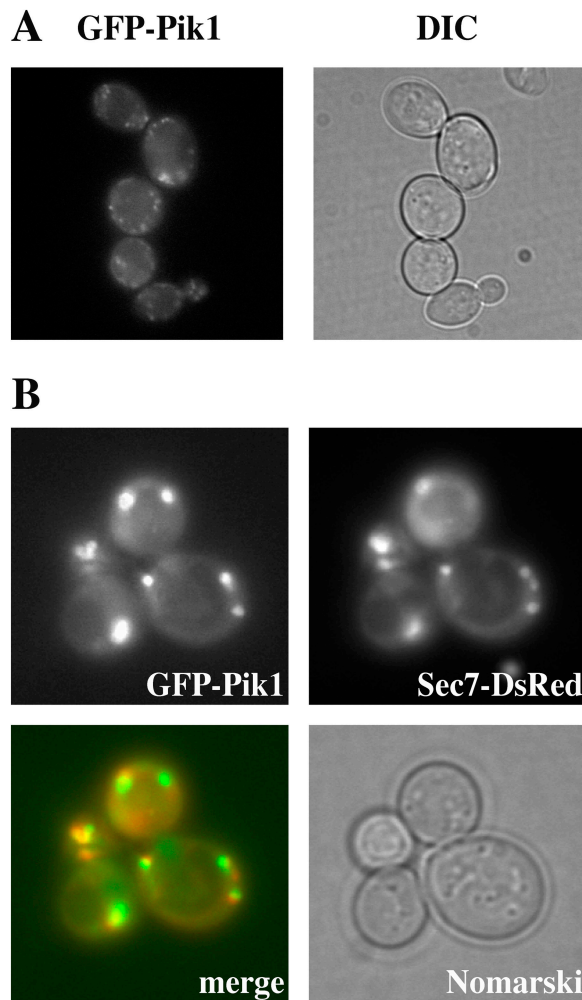
B

GFP-Pik1 Sec7-DsRed

merge Nomarski

Figure 1. **Pik1 localizes to cytoplasmic puncta enriched in a diagnostic Golgi marker.** (A) Strain BY4743 carrying pTS8 was grown to mid-exponential phase at 30°C and examined by fluorescence microscopy (left) or Nomarski (DIC) optics (right). (B) Strain YTS114 expressing Sec7-DsRed (from the *TRP1* locus) and GFP-Pik1 from pTS8 were grown to mid-exponential phase, immobilized in soft agar, and examined by fluorescence microscopy using band-pass filters optimized for the detection of GFP (top left) or DsRed (top right), and by Nomarski optics (bottom right). Overlay of the GFP and DsRed images is also shown (merge).

Genetic evidence implicates Pik1 in the synthesis of the Golgi pool of PtdIns4P. However, there is conflicting data concerning subcellular localization of Pik1. In cell lysates, the enzyme fractionates mainly like a soluble protein (Flanagan and Thorner, 1992; Huttner et al., 2003). Nevertheless, when examined by indirect immunofluorescence, epitope-tagged Pik1 localized to prominent cytosolic bodies, only some of which seemed congruent with a marker (Chs5) of the late Golgi (Schnieders, 1996; Walch-Solimena and Novick, 1999). In some cells, the nucleus was prominently stained (but it could not be discerned whether staining was inside or decorating the nuclear envelope). Yet another study that used an antibody of uncertain provenance and questionable utility for specific detection of Pik1, localized the protein exclusively to the nucleus upon subcellular fractionation (Garcia-Bustos et al., 1994). In contrast, a recent global analysis of yeast proteins using COOH-terminal GFP fusions

A GFP-Pik1 ($PIK1_{promoter}$)

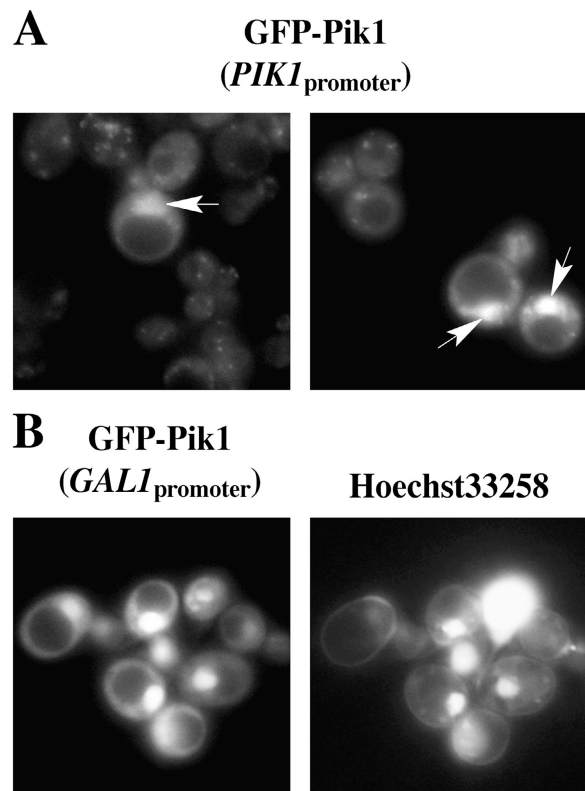

B GFP-Pik1 ($GAL1_{promoter}$) Hoechst33258

Figure 2. **Pik1 is also a resident in the nucleus.** (A) Two independent fields of the same culture that was described in Fig. 1 A. Some cells (typically those with the highest total fluorescence) exhibit a strong nuclear signal (arrows). (B) Strain BY4743 carrying pTS9 were induced with galactose for 3 h, counterstained for DNA with Hoechst 33258, and viewed under the fluorescence microscope with appropriate filters to visualize GFP (left) and the DNA dye (right).

concluded that Pik1 is localized uniformly in the cytosol (Huh et al., 2003). To further complicate matters, Pik1 binds and is positively regulated by the small, N-myristoylated, Ca^{2+}-binding protein, Frq1 (frequenin/neuronal calcium sensor-1; Hendricks et al., 1999; Ames et al., 2000); however, the role of Frq1 in subcellular localization of Pik1 has not been explored.

Given the evidence that PIP-derived products have roles in the nucleus in both yeast (Odom et al., 2000) and animal cells (Irvine, 2003), the potential duality in Pik1 localization was intriguing, suggesting that, the essential functions of this enzyme may involve generation of PtdIns4P pools both at the Golgi and in the nucleus. To this end, and to better understand the mechanisms that control its subcellular localization, we first examined the intracellular distribution and dynamics of Pik1 in vivo using a fully functional GFP-Pik1 fusion, which revealed that Pik1 undergoes nucleocytoplasmic shuttling. We then used genetic approaches to discern the requirements for nuclear import and export of Pik1. Moreover, using appropriate mutants, we also analyzed the contributions that Frq1 makes to intracellular localization of Pik1. Finally, using strategies to restrict Pik1 to either the cytosolic or nuclear compartments, we investigated whether Pik1 serves essential physiological functions at the Golgi and in the nucleus and whether its PI4K activity is required in either organelle.
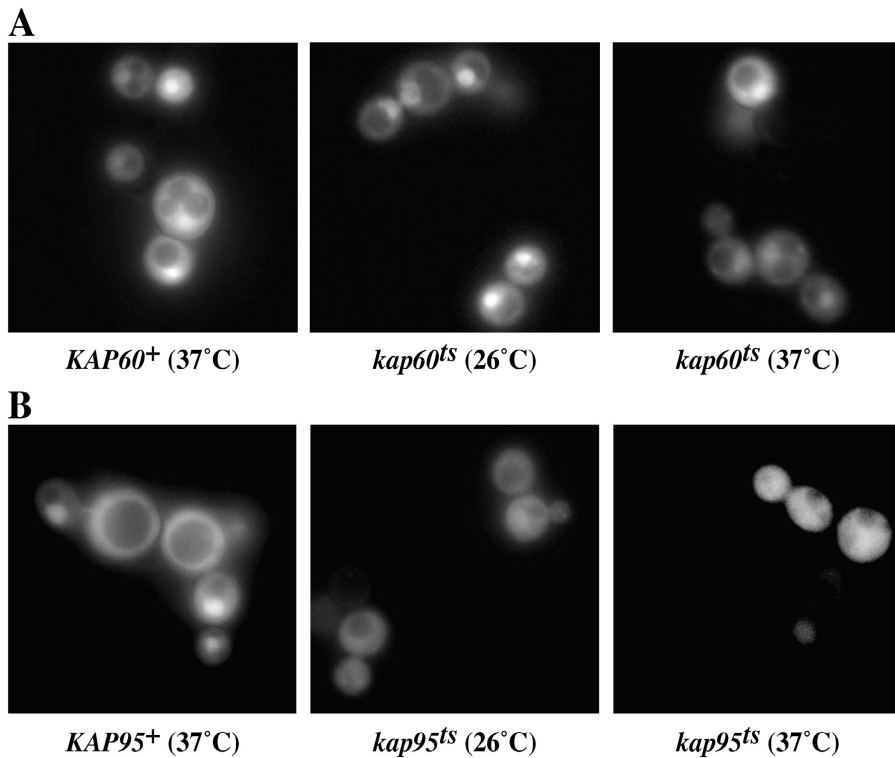
A

