Nucleocytoplasmic Shuttling of the Golgi Phosphatidylinositol 4-Kinase Pik1 Is Regulated by 14-3-3 Proteins and Coordinates Golgi Function with Cell Growth

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The yeast phosphatidylinositol 4-kinase Pik1p is essential for proliferation, and it controls Golgi homeostasis and transport of newly synthesized proteins from this compartment. At the Golgi, phosphatidylinositol 4-phosphate recruits multiple cytosolic effectors involved in formation of post-Golgi transport vesicles. A second pool of catalytically active Pik1p localizes to the nucleus. The physiological significance and regulation of this dual localization of the lipid kinase remains unknown. Here, we show that Pik1p binds to the redundant 14-3-3 proteins Bmh1p and Bmh2p. We provide evidence that nucleocytoplasmic shuttling of Pik1p involves phosphorylation and that 14-3-3 proteins bind Pik1p in the cytoplasm. Nutrient deprivation results in relocation of Pik1p from the Golgi to the nucleus and increases the amount of Pik1p–14-3-3 complex, a process reversed upon restored nutrient supply. These data suggest a role of Pik1p nucleocytoplasmic shuttling in coordination of biosynthetic transport from the Golgi with nutrient signaling.

INTRODUCTION

Phosphoinositides (PIs) are a family of lipids that include several derivatives distinguished by the phosphorylation status of their inositol headgroups. PIs play essential roles in the regulation of membrane traffic. The regulation of the activity and localization of PI kinases and phosphatases that modify PI headgroups is of central importance in the understanding of basic cellular processes such as secretion and growth or establishment of polarity.

The phosphatidylinositol 4-kinases (PI 4-kinase) Pik1p in yeast and PI 4-kinase IIβ in vertebrates are found at the trans-Golgi network (TGN) (Godi et al., 1999; Walch-Solimena and Novick, 1999; Sciorrà et al., 2005; Strahl et al., 2005), and they are key regulators of membrane transport and Golgi homeostasis. Mutants with defects in PI(4)P production by these lipid kinases showed reduced secretion, delayed vacuolar transport, defective protein retrieval from endosomes to the Golgi, and aberrant Golgi morphology (Godi et al., 1999; Hama et al., 1999; Walch-Solimena and Novick, 1999; Audhya et al., 2000). Recent work, mainly in mammalian cells, has revealed effectors of phosphatidylinositol 4-phosphate [PI(4)P] such as the PI(4)P adapter proteins FAPP1 and FAPP2, which are involved in the generation of transport carriers for exocytosis (Godi et al., 2004; Vieira et al., 2005) and the clathrin adaptor AP-1 (Wang et al., 2003; Heldwein et al., 2004) that participates in the formation of clathrin-coated vesicles. In the mammalian system, Golgi PI(4)P is not only generated by the type IIβ PI 4-kinase (Wong et al., 1997; Godi et al., 1999, 2004) but also by type Iα (Wei et al., 2002; Weixel et al., 2005).

In addition to their TGN localization, both Pik1p and PI 4-kinase IIβ also localize to the nucleus (Garcia-Bustos et al., 1994; Walch-Solimena and Novick, 1999; de Graaf et al., 2002; Strahl et al., 2005). Although the nuclear function of the two PI 4-kinases remains unknown, it has been demonstrated that they undergo nucleocytoplasmic shuttling (de Graaf et al., 2002; Strahl et al., 2005). Reversible relocation of Pik1p from the nucleus to the cytoplasm has been observed upon block of secretion in several sec (secretion) mutants as part of the so-called “arrest of secretion response” (ASR) (Walch-Solimena and Novick, 1999; Nanduri and Tartakoff, 2001). However, the physiologic significance and regulation of this process is not understood.

Nucleocytoplasmic shuttling is a behavior of many proteins, such as kinases, transcription factors, and cell cycle regulators (for review, see Nigg, 1997). There are several

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Abbreviations used: ASR, arrest of secretion response; λ-PPase, λ-protein phosphatase; GST, Glutathione S-transferase; PAP, peroxidase anti-peroxidase; PI, phosphoinositide; PI 4-kinase, phosphatidylinositol 4-kinase; PI(4)P, phosphatidylinositol 4-phosphate; TGN, trans-Golgi network; TAP, tandem affinity purification.

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examples in which this shuttling process is regulated by 14-3-3 proteins, a family of phosphorylation-dependent chaperones. 14-3-3 proteins are ubiquitous and highly conserved proteins, which alter protein localization or activity, modify protein–protein interactions, and induce conformational changes of their targets (for reviews, see Muslin and Xing, 2000; Tzivion and Avruch, 2002; Yaffe, 2002; Dougherty and Morrison, 2004; van Heusden and Steensma, 2006). The 14-3-3 proteins play a role in cellular processes such as cell cycle regulation, apoptosis, and cell proliferation (for review, see Dougherty and Morrison, 2004). In yeast, the two 14-3-3 proteins Bmh1p and Bmh2p are redundant and in combination essential proteins. They have been shown to play a role in the polarization of the actin cytoskeleton, cell cycle regulation at the G1/S transition, and regulation of transcription factors involved in nutrient signaling (Lottersberger et al., 2006; van Heusden and Steensma, 2006).

The only currently known binding partner of Pik1p is the calmodulin-like protein frequenin-1 (Frq1p), which functions in binding of Pik1p to the Golgi (Hendrickx et al., 1999; Ames et al., 2000; Strahl et al., 2005). We now report that Pik1p binds to the yeast 14-3-3 proteins Bmh1p and Bmh2p. The Pik1p–14-3-3 interaction requires phosphorylation, occurs primarily in the cytoplasm, and controls nucleocytoplasmic shuttling and availability of Pik1p for Golgi recruitment. This interaction interferes with secretion, because overexpression of BMH2 results in a phenocopy of pik1-101 mutant transport phenotypes in secretion. We further show that under nutrient deprivation the PI 4-kinase relocates from the TGN to the cytoplasm and into the nucleus. This relocation is rapidly reversed upon restoration of nutrient supply. Therefore, the nucleocytoplasmic shuttling of Pik1p and 14-3-3 interaction might be part of a regulatory circuit that coordinates cell growth cues with PI(4)P production at the TGN, possibly to adjust membrane transport to the growth requirements of the cell.

