Protein Phosphatase 2A (PP2A) Regulatory Subunits ParA and PabA Orchestrate Septation and Conidiation and Are Essential for PP2A Activity in Aspergillus nidulans

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Protein phosphatase 2A (PP2A) is a major intracellular protein phosphatase that regulates multiple aspects of cell growth and metabolism. Different activities of PP2A and subcellular localization are determined by its regulatory subunits. Here we identified and characterized the functions of two protein phosphatase regulatory subunit homologs, ParA and PabA, in Aspergillus nidulans. Our results demonstrate that ParA localizes to the septum site and that deletion of parA causes hyperseptation, while overexpression of parA abolishes septum formation; this suggests that ParA may function as a negative regulator of septation. In comparison, PabA displays a clear colocalization pattern with 4’,6-diamidino-2-phenylindole (DAPI)-stained nuclei, and deletion of pabA induces a remarkable delayed-septation phenotype. Both parA and pabA are required for hyphal growth, conidiation, and self-fertilization, likely to maintain normal levels of PP2A activity. Most interestingly, parA deletion is capable of suppressing septation defects in pabA mutants, suggesting that ParA counteracts PabA during the septation process. In contrast, double mutants of parA and pabA led to synthetic defects in colony growth, indicating that ParA functions synthetically with PabA during hyphal growth. Moreover, unlike the case for PP2A-Par1 and PP2A-Pab1 in yeast (which are negative regulators that inactivate the septation initiation network [SIN]), loss of ParA or PabA fails to suppress defects of temperature-sensitive mutants of the SEPH kinase of the SIN. Thus, our findings support the previously unrealized evidence that the B-family subunits of PP2A have comprehensive functions as partners of heterotrimeric enzyme complexes of PP2A, both spatially and temporally, in A. nidulans.

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found by using the human B\(^+\) type to BLAST search the yeast protein sequence database. In *Aspergillus nidulans*, according to homology and phenotypic analyses, there are two predicted genes for two PP2A catalytic subunits, designated *ppgA* (An0164.4) and *pphA* (An6391.4), which correspond to the *S. cerevisiae* homologs *ppg1* and *pph21*, respectively. Many studies have verified that the PP2A complex plays important roles during cell communication and differentiation (29–35).

The filamentous fungus *A. nidulans* is an excellent model organism for studying mitosis and cytokinesis because of its ability to generate septa in hyphal cells such that multinucleate hyphal cells are able to endure septum defects more strongly than are single yeast cells. To date, 28 protein phosphatase catalytic subunit genes have been identified in *A. nidulans* (36). Among them, a gene for the catalytic subunit of PP2A (R259/Q) leads to slow growth, delayed germ tube emergence, and mitotic defects at low temperatures (37). However, studies attempting to explicate potential functions of regulatory subunits of PP2A and their relationship with each other remain limited for fungi. In this study, we identified and characterized the functions of the protein phosphatase regulatory subunits encoded by *pabA* and *parA* by using systematic molecular approaches. Our results indicate that the B subunit *pabA* gene and the B’ subunit *parA* gene for PP2A in *A. nidulans* play very important roles in morphogenesis, conidiation, and self-fertilization. Moreover, relationships among PabA, ParA, and the septation initiation network (SIN) and between PabA, ParA, and formin SEPA during septation have been analyzed. Our findings support previously unrecognized evidence for the function of B-family subunits of PP2A and their own specific regulatory mechanisms in *A. nidulans*.

**MATERIALS AND METHODS**

**Strains, media, culture conditions, and transformation.** A list of *A. nidulans* strains used in this study is provided in Table S1 in the supplemental material. MM, YAG, YG (YAG without agar), YUU, YUUK, MMGPR, and MMGTPI (MMGPR with 100 mM threonine) media have been described in previous works (38–40). Growth conditions, crosses, and induction conditions for *alcA* (p)-driven expression were the same as those described previously (41). Expression of tagged genes under the control of the *alcA* promoter was regulated by different carbon sources, with repression on glucose, derepression on glycerol, and induction on threonine. Standard DNA transformation procedures were used for *A. nidulans* (42, 43).