KAP60⁺ (37°C) kap60ᵗˢ (26°C) kap60ᵗˢ (37°C)

B

KAP95⁺ (37°C) kap95ᵗˢ (26°C) kap95ᵗˢ (37°C)

Figure 3. **Nuclear import of Pik1 requires importin-β, but not importin-α.** (A) An importin-α (*kap60ᵗˢ*) mutant (YTS0012 *srp1-31ᵗˢ*) and its otherwise isogenic *KAP60⁺* parent (W303-1a) carrying pTS7 were grown in SCRaf-Trp at 26°C to $A_{600\,nm} \approx 0.7$, and a portion of each culture was shifted to 37°C. After 1 h, expression of GFP-Pik1 was induced by addition of galactose, and the cells were incubated for an additional 2 h at the indicated temperature before being inspected by fluorescence microscopy. (B) The identical experiment described in A was performed with an importin-β (*kap95ᵗˢ*) mutant (SWY1313 *rsl1ᵗˢ*) and its otherwise isogenic parent strain (SWY1312) carrying pTS7, except that, after galactose induction, the cells were incubated for 3 h at the indicated temperature before inspection.

Results

Pik1 localizes to both the Golgi and the nucleus

Prior work (Hama et al., 1999; Walch-Solimena and Novick, 1999; Audhya et al., 2000) indicated that Pik1 function is needed for the Golgi-to-PM stage of secretion. At a restrictive temperature, *pik1ᵗˢ* mutants (but not isogenic *PIK1⁺* cells) accumulate aberrant membranous structures (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200504104/DC1) that are similar in the EM to those accumulated by *sec* mutants blocked in Golgi-to-PM transport (Esmon et al., 1981; Fig. S1 B). The hypertrophied sacs that accumulate represent Golgi cisternae because they contain bona fide Golgi marker proteins, but not marker proteins of other compartments (Fig. S1 C). However, where Pik1 itself resides had not been resolved.

To examine subcellular localization in real time in live cells, GFP fusions were constructed. An NH₂-terminal fusion (GFP-Pik1) was functional, as judged, first, by its stable expression that was determined by immunoblotting (unpublished data) and, second, by its ability to fully restore viability to *pik1Δ* cells (unpublished data). A COOH-terminal fusion (Pik1-GFP) was stable, but not functional. When expressed from the native *PIK1* promoter on a low-copy (*CEN*) plasmid in a wild-type strain, GFP-Pik1 decorated numerous cytoplasmic puncta in the majority of cells (Fig. 1 A). To demonstrate that this compartment represents the Golgi, we exploited the properties of a chimera between a diagnostic marker for the late Golgi cisternae, Sec7 (Franzusoff et al., 1991), and *Discosoma spp.* red fluorescent protein (DsRed). Because DsRed tetramerizes, normally dispersed Golgi cisternae that contain Sec7-DsRed tend to clump into larger aggregates (Reinke et al., 2004).

When GFP-Pik1 and Sec7-DsRed were coexpressed, GFP-Pik1 also clumped into a few large aggregates (Fig. 1 B, top left), just like Sec7-DsRed (Fig. 1 B, top right); and, both proteins colocalized in the majority of these clusters (Fig. 1 B, top left). Hence, the cytosolic puncta decorated by GFP-Pik1 are the Golgi itself.

However, we noted consistently that, even when expressed at a near-endogenous level, GFP-Pik1 was found in the nucleus in 2–13% of the cells (depending on the strain examined; Fig. 2 A). No correlation between bud morphology and Pik1 distribution was seen, suggesting localization is not regulated by the cell cycle. When expression of GFP-Pik1 was elevated (Fig. 2 B), all cells displayed a very bright fluorescence that was congruent with the nucleus (stained with a vital DNA dye) and the cytosolic puncta were obscured by the general increase in cellular fluorescence.

Pik1 undergoes nucleocytoplasmic shuttling

The preceding results suggested that GFP-Pik1 is imported into the nucleus and, when overexpressed, accumulates there because its rate of entry exceeds its rate of export. Given its size, this nuclear import of GFP-Pik1 should require one (or more) of the known karyopherins (Strom and Weis, 2001). Therefore, we expressed GFP-Pik1 in strains carrying null mutations or temperature-sensitive alleles in all of the known importins. Nuclear accumulation of GFP-Pik1 was not impeded in mutants lacking any of the nonessential importins: Kap104, Sxm1, Mtr10, Kap114, Nmd5, Lph2, Pse1, Pdr6, and Yrb4 (unpublished data).

Kap60/Srp1/importin-α and Kap95/Rsl1/importin-β are each essential for viability. Typically, cargo proteins that use these karyopherins to enter the nucleus bind (via their NLS) to
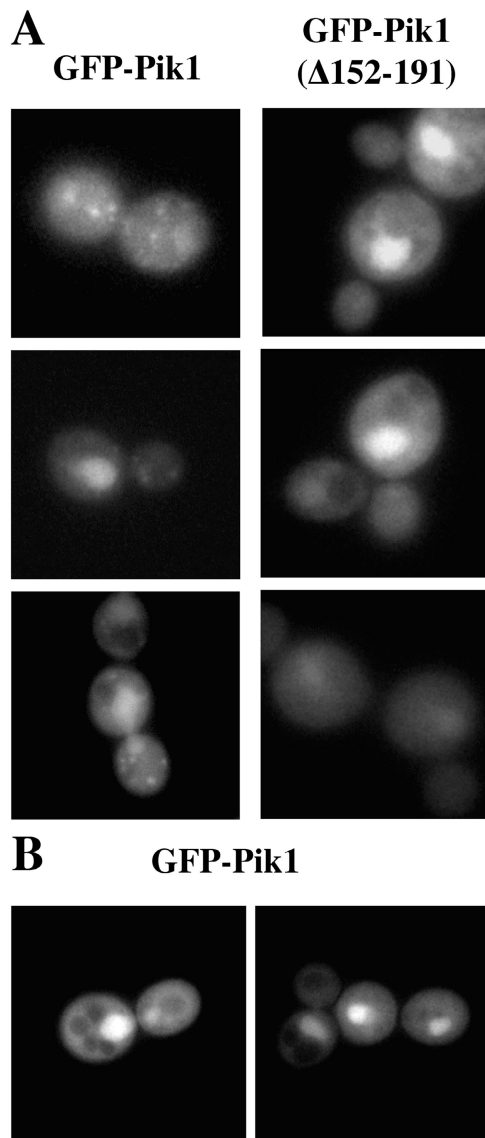
A GFP-Pik1   GFP-Pik1 (Δ152-191)

B GFP-Pik1

Figure 4. **Tethering of Pik1 at the Golgi requires Frq1.** (A) Transformants of strain BY4743 carrying pTS9 and pTS10 to express either GFP-Pik1 (left) or GFP-Pik1(Δ152-191) (right), respectively, were induced with galactose for a brief period followed by shift to glucose to repress further expression, before viewing by fluorescence microscopy. (B) A *frq1Δ* null mutant (YKBH9) is shown harboring both YEp352*GAL-PIK1* and pTS7, grown on galactose medium and viewed by fluorescence microscopy.

Frq1-GFP   Sec7-DsRed

Merge   Nomarski

Figure 5. **Frq1-GFP localizes to Golgi puncta in the cytosol.** Diploid strain YTS153, expressing *FRQ1-GFP* from its native promoter and integrated at its endogenous locus and *SEC7-DsRed* from the *TPI1* promoter and integrated at the *TRP1* locus, was grown and viewed by fluorescence microscopy, as in Fig. 1.