MATERIALS AND METHODS

Yeast Strains, Media, and Materials

The genotypes of the Saccharomyces cerevisiae strains used in this study are listed in Supplemental Table 2. Yeast strains were cultured in YPD, SC dropout media, or SD minimal media supplemented with necessary amino acids. Yeast were transformed using the lithium acetate method (Schiestl and Russell, 2000). Plasmid and Strain Constructions

Plasmid and Strain Constructions

Constructs used in this study are listed in Supplemental Table 3. General molecular biological methods were as described previously (Sambrook and Russell, 2001). Plasmid pMBL31 (LexA yeast two-hybrid vector) was constructed by replacing the GAL4 DNA-binding domain of vector pGBKT7 (Clontech, Mountain View, CA) by the coding region of LexA flanked by ADH promoter and terminator as described for pMBL33 (Brickley et al., 2005). Fusion constructs with the GAL DNA-binding domain and with LexA were obtained by in frame ligation of polymerase chain reaction (PCR) products. The pik1s396A and pik1s396D mutations have been generated by site-directed mutagenesis (QuikChange Multi kit; Stratagene, La Jolla, CA), and they were introduced into pRS415 containing the mutagenesis (QuikChange Multi kit; Stratagene, La Jolla, CA). Pik1p binds to the yeast 14-3-3 proteins Bmh1p and Bmh2p. The Pik1p–14-3-3 interaction requires phosphorylation, occurs primarily in the cytoplasm, and controls nucleocytoplasmic shuttling and availability of Pik1p for Golgi recruitment. This interaction interferes with secretion, because overexpression of BMH2 results in a phenocopy of pik1-101 mutant transport phenotypes in secretion. We further show that under nutrient deprivation the PI 4-kinase relocates from the TGN to the cytoplasm and into the nucleus. This relocation is rapidly reversed upon restoration of nutrient supply. Therefore, the nucleocytoplasmic shuttling of Pik1p and 14-3-3 interaction might be part of a regulatory circuit that coordinates cell growth cues with PI(4)P production at the TGN, possibly to adjust membrane transport to the growth requirements of the cell.

Interaction of Pik1p with 14-3-3 Proteins

The strain CSY370 was used for purification of Pik1p complexes and the Frq1p–TAP strain for Frq1p. TAP from 12 of 1 YPD culture was performed as described previously (Rigaut et al., 1997; Puig et al., 2001) with the modification that complete protease inhibitors were used throughout the procedure. When indicated, extracts were performed in the presence of phosphate inhibitors (25 mM NaF and 100 μM Na3VO4). Proteins were separated by SDS-PAGE (4–16% gradient) and visualized with Coomassie, and bands were excised and in-gel digested with trypsin and analyzed by a combination of matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS/MS) peptide mapping and nanoelectrospray (nanoES) tandem mass spectrometry (MS/MS) as described previously (Shevchenko et al., 1996) by using, respectively, REFLEX IV MALDI time of flight (TOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) and QSTAR Pulsar i hybrid quadrupole time-of-flight mass spectrometer (MDS Sciex, Concord, ON, Canada). Database searches were performed by Mascot, version 2.1 software (Matrix Science, London, United Kingdom) installed on a local server against the subset of S. cerevisiae proteins in MSDB protein sequence database.

Gap1p Activity Assay

The Gap1p activity was determined by measuring the uptake rate of 3H[14C]citrulline as described previously (Röberg et al., 1997).

Identification of Phosphorylation Sites by Mass Spectrometry

Phosphorylated Pik1p bands were excised. Disulfide bond reduction/alkylation, tryptic in-gel digestion, and sample preparation before analysis by MS were performed as described previously (Bauer and Krause, 2005). Separation of phosphorylated peptides was performed by selective binding on titanium.
Pik1p binds to the 14-3-3 proteins Bmh1p and Bmh2p

Pik1p has been reported to be present in a protein complex isolated by the TAP proteomic approach in which Bmh2p has been used as the bait (Gavin et al., 2002). Besides Pik1p, the Bmh2p complex contained Bmh1p, the second 14-3-3 protein found in yeast, the neutral trehalase proteins Nth1/2p, the transcription regulator Rtg2p, and the serine/threonine protein kinase Fun31p (Gavin et al., 2002). Using a yeast two-hybrid approach, we confirmed the interaction between Pik1p (amino acids [aa] 2–1066) and Bmh2p (Figure 1A, middle). Pik1p did not interact with other components of the Bmh2p complex in this assay (data not shown). The Pik1p–Bmh2p interaction was 10 times weaker than the interaction of Pik1p with Frq1p (Figure 1B). This interaction was lost upon deletion of the C-terminal catalytic domain of Pik1p (aa 770–1066) (Figure 1A, left). Bmh1p, which is highly similar to Bmh2p, also bound Pik1p in the yeast two-hybrid assay (Figure 1, A and B).

To confirm the observed interaction in vitro, we next tested the association of 14-3-3 GST fusions with endogenous Pik1p in GST pull-down experiments. Full-length Bmh1p and a construct encompassing a region of ~97% identity between Bmh1p and Bmh2p (GST-Bmh11–198) interacted with Pik1p from yeast lysates (Figure 1C). We then asked whether the Pik1p interaction with 14-3-3 is direct. Full-length Pik1p was synthesized by in vitro transcription and translation in the presence of [35S]methionine. Radiolabeled Pik1p was incubated with GST-Bmh1p or GST-Bmh2p. As shown in Figure 1D, either GST fusion protein was able to pull down Pik1p, suggesting a direct interaction between the tested partners.
To test whether this interaction occurs in vivo, we performed a TAP experiment. The chromosomal copy of *PIK1* was fused to the TAP tag. The fusion protein of the essential PI 4-kinase was functional only when we inserted a six-amino acid linker (SGPGS) at the C terminus of Pik1p as suggested by normal growth of the resulting strain (data not shown). The identification of coisolated proteins by mass spectrometry revealed interaction of Pik1p, Bmh1p/Bmh2p, and Frq1p (Figure 1E), whereas other proteins represented common contaminants of the TAP procedure (Shevchenko et al., 2002). We conclude that the Pik1p–14-3-3 complex occurs in vivo and includes both Bmh1p and Bmh2p, which often function as a heterodimer (Jones et al., 1995).