**Construction of parA and pabA deletion strains.** A strain containing a *parA*-null mutation was created by double-joint PCR (44). The *Aspergillus fumigatus* pyrG gene in plasmid pXDRFP4 was used as a selectable nutritional marker for fungal transformation. The linearized DNA fragment 1, which included a sequence of about 725 bp that corresponded to the region immediately upstream of the *parA* start codon, was amplified with primers 5’-ParA-For and 5’-ParA-Rev+Tail (see Table S2 in the supplemental material). Linearized DNA fragment 2, including a sequence of about 688 bp that corresponded to the region immediately downstream of the *parA* stop codon, was amplified with primers 3’-ParA-For+Tail and 3’-ParA-Rev (see Table S2). Lastly, purified linearized DNA fragments 1 and 2 plus the pyrG gene were mixed and used in a fusion PCR with primers Nested-parA5’ and Nested-parA3’. The final, 3,204-bp fusion PCR products were purified and used to transform *A. nidulans* strain TN02A7. A similar strategy was applied to construct the *pabA* deletion strain, using primers 5’-PabA-For and 5’-PabA-Rev+Tail for linearized DNA fragment 1, primers 3’-PabA-For+Tail and 3’-PabA-Rev for linearized DNA fragment 2, and primers Nest-pabA5’ and Nest-pabA3’ for the fusion product. The final, 3,665-bp 5’-pabA-AfpypG-pabA-3’ cassette was purified and used to transform *A. nidulans* strain TN02A7 (see Fig. S1).

**Tagging of ParA and PabA with GFP.** To generate an *alcA* (p)-*gfp-parA* fusion construct, a 1,192-bp fragment of *parA* was amplified from TN02A7 genomic DNA by use of primers Rec-parA-5’ (NotI site included) and Rec-parA-3’ (XbaI site included) (see Table S2 in the supplemental material). The 1,192-bp amplified DNA fragment was cloned into the corresponding sites of pLB01, yielding pLB-parA 5’ (45). This plasmid was transformed into TN02A7. Homologous recombination of this plasmid into the *parA* locus should result in an N-terminal green fluorescent protein (GFP) fusion with the product of the entire *parA* gene under the control of the *alcA* promoter and a fragment of *parA* under the control of its own promoter. Most transformants displayed identical phenotypes, forming normal colonies under inducing conditions but showing growth defects at 37°C under repressing conditions. One transformant was subjected to diagnostic PCR analysis using a forward primer (GFP-up) designed to recognize the *gfp* sequence and a reverse primer (ParA-down-3’) designed to recognize the 3’ *parA* sequence. A similar strategy was used to construct the *alcA* (p)-gfp-pabA strain.

**Immunoblotting experiment and Southern hybridization.** To extract proteins from *A. nidulans* mycelia, conidial spores from the *alcA* (p)-*gfp-parA*, *alcA* (p)-*gfp-pabA*, and wild-type (WT) parent control strains were inoculated into MMGPR liquid medium and then shaken at 220 rpm on a rotary shaker at 37°C for 20 h. Tissue was ground in liquid nitrogen with a mortar and pestle and suspended in an ice-cold extraction buffer (50 mM HEPES [pH 7.4], 137 mM KCl, 10% glycerol, 1 mM EDTA, 1 μl/ml pepstatin A, 1 μl/ml leupeptin). Equal amounts of protein (40 μg) in gel lanes were subjected to 10% SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore) in 384 mM glycine, 50 mM Tris (pH 8.4), and 20% methanol at 250 mA for 1.5 h. The membrane was then blocked with phosphate-buffered saline (PBS) containing 5% milk and 0.3% Tween 20. Next, the membrane was probed sequentially with a 1:1,000 dilution of anti-GFP antibody (Roche Applied Science) and goat anti-rabbit IgG–horseradish peroxidase diluted in PBS including 5% milk and 0.3% Tween 20. The blot was developed by enhanced chemiluminescence (ECL; Amersham). Other procedures in immunoblotting experiments were carried out as previously described (46). To perform Southern blotting, genomic DNA was digested with EcoRV or BamH I and then separated by electrophoresis and transferred to a nylon membrane (Zeta-probe G; Bio-Rad). The fragment amplified with primers Para-s-up and Para-s-down (see Table S2 in the supplemental material) was used as a probe to detect different *parA* and parent control wild-type strains, respectively. Meanwhile, the fragment amplified with primers PabA-s-up and PabA-s-down (see Table S2) was used as a probe to detect the *pabA* and parent wild-type strains (see Fig. S1). Labeling and visualization were performed using a digoxigenin (DIG) DNA labeling and detection kit according to the manufacturer’s instructions (Roche Applied Science, Indianapolis, IN).