Kap60, which, in turn, binds to Kap95 (Enenkel et al., 1995). If Pik1 has an essential role in the nucleus, then these karyopherins may mediate its entry. In a *kap60^{ts}* mutant (Loeb et al., 1995), GFP-Pik1 still localized to the nucleus, even after a prolonged incubation at a restrictive temperature, which was no different from *KAP60$^+$* cells at the same high temperature (Fig. 3 A). In contrast, nuclear accumulation of GFP-Pik1 was largely abrogated in a *kap95^{ts}* mutant (Iovine and Wente, 1997), even at permissive temperature, whereas robust nuclear accumulation of GFP-Pik1 was seen in otherwise isogenic *KAP95$^+$* cells even at restrictive temperature (Fig. 3 B). Thus, although not unprecedented (Nikolaev et al., 2003), Pik1 is among the rare cargo whose nu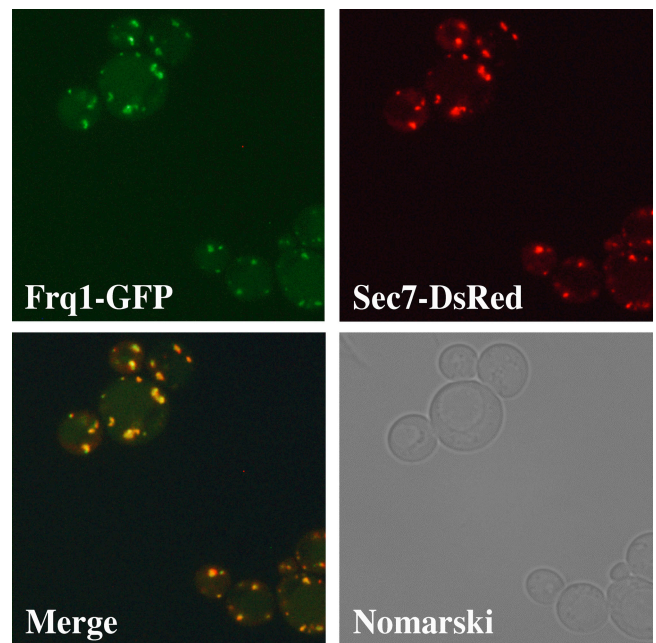clear import is mediated by Kap95 directly. Consistent with this conclusion, mutagenesis of the only close match in Pik1 to the classical NLS recognized by Kap60 (^{284}KLPKRKPK^{291} to ^{284}KLPKLLLK^{291}) did not prevent its nuclear import or otherwise alter its intracellular distribution (unpublished data).

For confirmation, the ability of Pik1 to associate directly with Kap95 was examined in vitro. Empty nickel agarose beads or beads coated with purified Kap95-His$_6$ were incubated with the soluble fraction of extracts from yeast cells carrying an empty vector or expressing NH$_2$ terminally c-Myc epitope–tagged Pik1 or a truncation, Pik1(Δ793-1066), lacking its catalytic domain. Bound proteins were analyzed by SDS-PAGE and immunoblotting. No species cross-reactive with the anti-c-Myc (αMyc) mAb was detected in extracts from cells carrying vector alone (Fig. S2, top, lanes 2 and 5; bottom, lane 2). In contrast, ∼80% of the input mycPik1 bound to the Kap95-His$_6$-coated beads, whereas <5% of the input mycPik1 bound to empty beads (Fig. S2, top panel, compare lane 1 with lane 4). Although expressed at a lower level (Fig. S2, bottom, compare lane 3 with lane 1), ∼80% of the input mycPik1(Δ793-1066) bound to the Kap95-His$_6$ beads, whereas no detectable binding to empty beads was observed (Fig. S2, top, compare lane 3 with lane 6). Thus, Pik1 binds to Kap95 via an interaction site within its NH$_2$-terminal regulatory domain.

Role of Frq1 in subcellular localization of Pik1

The NH$_2$-terminal regulatory domain of Pik1 also contains a high-affinity binding site for Frq1, and Frq1 is required for optimal activity of Pik1 and for normal Pik1 function in vivo (Hendricks et al., 1999; Ames et al., 2000; Huttner et al., 2003).

Based on its fractionation properties and its tight binding to Pik1, Frq1 might promote membrane interaction of Pik1, which lacks any known membrane-targeting motifs or domains. To determine whether Frq1 plays any role in subcellular localization of Pik1, we first examined a small deletion mutant, Pik1(Δ152-191), that retains considerable catalytic function (~80% of wild-type Pik1), but lacks nearly all Frq1-binding activity (Huttner et al., 2003). Compared with GFP-Pik1, which displayed the expected distribution between the Golgi puncta and the nucleus (Fig. 4 A, left), GFP-Pik1(Δ152-191) showed a somewhat enhanced nuclear accumulation, diffuse cytoplasmic distribution, and never any detectable cytoplasmic puncta, even in cells expressing it at a low level (Fig. 4 A, right). As independent confirmation, we took advantage of the fact that inviability of frq1Δ cells can be rescued when Pik1 is highly overexpressed. Although overexpression tends to obscure visualization of the Golgi puncta, such bodies are still seen in a fraction of FRQ1^{+} cells where GFP-Pik1 is produced from the GAL1 promoter (not depicted), but never observed in frq1Δ cells (Fig. 4 B).

If Frq1 is required for Golgi recruitment of Pik1, then Frq1 itself should localize to the Golgi. Hence, Frq1 was tagged at its COOH terminus with GFP (because its NH$_2$-terminal myristoyl group is important for its function) and was fully functional (unpublished data). Frq1-GFP displayed prominent cytoplasmic puncta, congruent with Sec7-DsRed (Fig. 5). As in pik1[ts] mutants at restrictive temperature, secretion of invertase (Suc2) is largely blocked in frq1[ts] mutants (even at permissive temperatures; Fig. S3 A, available at http://www.jcb.org/cgi/content/full/jcb.200504104/DC1) and aberrant Golgi-like structures are seen in frq1[ts] cells at the restrictive temperature (Fig. S3 B). Thus, Frq1 plays a critical role in secretion because it is necessary for recruitment of Pik1 to the Golgi, but not for its nuclear import or export.

Nuclear export of Pik1 is dependent on Msn5

A hallmark of proteins that undergo continuous nucleocytoplasmic shuttling (as opposed to those bound and sequestered in the nucleus) is that they accumulate dramatically in the nucleus if the karyopherin responsible for their active export is removed. To demonstrate that Pik1 cycles between the nucleus and the cytosol and to identify the exportin responsible, we expressed GFP-Pik1 in strains that carry temperature-sensitive or null mutations in the four known exportins (Strom and Weis, 2001). No change in distribution of GFP-Pik1 was observed in cse1[ts] or xpo1[ts] mutants at the restrictive temperature, or in a los1Δ mutant under normal growth conditions (Fig. 6), or in the corresponding parental strains (not depicted). In contrast, a majority (≥70%) of msn5Δ cells displayed prominent nuclear accumulation of GFP-Pik1 (Fig. 6), but the isogenic MSN5^{+} parental strain did not (see Fig. 7). Thus, Pik1 continually shuttles between the nucleus and cytoplasm, and Msn5 mediates its nuclear export.

Retention of Pik1 in the cytosol

To test whether nuclear entry is essential for its function, we devised means to restrain Pik1 in the cytosol. There is no

GFP-Pik1   **DIC**

cse1-1[ts] (37°C)

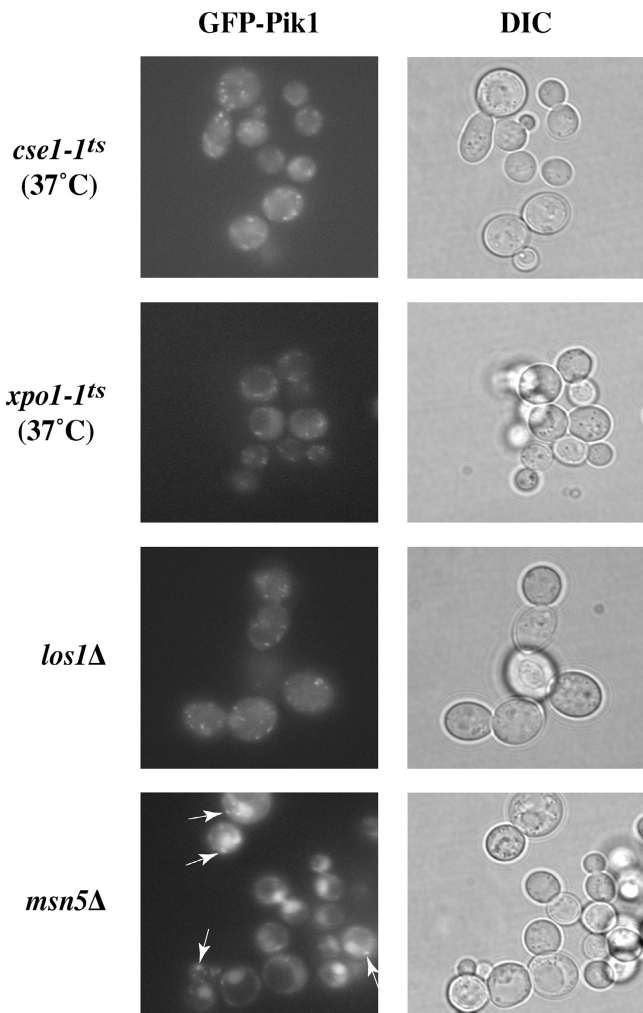xpo1-1[ts] (37°C)

los1Δ

msn5Δ

Figure 6. **Pik1 is exported from the nucleus in an Msn5-dependent manner.** Strains carrying conditional or null mutations in the exportin genes indicated (see Table II), and their otherwise isogenic parental strains, were transformed with either pTS6 or pTS8, as necessary, and inspected by fluorescence microscopy. Pronounced nuclear accumulation occurred only in the msn5Δ mutant, but cytosolic puncta were also present (arrows).