**Pik1p, Bmh1/2p, and Frq1p Occur in a Common Complex**

The interaction of Pik1p with both Bmh1/2p and Frq1p opens the question whether these binding partners can occur in a common complex or bind in a mutually exclusive manner. We performed glycerol velocity gradient centrifugation to determine size and composition of Pik1p–14-3-3–containing protein complexes providing support for the existence of a heterotetrameric complex (Pik1p, Frq1p, and Bmh1/2p). As shown in Supplemental Figure 1, Pik1p and Frq1p were mainly present in fractions 3–10, with a peak in fractions 4–5 corresponding to ~230 kDa. The major pool of Bmh1p seems to be present in oligomeric complexes, because this protein is only weakly detectable in the monomer and dimer fractions 1–3. An antibody detecting both Bmh1p and Bmh2p revealed an identical behavior in the gradients, supporting involvement of heterodimers in the complex with Pik1p. This is in agreement with published data reporting that Bmh1/2p mainly occur as heterodimers (Chaudhri et al., 2003). All four proteins together were found in a peak in fractions 5–7 that runs between 230 and 670 kDa, suggesting the presence of a heterotetrameric complex involving Pik1p, Frq1p, Bmh1p, and Bmh2p (Supplemental Figure 1).

For further confirmation of this hypothesis, we then isolated Frq1p–TAP-containing protein complexes using the TAP purification protocol (Figure 2). Western blotting of purified Frq1p–TAP complexes (Figure 2A) and mass spectrometry (Figure 2B and Supplemental Table 4) provided evidence for copurification of Bmh1/2p and thus for the presence of these proteins in a common protein complex.

**BMH Function Interferes with the Late Secretory Pathway**

The observed interaction of Bmh1p/Bmh2p with Pik1p prompted us to address a possible role of this complex in the regulation of secretion. Although we did not find synthetic
genetic interaction of bmh1Δ or bmh2Δ with an allele of pik1-101 impaired in catalytic activity (data not shown), we did observe enhancement of the growth defect of pik1-101 by overproduction of Bmh proteins (2μ BMH1 or BMH2) (Figure 3A). We further tested the effect of the overproduction of 14-3-3 in mutants along the secretory pathway (Supplemental Table 1). Synthetic enhancement of the growth defect by 14-3-3 overproduction was found in mutants of genes involved in Golgi vesicle formation (act1Δ, rps1Δ, ypt31Δ ypt32ts, and sec14-3) and in mutants of genes required for TGN-to-plasma membrane transport (sec1-1, sec2-41, sec2-59, sec3-2, sec4-8, and sec15-1). This type of interaction was also found in mutants of cytoskeleton genes required for polarized transport of secretory vesicles (act1-2 and myo2Δ-66). No or a very weak genetic interaction was detected upon overexpression of BMH1 or BMH2 in mutants of genes in the general secretion gene SEC19 (sec19-1) or genes of endoplasmic reticulum-to-Golgi transport (sec21-1, sec23-1). Only the N-ethylmaleimide sensitive factor homologue SEC18 (sec18-1) showed suppression of the growth phenotype upon 14-3-3 overexpression. In most cases, the synthetic genetic interaction of BMH1/2 was found with mutant alleles of those sec mutants, which show synthetic sickness or lethality when combined with pik1-101 (Walch-Solimena and Novick, 1999). These data suggested that both Pik1p and 14-3-3 might be involved in regulation of post-Golgi trafficking and that 14-3-3 overproduction counteracts Pik1p function.

To test more directly for a role of 14-3-3 proteins in membrane transport from the TGN together with Pik1p, we next used a visual assay for exocytosis. In pik1-101 cells, surface transport of a GFP-tagged mutant version of the general amino acid permease Gap1p (Gap1p-GFP lacking sites for ubiquitylation) is inhibited. This inhibition can be seen as dot-like accumulations, which could represent Golgi or endosomal structures (data not shown). Using this assay, we found an accumulation of Gap1p-GFP compared with wild-type cells upon overexpression of BMH2, as we had detected in pik1-101 (Figure 3, B and C). For a more quantitative evaluation of the Gap1p-GFP transport defect, we measured Gap1p permease activity by uptake of [14C]citrulline, an amino acid exclusively taken up through Gap1p (Roberg et al., 1997). In this assay, Gap1p-GFP activity was fivefold reduced upon increased Bmh2p level (Figure 3D). Thus, in agreement with the described genetic interaction, Bmh2p overexpression seems to inhibit secretion, and likely negatively regulates Pik1p in this process as it shows similarities to loss of Pik1p activity. To understand how 14-3-3 proteins regulate Pik1p function, we first asked about the localization of the interaction.

**Pik1p–14-3-3 Interaction Occurs in the Cytoplasm**

Pik1p has been found at the TGN and in the nucleus (Walch-Solimena and Novick, 1999; Sciorra et al., 2005; Strahl et al., 2005). 14-3-3 proteins have a more widespread distribution. Fluorescence microscopy revealed a general cytoplasmic distribution of Bmh1p-myc with nuclear exclusion (Figure 4A). Bmh2p-myc was found in a punctate pattern throughout the entire cell. There was no obvious colocalization of Bmh proteins with Pik1p at the TGN (Figure 4A, insets). Because Pik1p immunofluorescence did not perfectly overlap with DAPI staining of DNA, we asked whether Pik1p could be concentrated in the nucleolus. As shown in Figure 4B, Pik1p colocalizes with a nucleolar marker (detected by monoclonal antibody [mAb] 2.3b; Yang et al., 1989). Due to the general cytoplasmic labeling for Bmh1p-myc and Bmh2p-myc, we could not exclude that there could be a pool of these proteins that localizes to this compartment. Therefore, we further investigated the subcellular localization by differential fractionation.

