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Novel Mechanism Coupling Cyclic AMP-Protein Kinase A Signaling and Golgi Trafficking via Gyp1 Phosphorylation in Polarized Growth

Zhen-Xing Huang,[a] Haitao Wang,[a] Yan-Ming Wang,[a] Yue Wang[a,b]

Institute of Molecular and Cell Biology, Agency for Science, Technology and Research, Singapore[a]; Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore[b]

The cyclic AMP (cAMP)-protein kinase A (PKA) signaling activates virulence expression during hyphal development in the fungal human pathogen *Candida albicans*. The hyphal growth is characterized by Golgi polarization toward the hyphal tips, which is thought to enhance directional vesicle transport. However, how the hypha-induction signal regulates Golgi polarization is unknown. Gyp1, a Golgi-associated protein and the first GTPase-activating protein (GAP) in the Rab GAP cascade, critically regulates membrane trafficking from the endoplasmic reticulum to the plasma membrane. Here, we report a novel pathway by which the cAMP-PKA signaling triggers Golgi polarization during hyphal growth. We demonstrate that Gyp1 plays a crucial role in actin-dependent Golgi polarization. Hyphal induction activates PKA, which in turn phosphorylates Gyp1. Phosphomimetic mutation of four PKA sites identified by mass spectrometry (Gyp1^{4E}) caused strong Gyp1 polarization to hyphal tips, whereas nonphosphorylatable mutations (Gyp1^{4A}) abolished it. Gyp1^{4E} exhibited enhanced association with the actin motor Myo2, while Gyp1^{4A} showed the opposite effect, providing a possible mechanism for Golgi polarization. A GAP-dead Gyp1 (Gyp1^{R292K}) showed strong polarization similar to that seen with Gyp1^{4E}, indicating a role for the GAP activity. Mutating the PKA sites on Gyp1 also impaired the recruitment of a late Golgi marker, Sec7. Furthermore, proper PKA phosphorylation and GAP activity of Gyp1 are required for virulence in mice. We propose that the cAMP-PKA signaling directly targets Gyp1 to promote Golgi polarization in the yeast-to-hypha transition, an event crucial for *C. albicans* infection.

Polarized cell growth is essential for cell division and morphogenesis in all organisms and is largely achieved through directional transport of secretory vesicles from the Golgi compartment to the growth sites in eukaryotes. In the budding yeast *Saccharomyces cerevisiae*, the transport from the endoplasmic reticulum (ER) to the plasma membrane (PM) involves a cascade of three Rab GTPases, Ypt1, Ypt31/32, and Sec4 (1, 2). Ypt1 controls ER-Golgi and intra-Golgi transport, Ypt31/32 regulates the transport from late-Golgi to secretory vesicles, and Sec4 drives the transport further to the PM. When Ypt32 is activated, it recruits the guanine nucleotide exchange factor (GEF) Sec2 to activate the downstream Rab, Sec4 (3). Meanwhile, Ypt32 also recruits the GTPase-activating protein (GAP), Gyp1, to deactivate its upstream Rab, Ypt1, by stimulating the hydrolysis of its bound GTP to GDP (4–6). Gyp1 plays a central role in this cascade mechanism to maintain the boundary between the Ypt1 and Ypt32 domains on Golgi compartments (4).

Golgi-derived secretory compartments migrate to the growth sites largely by riding on polarized cytoskeletal tracks (7). The unconventional myosin V motor proteins play a crucial role in moving these secretory vesicles along actin cables (8). In yeasts, Rab GTPases associated with different stages of the secretory vesicles interact with Myo2, a myosin V, which is thought to facilitate the loading and efficient transport of the respective compartments along actin cables during polarized growth (9–12).

The hyphal development of the fungal human pathogen *Candida albicans* provides a striking example of highly polarized morphogenesis. In response to environmental cues, *C. albicans* switches from a budding yeast growth form to a highly elongated hyphal growth form (13). This ability to switch is a key virulent trait of this pathogen (14). Upon switching to hyphal development, cell growth is focused in a narrow space of the hyphal tip, leading to rapid hyphal extension. To achieve this remarkable polarized growth, *C. albicans* has evolved hypha-specific means to facilitate rapid, long-distance vesicular transport to the hyphal tip. For example, a vesicular structure called the Spitzenkörper is formed at the subapical region immediately behind the hyphal apex, acting as a center for supply of secretory vesicles to the growth site (15, 16). Another example is the polarization of Golgi compartments toward the hyphal tip in a manner dependent on polarized actin cables (17).

A range of environmental signals activate *C. albicans* hyphal growth mainly through two signal transduction pathways, a mitogen-activated protein kinase pathway and the cyclic AMP (cAMP)-protein kinase A (PKA) pathway, although the latter plays a more prominent role (18). A central component of the cAMP-PKA pathway is Cyr1, the sole adenylyl cyclase in this fungus. In response to hyphal induction, Cyr1 increases the synthesis of cAMP, which in turn activates PKA (19). Once activated, PKA phosphorylates the Efg1 transcription factor to switch on the expression of hypha-specific genes (20, 21).

In the past 2 decades, tremendous progress has been made in elucidating the molecular mechanisms by which the hypha-induction signaling pathways regulate the cell's polarity machinery to achieve polarized growth during *C. albicans* hyphal develop-

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Address correspondence to Zhen-Xing Huang, zxhuang@imcb.a-star.edu.sg, or Yue Wang, mcbwangy@imcb.a-star.edu.sg.