known consensus sequence for Kap60-independent, Kap95-dependent import. Hence, we exploited the COOH-terminal CCAAX motif of S. cerevisiae Ras2, which is S-prenylated, S-palmitoylated and carboxymethylated (Hancock, 2003) and serves as a constitutive membrane anchor (Pryciak and Huntress, 1998) that traffics proteins from the ER to the Golgi and then to the PM (Hancock, 2003). The five COOH-terminal residues of Pik1 (QGYIS-COOH) were replaced with the five COOH-terminal residues of Ras2 (CCIIS-COOH), designated Pik1-CCAAX. When expressed briefly from the GAL1 promoter on a CEN plasmid in normal cells, both GFP-Pik1 and GFP-Pik1-CCAAX decorated the Golgi puncta (although the latter was expressed at a somewhat lower level; Fig. 7 A), but GFP-Pik1-CCAAX was never observed in the nucleus, even upon prolonged expression from the GAL1 promoter (not depicted). When expressed in the same way in msn5Δ cells, GFP-Pik1 accumulated detectably in the nucleus, whereas GFP-Pik1-CCAAX did not (Fig. 7 B), confirming that GFP-
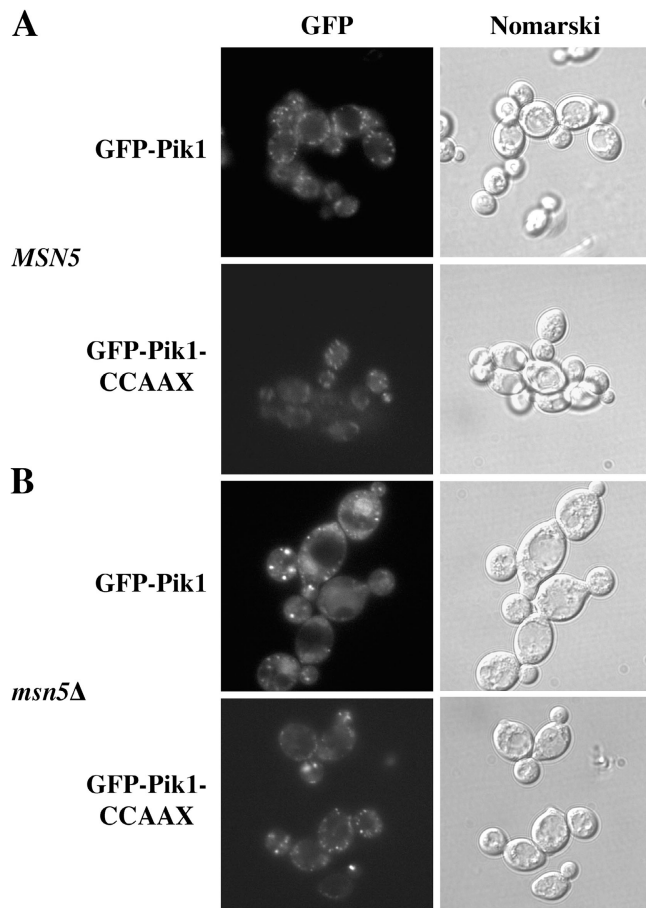
A

B

Figure 7. **GFP-Pik1-CCAAX is restrained in the cytosol.** A wild-type (*MSN5⁺/MSN5⁺*) diploid (BY4743) (A) and a homozygous *msn5Δ/msn5Δ* derivative (YTS002) (B) were transformed with pTS9 (GFP-Pik1) or pTS11 (GFP-Pik1-CCAAX), as indicated, grown to mid-exponential phase, induced with galactose for 45 min, returned to glucose medium to repress further expression, and were viewed in the fluorescence microscope (left) or under Nomarski optics (right) after 1 h.

Pik1-CCAAX does not enter the nucleus. Thus, attachment of the Ras2 CCAAX motif to the Pik1 COOH terminus did not interfere with Golgi localization, but prevented nuclear import.

Retention of Pik1 in the nucleus

A 28-residue site (147–172) in Pik1 is necessary and sufficient for Frq1 binding (Huttner et al., 2003; Strahl et al., 2003), as judged by deletion mutants that remove these residues. To determine how such mutations affect subcellular localization, we examined Pik1 derivatives tagged with an NH$_2$-terminal c-Myc epitope by indirect immunofluorescence. Generally, localization in fixed cells observed using the epitope-tagged constructs gave the same result as for GFP-Pik1 fusions in live cells, for example, GFP-Pik1-CCAAX (Fig. 7) and mycPik1-CCAAX (Fig. 8). A lack of the Frq1-binding site alone prevented Golgi targeting of GFP-Pik1 (Fig. 4 A) or mycPik1 (not depicted). A larger deletion, mycPik1(Δ10-192), compromised Golgi decoration, but also displayed prominent nuclear accumulation under conditions where mycPik1 did not (Fig. 8). Other deletions of a similar or greater size elsewhere, e.g., Pik1(Δ411-544), did not exhibit nuclear accumulation (unpublished data).
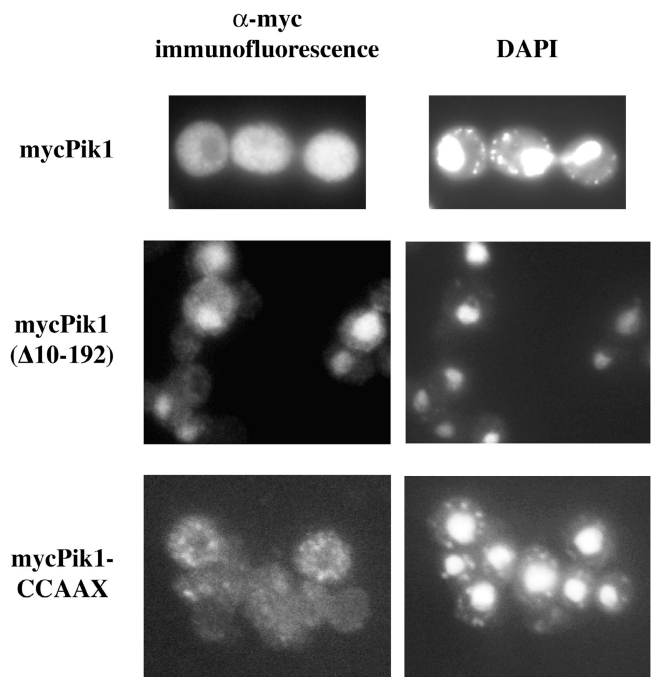

Figure 8. **Pik1(Δ10-192) accumulates in the nucleus.** Diploid strain (BYB67) expressing from pRS314*GAL-mycPIK1*, pTS2 and pTS3, respectively, mycPik1 (top), mycPik1(Δ10-192) (middle) or mycPik1-CCAAX (bottom) were fixed and examined by indirect immunofluorescence using αMyc mAb 9E10 (left), after counterstaining with DAPI to reveal the nucleus (right).

No consensus for cargo recognition by Msn5 is known, yet Pik1(Δ10-192) must lack not only its Frq1-binding site, but also some sequence required for its Msn5-dependent export, confining it in the nucleus.

Pik1 has essential functions inside and outside of the nucleus

Pik1-CCAAX does not enter the nucleus, whereas Pik1(Δ10-192) accumulates in the nucleus. Hence, we examined whether Pik1 restricted to these compartments can provide its essential function, when present as the sole source of this enzyme. Each variant was expressed from the *GAL1* promoter on a *TRP1*-marked *CEN* plasmid in a heterozygous *pik1Δ::KanMX4/PIK1⁺* diploid. The resulting transformants were sporulated, and the tetrads were dissected on Gal medium at 26°C. If a Pik1 variant is functional, it permits growth of the otherwise inviable *pik1Δ* spores. Expression of mycPik1 rescued inviability of the *pik1Δ* spores because most tetrads yielded three or four viable colonies (Fig. 9 C) and many of the spore clones were both kanamycin (G418) resistant and Trp⁺ (not depicted). Neither mycPik1-CCAAX nor mycPik1(Δ10-192) supported growth of the *pik1Δ* spores because only two viable spores were produced from every tetrad (Fig. 9, A and B) and both spores were always G418-sensitive (not depicted).