**Overexpression of parA and pabA.** To clone the open reading frame (ORF) of the *parA* gene into the inducible expression plasmid pAL5 (41), a KpnI site was introduced by PCR before the upstream sequence of the first codon and after the downstream sequence of the stop codon, using primers Pal-parA5’ and Pal-parA3’. A 2,389-bp PCR product confirmed by sequence analysis was cut with KpnI and then ligated with a KpnI-cut pAL5 vector to produce the plasmid pAL-parA. This plasmid was transformed into TN02A7. Transformants embedding the entire *parA* gene under the control of the *alcA* promoter and another entire *parA* gene under the control of its own promoter were selected. Overexpression of *pabA* was carried out in the *alcA* (p)-*gfp-pabA* strain ZGB04 under induc tion conditions with threonine (100 mM). Quantification of overexpression was confirmed by real-time PCR analysis. The plasmids pAL5 and pFNO3, carrying the GFP gene, were purchased from FGSC (http://www.fgsc.net).

**Microscopy and image processing.** Several sterile glass coverslips were placed on the bottom of petri dishes and gently overlaid with liquid
medium containing conidia. Strains were grown on the coverslips at related temperatures prior to observation under a microscope. The GFP-ParA and GFP-PabA signals were observed in live cells by placing the coverslips on a glass slide. DNA and chitin were stained using 4',6-diamidino-2-phenylindole (DAPI) and calcofluor white (CFW) (Sigma-Aldrich, St. Louis, MO), respectively, after the cells had been fixed with 4% amidino-2-phenylindole (DAPI) and calcofluor white (CFW) (Sigma-Aldrich). Differential interference contrast (DIC) images of the cells were collected with a Zeiss Axio Imager A1 microscope (Zeiss, Jena, Germany). These images were then collected and analyzed with a Sensicam QE cooled digital camera system (Cooke Corporation, Germany) with the MetaMorph/MetaFluor combination software package (Universal Imaging, West Chester, PA), and the results were assembled in Adobe Photoshop 7.0 (Adobe, San Jose, CA).

Assay of PP2A activity. Phosphatase activity was measured using a calceinurin assay kit (Biomol, Plymouth Meeting, PA) (48). Total proteins were extracted from wild-type and related mutant strains grown on YAG for 18 h. PP2A activity was measured as the dephosphorylation rate of a synthetic phosphopeptide substrate (RIL peptide) in the presence or absence of 10 mM EGTA buffer. The amount of liberated PO$_4^{3-}$ was determined colorimetrically. Results were normalized on the basis of the protein concentration in each sample. The differences in PP2A activity between the wild-type, ΔparA, ΔpabA, and ΔpabA ΔparA strains were analyzed by the $t$ test ($P$ values < 0.05 were considered significant). Each activity assay was performed in triplicate. The soluble protein content of the supernatant was determined using a dye-binding assay (49).

Quantitative real-time PCR analysis. The samples were cultured for 18 h in the relevant media and then were purified to a fine powder in the presence of liquid nitrogen. The total RNA was extracted using TRIzol (Roche) following the manufacturer’s instructions. The samples were treated with DNase I (TaKaRa), and cDNA was generated using an iScript select cDNA synthesis kit (Bio-Rad). Real-time PCR was performed using an ABI one-step fast thermocycler (Applied Biosystems), and the reaction products were detected with SYBR green (TaKaRa). PCR was accomplished by a 10-min denaturation step at 95°C followed by 40 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. Transcript levels were calculated by the comparative $ΔC_T$ method and normalized against the expression of the tubulin gene in A. nidulans. Primer information is provided in Table S2 in the supplemental material.