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ment. The discovery of the hypha-specific Hgc1-Cdc28 kinase has led to findings of direct molecular links to several key regulators governing different aspects of polarized growth (22, 23). For example, Hgc1-Cdc28 activates the Cdc42 GTPase polarity promoter at the hyphal tip by phosphorylating and inhibiting Rga2, the negative regulator of Cdc42 (24). Hgc1-Cdc28 also phosphorylates Sec2 and the Exo84 exocyst component to facilitate the transport of secretory vesicles to the hyphal tip (25, 26). Efg1 is also a substrate of Hgc1-Cdc28, and this phosphorylation converts Efg1 to a transcription repressor to switch off genes normally required for cell separation during yeast growth, which ensures that hyphal cells remain attached after cytokinesis (27). In addition to these Hgc1-dependent posttranscriptional regulations, modifications of some polarity-related proteins have been found to occur immediately after hyphal induction in manners not requiring Hgc1, such as phosphorylation of the septin Cdc11 (28) and the dephosphorylation of actin patch protein SlaI (29). Indeed, *hgc1*Δ mutants show an immediate response to hyphal induction, although they are able to form only a wide surface protrusion that is unlike the thin germ tube typically seen in wild-type (WT) cells (22). These observations indicate that the hypha-induction signaling pathways may directly activate some early events of hyphal growth by as-yet-unknown mechanisms.

By screening a *C. albicans* haploid mutant library deleted of genes encoding GTPases and their regulators, we discovered that *GYP1* is essential for *C. albicans* hyphal growth. Further characterizations revealed that hyphal induction activates rapid Gyp1 phosphorylation by PKA through the cAMP signaling pathway and that this phosphorylation enhances Gyp1's affinity to the Myo2 actin motor protein and determines the polarization of Golgi compartments. Deleting *GYP1* or mutating the PKA phosphorylation sites on Gyp1 attenuated *C. albicans* virulence in mice. Our results establish a novel early event linking the cAMP-PKA pathway to direct regulation of membrane trafficking in *C. albicans* hyphal development.

MATERIALS AND METHODS

Plasmids, strains, and culture conditions. Plasmids and *C. albicans* strains used in this study are listed in Table S1 and Table S2 in the supplemental material. Yeast cells were routinely grown at 30°C in yeast extract-peptone-dextrose (YPD) medium containing 2% yeast extract, 1% peptone, and 2% glucose or in glucose minimal medium (GMM) containing 6.79 g/liter yeast nitrogen base without amino acids and 2% glucose with appropriate supplementation (uridine at 80 μg/ml, arginine at 40 μg/ml, or histidine at 40 μg/ml) for auxotrophic mutants. For hyphal induction, yeast cells were inoculated into YPD medium or GMM supplemented with 20% fetal bovine serum (HyClone Laboratories) and incubated at 37°C.

Hyphal growth on solid medium. Overnight cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.1, and 2 μl of diluted cultures was spotted onto the Spider medium plates (30) or the Lee's medium plates (31). The plates were incubated at 30°C for 3 and 6 days, respectively.

Protein extraction, immunoprecipitation, and Western blotting. Experiments were performed as described previously (32), except that an anti-PKA substrate antibody (Cell Signaling; product no. 9621) was used to detect PKA-phosphorylated proteins. Immunoprecipitated Gyp1-green fluorescent protein (Gyp1-GFP) was subjected to liquid chromatography-tandem mass spectrometry (LC/MS/MS) phosphorylation site identification, a service provided by the Proteomics Core Facility of University at Albany, Rensselaer, NY, USA.

***In vitro* kinase assay.** Immunoprecipitated WT or mutant Gyp1 proteins were incubated in the reaction buffer containing 40 mM Tris-HCl (pH 7.4), 20 mM $MgCl_2$, and 500 μM ATP with or without 30 U of bovine PKA catalytic subunit (Promega) at 30°C for 5 min. The level of phosphorylation was determined by Western blot analysis using the anti-PKA substrate antibody.

Microscopy. Colony morphologies on solid plates were examined using a Leitz DMRB inverted microscope (Leica) with a 5× objective lens. Cell morphology in liquid media and the subcellular localization of GFP-tagged proteins were examined using a Leica digital module R (DMR) fluorescence microscope with a 100× objective lens.

Virulence assays. Mouse survival assays were performed as described previously (32). Periodic acid-Schiff staining of formaldehyde-fixed kidneys from infected mice and the slide scanning procedures were carried out by the Advanced Molecular Pathology Laboratory of the Institute of Molecular and Cell Biology, Singapore.

RESULTS

Gyp1 is required for the hyphal growth in *C. albicans*. In an effort to generate and screen a mutant library of GTPase-related genes using *C. albicans* haploids (33), we found that among three reported Ypt1 GAPs, namely, Gyp1, Gyp5 and Gyp8 (5, 34), only Gyp1 was required for the hyphal growth (unpublished data). In this study, we deleted *GYP1* (orf19.3811), *GYP5* (orf19.5340), or *GYP8* (orf19.4315) in diploid *C. albicans* and tested the mutants for hyphal growth. As in the case of haploid *C. albicans*, we again found Gyp1 to be the only Ypt1-GAP essential for hyphal growth on solid Spider medium (30) and Lee's medium (31), whereas *gyp5*Δ/Δ and *gyp8*Δ/Δ cells exhibited normal hyphal growth (Fig. 1A). Consistently, under conditions of induction with 20% serum at 37°C in liquid media, although *gyp1*Δ/Δ cells could form germ tubes, the hyphal growth was much slower than that seen in wild-type (WT) cells (Fig. 1B). Reintegration of a copy of WT *GYP1* restored normal hyphal growth in *gyp1*Δ/Δ cells (Fig. 1A). Du and Novick (5) have reported that mutating arginine 343 (R343) of *Saccharomyces cerevisiae* Gyp1 to lysine (K) abolishes its GAP activity without disrupting the protein folding. To determine whether the GAP activity is required for Gyp1's function in *C. albicans* hyphal growth, we mutated the corresponding arginine residue (R292) to K and found that the GAP-dead $gyp1^{R292K}$ failed to restore the hyphal growth in *gyp1*Δ/Δ cells (Fig. 1A). Thus, Gyp1's GAP activity is required for hyphal growth.