Failure to rescue *pik1Δ* spore viability could arise if Pik1-CCAAX and Pik1(Δ10-192) were expressed at a much lower level than normal Pik1; yet, immunoblotting indicated that mycPik1, mycPik1(Δ10-192), and mycPik1-CCAAX were produced at similar levels (Fig. S4, bottom; available at http://
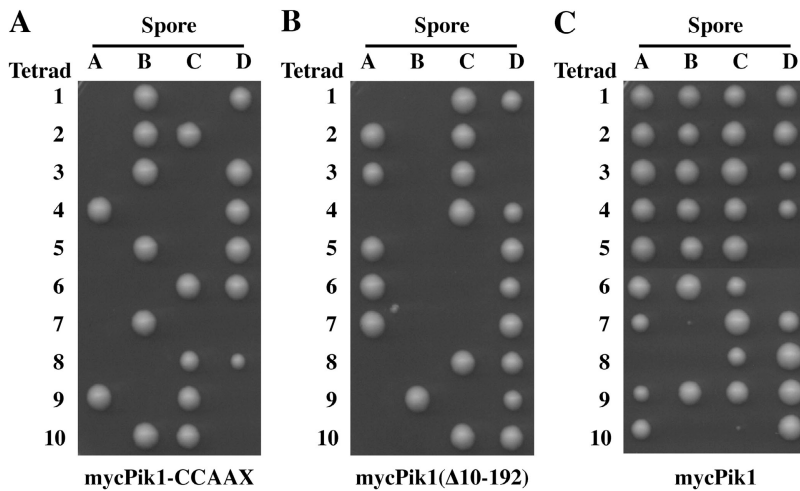
Figure 9. **Neither Pik1-CCAAX nor Pik1(Δ10-192) can rescue the inviability of _pik1Δ_ cells.** Heterozygous _pik1Δ::KanMX4/PIK1_ diploid strain (YTS68) was transformed with pTS3, pTS2 and pRS314GAL-mycPIK1 expressing, respectively, Myc-tagged Pik1-CCAAX (A), mycPik1(Δ10-192) (B), or mycPik1 (C). Each of the transformants was induced to undergo meiosis and sporulation, and the resulting tetrads were dissected and germinated on galactose medium. Spore clones (A–D) from 10 representative tetrads (1–10) of each strain are shown.

www.jcb.org/cgi/content/full/jcb.200504104/DC1). Another explanation could be that the modifications in Pik1-CCAAX or Pik1(Δ10-192) severely compromised catalytic activity. To examine this possibility, epitope-tagged proteins were recovered from extracts by immunoprecipitation and used as the enzyme source in in vitro PI4K assays. For cells expressing mycPik1, robust PtdIns4P production was found (Fig. S4, top, lane 1). A trace of PtdIns4P was generated when untagged Pik1 was overexpressed (Fig. S4, top, lane 2), presumably because, when a Myc-tagged protein is absent, some of the abundant untagged Pik1 binds nonspecifically to the c-Myc mAb-coated beads. This background disappeared when a Myc-tagged protein was present and Pik1 was at its endogenous level. For example, immunoprecipitation of a known catalytically inactive derivative, mycPik1(Δ302-785), produced no PtdIns4P (Fig. S4, top, lane 3). By contrast, mycPik1-CCAAX exhibited a specific activity indistinguishable from mycPik1 (Fig. S4, top, lane 5), whereas mycPik1(Δ10-192) displayed a specific activity about ∼10% of mycPik1 (Fig. S4, top, lane 4). Thus, the inability of Pik1(Δ10-192) to complement a _pik1Δ_ mutation might be caused by its lower catalytic potency. However, several observations suggest that this is not the case. First, very low expression of normal Pik1, for example, from the _GAL1_ promoter under repressing conditions (i.e., on glucose medium) is sufficient to support the growth of _pik1Δ_ cells (Schnieders, 1996). Second, expression of Pik1(Δ10-192) from the strong _GAL1_ promoter on a _CEN_ plasmid is still unable to rescue the inviability of _pik1Δ_ cells (Fig. 9).

As a further test that the inability of Pik1(Δ10-192) to complement is caused by its restricted compartmentalization and not its lower specific activity, mycPik1(Δ10-192) and mycPik1-CCAAX were coexpressed from the _GAL1_ promoter on differentially marked plasmids in the _pik1Δ::KanMX4/PIK1_+ diploid. _GAL1_-expressed mycPik1-CCAAX was introduced into the diploid on the two differentially marked plasmids as a control for the effect of elevated Pik1 level because of its expression from two separate plasmids. When nuclearly localized mycPik1(Δ10-192) was coexpressed with cytoplasmically localized mycPik1-CCAAX, the _pik1Δ_ spores were able to survive, as judged by recovery of tetrads with three or

four viable spores (Fig. 10 A), and Trp+ Leu+ KanR spores were recovered at a reasonable frequency (not depicted). As judged by immunoblotting, the viable Trp+ Leu+ KanR spores produced both mycPik1-CCAAX and mycPik1(Δ10-192), albeit sometimes not in equal proportions, and did so in a galactose-dependent manner (Fig. 10 B). Despite the fact that Pik1-CCAAX has a catalytic activity indistinguishable from wild-type Pik1, expression of this derivative alone from two different plasmids did not rescue inviability of the _pik1Δ_ spores because only two viable (and both G418 sensitive) spore colonies were recovered in every tetrad (Fig. 10 A). Because only those _pik1Δ_ spores that expressed Pik1 in both the nucleus and the cytosol survived, it suggests that discrete PtdIns4P pools must be generated for nuclear functions and for secretory transport. The data presented here demonstrate that this is normally achieved by nucleocytoplasmic shuttling of Pik1 and its Frq1-dependent tethering to the Golgi.

To determine whether the PI4K activity of Pik1 is required for its function at the Golgi and in the nucleus, we mutated a residue (D918A) that is invariant in all PI4Ks (and PI3Ks). The crystal structure of a type IIβ PtdIns-phosphate kinase shows the equivalent position makes contact with both ATP and the substrate (Rao et al., 1998). The corresponding mycPik1(D918A), mycPik1(D918A)-CCAAX and mycPik1(Δ10-192 D918A) proteins were stably expressed (not depicted) and upon immunoprecipitation (Fig. S5 A, bottom, available at http://www.jcb.org/cgi/content/full/jcb.200504104/DC1) none of them displayed detectable PI4K activity, whereas mycPik1 displayed robust activity under the same conditions (Fig. S5 A, top). In addition, neither mycPik1(D918A) nor GFP-Pik1(D918A) was able to complement the inviability of _pik1Δ_ spores (Fig. S5 B). Moreover, the mutation did not cause mislocalization because GFP-Pik1(D918A) was readily detectable both at Golgi puncta and in the nucleus (Fig. S5 C).

Finally, to determine whether Pik1 catalytic activity is needed for its essential functions in either compartment, either mycPik1-CCAAX and mycPik1(Δ10-192 D918A) or mycPik1(D918A)-CCAAX and mycPik1(Δ10-192) were coexpressed on differentially marked plasmids in the _pik1Δ::KanMX4/PIK1_+ diploid, which was subjected to tetrad analysis.
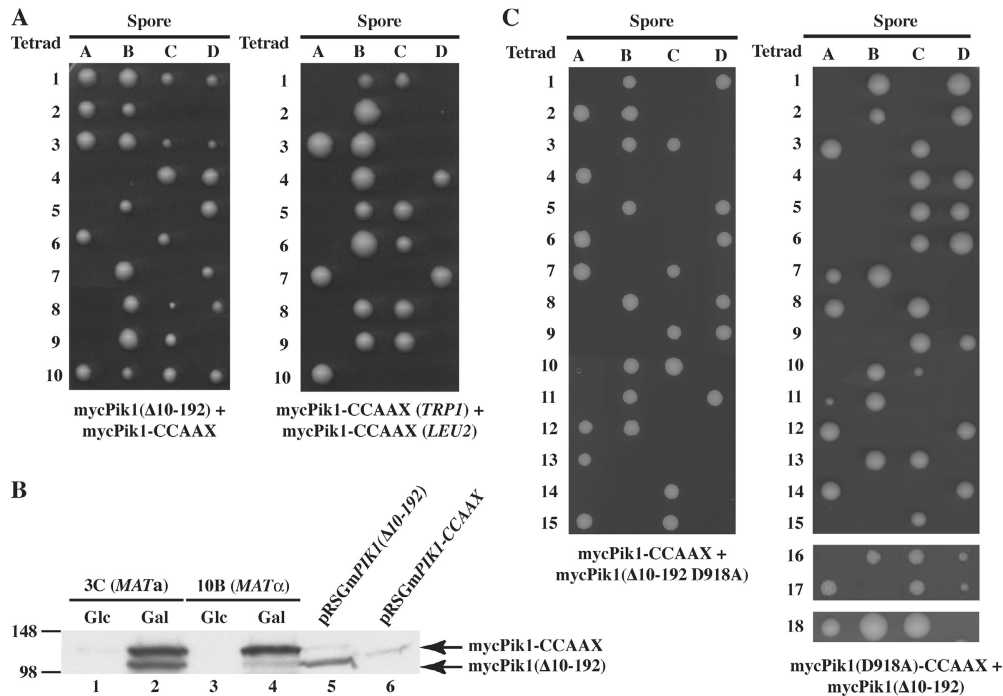
Figure 10. Interallelic complementation of the inviability of *pik1Δ* cells by Pik1-CCAAX and Pik1(Δ10-192). (A) Heterozygous *pik1Δ::KanMX4/PIK1* diploid (YTS68) cotransformed with either pTS2 and pTS4 expressing mycPik1(Δ10-192) and mycPik1-CCAAX (left) or pTS3 and pTS4 both expressing mycPik1-CCAAX (right) was sporulated and the resulting tetrads were dissected and germinated on galactose medium. Spore clones (A–D) from 10 representative tetrads (1–10) are shown. (B) Two representative G418-resistant colonies of opposite mating type were picked, propagated on galactose (Gal) or then shifted to glucose medium (Glc), and samples of the cultures were lysed and analyzed by SDS-PAGE and immunoblotting with αMyc mAb 9E10. As markers, samples of lysates from cells expressing only mycPik1(Δ10-192) (lane 5) or mycPik1-CCAAX (lane 6) were examined. (C) Tetrad analysis, as in A, was conducted with YTS68 coexpressing either mycPik1-CCAAX and mycPik1(Δ10-192 D918A) (left) or mycPik1(D918A)-CCAAX and mycPik1(Δ10-192) (right).