Pik1p-TAP and Bmh2p-myc were similarly distributed in fractions separated in a 13,000 × g and a 100,000 × g centrifugation step whereby both proteins were more abundant in the cytosol than in membrane fractions (Figure 4C). To determine whether Pik1p–14-3-3 interaction occurs in the cytosol or at the membrane, we performed immunoprecipitation experiments from either the supernatant or the pellet of the 100,000 × g centrifugation. Pik1p-myc coprecipitates Bmh1p-ha mainly from the cytosol, whereas little of the 14-3-3 protein binds to Pik1p-myc from the membrane pellet (Figure 4D). This suggests that Pik1p-14-3-3 interaction occurs mainly in the cytoplasm. We did not find significant differences in the PI 4-kinase activity of Pik1p-13myc immunoprecipitated from cytosol or membranes prepared as in Figure 4D (Figure 4E). Therefore, we hypothesized that 14-3-3 proteins might serve to regulate the localization of Pik1p, e.g., by formation of a Pik1p–14-3-3 complex in the cytoplasm, and this way might influence not only the availability of Pik1p at the TGN but also the amount of nuclear Pik1p.

**Pik1p Is Primarily Nuclear upon Loss of 14-3-3 Function**

To address a possible role of 14-3-3 in regulation of Pik1p distribution between cytosol and nucleus, we studied the localization of GFP-Pik1p in the background of bmh mutant alleles. Although GFP-Pik1p localized to the TGN and to the nucleus in wild-type cells (Figure 5, A–C), we observed an increased number of cells with nuclear Pik1p and decreased Pik1p levels at the TGN in bmh1Δ or bmh1Δ bmh2ts mutants at 25°C, an effect more pronounced at 37°C (Figure 5, A–C). Whole cell Pik1p levels were unchanged in bmh mutants at 25°C and in bmh1Δ at 37°C. There was a slight decrease at the restrictive temperature in the bmh1Δ bmh2ts double mutant (Supplemental Figure 2). Thus, the microscopy data indicate a true redistribution of Pik1p, suggesting that 14-3-3 interaction is required for maintenance of a pool of Pik1p in the cytoplasm and thus might make it accessible to the TGN. This idea is supported by our finding that in bmh mutants,
GFP-Pik1p still localizes to the Golgi; however, it does so with strongly decreased fluorescence intensity (Figure 5D and Supplemental Figure 3A). For quantification of the fluorescence intensity, we measured only structures smaller than 0.95 μm, thus excluding nuclei (Supplemental Figure 3B).

**Figure 3.** 14-3-3 overproduction interferes with late secretory pathway function. (A) Synthetic enhancement of pik1-101 temperature-sensitive growth defect by high copy BMH1/2. The pik1-101 mutant (CSY212) was transformed with 2μ BMH1 (pMB180), 2μ BMH2 (pMB181), 2μ Pik1 (pCS8), or control vector (pRS426), and serial dilutions of cells were tested for growth at 25, 32, or 34°C. (B) Gap1p-GFP surface transport is similarly impaired in pik1-101 and wild-type cells overexpressing BMH2 (pMB263). Wild-type (NY604) and pik1-101 (YMB064) cells were transformed with Gap1p-GFP (TPQ99). Cells were grown overnight at 25°C in SCaf-URA and induced with 3% galactose for 3 h. The temperature shift was performed during the last hour of induction. Cells were analyzed by fluorescence microscopy. Gap1p-GFP accumulation is indicated by arrowheads. (C) Gap1p-GFP accumulating structures were counted for pik1-101 (n = 68), wild-type (pRS425; n = 256), wild-type cells overexpressing BMH1 (pMB262; n = 54) or overexpressing BMH2 (pMB263; n = 243). Data are mean ± SEM. (D) Gap1p-GFP surface activity is reduced in cells overexpressing BMH2. Cells were grown, and Gap1p-GFP expression was induced as described in B. Gap1p-GFP activity was determined by measuring the uptake of [14C]citrulline. Data are mean ± SD (n = 3).

**Pik1p–14-3-3 Interaction Involves Phosphorylation**

The negative genetic interaction of 14-3-3 with Pik1p, the negative regulation of secretion by 14-3-3 overproduction, and the interaction between these proteins in the cytoplasm rather than at the TGN suggested that 14-3-3 might alter the availability of Pik1p for its function at the TGN. To further investigate the mechanisms underlying this regulation, we next asked how Pik1p–14-3-3 interaction itself is regulated. Because 14-3-3 proteins often bind to their targets through phosphorylated residues (Tzivion and Avruch, 2002; Yaffe, 2002; Dougherty and Morrison, 2004), we decided to investigate a possible phosphorylation dependence of the Pik1p–14-3-3 interaction.

GST pull-down experiments (GST-14-3-3) of genomically tagged Pik1p-TAP from yeast lysates were performed in the presence or absence of λ-PPase or phosphatase inhibitors. As shown in Figure 6A, Pik1p-TAP only bound to either GST-Bmh1p or GST-Bmh2p in the presence of phosphatase inhibitors, thus, Pik1p–14-3-3 interaction does require phos-
phorylation. This finding is consistent with experiments in Figure 1, C and E, which were performed in the presence of phosphatase inhibitors. DNA was stained with DAPI. Cells were viewed by fluorescence microscopy. (B) Pik1p is localized to the nucleolus. Wild-type cells (NY13) were labeled with anti-Pik1p antibody, mAb clone 2.3b for nucleoli, and DAPI. (C) Pik1p and Bmh1p/2p distribution in subcellular fractions. Cells containing PIK1-TAP and BMH2-13myc (YMB119) were subjected to 13,000 × g and 100,000 × g centrifugation, and fractions examined by Western blotting. Pik1p-TAP was detected with PAP complex and Bmh1p-13myc or Bmh2p-13myc with anti-myc antibody (9E10). Coprecipitated Bmh1p-3ha was detected using anti-HA antibody (Y11). Loading controls represent 0.5% of the fractions. (D) Pik1p and 14-3-3 interact in the cytoplasm. Cells containing PIK1-13myc and BMH1-3ha (YMB148) were subjected to 100,000 × g centrifugation. After detergent lysis of the cytosolic or the membrane fraction, Pik1p-13myc was immunoprecipitated using anti-myc antibody (9E10). Coprecipitated Bmh1p-3ha was detected using anti-HA antibody (Y11). Loading controls represent 0.5% of the fractions. (E) PI 4-kinase activity of immunoprecipitated Pik1p from cytosol or membrane fractions prepared as described in D. Pik1p-13myc was precipitated with anti-myc antibody (9E10) and subjected to lipid-kinase assay by using phosphatidylinositol as substrate. Shown are results of two representative experiments. The amount of radioactive labeled PI(4)P was normalized to Pik1p-13myc protein levels.