RESULTS

Identification and analysis of two PP2A regulatory subunit homologs (B and B') in A. nidulans. A BLASTp search using the Homo sapiens PP2A-B and PP2A-B' subunits as queries in the NCBI and CADRE databases produced one result each for putative PP2A regulatory subunit homologs of yeast par1 and pab1 in A. nidulans, which were referred to as pabA (GenBank accession no. AN1545.4 and CADRE accession no. ANIA_01545) and parA (GenBank accession no. AN9467.4 and CADRE accession no. ANA_09467), respectively. parA encodes a protein with a total length of 636 amino acids (aa), in which a typical conserved PP2A regulatory subunit domain, B56, is included, between aa 166 and 573, by NCBI conserved domain BLASTp analysis. Moreover, we found that ParA shares 48.2% and 38.3% amino acid sequence identities with SpPar1 and SpPar2, respectively. In comparison, PabA is a predicted SpPab1 ortholog in A. nidulans, and PabA shares 68.0% amino acid sequence identity with SpPab1. Theoretically, the pabA gene translates into a 472-amino-acid protein with a WD40 domain located in the region between aa 30 and 378.
FIG 2 Phenotypic characterizations of deletion and conditional mutants. (A) PCR analysis showed that the full-length sequences of parA and pabA (ZGB01 and ZGB02) were deleted in the ΔparA and ΔpabA mutants and that, in conditional strains ZGB03 and ZGB04, the original loci of parA and pabA were replaced by alcA(p)-gfp-parA and alcA(p)-gfp-pabA, respectively. For lanes 1 and 3, the PCR primers were ParA-5′ and ParA-3′ to detect whether parA still existed in the genome, and the expected size was 0.93 kb. For lanes 2 and 4, the PCR primers were ParA-5′ and AfpyrG-3′ to detect whether parA was replaced by the auxotrophy gene AfpyrG in the genome, and the expected size was 1.67 kb. For lanes 5 and 7, the PCR primers were PabA-5′ and PabA-3′ to detect whether pabA still existed in the genome, and the expected size was 1.99 kb. For lanes 6 and 8, the PCR primers were PabA-5′ and AfpyrG-3′ to detect whether there was a homologous recombination to replace pabA with the auxotrophy gene AfpyrG in the genome, and the expected size was 1.79 kb. For lanes 9 and 11, the PCR primers were ParA-up-5′ and ParA-down-3′ to detect whether parA still existed in the genome, and the expected size was 2.12 kb. For lanes 10 and 12, the PCR primers were GFP-up-5′ and ParA-down-3′ to detect whether there was a homologous recombination to replace parA with the auxotrophy gene AfpyrG in the genome, and the expected size was 1.67 kb. For lanes 13 and 15, the PCR primers were PabA-up-5′ and PabA-down-3′ to detect whether pabA still existed in the genome, and the expected size was 1.32 kb. For lanes 14 and 16, the PCR primers were GFP-up and PabA-down-3′ to detect whether there was a homologous recombination to replace pabA with the auxotrophy gene AfpyrG in the genome, and the expected size was 1.48 kb. (B) Identification of homologous recombination by Southern blotting. Only one copy of the AfpyrG selectable marker existed in the chromosome of the ΔparA and ΔpabA mutants. (C) Colony morphologies of wild-type (WT) parent control strain WJA01 and the ΔparA (ZGB01) and ΔpabA (ZGB02) mutants grown on rich medium (YAG) at 37°C for 3 days. (D) Colony phenotypic comparison of the wild type (WJA01) and the ΔparA (ZGB03) and ΔpabA (ZGB04) conditional strains on derepressing medium (MMGPR) and repressing medium (YAG) at 37°C for 3 days. (E) Western blotting indicated that the GFP-ParA and GFP-PabA fusion proteins were detected by the anti-GFP antibody at the predicted sizes of about 95 and 80 kDa, respectively.