For the former combination, only two viable (and G418-sensitive) spores were recovered from every tetrad (Fig. 10 C, left panel), indicating that the kinase activity of Pik1 is essential for its nuclear function. For the latter combination, the vast majority of the tetrads (∼90 total) also yielded only two viable (and G418-sensitive) spores; however, in each of three tetrads, one of the *pik1Δ* (Kan[R]) spores was recovered and carried both plasmid markers (Leu[+] and Trp[+]). Nonetheless, when subsequently propagated, these cells grew very slowly (especially on glucose, where wild-type Pik1 expressed from the *GAL1* promoter supports a normal growth rate, even when repressed), appeared grossly enlarged, were highly vacuolated and/or accumulated apparent vesicular material in the cytosol (data not shown). Clearly, therefore, the kinase activity of Pik1 is also important for its function at the Golgi.

Discussion

This is the first comprehensive study of Pik1 localization in live cells. Our findings confirm our own prior observations (Schnieders, 1996; Hama et al., 1999) and those of others (Walch-Solimena and Novick, 1999; Sciorra et al., 2005), but significantly extend those conclusions and provide new insights about the factors that regulate subcellular localization of this PI4K. In particular, we demonstrated that Pik1 enters the nucleus and undergoes active nucleocytoplasmic shuttling. Moreover, we showed that a primary role of its tightly bound

regulatory subunit, Frq1, is to assist with targeting of Pik1 to the cytoplasmic face of the Golgi. By preventing cycling of Pik1 between the nucleus and the cytosol and, conversely, by coexpressing Pik1 derivatives restricted to each of those compartments, we found that execution of the cellular functions of Pik1 requires its presence in catalytically active form both in the nucleus and at the Golgi.

Pik1 and Stt4 catalyze the same biochemical reaction, but play nonoverlapping roles, as deletion of either gene is lethal and overexpression of each gene cannot compensate for the loss of the other. This lack of redundancy is now explained by localization of each essential PI4K to distinct compartments: Stt4 to the PM (Audhya and Emr, 2002) and Pik1 at the Golgi and in the nucleus. Dual localization of Pik1 is not an artifact of overexpression because it was observed when cells expressed GFP-Pik1 from its own promoter on a low-copy-number vector. Moreover, nuclear entry of Pik1 was blocked by the loss of a specific importin (Kap95), and nuclear exit was blocked by the loss of a specific exportin (Msn5). But, we cannot exclude the possibility that other karyopherins may make minor contributions to nucleocytoplasmic transport of Pik1.

Cytosolic Pik1 localizes to the Golgi. Residence of Pik1 on this compartment is in accord with prior evidence that Pik1-generated PtdIns4P is important for efficient formation of Golgi-derived secretory vesicles destined for exocytosis at the PM (Hama et al., 1999; Walch-Solimena and Novick, 1999; Audhya et al., 2000). The Pik1-containing cytosolic puncta

were identified as Golgi via colocalization with an Arf-GEF, Sec7, a well-accepted marker for this organelle in yeast (Franzusoff et al., 1991). Reportedly, Arf itself has a role in recruitment of PI4KIIIβ to the Golgi in tissue culture cells (Godi et al., 1999). Treatment of yeast cells with brefeldin A, which inhibits Sec7 GEF activity in vitro (Sata et al., 1998), causes aberrant Golgi to accumulate and blocks secretory transport (Chang et al., 2004), closely resembling the abnormal structures accumulated and the marked reduction in protein secretion seen in temperature-sensitive *pik1* mutants at the restrictive temperature. However, we have no direct evidence that any yeast Arf involved in anterograde secretory transport is required for Pik1 function (unpublished data). Rather, as shown here, its regulatory subunit, Frq1, is targeted to the Golgi and is required for recruitment of Pik1 to the Golgi. Indeed, after our discovery of Frq1 as a permanently bound positive regulator of Pik1 in yeast (Hendricks et al., 1999), evidence has accumulated that its mammalian orthologue plays a similar role in localizing PI4KIIIβ at the Golgi (Bourne et al., 2001; Kapp-Barnea et al., 2003; Haynes et al., 2005).

PtdIns4P generated by Pik1 at the Golgi is not efficiently converted to PtdIns4,5P$_2$ presumably because the PtdIns4P 5-kinase, Mss4, localizes to the PM and to the nucleus, but not to the Golgi (Desrivieres et al., 1998; Audhya and Emr, 2003). In addition, Inp53/Sjl3, a PIP 5-phosphatase that operates at the Golgi (Ha et al., 2003), may prevent inadvertent or premature conversion of PtdIns4P to PtdIns4,5P$_2$ before secretory vesicles reach the PM. It is assumed that Pik1-generated PtdIns4P serves as a specific docking epitope on the Golgi membrane for recruitment of other proteins that are involved in vesicle formation and trafficking. Although certain yeast proteins have PH domains (Levine and Munro, 2002) and PH-like domains (Li et al., 2002) that mediate their Pik1-dependent and PtdIns4P-specific localization to the Golgi, these polypeptides are not individually required for secretion. Recently, an automated global screen for mutations synthetically lethal with a *pik1^{ts}* allele implicated Gyp2, the cognate GAP for a Golgi-specific Rab family member (Ypt31), as a potential candidate (Sciorra et al., 2005). Gyp2 contains a conserved GRAM domain (Doerks et al., 2000), which is a structural motif that resembles that of PH domains (Begley et al., 2003). Whether GRAM domains bind PIPs is, however, somewhat controversial (Begley et al., 2003). Moreover, mutations in the GRAM domain that make Gyp2 nonfunctional in vivo did not alter the subcellular distribution of GFP-Gyp2 nor is a *gyp2Δ* mutant inviable. Hence, the identity of the PtdIns4P-binding protein(s) required for secretion remains elusive.

In this regard, one study showed that PI4KIIIβ (but not its kinase activity) is necessary to recruit Rab11 (the mammalian Ypt31 orthologue) to the Golgi, and that this interaction is important for subsequent membrane traffic from the Golgi to the PM (de Graaf et al., 2004). However, in addition to the tagged PI4KIIIβ derivatives followed, catalytically active endogenous PI4KIIIβ was present in the same cells. In yeast, it is claimed that Lsb6 contributes to endosome motility independent of its own ability to generate PtdIns4P (Chang et al., 2005), but Stt4 at the PM could supply PIPs for endocytosis and control of actin dynamics.

In any event, it seems well established that one vital function of Pik1 is executed at the Golgi. However, as shown here, it is not sufficient for cell viability to restrain Pik1 to the cytosol or even to Golgi membranes. When expressed alone, Pik1-CCAAX, which is stable, fully catalytically active, present abundantly at the Golgi, and able to generate copious amounts of PtdIns4P at the Golgi—as judged by decoration with a PtdIns4P-specific reporter, GFP-2X-PH-domain[Osh2] (Levine and Munro, 2002; unpublished data)—was unable to support the viability of *pik1Δ* cells. Thus, the essential functions of Pik1 are not confined to the Golgi and to the secretory process. Indeed, when Pik1-CCAAX was coexpressed with Pik1(Δ10-192) (which is only feebly active, unable to complement a *pik1Δ* mutation, and largely confined to the nucleus), the simultaneous presence of these two proteins was able to rescue the inviability of *pik1Δ* cells. When Pik1-CCAAX was coexpressed with catalytically inactive Pik1(Δ10-192), the cells were dead, confirming that PtdIns4P supplied by Pik1 in the nucleus is essential for cell function.