Gyp1 localizes to and regulates hypha-specific translocation of the Golgi apparatus in *C. albicans*. Unlike their polarized localization toward the tip of a growing bud in *S. cerevisiae* (35), the Golgi compartments appear as random puncta in *C. albicans* yeast cells. However, the Golgi compartment is polarized toward the tip of growing hyphae, which is thought to facilitate the long-distance directional vesicle transport required for rapid hyphal extension (17). To reveal the subcellular localization of Gyp1 in *C. albicans*, we expressed Gyp1-GFP from its endogenous promoter for examination by fluorescence microscopy. We observed that, consistent with its Golgi association (5), Gyp1 localized as random puncta in the cytoplasm in yeast cells and showed polarization toward the growing tip in hyphal cells (Fig. 2A). Golgi polarization is dependent on the formin Bni1 and actin cables in both *S. cerevisiae* (35) and *C. albicans* (17). Consistently, the polarized Gyp1 localization in hyphae was completely abolished when *BNI1* was deleted (Fig. 2A). Interestingly, we observed that the polarized localization of the Vrg4-labeled early Golgi compartments (17, 36) was largely lost in *gyp1*Δ/Δ hyphal cells (Fig. 2B). Thus, the data demonstrate

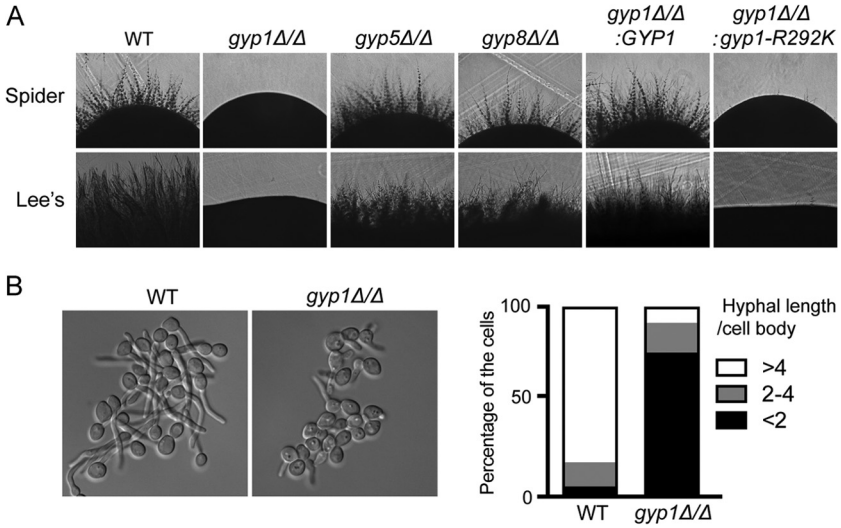

FIG 1 Gyp1 functions in *C. albicans* hyphal growth. (A) *gyp1*Δ/Δ and *gyp1*^{R292K} strains have defects in hyphal development. Yeast cells of BWP17 (WT), HZX201 (*gyp1*Δ/Δ; for details on strain genotypes, please see Table S2 in the supplemental material), HZX250 (*gyp5*Δ/Δ), HZX251 (*gyp8*Δ/Δ), HZX202 (*gyp1*Δ/Δ:*GYP1*), and HZX203 (*gyp1*Δ/Δ:*gyp1R292K*) were grown overnight in YPD medium at 30°C, adjusted to an OD_{600} of 0.1, and spotted onto Spider medium plates or Lee's medium plates. The plates were incubated for 3 days or 6 days, respectively. (B) The *gyp1*Δ/Δ mutant shows markedly slower hyphal extension in liquid media. As shown in the left panel, cells of BWP17 (WT) and HZX201 (*gyp1*Δ/Δ) were induced for hyphal development in GMM–20% fetal bovine serum (FBS) at 37°C for 2 h before imaging. The right panel shows the distributions of hyphae with different ratios of hyphal length to the diameter of the mother cell body at 2 h of hyphal induction ($n = 120$).

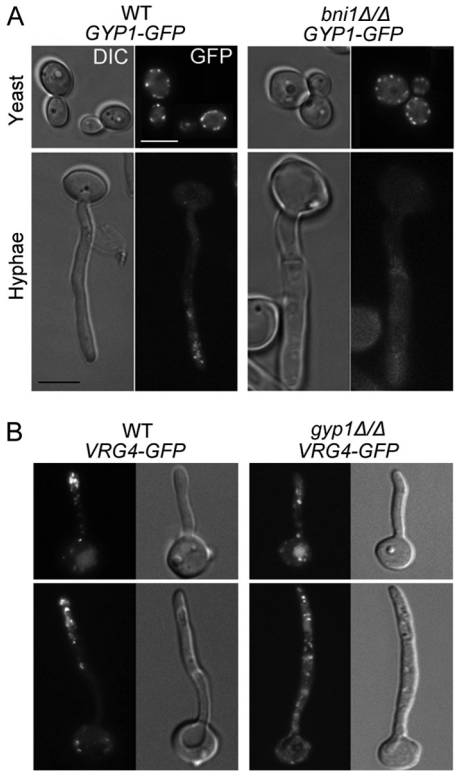

FIG 2 Gyp1 localizes to the Golgi compartment and is required for Golgi polarization in *C. albicans*. (A) Localization of Gyp1-GFP in WT (HZX202) and *bni1*Δ/Δ (HZX225) yeast and hyphal cells. Yeast cells were grown in GMM to the log phase at 30°C. Hyphal cells were grown in GMM–20% FBS at 37°C for 2 h. Size bar = 5 μm. Images of differential interference contrast (DIC) and GFP fluorescence microscopy results are shown. (B) Localization of Vrg4 in short and long hyphae in WT (HZX210) and *gyp1*Δ/Δ (HZX211) backgrounds. Hyphal cells were grown in GMM–20% FBS at 37°C for 2 to 3 h. Although *gyp1*Δ/Δ cells are defective in hyphal growth, a small percentage of long hyphae can be found. Size bar = 5 μm in all images.

that *C. albicans* Gyp1 is a Golgi-associated protein required for the polarization of early Golgi compartments during hyphal development.