The same method (BLASTp) was used to search both the structural subunit and the catalytic subunit of PP2A. Consequently, the potential homologs of the catalytic subunit (yeast pphA homolog) and the structural subunit were found to be AN4085.4 and AN6391.4, respectively. Based on information on the PP2A holoenzyme in yeasts and mammals, a putative structural complex of PP2A in A. nidulans was predicted (Fig. 1B).

Phylogenetic relationships were compared among putative orthologs of ParA and PabA from different fungi. The results suggested that based on full-length alignment, ParA in A. nidulans most closely resembled its counterpart from A. fumigatus, with 79.8% identity, and least resembled its counterpart from Candida albicans, with 37.2% identity. Comparatively, PabA exhibited the highest identity with A. fumigatus (90.6%) and the lowest identity (54.0%) with S. cerevisiae (Fig. 1C). In addition, based on comparisons of conserved domain B36 or WD40, both ParA and PabA orthologs showed highly conserved characterization among the selected fungi, even with orthologs in Homo sapiens, as shown in Fig. 1C.

Deletion and tagging of ParA and PabA. To gain insight into the functions of ParA and PabA, we made full-length deletion mutants of the parA and pabA genes, respectively. Among many obtained transformants, most displayed identical phenotypes.

One transformant was subjected to diagnostic PCR analysis and Southern blotting. As shown in Fig. 2A and B, diagnostic PCR analysis and Southern blotting data showed that the parA and pabA genes were successfully replaced by AfpyrG through the DNA homologous replacement approach. We referred to the ΔparA strain as ZGB01 and the ΔpabA strain as ZGB02 (see Table S1 in the supplemental material). In addition, backcrossing ZGB01 or ZGB02 with the strain having the pyrG marker demonstrated cosegregation of the colony morphology defects with the pyrG marker, indicating that the parA and pabA genes were successfully replaced by one copy of AfpyrG through the DNA homol-
ogous replacement approach. On the rich medium YAG, the colony size of the ΔparA mutant was reduced about 50% compared to that of the wild type, indicating that the loss of parA significantly reduced the vegetative growth rate, as shown in Fig. 2C. In comparison, under the same culture conditions, compared to the wild type, the loss of pabA caused much more severe growth defects than those of the ΔparA mutant, resulting in less than half of the colony size and an irregular colony edge (Fig. 2C). Furthermore, as shown in Fig. 2C, colonies of both the ΔparA and ΔpabA mutants were notably devoid of conidia on agar media.

To further confirm and identify the phenotypes caused by parA or pabA deletion, two conditional strains, the alcA(p)-gfp-parA and alcA(p)-gfp-pabA strains, were generated (Fig. 2D), in which the expression of parA or pabA was able to be repressed by glucose on YAG medium, nonrepressed by glycerol on MMGPR, and induced by glycerol plus threonine on MMGTPr. We referred to the alcA(p)-gfp-parA strain as ZGB03 and the alcA(p)-gfp-pabA strain as ZGB04. As shown in Fig. 2A, diagnostic PCR analysis showed that the gene cassettes were integrated into the predicted site in these conditional strains. To further test the functionality of these two conditional strains (ZGB03 and ZGB04), we next inoculated them on nonrepressing medium for 3 days at 37°C. As expected, both the ZGB03 and ZGB04 strains displayed almost normal colony phenotypes compared to the wild-type strain, indicating the functionalities of the GFP-ParA and GFP-PabA fusion proteins. In comparison, when grown on repressing medium (YAG), the two conditional mutants produced tiny, fluffy colony phenotypes, which were consistent with those displayed by the ΔparA and ΔpabA deletion mutants (Fig. 2D). Moreover, by Western blotting, GFP-ParA and GFP-PabA were detected as bands of about 95 and 80 kDa, respectively, by use of anti-GFP antibody under derepressed conditions (Fig. 2E). Because GFP is a 27-kDa protein, this suggests that the molecular masses of ParA and PabA are about 70 and 50 kDa, respectively, which is consistent with the predicted sizes of these two proteins based on protein sequence information. Compared to the case for GFP-tagged strains, the band was completely absent from the control parent wild-type strain, WJA01. Thus, the above data suggest that both ParA and PabA were tagged with GFP at the predicted site in conditional strains ZGB03 [alcA(p)-gfp-parA] and ZGB04 [alcA(p)-gfp-pabA].