Figure 4. Localization of the Pik1p–14-3-3 interaction. (A) Subcellular localization of Pik1p and Bmh1/2p. BMH1-13myc (YMB054) and BMH2-13myc (YMB058)–expressing cells were subjected to fixation, and Bmh proteins were labeled using anti-myc antibody (9E10) and Pik1p with anti-Pik1p antibody. DNA was stained with DAPI. Cells were viewed by fluorescence microscopy. (B) Pik1p is localized to the nucleolus. Wild-type cells (NY13) were labeled with anti-Pik1p antibody, mAb clone 2.3b for nucleoli, and DAPI. (C) Pik1p and Bmh1p/2p distribution in subcellular fractions. Cells containing PIK1-TAP and BMH2-13myc (YMB119) were subjected to 13,000 × g and 100,000 × g centrifugation, and fractions examined by Western blotting. Pik1p-TAP was detected with PAP complex and Bmh1p-13myc or Bmh2p-13myc with anti-myc antibody (9E10). (D) Pik1p and 14-3-3 interact in the cytoplasm. Cells containing PIK1-13myc and BMH1-3ha (YMB148) were subjected to 100,000 × g centrifugation. After detergent lysis of the cytosolic or the membrane fraction, Pik1p-13myc was immunoprecipitated using anti-myc antibody (9E10). Coprecipitated Bmh1p-3ha was detected using anti-HA antibody (Y11). Loading controls represent 0.5% of the fractions. (E) PI 4-kinase activity of immunoprecipitated Pik1p from cytosol or membrane fractions prepared as described in D. Pik1p-13myc was precipitated with anti-myc antibody (9E10) and subjected to lipid-kinase assay by using phosphatidylinositol as substrate. Shown are results of two representative experiments. The amount of radioactive labeled PI(4)P was normalized to Pik1p-13myc protein levels.

The sites of phosphorylation within these fragments were unambiguously identified by tandem mass spectrometry (Table 1). Two distinct 14-3-3 binding motifs have been described in the literature: RSXS/TXp (mode 1; pS represents phosphorylated Ser/Thr and X any amino acid) and RXXSxS/TXP (mode 2) (Yaffe et al., 1997; Rittinger et al., 1999). Among the identified sites for phosphorylation, two (S396 and S605) are part of a predicted 14-3-3 target interaction motif according to Motif Scan (http://scansite.mit.edu). A third putative
14-3-3 binding motif was found at S780, a site that has not been identified by our mass spectrometry analysis. We generated mutants in these sites, tested for interaction of these mutated Pik1p proteins with Bmh1p and Bmh2p in the yeast two-hybrid assay and found that S396 is required for 14-3-3 interaction (Figure 6B). Together with the observed loss of interaction between Pik1p and 14-3-3 upon deletion of the catalytic domain of the PI 4-kinase (Figure 1A), we suggest that S396 is necessary but not sufficient for this interaction.

Pik1p Phosphorylation Correlates with Nucleocytoplasmic Relocalization

We next asked whether phosphorylation of Pik1p shows any correlation with its subcellular localization. We have previously described that Pik1p rapidly and reversibly relocalizes from the nucleus to the cytoplasm upon block of secretion, e.g., in a sec6-4 mutant. The ASR occurs in sec mutants along the entire secretory pathway and also results in relocation of other nuclear proteins (Walch-Solimena and Novick, 1999; Nanduri and Tartakoff, 2001), probably by nuclear import inhibition (Nanduri et al., 1999). We took advantage of the ASR and analyzed Pik1p phosphorylation in wild-type and sec6-4 mutant cells after a 30-min shift to the restrictive temperature (Figure 6C). Under these conditions, a massive relocalization of nuclear Pik1p to the cytoplasm has been observed previously (Walch-Solimena and Novick, 1999).

To resolve the phosphorylated and nonphosphorylated form of Pik1p by SDS-PAGE, we empirically determined an acrylamide:bisacrylamide ratio of 500:1 as the best composition. On ASR in sec6-4, we detected a shift of Pik1p to a higher molecular weight form in lysates from sec6-4 cells at the restrictive temperature compared with wild type. This shift was reversed upon treatment with λ-PPase, indicating that the higher molecular weight form is phosphorylated (Figure 6C). Together, the increase in phosphorylation and shuttling out of the nucleus of Pik1p during the ASR suggested that this modification might play a role in regulation of Pik1p distribution.