Gyp1 is phosphorylated by PKA upon hyphal induction. Global phosphoproteome analysis indicated that *C. albicans* Gyp1 is phosphorylated *in vivo* (37) at serine residues predicted to be PKA substrates (38). PKA activity is strongly activated by cAMP upon hyphal induction and plays a major role in transducing extracellular hypha-induction signals to the nucleus to activate hypha-specific gene expression (15). We hypothesized that Gyp1 could be a novel PKA substrate/effecter in cytoplasm that couples the hyphal signal to Golgi polarization. To test this hypothesis, we first demonstrated that Gyp1 was phosphorylated by PKA upon hyphal induction. We immunoprecipitated GFP-tagged Gyp1 at timed intervals after hyphal induction and then probed it with an antibody that specifically recognizes PKA-phosphorylated serine residues (PKA substrate antibody) in Western blot analysis. We detected a low level of PKA-phosphorylated Gyp1 in yeast cells. Strikingly, within 5 min of hyphal induction, a markedly higher level of PKA phosphorylation was detected which persisted for at least 2 h (Fig. 3A). Furthermore, immunoprecipitated Gyp1 can be phosphorylated by bovine PKA *in vitro* (Fig. 3B), strongly suggesting that Gyp1 is a direct substrate of PKA. PKA is activated by cAMP produced by adenylyl cyclase in response to hyphal induction (39). *C. albicans* has a single adenylyl cyclase, Cyr1 (39), and two isoforms of PKA catalytic subunits, Tpk1 and Tpk2 (40). As expected, PKA phosphorylation of Gyp1 is largely abolished when *CYR1* is deleted (Fig. 3C). Furthermore, consistent with a previous report that the overall cellular PKA activity is lower in *tpk2*Δ/Δ cells than in WT and *tpk1*Δ/Δ cells (41), we detected markedly reduced PKA phosphorylation of Gyp1 in *tpk2*Δ/Δ cells in comparison with WT and *tpk1*Δ/Δ cells (Fig. 3D). Together, these results demonstrate that Gyp1 is phosphorylated by cAMP-activated PKA in response to hyphal induction.

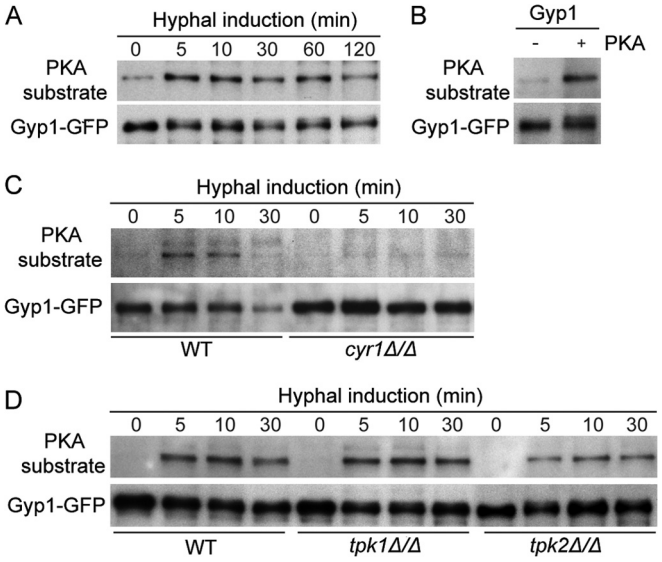

FIG 3 *C. albicans* Gyp1 is a PKA substrate. (A) Gyp1 is phosphorylated by PKA upon hyphal induction. Log-phase yeast cells expressing Gyp1-GFP (HZX202) were subjected to hyphal induction by switching from GMM at 30°C to prewarmed GMM–20% serum at 37°C. Aliquots were harvested at the indicated times after hyphal induction for preparation of cell lysates. PKA phosphorylation of Gyp1 was detected by Western blot analysis using the anti-PKA substrate antibody, and Gyp1 was probed with anti-GFP antibody. (B) Gyp1 can be phosphorylated by bovine PKA *in vitro*. Gyp1-GFP immunoprecipitated from yeast cells (HZX202) was subjected to *in vitro* kinase assay in the presence (+) or absence (−) of bovine PKA and then subjected to Western blot analysis using anti-PKA substrate antibody and anti-GFP antibody. (C and D) Gyp1 PKA phosphorylation levels are decreased in *cyr1*Δ/Δ and *tpk2*Δ/Δ cells upon hyphal induction. WT (HZX202), *cyr1*Δ/Δ (HZX232), *tpk1*Δ/Δ (HZX226), and *tpk2*Δ/Δ (HZX227) cells expressing Gyp1-GFP were processed and PKA phosphorylation of Gyp1 was analyzed as described for panel A.

PKA phosphorylation of Gyp1 dictates its polarized translocation. Mass spectrometry detected six phosphorylated serines in Gyp1, four of which, namely, S32, S96, S105, and S196, have one or two basic residues at the −3 position or at both the −3 and −2 positions (Fig. 4A), representing highly preferred PKA substrates (42, 43). Furthermore, the four serines are all located in the N-terminal regulatory domain (4), suggesting a regulatory function (Fig. 4A). To uncover the function of phosphorylation at these potential PKA sites, we mutated them to either the nonphosphorylatable alanine (*gyp1*^{4A}) or the phosphomimetic glutamic acid (*gyp1*^{4E}). We found that Gyp1^{4A} was significantly less phosphorylated by bovine PKA than the WT Gyp1 *in vitro* (Fig. 4B), indicating that these four sites are indeed phosphorylated by PKA. The observation that bovine PKA can still cause weak phosphorylation of Gyp1^{4A} suggests the existence of other unidentified PKA sites. Interestingly, Gyp1^{4A} was able to restore the hyphal growth in *gyp1*Δ/Δ cells on solid media, suggesting that a redundant pathway may exist that can compensate the function of Gyp1 phosphorylation in hyphal development. In contrast, Gyp1^{4E} was unable to restore the hyphal growth defect, indicating that constitutive Gyp1 phosphorylation by PKA blocks hyphal morphogenesis (Fig. 4C). This notion is supported by the report that mutants with constitutive activation of PKA are defective in hyphal growth (44, 45). However, neither *gyp1*^{4A} cells nor *gyp1*^{4E} cells exhibited significant hyphal defects under serum induction conditions.