ParA and PabA are required for conidiation and self-fertilization. With the aim of understanding the functions of ParA and PabA during conidiation, ΔparA and ΔpabA mutants were analyzed under a dissecting microscope. As shown in Fig. 3A and C, the vegetative mycelia of the control parent strain TN02A7 were capable of developing into conidiophores with visible phialides connected with numerous conidia. In contrast, the ΔparA and ΔpabA mutants had almost completely abolished conidiation. Through quantitative testing, numbers of conidia produced by the ΔparA mutant were approximately 148-fold lower than those of the wild type (2.8 × 10^7 ± 0.28 ± 10^7 conidia per cm^2) for the WT versus 1.9 × 10^5 ± 0.31 ± 10^5 conidia per cm^2 (for the ΔparA mutant) on YAG. Moreover, under the same culture conditions as those described above, the ΔpabA mutant showed more severe conidiation defects than those of the parA mutant, resulting in a nearly 772-fold decrease in the number of conidia per cm^2 (2.8 × 10^7 ± 0.28 ± 10^7 conidia per cm^2 for the WT versus 3.6 × 10^4 ± 0.22 ± 10^4 conidia per cm^2 for the ΔpabA mutant) relative to that of the wild-type strain (Fig. 3B). To dissect the details of these conidiation defects, the morphology of conidiophores in both mutant and wild-type strains was observed. Occasionally, the parA deletion strain could still develop a few irregular metulae and phialides, but most parA deletion hyphae were unable to form these structures, and even had no normal developed vesicles, as shown in the right panel for the parA deletion strain in Fig. 3C. Interestingly, there were multiple septa in the stalk of the ΔparA mutant, which was not found in the wild-type or ΔpabA strain under the same conditions (Fig. 3C). To further test the ability of the mutant strains to self-fertilize for fruiting body formation, the ΔparA and ΔpabA mutants and their parent strain (TN02A7) were point inoculated onto minimal or rich medium. After cultivation for 2 days at 37°C, all the above-described agar plates were sealed to be induced for sexual development. As a result, deletion of parA did not produce any visible cleistothecia, but the parA deletion strain still developed a few aggregated Hülle cells (Fig. 3D). In comparison, deletion of pabA was able to formed aggregated Hülle cells and produced tiny cleistothecia without any ascospores and asci enclosed (Fig. 3D and E). When the ΔparA or ΔpabA mutant was outcrossed with the wild type, all crosses were able to form normal cleistothecium-containing ascospores with normal viability. These data suggest that both the parA and pabA genes are essential for self-fertilization but not for heterothallic sexual development in A. nidulans.

Deletion of parA or pabA results in abnormal distribution of nuclei and septa. Because previous studies verified that abnormally conidiation was induced mostly by the septation defect during cell division (45, 50), we examined the phenotypes of cell division and septation in the ΔparA and ΔpabA mutants. When conidial spores were inoculated into YG liquid medium for 12 h at 37°C and hyphae were stained with DAPI and CFW, the ΔparA mutant showed an irregular shape of abnormal nucleus distribution in both germlings and mature cells. However, in control parent strains, nuclei were distributed normally along hyphal cells (Fig. 4). In comparison, under the same culture conditions, most spores of the ΔpabA mutant failed to germinate and resulted in multiple nuclei localized in the spore, some of which showed very severe growth defects with a lot of fiber-like nuclear structures or DAPI-stained fragments over a prolonged culture time. When the ΔpabA strain was grown in YG at 37°C for 16 h, it germinated very slowly and showed more than 8 nuclei in short germlings, without any sign of septa. However, in the wild type, most germlings formed the first septa after three rounds of mitosis (8 nuclei). This suggested that the pabA mutant had a delayed-septation phenotype. Because the shape of the nucleus in the ΔparA or ΔpabA mutant was so abnormal, it was hard to quantify properly the number of nuclei in hyphal cells of mutants (Fig. 4). Moreover, the ΔparA mutant also displayed an abnormal septum distribution compared to that of the wild type. As a result, the distance between septa in the ΔparA mutant was 13.99 ± 6.37 μm (n = 110), and that in the ΔpabA mutant was 24.26 ± 8.04 μm (n = 120), instead of the distance of 20.91 ± 5.43 μm (n = 110) seen in the wild type for the same length of hyphae. Thus, according to the average distance between septa, we concluded that the ΔparA mutant had a hyperseptation phenotype, whereas the ΔpabA mutant had a delayed-septation defect. This suggests that ParA and PabA may have opposite functions during septation. It is also possible that the septation defects observed in these mutants may be a consequence of the altered morphology.