There is considerable evidence that PtdIns4,5P$_2$ is present in the *S. cerevisiae* nucleus and is cleaved by the PLCδ1 orthologue, Plc1 (Flick and Thorner, 1993), to generate IP$_3$ and other more highly phosphorylated IP$_x$s derived from it, which have apparent roles in regulating mRNA export (York et al., 1999), modulating transcription (Odom et al., 2000) perhaps via chromatin-remodeling complexes (Shen et al., 2003; Steger et al., 2003), and influencing telomere length (York et al., 2005). Our results pinpoint Pik1 as the PI4K that is responsible for supplying the nuclear pool of PtdIns4P and, because Mss4 also traffics to the nucleus, at least some of this lipid is presumably converted to PtdIns4,5P$_2$ to supply the substrate for Plc1. Like Pik1 and Mss4, Plc1 also undergoes active nucleocytoplasmic shuttling (Huynh, 1998). Thus, the cell brings the necessary machinery into the nucleus to ensure that these products are generated in situ. Given that IP$_x$s are small enough to freely traverse the nuclear pore complex by passive diffusion, it is perhaps not surprising that generation of IP$_x$s in the cytosol by contrived means is sufficient to support their nuclear functions (Miller et al., 2004). However, we find that when Plc1 is confined to the nucleus by point mutations that ablate its Xpo1-dependent nuclear export sequence, the rate and extent of IP$_3$ production and generation of its more highly phosphorylated derivatives is elevated (unpublished data). Thus, most likely, soluble IP$_x$s normally are generated locally within the nucleus via Plc1-dependent cleavage of the PtdIns4,5P$_2$ supplied by Pik1 and Mss4 action.

However, *plc1Δ* mutants are viable (under nonstressful conditions; Flick and Thorner, 1993), whereas *pik1Δ* mutations are lethal under all conditions (Flanagan et al., 1993). Therefore, the PtdIns4P (and/or PtdIns4,5P$_2$) derived from Pik1 action in the nucleus is not solely used as the substrate for Plc1, but must have some other essential function(s). Current evidence suggests that this essential role might be transcription. Mammalian tumor suppressor protein, ING2, is a member of a family of at least six, highly related chromatin-associated proteins and contains a plant homeodomain (PHD) motif that reportedly binds PtdIns4,5P$_2$ and PtdIns5P (Gozani et al.,

2003). Four close relatives of ING2 exist in the *S. cerevisiae* genome, all contain PHD motifs, and all are components of nuclear chromatin remodeling machines involved in gene regulation. Whether yeast PHD proteins bind PIPs is not known.

Also, tethering of transcription factors to integral membrane proteins on the inner face of the yeast nuclear envelope seems to have a critical role in regulating the expression of certain essential genes during the unfolded protein response and presumably during adaptation to other cellular stresses (Brickner and Walter, 2004). Perhaps Pik1-generated PIPs affect organization, stability, or function of the nuclear envelope proteins required for this transcriptional control. However, like other secretory blocks, shift of *pik1^ts* mutants to restrictive temperature induces robust *UPR-lacZ* reporter expression (unpublished data); hence, sustained Pik1-dependent PIP production is not needed, at least for the unfolded protein response. Potential involvement of Pik1-derived products in transcriptional regulation may be coupled to a perhaps related phenomenon. Certain proteins that reside in the nucleus rapidly relocalize to the cytosol when the secretory pathway is compromised (Nanduri et al., 1999). The so-called cell integrity MAPK signaling pathway initiated by Pkc1 is reportedly necessary for this response (Li et al., 2000; Nanduri and Tartakoff, 2001). When a secretion-defective mutant (*sec6-4*) was shifted to restrictive temperature for only 10 min, all nuclearly associated Pik1 was lost (Walch-Solimena and Novick, 1999). Hence, Pik1 may be a protein that rapidly relocalizes to the cytosol upon secretory stress. Nucleocytoplasmic shuttling of Pik1 may, therefore, be under regulation and Pik1 itself (or some component involved in its nucleocytoplasmic transport) may be a target of Pkc1 or of the MAPK (Mpk1/Slt2) it activates.

Materials and methods

Construction of plasmids

Plasmids (Table I) were constructed using standard recombinant DNA methods. *Escherichia coli* strains DH5α and NM522 were used to manipulate and propagate plasmids. PCR was performed using *Pfu* DNA polymerase (Stratagene); all constructs were verified by dideoxy chain termination sequencing. To produce kinase-dead Pik1, DNA fragments encoding a D918A mutation were generated by PCR using appropriate primers and incorporated by homologous recombination-mediated double strand break repair in vivo via cotransformation with acceptor vectors linearized with PflMI and BspEI (pTS12 and pTS16), BglII and BspEI (pTS13) or NheI and SacI (pTS15) in strain BJ2407. Plasmid DNA recovered from the resulting Leu⁺ or Trp⁺ transformants was rescued and amplified in *E. coli*. A YEp24-based plasmid encoding *PEP4* (pBJ2802; gift of E.W. Jones, Carnegie Mellon University, Pittsburgh, PA) was used to allow sporulation of the protease-deficient diploid strain BJ2407. GEX2TK-*KAP95-HIS₆* (gift of M. Rexach, Stanford University, Stanford, CA) was used to prepare GST-Kap95-His₆ in *E. coli*.

Yeast strains, growth conditions, and tetrad dissections

Yeast strains used in this study are listed in Table II. Standard rich (YP) and synthetic complete media (Sherman et al., 1986) were supplemented with either 2% glucose, 2% raffinose, or 2% galactose and with appropriate nutrients to maintain selection for plasmids. Yeast was cultivated at 30°C, unless otherwise stated. Standard methods for DNA-mediated transformation, sporulation, tetrad dissection, and other genetic manipulations of yeast cells were used (Guthrie and Fink, 1991). To generate a novel *pik1* null allele, a *KANMX4* cassette, flanked by short stretches (45 bp) of homology to sequences immediately upstream and downstream of the *PIK1* coding region in the yeast genome, was amplified in a standard PCR reaction using pFA6a-*KANMX4* as the template. The resulting DNA fragments were introduced by DNA-mediated transformation into strain BJ2407, and the cells were plated on YPD medium containing 200 μg/liter G418 sulfate (Mediatech Inc.). Candidate antibiotic-resistant integrants were restreaked on the same medium containing G418 and authentic transplacement of the *PIK1* locus by the *pik1Δ::KANMX4* allele on one homologue was verified by analytical PCR using appropriate primers. Strains YTS129 and YTS131 were generated in a similar manner, using strain BY4743 and

Table I. **Plasmids used in this study**

Plasmid	Marker	Promoter	Insert	Reference or source
pRS314myc*PIK1*	*TRP1*	*PIK1*	myc*PIK1*	Hendricks et al., 1999
pRS314*GAL*-myc*PIK1*	*TRP1*	*PIK1*	myc*PIK1*	Hendricks et al., 1999
pTS12[a]	*TRP1*	*GAL1*	myc*PIK1(D918A*	This study
pTS1	*TRP1*	*GAL1*	myc*PIK1(Δ302-785)*	This study
pTS2	*TRP1*	*GAL1*	myc*PIK1(Δ10-192)*	This study
pTS13	*LEU2*	*GAL1*	myc*PIK1(Δ10-192)*	This study
pTS14	*LEU2*	*GAL1*	*mycPIK1 (Δ10-192 D918A)*	This study
pTS3	*TRP1*	*GAL1*	*mycPIK1-CCAAX*	This study
pTS15	*TRP1*	*GAL1*	*mycPIK1(D918A)-CCAAX*	This study
pTS4	*LEU2*	*GAL1*	*mycPIK1-CCAAX*	This study
pRS314*GAL-mycPIK1(Δ793-1066)*	*TRP1*	*GAL1*	myc*PIK1(Δ793-1066)*	Hendricks et al., 1999
pTS6	*TRP1*	*PIK1*	*GFP-PIK1*	This study
pTS7	*TRP1*	*GAL1*	*GFP-PIK1*	This study
pTS8	*LEU2*	*PIK1*	*GFP-PIK1*	This study
pTS9	*LEU2*	*GAL1*	*GFP-PIK1*	This study
pTS16	*LEU2*	*GAL1*	*GFP-PIK1(D918A)*	This study
pTS10	*LEU2*	*GAL1*	*GFP-PIK1(Δ152-191)*	This study
pTS11	*LEU2*	*GAL1*	*GFP-PIK1-CCAAX*	This study
pFA6a-kanMX4	kan^r	*TEV*	*KanMX4*	Longtine et al., 1998
pFA6a-GFP(S65T)-kanMX6	kan^r	*TEV*	*GFP(S65T)-KanMX6*	Longtine et al., 1998
BJ2802	*URA3*	*PEP4*	*PEP4*	E.W. Jones
pGST-Kap95-His₆	Amp^R	*tac*	*GST-KAP95-His₆*	M. Rexach
YEp352*GAL-PIK1*	*URA3*	*GAL1*	*PIK1*	Hendricks et al., 1999
YIplac204-T/C-*SEC7*-DsRed.T4	*TRP1*	*TPI1*	*SEC7*-DsRed	Reinke et al., 2004

[a]All pTS plasmids are based either on pRS314 (*TRP1*) or pRS315 (*LEU2*) (Sikorski & Hieter, 1989).

appropriate primers to replace the *PSE1* or *MTR10* chromosomal loci, respectively, with a *KANMX4* cassette. A one-step PCR-based technique for targeted modification of chromosomal loci (Longtine et al., 1998) was used to generate *FRQ1-GFP*. In brief, oligonucleotide primers with 5'-ends corresponding, respectively, to the last 40 bp of the *FRQ1* ORF (forward) or to the first 40 bp downstream of the respective stop codons (reverse), and with 3'-ends to allow in-frame amplification of the *GFP (S65T)-KanMX6* cassette, were used for PCR with pFA6a-*GFP(S65T)-KanMX6* as the template. The resulting DNA products were purified, introduced by transformation into strain BY4743, and G418-resistant transformants were selected. Genomic DNA was prepared from candidate clones to verify its integration by homologous recombination at the *FRQ1* locus and sequenced to confirm the integrity of the construct. For integration of YIplac204-T/C-*SEC7*-DsRed.T4, the plasmid was cut with Bsu36 I before transformation.