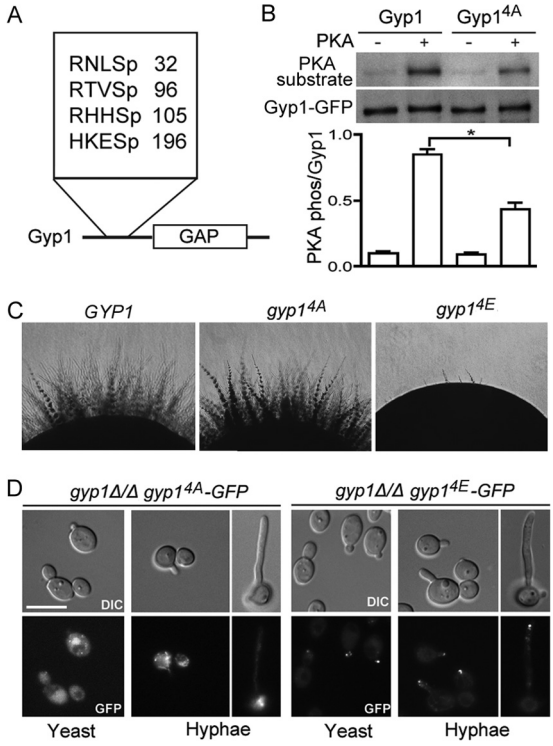

FIG 4 PKA phosphorylation of Gyp1 controls its polarized localization. (A) The diagram depicts the location of and amino acid sequences around four PKA-phosphorylated serines (Sp) identified by mass spectrometry in this study. (B) Gyp1^{4A} is less phosphorylated than WT Gyp1 by bovine PKA *in vitro*. A kinase assay was performed, and PKA phosphorylation of Gyp1 was analyzed as described for Fig. 2C. PKA phosphorylation of Gyp1 was quantified by dividing the density of the PKA substrate band (PKA phos) by that of the Gyp1 band (lower panel). The experiment was done three times, and the difference between PKA phosphorylation of Gyp1 and that of Gyp1^{4A} was statistically significant (*, $P < 0.05$ [*t* test]). Error bars represent standard errors of the means (SEM). (C) Hyphal development of *GYP1* phosphorylation mutants (phosphomutants) on solid medium. A hyphal development assay was performed on Spider medium plates as described for Fig. 1A. The strains used were HZX202 (*gyp1*Δ/Δ *GYP1*), HZX204 (*gyp1*Δ/Δ *gyp1*^{4A}), and HZX205 (*gyp1*Δ/Δ *gyp1*^{4E}). (D) Cellular localization of Gyp1^{4A}-GFP (HZX204) and Gyp1^{4E}-GFP (HZX205) in yeast and hyphal cells.

To test our hypothesis that Gyp1 PKA phosphorylation controls Golgi polarization, GFP-tagged Gyp1^{4A} and Gyp1^{4E} were expressed in *gyp1*Δ/Δ cells and their localization in yeast and hyphal cells was examined. The two mutant versions of Gyp1 exhibited greatly distinct localization patterns. Gyp1^{4A} showed a random punctate pattern similar to that of WT Gyp1 in yeast cells. In hyphal cells, however, Gyp1^{4A} failed to polarize toward the hyphal tip, a result that was similar to the localization of WT Gyp1 in *bni1*Δ/Δ cells (Fig. 4D). In sharp contrast, Gyp1^{4E} was intensely polarized to the growing tips even during yeast budding, which resembles the behavior of WT Gyp1 in hyphal cells. During hyphal growth, Gyp1^{4E} was not only polarized but also highly concentrated at the hyphal tip (Fig. 4D). Taken together, our results demonstrate that PKA phosphorylation of Gyp1 governs its polarization to the growing site.

PKA phosphorylation of Gyp1 enhances its association with Myo2. As mentioned above, Golgi polarization toward the hyphal tips depends on the presence of polarized actin cables in *C. albicans* (Fig. 2B). Actin cables, however, are oriented toward the

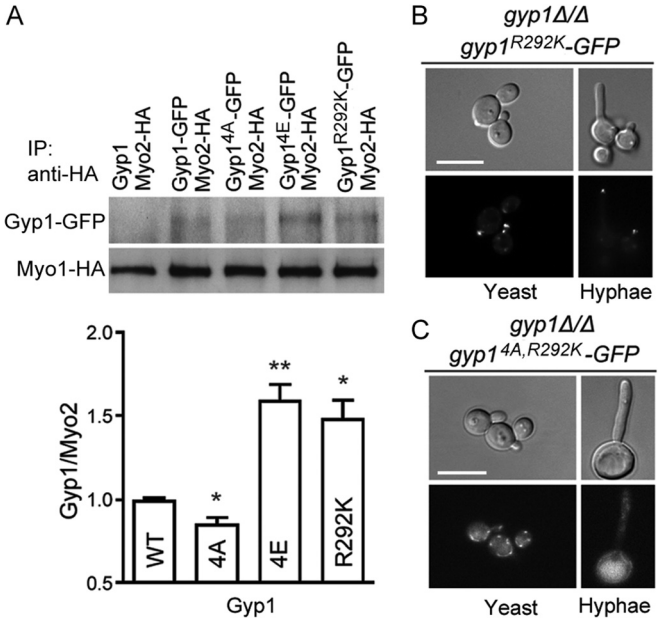

FIG 5 PKA phosphorylation and GAP activity inactivation of Gyp1 enhance its association with Myo2. (A) Log-phase yeast cells coexpressing Myo2-hemagglutinin (Myo2-HA) with Gyp1 (HZX232), Gyp1-GFP (HZX228), Gyp1^{4A}-GFP (HZX230), Gyp1^{4E}-GFP (HZX231), or Gyp1^{R292K}-GFP (HZX229) were subjected to immunoprecipitation (IP) with anti-HA antibody to pull down Myo1, and the precipitation products were analyzed by Western blotting using anti-GFP and anti-HA antibody. The band intensity of the GFP-tagged Gyp1 protein was normalized to the band intensity of Myo2-HA in the same sample (lower panel). The experiment was done three times, and the differences between WT Gyp1 and mutant Gyp1 proteins were statistically significant (*, $P < 0.05$; **, $P < 0.01$ [*t* test]). Error bars represent SEM. (B and C) Localization of Gyp1^{R292K}-GFP (HZX203) and Gyp1^{4A,R292K}-GFP (HZX233) in yeast and hyphal cells.