To test this further, we investigated the phosphorylation status of Pik1p in a mutant version of the protein showing a localization defect on its own. For this, we focused on the N-terminal regulatory domain of Pik1p, and we generated...
temperature-sensitive pik1 mutant alleles by error-prone PCR. Among the mutants we identified a new allele, pik1-130, which had a defect in nuclear but not the cytoplasmic punctate labeling for Pik1p (Figure 6D). Like Pik1p in Figure 4B, at the permissive temperature, mutant Pik1-130p localized immediately adjacent to DAPI, suggesting nucleolar localization (Figure 6D). At the restrictive temperature, when Pik1-130p no longer is nuclear, the protein was in part shifted to a higher molecular weight, phosphorylated form (Figure 6C) that could be detected even though the stability of the mutant protein was decreased. We con-

Table 1. Phosphorylation sites of Pik1p

<table>
<thead>
<tr>
<th>Peptide sequence</th>
<th>Position (amino acids)</th>
<th>Phosphorylation at</th>
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<tbody>
<tr>
<td>pSFDDTTIELKK</td>
<td>10–19</td>
<td>Ser10</td>
</tr>
<tr>
<td>S1STPpSPIDLIDPIK</td>
<td>231–245</td>
<td>Ser236</td>
</tr>
<tr>
<td>TNpSQPLSR</td>
<td>394–401</td>
<td>Ser396</td>
</tr>
<tr>
<td>SDpSASTAHVR</td>
<td>603–612</td>
<td>Ser605</td>
</tr>
</tbody>
</table>

To identify the sites of phosphorylation, Pik1p was isolated by TAP purification from a strain expressing PIK1-TAP. After in-gel digestion with trypsin, the phosphorylated sequences were identified using a nanoLC ESI MS/MS.

Figure 6. Pik1p interaction with 14-3-3 and localization is regulated by phosphorylation. (A) Pik1p binding to 14-3-3 requires phosphorylation. Extracts from cells containing Pik1p-TAP (CSY513) were either treated with α-PPase or with phosphatase inhibitors followed by incubation with GST alone, GST-Bmh1p, or GST-Bmh2p immobilized on glutathione-Sepharose. Immunoblot analysis was with PAP complex. The input represents 5% of lysate. (B) Liquid β-galactosidase assay and yeast two-hybrid interactions of Pik1p phospho-mutants (pik1-145 to 148, pMB365-368, respectively) with Bmh1p (pMB116) and Bmh2p (pMB125) were quantified. Interaction between Pik1p (pMB138) and Bmh1p or Bmh2p serves as positive control, and interactions with pGADT7 as negative control. Values were from nine independent experiments. (C) Pik1p phosphorylation upon nucleocytoplasmic relocation. Wild-type (NY1211), sec6-4 (NY778) and pik1-130 (YMB087) cells were grown at 25°C, incubated for 30 min at 25 or 37°C as indicated, and lysed in the presence or absence of phosphatase inhibitors. Lysates without phosphatase inhibitors were incubated with α-PPase. Samples were analyzed by SDS-PAGE (acylamide: bisacylamide ratio, 500:1) to resolve phosphorylated and nonphosphorylated Pik1p and immunoblotting with anti-Pik1p antibody. (D) The pik1-130 mutant cells lack nuclear localization at the restrictive temperature. Cells carrying the pik1-130 mutation (YMB087) were processed for immunofluorescence microscopy by using anti-Pik1p antibody and DAPI after incubation for 60 min at 25 or 37°C as indicated. Arrowheads indicate nuclear exclusion of Pik1-130p.
Figure 7. Pik1p relocalizes from the TGN to the cytoplasm and nucleus under growth-limiting conditions. (A) Pik1p is lost from the TGN in late log phase. Cells (NY1211) expressing GFP-Pik1 from the endogenous promoter on a CEN-based plasmid were observed by laser
clude that the distribution of Pik1p correlates with its phosphorylation status. Together with the finding that Pik1p-14-3-3 interaction occurs in the cytoplasm and requires phosphorylation, we propose a model in which 14-3-3 proteins modulate nucleocytoplasmic shuttling of Pik1p. What could be the physiological significance of such a shutting mechanism?

Pik1p Relocates from the TGN to the Nucleus under Conditions of Nutrient Limitation

We have shown that Pik1p redistributes from the nucleus to the cytosol during the ASR (Walch-Solimena and Novick, 1999), a condition where a rapid adjustment of the protein synthesis machinery occurs in response to a block in the secretory pathway (Mizuta and Warner, 1994; Nanduri and Tartakoff, 2001). Therefore, we asked whether a change in localization of Pik1p could also be observed under different growth conditions. For this, we observed GFP–Pik1p localization first in either early or late log phase growth. In early log phase cultures, only a small percentage of cells (9.0 ± 9.1%) showed nuclear GFP–Pik1p (Figure 7, A and E). Instead, all cells exhibited GFP–Pik1p labeling at the TGN (Figure 7, A and G). There was no obvious change in localization during the cell cycle (as judged by the size of the bud, data not shown). In late log phase, GFP–Pik1p became mostly cytoplasmic (Figure 7, A and G) without major change in nuclear (8.1 ± 9.1%) labeling (Figure 7, A and E). The TGN integrity was unchanged under these conditions since labeling with the TGN marker Sec7p-GFP was unaffected in late log phase cells (Figure 7D).

The change in Pik1p localization in response to growth prompted us to investigate the behavior of GFP–Pik1p in log phase cells under conditions of nutrient deprivation. In the absence of glucose, a strong increase in the nuclear labeling for GFP–Pik1p (37.1 ± 10.2%) and a concomitant loss of the lipid kinase from TGN membranes was observed (Figure 7, B, F, and H). Similar results were obtained for deprivation of raffinose or galactose (Supplemental Figure 4). No such effect was observed upon depletion of the nonfermentable carbon source glycerol (Figure 7, B, F, and H) or nitrogen starvation (data not shown), suggesting that only deprivation of fermentable carbon sources results in signaling that changes intracellular distribution of Pik1p. The TGN integrity was not disturbed under the nutrient deprivation conditions used as we observed normal labeling with Sec7p-GFP (Figure 7D). Also, ultrastructural analysis of glucose-deprived cells did not reveal any abnormal accumulation of the TGN (data not shown).

The relocalization of Pik1p from the TGN to the nucleus might represent a mechanism by which cells can quickly adjust both PI(4)P production and vesicle formation from the late Golgi to growth conditions. If such a signaling pathway existed, one would expect rapid restoration of the TGN localization of Pik1p if the appropriate carbon source (glucose, galactose or raffinose) is added back to the cells. As shown in Figure 7C, we did observe such a restoration of TGN localization of Pik1p within minutes after glucose addition. We obtained similar results using galactose (Supplemental Figure 5).