Localization of GFP-ParA and GFP-PabA. Next, we studied
the subcellular locations of ParA and PabA by using live-cell imaging of GFP-ParA and GFP-PabA fusions in two conditional strains and found that, in germlings, GFP-ParA localized mostly to the middle of the septum and then extended to the whole septum in mature cells. Later on, GFP-ParA was able to diffuse to the cytosol or aggregated in some cellular particles in old hyphal cells (Fig. 5A; see Fig. S2 in the supplemental material). Notably, no detectable signal of GFP-ParA was found prior to the appearance of a CFW-stained septum. To gain insight into the exact location of ParA at the septum, a three-dimensional scanning image was obtained using confocal microscopy (Fig. 5A). The result clearly showed that GFP-ParA localized just right of the center of the septum, inside the area of chitin staining, in germlings. Additionally, GFP-ParA also localized to the junction between the vesicle and metulae (Fig. 5B). These results suggest that parA may function not only during septation but also during septation, we made another recombination strain, placing GFP at the C termi-

![FIG 3](image-url) ParA and PabA are involved in asexual development and self-fertilization. (A) Conidiation phenotypes of the wild-type (WT) strain TN02A7 and the ΔparA and ΔpabA mutants (ZGB01 and ZGB02), showing that the vegetative mycelia of the parent control strain TN02A7 could be developed into conidiophores with visible phialides connected with numerous conidia, while the ΔparA and ΔpabA mutants had almost completely abolished conidiation. Bars, 10 μm. (B) Quantitative data for numbers of conidia determined using the images in panel A. (C) Conidiophores of the parent control strain and the ΔparA (ZGB01) and ΔpabA (ZGB02) mutants, showing that multiple septa existed in the stalk of the ΔparA mutant. Bars, 10 μm. (D) Comparison of cleistothecium development with and without ascosporulation during self-fertilization in the wild type and the mutants. The left panel shows that the parent control wild-type strain TN02A7 produced many dark, large cleistothecia near the white Hülle cells (arrows indicate developed cleistothecia). The middle panel displays that there were a few aggregated Hülle cells in the ΔparA mutant, but no detectable cleistothecia, and in the right panel, white arrows indicate small cleistothecia in the ΔpabA mutant under normal sexually induced conditions. (E) Cleistothecia with or without ascii in the wild type and the ΔpabA mutant. Bars, 10 μm.

![FIG 4](image-url) Abnormal distributions of nuclei and septa in the ΔparA and ΔpabA mutants. Comparison of septum and nucleus distributions in hyphal cells between the wild-type, ΔparA, and ΔpabA strains. The ΔparA strain was grown in YAG liquid medium at 37°C for 12 h, and the ΔpabA strain was grown for 12 h, 16 h, and 20 h. Nuclei were visualized with DAPI, and septa were stained by calcofluor white. Arrows indicate the locations of septa. Bars, 10 μm.
nus of ParA under the control of the native parA promoter. In contrast to our expectations, although the strain was constructed successfully according to diagnostic PCR assay (see Fig. S3), there were no detectable ParA-GFP signals in this strain. We assume that other interaction proteins or unknown reasons possibly interrupted the GFP signal fused at the C terminus of ParA.

We also checked the localization pattern of GFP-PabA in the alcA(p)-gfp-pabA strain under the same culture conditions as those described above. In this case, the fluorescence of the GFP-PabA fusion was always highly accumulated as spots with nuclei along mature hyphal cells, such that GFP-PabA displayed almost complete colocalization with DAPI staining (Fig. 5C).