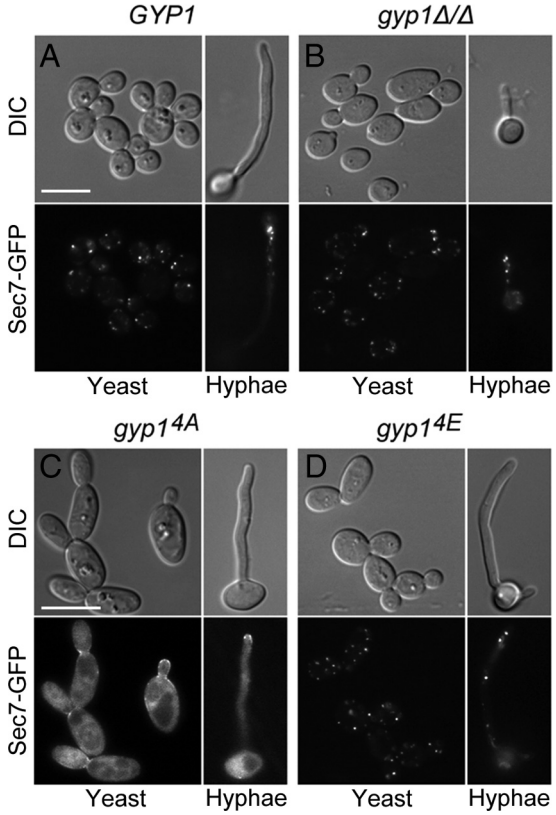

FIG 6 Recruitment of Sec7 to late Golgi compartments requires PKA phosphorylation of Gyp1. Actively growing yeast and hyphal cells of HZX215 (*GYP1* SEC7-GFP) (A), HZX216 (*gyp1*Δ/Δ SEC7-GFP) (B), HZX218 (*gyp1^{4A}* SEC7-GFP) (C), and HZX219 (*gyp1^{4E}* SEC7-GFP) (D) were examined for Sec7-GFP localization by fluorescence microscopy. Yeast cells were grown in GMM at 30°C, and hyphae were induced in GMM–20% FBS at 37°C for 2 h.

growth sites during both yeast and hyphal growth in *C. albicans*. Thus, there must be a mechanism to ensure that the Golgi compartments ride only on the polarized actin cables during hyphal but not yeast growth. In *S. cerevisiae*, the myosin V motor protein, Myo2, is required for transporting Golgi compartments toward the growth sites by binding to proteins on Golgi compartments and Golgi-derived vesicles, such as Ypt11 (46), Ypt32 (11, 12), and Sec4 (10). In vertebrates, phosphorylation has been suggested to be a mechanism to regulate the affinity between myosin V and its cargoes (47). To test whether PKA phosphorylation of Gyp1 enhances its association with Myo2, we performed coimmunoprecipitation to compare the affinities of Myo2 with WT and mutant Gyp1 proteins. Consistent with the distinct localization patterns described above, Myo2 pulled down significantly more Gyp1^{4E} than WT Gyp1 and Gyp1^{4A} (Fig. 5A). The results lend strong support to our hypothesis that PKA phosphorylation of Gyp1 strengthens its association with Myo2, which facilitates Golgi transport toward the hyphal tips.

We observed that, similarly to Gyp1^{4E}, the GAP-dead Gyp1^{R292K} also showed enhanced association with Myo2 (Fig. 5A). Consistently, the localization of Gyp1^{R292K} was highly polarized to the growing sites during both yeast and hyphal growth, closely resembling that of Gyp1^{4E} (Fig. 5B). If PKA phosphorylation controlled Gyp1's localization only by inactivating its GAP activity, the nonphosphorylatable and GAP-dead Gyp1 (Gyp1^{4A,R292K}) would exhibit a localization pattern similar to those of Gyp1^{R292K} and Gyp1^{4E}. However, the localization of Gyp1^{4A,R292K} was found to resemble that of Gyp1^{4A} (Fig. 5C), suggesting that PKA phosphorylation and the GAP activity of Gyp1 do not function in a simple linear pathway. Future research is needed to elucidate the interplay between these two important pathways.

PKA phosphorylation of Gyp1 is required for the recruitment of Sec7 to late Golgi compartments. To investigate whether PKA phosphorylation of Gyp1 affects downstream Golgi-derived secretory compartments, we examined the localization of a marker protein, Sec7 (35, 48). As expected, GFP-tagged Sec7 markers appeared as random puncta in WT cells (Fig. 6A). Unlike the early Golgi marker Vrg4, the localization of Sec7 was apparently not affected by the deletion of *GYP1* (Fig. 6B). Sec7 localization also appeared to be normal in the *gyp1^{4E}* mutant (Fig. 6D). However, in the *gyp1^{4A}* mutant, localizations of Sec7 became diffuse throughout the cytoplasm, with only weak enrichment at the site of growth (Fig. 6C). This localization pattern suggested a failure in Sec7 recruitment to the Golgi compartment. It has been recently reported that stable Sec7 membrane recruitment requires the initial GTP-bound Arf1 (Arf1-GTP) that is activated on early Golgi compartments to kick off a positive-feedback mechanism (49). We speculate that this initial Arf1-GTP activation may require signals from PKA-phosphorylated Gyp1. Future experi-