Fluorescence microscopy and indirect immunofluorescence

To view GFP and/or DsRed fusion proteins, cells were grown to mid-exponential phase and examined directly under a fluorescence microscope equipped with appropriate band-pass filters for detection of each protein. To counterstain for DNA, cells were incubated in growth medium with 2 µg/ml Hoechst 33258 (Invitrogen) for 15 min before viewing. Unless otherwise noted, expression of GFP fusion proteins under control of the *GAL1* promoter was induced by adding galactose (2% final concentration) to the culture and, after 45 min, glucose was added (2% final concentration) to repress further expression and the cells were grown for 1 h. To localize GFP-Pik1 in strains SWY1312 and SWY1313, cells first were grown at 26°C, shifted to 37°C for 1 h, and then galactose was added to induce *GAL1* promoter-driven expression; after 3 h, the cells were viewed by fluorescence microscopy. To localize GFP-Pik1 in strains YTS0012 and W303-1a (*kap60*[ts] and *KAP60*^{+}, respectively), cells were

Table II. **Strains used in this study**

Strain	Genotype	Reference or source
BJ2168	*MATa leu2 trp1 ura3-52 prb1-1122 prc1-40* pep4-3 gal2	Jones, 1991
CFY13	*hsc83∆::LEU2 pep4∆::URA3* (derived from BJ2168)	Flanagan and Thorner, 1992
BJ2407	*MATa/MATα* (otherwise isogenic to BJ2168)	Jones, 1991
YTS68	*pik1∆::KanMX4/PIK1* [pBJ2802] (otherwise isogenic to BJ2407)	This study
YTS153	*FRQ1-GFPkanMX6::FRQ1/FRQ1 SEC7-*DsRed::TRP1/TRP1 (otherwise isogenic to BJ2407)	This study
BY4743	*MATa/MATα his3-∆1/his3-∆1 leu2-∆0/leu2-∆0 lys2-∆0/lys2-∆0 ura3-∆0/ura3-∆0*	Brachmann et al., 1998
YTS001	*los1∆/los1∆* (otherwise isogenic to BY4743)	Brachmann et al., 1998
YTS002	*msn5∆/msn5∆* (otherwise isogenic to BY4743)	Brachmann et al., 1998
YTS005	*kap108∆/kap108∆* (otherwise isogenic to BY4743)	Brachmann et al., 1998
YTS006	*kap114∆/kap114∆* (otherwise isogenic to BY4743)	Brachmann et al., 1998
YTS007	*kap120∆/kap120∆* (otherwise isogenic to BY4743)	Brachmann et al., 1998
YTS008	*kap122∆/kap122∆* (otherwise isogenic to BY4743)	Brachmann et al., 1998
YTS009	*kap123∆/kap123∆* (otherwise isogenic to BY4743)	Brachmann et al., 1998
BY4741	*MATa* (otherwise isogenic to BY4743)	Brachmann et al., 1998
YTS129	*pse1∆::KanMX4* (otherwise isogenic to BY4741)	This study
YTS131	*mtr10∆::KanMX4* (otherwise isogenic to BY4741)	This study
KWY12	*MATa ade2-101 his3-11,15 trp1-∆901 ura3-52*	Xiao et al., 1993
KWY125	*cse1-1* (otherwise isogenic to KWY124)	Xiao et al., 1993
BYB67	*MATa/MATα ADE2/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,11 LYS2/lys2∆::hisG trp1-1/trp1-1 ura3-1/ura3-1* (derived from W303)	Shulewitz et al., 1999
W303-1a	*MATa ade2-1 LYS2* (derived from W303)	Loeb et al., 1995
KWY121	*MATα ade2-1 LYS2 xpo1∆::LEU2* [pKW457/pRS313-*xpo1-1*[ts]] (otherwise isogenic to W303-1a)	Stade et al., 1997
YKBH9	W303-1a [YEp352*GAL-PIK1*]	Hendricks et al., 1999
RSY298	*sec7-1*[ts] (otherwise isogenic to W303-1a)	Esmon et al., 1981
YTS001	*srp1-31*[ts] (otherwise isogenic to W303-1a)	Loeb et al., 1995
YTS0010	*MATa his3-∆200 leu2-3, 2-112 lys2-801 trp1-1 ura3-52 kap104∆::URA3*	Aitchison et al., 1996
YTS0011	*MATα his3-∆200 leu2-3, 2-112 lys2-801 trp1-1 ura3-52 nmd5∆::HIS3*	Albertini et al., 1998
SWY1312	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 kap95∆::HIS3* [pSW503/pRS315-*KAP95*]	Iovine and Wente, 1997
SWY1313	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 kap95∆::HIS3* [pSW509/pRS315-*kap95(L63A)*[ts]]	Iovine and Wente, 1997
YPH499	*MATa ade2-101*[och] *his3-∆200 leu2-∆1 lys2-801*[am] *trp1-∆63 ura3-52*	Sikorski and Hieter, 1989
YKBH4	*frq1-1::HIS3* (otherwise isogenic to YPH499)	Hendricks et al., 1999
YES32	*PIK1::TRP1* (otherwise isogenic to YPH499)	Schnieders, 1996
YES102	*pik1-83::TRP1* (otherwise isogenic to YPH499)	Schnieders, 1996
YTS114	*prom*[TPI1]-*SEC7-*DsRed::TRP1 [pTS8] (otherwise isogenic to YPH499)	This study
CTY1-1a	*MATa his3-∆200 lys2-801 ura3-52 sec14-3*	Hama et al., 1999

treated as just described, but incubated at 37°C for as long as 5 h after galactose induction.

BYB67 cells carrying plasmids for the *GAL1* promoter-driven expression of either mycPik1, mycPik1(Δ10-192) or mycPik1-CCAAX were grown to A$_{600\ nm}$ ≈ 0.5, induced by addition of galactose (2% final concentration) for 90 min, fixed, and prepared for indirect immunofluorescence. Cells were incubated with a solution (1 mg/ml) of affinity-purified αMyc mAb 9E10 that was diluted 1:150 in PBS, pH 7.3, containing 1 mg/ml BSA (PBSA) for 2 h at RT, washed three times with PBSA, and then incubated for 1 h with FITC-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) that was diluted 1:700 in PBSA. To counterstain for DNA, DAPI was added (1 μg/ml final concentration) during the last 2 min of incubation. Subsequently, cells were washed six times with PBS, mounted in Citifluor (Ted Pella Inc.), examined under either a Plan Apo 100×/1.4 oil objective on a fluorescence microscope (Eclipse TE300; Nikon) equipped with a charged-coupled device camera (Orca 100; Hamamatsu) and compatible software (Phase 3 Imaging Systems, Inc.) or a D Plan Apo UV 100×/1.3 oil objective on a BH-2 fluorescence microscope equipped with a MagnaFire SP charged-coupled device camera and software (Olympus).

Preparation of yeast cell extracts and immunoprecipitation
Yeast cell lysis, preparation of extracts, and immunoprecipitations were performed as described previously (Hendricks et al., 1999; Huttner et al., 2003).

Online supplemental material
Supplemental materials and methods section describes the following protocols: negative stain and immunogold EM; preparation of Kap95-His6 and in vitro binding studies; invertase secretion measurement; and measurement of PI4K activity. Fig. S1 shows aberrant membrane structures accumulated in *pik1*^{ts} mutants at restrictive temperature are hypertrophied cisternae enriched in Golgi marker proteins. Fig. S2 shows purified importin-β binds Pik1. Fig. S3 depicts a *frq1*^{ts} mutant is defective in secretory transport and accumulates aberrant membrane structures at restrictive temperatures. Fig. S4 shows that both Pik1-CCAAX and Pik1(Δ10-192) are catalytically active. Fig. S5 characterizes the catalytically inactive mutant, Pik1(D918A). Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200504104/DC1.

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