Pik1p Relocation under Nutrient Deprivation Involves Phosphorylation and 14-3-3 Binding

We next asked whether the observed relocation of Pik1p from the TGN to the cytoplasm and into the nucleus during nutrient deprivation might be mediated by Pik1p phosphorylation and 14-3-3 binding. For this, we performed a glucose deprivation experiment as shown in Figure 7, but this time we lysed cells after 45 min without glucose and 20 min after glucose readdition. As shown in Figure 8, more phosphorylated Pik1p was recovered after glucose deprivation than in control cells or after glucose readdition. Also, glucose deprivation resulted in an increase of Bmh1p in complex with Pik1p (Figure 8). Phosphorylation and 14-3-3 complex formation thus correlates with the observed loss of Pik1p from the TGN that results in relocation to the cytoplasm and to the nucleus.

We next investigated the role of Pik1p-14-3-3 interaction in the relocalization of Pik1p from the TGN to the nucleus. We observed that in cells co-overexpressing BMH1 and BMH2, but not either gene alone, GFP–Pik1p no longer accumulated in the nucleus (Figure 9, A and B). TGN localization was unchanged compared with control cells both in the presence or absence of glucose (Figure 9, A and C). Thus, 14-3-3 levels seem to determine the amount of Pik1p kept in the cytoplasm after it has been released from the TGN. The Pik1p-14-3-3 complex might need to dissociate to allow for nuclear accumulation of Pik1p, because loss of 14-3-3 function results in increased nuclear accumulation (Figure 5). Together, these data suggest that nucleocytoplasmic shuttling of Pik1p is directly regulated by 14-3-3 interaction.
Role of S396 Phosphorylation in Control of Cellular Pik1p Pools

To directly address the role of S396 phosphorylation in this process, we generated S396A and a corresponding phospho-mimicking mutation S396D. GFP-Pik1S396A or GFP-Pik1S396D, like wild-type GFP-Pik1p, localized to the Golgi in the presence of glucose and they were lost from this compartment upon glucose deprivation (Figure 10, A and C). The GFP-Pik1S396A protein was more nuclear in full media, and it accumulated approximately threefold more than wild type when glucose was removed (Figure 10, A and B). GFP-Pik1S396D did behave like wild-type GFP-Pik1p with glucose, but it also accumulated more in the nucleus in the absence of glucose. Thus, S396 phosphorylation (and by extension 14-3-3 interaction) does not directly regulate Golgi association, but it does affect nucleocytoplasmic shuttling. The increased GFP-Pik1S396D nuclear accumulation upon glucose deprivation might indicate that reversibility of phosphorylation is needed for the shuttling process (e.g., for nuclear exit).

We also measured PI 4-kinase activity of immunoprecipitated GFP-Pik1p compared with GFP-Pik1S396A or GFP-Pik1S396D, and we found no significant differences when equal amounts of immunoprecipitated GFP-Pik1 proteins were used for the assay (Figure 10, D and E). Thus, this phosphorylation (and 14-3-3 interaction) does not have a direct effect on the enzymatic activity of the lipid kinase.

We further took advantage of the S396 mutants to ask whether phosphorylation at this site regulates Pik1p–Frq1p interaction. In the yeast two-hybrid assay, we found no differences in the interaction of mutant versus wild-type Pik1p (Supplemental Figure 6). Together with our velocity gradient (Supplemental Figure 1) and Frq1p–TAP experiments (Figure 2), this suggests that S396 phosphorylation and 14-3-3 interaction do not function to regulate the Pik1p–Frq1p complex.

We have already shown that BMH overexpression can interfere with trafficking (Figure 3) and with nucleocytoplasmic shuttling (Figure 9). To investigate more directly the role of Pik1–14-3-3 interaction through phosphorylated S396 and trafficking, we measured endogenous Gap1p permease activity with the [14C]citrulline uptake assay in yeast strains carrying pik1S396A or pik1S396D, respectively, as the only PIK1 copy of the cell compared with cells carrying PIK1 on the same plasmid (Supplemental Figure 7). We found a decrease in surface transport for S396A. These data further support the hypothesis that regulated nucleocytoplasmic shuttling and Pik1p function during trafficking are closely connected.

Figure 9. Co-overexpression of BMH1 and BMH2 prevents relocation of Pik1p to the nucleus upon glucose deprivation. (A) Early log phase cultures of cells (NY1211) expressing GFP-PIK1 from the endogenous promoter on a CEN-based plasmid, and BMH1 (pMB180), BMH2 (pMB263), or BMH1 and BMH2 on a 2μ-based plasmid were depleted of glucose for 45 min. Cells were observed by confocal microscopy. (B and C) Number of cells with nuclear GFP-Pik1p (A) and with GFP-Pik1p at the Golgi (C) were plotted (mean ± SD).
DISCUSSION

Regulated Localization of PI-metabolizing Enzymes to Control Traffic

Our data suggest that the Golgi PI 4-kinase Pik1p is at least in part controlled by regulated localization. Previous studies reported that this enzyme localizes to the TGN and the nucleus (Garcia-Bustos et al., 1994; Walch-Solimena and Novick, 1999; Sciorra et al., 2005; Strahl et al., 2005). A similar dual localization has been found for PI 4-kinase III (Godi et al., 1999; de Graaf et al., 2002). Pik1p shuttles constitutively between nucleus and cytoplasm (Strahl et al., 2005). We provide evidence that 14-3-3 proteins regulate nucleocytoplasmic shuttling of this enzyme.

Bmh1p and Bmh2p bind to Pik1p in vitro and in vivo depending on phosphorylation, a modification involved in 14-3-3 target binding (Tzivion and Avruch, 2002; Yaffe, 2002; Dougherty and Morrison, 2004). During the ASR, Pik1p rapidly relocates from the nucleus to the cytoplasm and undergoes a shift to the phosphorylated form, suggesting that Pik1p needs phosphorylation for cytoplasmic localization. Consistent with this model, we found an increase in phosphorylated Pik1p and Pik1p–14-3-3 complex during nutrient deprivation, when Pik1p is no longer TGN-bound but becomes cytoplasmic, before it accumulates in the nucleus.