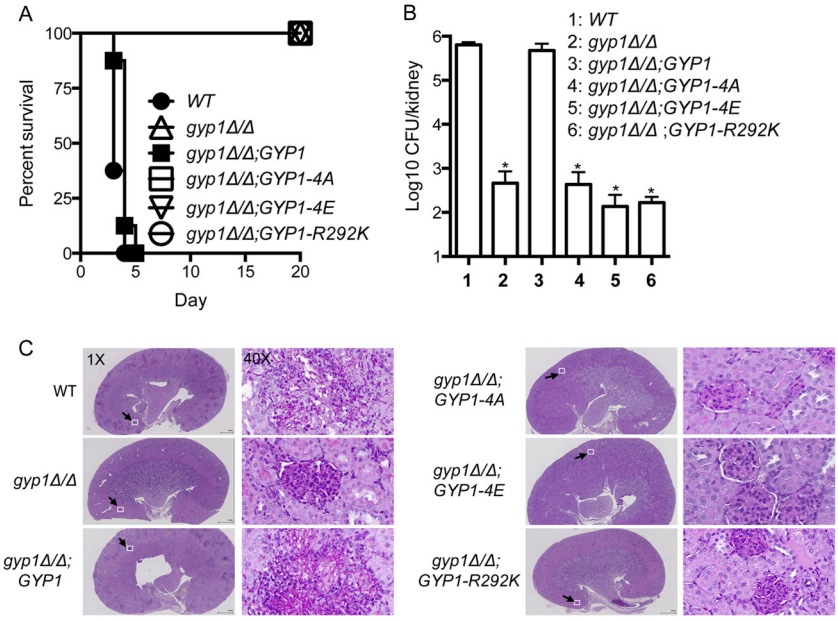

FIG 7 Gyp1 function is required for virulence of *C. albicans*. (A) Survival curves of mice inoculated with 1×10^6 yeast cells with the indicated genotype. Eight mice were used for each *C. albicans* strain (SC5314, HZX201, HZX202, HZX204, HZX205, and HZX203). (B) Kidney fungal burden of the infected mice. Two mice for each *C. albicans* strain were sacrificed 2 days after injection to determine the $\log_{10}$CFU/kidney (*, $P < 0.01$ [*t* test]). (C) Histological examination of the kidneys of infected mice. The kidneys of mice infected with *C. albicans* with the indicated genotypes were removed 2 days after infection. Kidney sections were stained using the periodic acid-Schiff staining method, which stains *C. albicans* cells dark magenta. The square in the 1× image marks that region shown in the 40× image.

ments, however, are required to elucidate the exact mechanisms. Nevertheless, the data suggest that PKA phosphorylation of Gyp1 is important for some downstream events in the Golgi trafficking pathway.

PKA phosphorylation of Gyp1 plays an important role in the virulence of *C. albicans*. The cAMP-PKA signaling activates the expression of a range of virulence factors in *C. albicans*, including hyphal morphogenesis and host cell adhesion (50). Gyp1's role in coupling cAMP-PKA signals to the Golgi-mediated secretory pathway suggests that it could be explored as a potential therapeutic target. To test the impact of altering the PKA phosphorylation of Gyp1 on *C. albicans* virulence, we employed a mouse model of systemic infection in which *C. albicans* yeast cells were injected via the tail vein (32). All mice infected with the WT or the *GYP1*-reintegrant strain died within 5 days after inoculation (Fig. 7A). Similar results were observed in mice infected with the *gyp5*Δ/Δ mutant or the *gyp8*Δ/Δ mutant, both of which showed normal hyphal growth (see Fig. S1 in the supplemental material). In contrast, mice infected with the $gyp1^{4A}$ mutant, the $gyp1^{4E}$ mutant, or the $gyp1^{R292K}$ mutant all survived to the end of the experiment (Fig. 7A). Consistently, there was no significant difference in kidney fungal burdens between mice infected with the WT and *GYP1*-reintegrant cells 2 days after inoculation, whereas mice infected with $gyp1^{4A}$, $gyp1^{4E}$, or $gyp1^{R292K}$ mutant cells showed greater than 1,000 times less fungal burden in the kidney (Fig. 7B). Histological examination revealed that WT and *GYP1*-reintegrant cells in the infected kidney and brain were largely in the hyphal form, whereas few hyphae was observed in the kidney and brain of the mice infected with the mutant cells (Fig. 7C; see also Fig. S2). Taken together, our results show that proper PKA phosphorylation of Gyp1 is essential to the virulence of *C. albicans*. Thus, Gyp1 provides an attractive therapeutic target for the treatment of *C. albicans* infection.

DISCUSSION

In this study, we have discovered a novel role for Gyp1 in transducing hypha-induction signals to the Golgi-derived vesicular transport during the yeast-to-hypha transition in *C. albicans*. Gyp1 is the only Rab1/Ypt1 GAP that is essential for the hyphal growth and virulence in this pathogen. Gyp1 receives the hypha-induction signal via direct phosphorylation by cAMP-activated PKA. This phosphorylation triggers a switch of the transport machinery from the yeast mode to the hyphal mode by, at least in part, enhancing the interaction between the proteins on Golgi compartments to the actin motor Myo2 on polarized actin cables. Altering PKA phosphorylation of Gyp1 has dramatic effects on Golgi polarization, impairs the hyphal growth, and attenuates the virulence of this pathogen. The idea of the importance of Gyp1 in the hyphal growth is supported by previous reports that the transcript of *GYP1* is coregulated by three hyphal gene repressors, Tup1, Nrg1, and Mig1 (51), and several prevacuolar Rab GTPases have recently been reported to impact the hyphal growth (52, 53). Thus, our results provide the first direct link between the hypha-induction signal and the vesicular transport machinery, which is essential for the morphogenetic transition and virulence in *C. albicans*.