Membrane-associated Pik1p binds to 14-3-3 only to a small extent.

We were unable to disrupt Pik1p–14-3-3 complexes from cell lysates either with phosphatases or by competition with the R18 peptide, a peptide which competes with 14-3-3 interactors (Wang et al., 1999; data not shown), suggesting that dissociation of 14-3-3 from Pik1p is regulated and might precede dephosphorylation. This interaction might thus protect cytoplasmic Pik1p from dephosphorylation, possibly to offer phosphorylated Pik1p to the TGN. According to our data, membrane binding of Pik1p and Pik1p–Frq1p interaction occurs by a mechanism not requiring 14-3-3 function (see model in Figure 11). Instead, Pik1p binding to the Golgi does require Frq1p (Strahl et al., 2005). Such a model would be consistent with our finding that 14-3-3 overproduction interferes with function of the late secretory pathway.

What is the function of cytoplasmic Pik1p–14-3-3 complexes? We have developed a kinetic model for Pik1p localization (see Supplemental Material) in which, in agreement with our microscopic observations, release of Pik1p from the Golgi results in an increase of both cytoplasmic and nuclear Pik1p. Our biochemical data provide some evidence for an increase of the cytosolic pool, because we find an increase in Pik1p–14-3-3 complex, probably caused by increased cytosolic concentration of Pik1p. The 14-3-3 interaction appar-
Interaction of Pik1p with 14-3-3 Proteins

which such shuttling of a PI kinase might be controlled: formation of a complex with 14-3-3 proteins.

Do 14-3-3 Proteins Coordinate Golgi Function with Nutrient Signaling?

Use of a phosphorylation-dependent and 14-3-3–dependent mechanism for regulated subcellular distribution of Pik1p provides in addition to rapid and local control of PI(4)P production the possibility of coupling Pik1p function to signaling processes, which might need to be coordinated with membrane transport. We did indeed find that 14-3-3 proteins affect secretion, and that Pik1p–14-3-3 interaction mediates relocation of Pik1p in response to nutrient limitation. We propose that 14-3-3 proteins act as a gatekeeper for Golgi Pik1p through control of cytoplasmic Pik1p levels and nucleocytoplasmic shuttling.

14-3-3 proteins have previously been implicated in membrane transport regulation (Gelperin et al., 1995; Vasara et al., 2002) and cytoskeleton organization (Roth et al., 1999), but so far little is known about the targets of these proteins. We found here that overexpression of BMH1 or BMH2 was lethal in the actin mutant act1-2 and a mutant of the myosin motor of secretory vesicles (myo2-66), together suggesting that 14-3-3 proteins, on their own or with Pik1p, could regulate the actin cytoskeleton. This regulation is likely to contribute to the effects of 14-3-3 on post-Golgi transport. Besides a defect in actin polarization, bmh and pik1 mutants both exhibit defects in budding and cell wall integrity (Lottersberger et al., 2006; data not shown), which could be related to membrane transport but could also point toward additional roles of both proteins.

We found that phosphorylation of Pik1p and Pik1p–14-3-3 complex formation is subject to regulation both in the ASR and under nutrient deprivation conditions. Therefore, we propose that this interaction could be part of a signaling pathway that coordinates growth conditions with Golgi function. 14-3-3 proteins have been implicated in many cellular processes. In yeast, they participate in the Ras/mitogen-activated protein kinase cascade of pseudohyphal development (Roberts et al., 1997), the target of rapamycin (TOR) pathway (Gelperin et al., 1995; Bertram et al., 1998; Beck and Hall, 1999), the RAS–CAMP pathway (Gelperin et al., 1995), and possibly in the cell wall integrity pathway (Lottersberger et al., 2006). Because we found that deprivation of fermentable carbon sources results in rapid and reversible relocation of Pik1p and increased Pik1p–14-3-3 interaction, the TOR and the Ras pathways are possible candidates for upstream signaling. It will now be important to identify the kinases for Pik1p phosphorylation.

Is there a coordination of growth with secretion via PI(4)P signaling? Interestingly, we found Pik1p loss from the TGN under similar conditions that caused relocation of the lipid phosphatase Sac1p from the ER to the Golgi (Faulhammer et al., 2005, 2007). Because Pik1p generates PI(4)P and Sac1p dephosphorylates this lipid, the coordinated relocation of both enzymes would result in a drastic and rapid reduction of PI(4)P on the TGN membrane. This in turn would reduce PI(4)P-dependent membrane transport and coordinate SV production with growth conditions. Indeed, it has been reported that a fluorescent PI(4)P-binding PH domain no longer localized to the Golgi under conditions of glucose deprivation (Faulhammer et al., 2005) similar to the conditions used in our study. It is now of great interest whether this signaling pathway is conserved in vertebrates. Interestingly, Frq1p, a positive regulator of function of the fission yeast Pik1p homologue, participates in nutrient-regulated signaling pathways (Hamasaki-Katagiri et al., 2004).

Figure 11. Model of the role of 14-3-3 interaction in the regulation of cellular Pik1p pools. A cytoplasmic Pik1p–14-3-3 complex maintains a pool of Pik1p that can be recruited to the TGN, where Pik1p binds to the membrane through the action of Frq1p and produces PI(4)P. Cytoplasmic Pik1p is protected from dephosphorylation and nuclear import through the action of 14-3-3. Glucose deprivation releases Golgi Pik1p, increases Pik1p–14-3-3 complex formation, and, possibly due to an effective increase in Pik1p concentration, increases also the nuclear pool. This regulation might provide a very rapid mode of adjustment of PI(4)P levels and thus transport capacity of the Golgi to coordinate nutrient signaling with growth. The k-values represent hypothetical reaction constants (see Discussion and Supplemental Material).
The Golgi apparatus is not only a major hub for transport in the secretory pathway but also an important signaling platform. Further work on the role of the Ptk1p–14-3-3 interaction will provide new insights of how membrane transport at the Golgi is coordinated with growth.

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