The cAMP-PKA pathway is the major signaling pathway that transduces hyphal inducing signals to its downstream effectors to turn on the hyphal program (18). Studies of the role of PKA in the yeast-to-hypha transition have been focused on its downstream transcription factors, including Efg1 and Flo8 (54–57). Our results identify Gyp1 as the first PKA effector in the vesicular trans-

port pathway. Our findings also suggest that the role of the cAMP-PKA signaling pathway is broader than just regulating hyphal gene expression. As Gyp1 functions in the early steps of the vesicular transport pathway from the ER to the PM, it could act as a receiver of hypha-induction signals, which are then passed on along the pathway via established signaling cascades. Our finding that localization of the late Golgi marker Sec7 requires PKA phosphorylation of Gyp1 (Fig. 6) supports this model. Another possible model could be that switching to the hyphal mode would require PKA to simultaneously phosphorylate its effectors at multiple steps of the vesicular transport pathway to alter their functions, such as increasing vesicle binding to Myo2 to meet the need for rapid hyphal growth. The recent discovery that *S. cerevisiae* Myo2 is a potential PKA substrate and that its phosphorylation regulates its polarized localization to the growing bud seems to support this model (58). A third possibility is the combination of these two models, i.e., that PKA phosphorylates Gyp1 to activate the signaling cascade along the transport pathway and at the same time PKA also phosphorylates other effectors along the pathway to reinforce the signal initiated from Gyp1. Further efforts are justified to identify more PKA effectors and elucidate how the hypha-induction signals are integrated into the Rab GTPase cascade in vesicular transport.

Besides PKA, other kinases, such as the cyclin-dependent kinases (CDKs), also play critical roles in *C. albicans* hyphal growth. In the past decade, a series of studies have shown that CDKs control *C. albicans* hyphal growth by phosphorylating components in key cellular machines that execute polarized growth (23), including the Cdc42 GTPase module (24), actomyosin ring components (59), septins (28, 60), endocytic machinery (29), and the Cbk1/Mob2 complex in the regulation of Ace2 and morphogenesis network (61). Recently, Sudbery and his colleagues have reported that the hypha-specific Hgc-Cdc28 phosphorylates and regulates Sec2 and Exo84, two downstream components of the vesicular transport pathway during *C. albicans* hyphal growth (25, 26). Together with the results in this study, we propose that PKA and CDK work in a coordinated manner to control early and late events in the vesicular transport pathway during hyphal growth. As PKA is activated first by hyphal induction, it phosphorylates some upstream components on Golgi compartments, such as Gyp1, to initiate changes in the transport machinery, and at the same time PKA phosphorylates a transcription factor(s) to turn on the expression of the hypha-specific G1 cyclin Hgc1 (22). After Hgc1 has been synthesized, the Hgc1-CDK complex starts to phosphorylate some downstream components on Golgi-derived secretory vesicles, such as Sec2 and Exo84 (25, 26), to complete the transition of the entire vesicular transport machinery from the yeast mode to the hyphal mode. This PKA-CDK relay would ensure rapid establishment of the hyphal growth in response to environmental cues.

There have been a number of reports on a strategy used in the evolution of several key traits in *C. albicans* to specifically meet the needs of its commensal/pathogen life style in the human host: it has modified and converted some existing developmental pathways for specific purposes. One example is the evolution of Hgc1 from a cell cycle regulator to a specific promoter of hyphal growth (22). In spite of its homology to G1 cyclins, Hgc1 has nearly completely lost its function in cell cycle control but has specifically retained and possibly enhanced the ability of the yeast G1 cyclins such as Cln1 and Cln2 to promote apical growth (62, 63). Furthermore, the expression of *HGC1* has also been uncoupled from cell cycle control and placed under the control of the hypha-induction signals to ensure the continuous polarized growth in hyphal development (22). Furthermore, Hgc1-Cdc28 phosphorylation of Rga2 (24), Efg1 (27), Sec2 (25), Exo84 (26), and Cdc11 (60) leads to modification of highly conserved cellular processes to suit the requirement by different aspects of hyphal development. The evolution of *C. albicans* has also involved extensive rewiring of existing transcription regulation circuits (64–67). The phosphoregulation of Gyp1 by PKA found in this study may provide another example of this evolutionary strategy. In *S. cerevisiae*, the Golgi compartments are polarized toward the growing bud (35), whereas in *C. albicans* budding yeast growth, the Golgi compartments exhibit no such polarity. Interestingly, Gyp1 in *S. cerevisiae* has been reported to be among the proteins that are most highly phosphorylated by CDK1 in the secretory pathways (37, 68). In contract, *C. albicans* Gyp1 does not have a CDK consensus site; instead, it has multiple PKA sites. Budding is tightly coupled with the cell cycle, while the hyphal extension is independent of it (69) but dependent on PKA activation by cAMP (50, 70). We speculate that the mechanism by which Gyp1 phosphorylation regulates Golgi polarization may be evolutionarily conserved. However, Gyp1 has evolved different regulatory targets for different kinases, thus linking Golgi polarization to different upstream signaling cascades to adapt to various developmental programs in different species.

Several proteins associated with post-Golgi secretory vesicles have been reported to play important roles in *C. albicans* hyphal growth and virulence (15, 25, 26, 71), which underscores the importance of the vesicular transport pathway in these processes. The formation of the hypha-specific Spitzenkörper and the polarization of the Golgi compartment toward hyphal tips are clear indications that hypha-induction signals regulate the vesicular transport machinery (16). Our discovery of Gyp1 as a direct substrate of PKA reveals an important signaling node for this control. Interestingly, it has been reported that the bacterial pathogen *Legionella pneumophila* secrets proteins that mimic the activity of eukaryotic Rab1 GAP to manipulate the host secretory pathway (72, 73). These findings suggest that Rab1/Ypt1 GAPs could be targets for effective regulation and manipulation of the eukaryotic secretory process throughout evolution. Moreover, as the activity of Gyp1 is crucial for the virulence of *C. albicans*, it may represent an attractive target for developing new antifungal strategies.

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