**Synthetic defects caused by ΔparA sepH1, ΔpabA sepH1, ΔparA sepA1, and ΔpabA sepA1 double mutants.** Previous studies have verified that the septation initiation network (SIN) is a primary regulatory pathway that controls septation; it includes the serine/threonine protein kinase SEPH, a key component, in both fission yeast and *A. nidulans* (40, 51). In fission yeast, Pab1 and Par1, as orthologs of PabA and ParA, physically interact with the components of the SIN cascade, and loss of either of them suppresses the defects of SIN mutants (17, 22, 24). Thus, loss of *pab1* or *par1* is able to rescue the lethality of these SIN mutants in *S. pombe*. Similarly, SIN kinases may also interact with the PP2A heterotrimeric complex, consisting of PPH1, PP2A-A, and RGB-1, in *Neurospora crassa* (52, 53). In addition, formin SEPA in *A. nidulans* is required for the formation of actin rings at septation sites (54, 55). To identify the relationships between PabA/ParA and the SIN and between PabA/ParA and formin SEPA during

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**FIG 5** Localization patterns of GFP-ParA and GFP-PabA. (A) GFP-ParA showed dot-like structures localized near the middle of septum sites (a to c). (d) Three-dimensional scanning image of calcofluor white-stained septa and GFP-ParA, obtained using a confocal microscope. (Inset) Enlarged view of the localization of GFP-ParA inside the area of chitin staining by calcofluor white. (B) GFP-ParA was localized in the entire region of septum sites (a to c) and in junctions between the sites of the vesicle and phialides in mature conidiophores (d to f) in the alcA(p)-gfp-para conditional strain (ZGB03) under inducing conditions. Arrows indicate GFP-ParA localized to junctions between the vesicle and phialides in the mature conidiophore. (C) GFP-PabA was colocalized with nuclei in the alcA(p)-gfp-pabA conditional strain (ZGB04) under inducing conditions. Bars, 10 μm.
septation, we generated \( \Delta \)parA sepH1, \( \Delta \)parA sepA1, \( \Delta \)pabA sepH1, and \( \Delta \)pabA sepA1 double-mutant strains by crossing temperature-sensitive sepH1 and sepA1 mutants with a pabA or parA deletion mutant. According to previous research, sepH1 and sepA1 mutants are temperature-sensitive cytokinesis mutants; when the mutants are cultured at the restrictive temperature of 42°C, sepH1 and sepA show losses of functionality. In contrast, 30°C is a permissive temperature for these strains (Fig. 6A). As shown in Fig. 6B, after culturing on a rich medium (YUU) at 42°C for 3 days, almost no detectable conidiation was found in all crossed progenies. In addition, the \( \Delta \)parA sepH1 double mutant had reduced colony sizes with synthetic defects compared to the parent strains; the \( \Delta \)pabA sepH1 mutant was a little similar to the sepH1 mutant at 42°C (Fig. 6B). Further microscopic studies indicated that both gernilms and mature hyphal cells of the \( \Delta \)parA sepH1 and \( \Delta \)pabA sepH1 double mutants cultured in liquid medium could not show any clear CFW-stained septa (see Fig. S4 in the supplemental material). Moreover, the \( \Delta \)parA sepA1 double mutant even caused a synthetic lethal colony phenotype compared to either the sepA1 or \( \Delta \)parA single mutant. The \( \Delta \)pabA sepA1 double mutant was a little similar to the sepA1 mutant. These data indicate that the \( \Delta \)parA or \( \Delta \)pabA mutation failed to suppress the defects of the sepH1 or sepA1 mutant, which was totally different from the case in yeasts.

**Coordination between parA and pabA and their contributions to PP2A phosphatase activity.** With the aim of exploring the relationship between two regulatory subunits—ParA and PabA—a genetic crossing between the \( \Delta \)parA and \( \Delta \)pabA strains (ZGB01 and ZGB02) was carried out as described previously. As a result, compared to the \( \Delta \)parA or \( \Delta \)pabA single mutant, the \( \Delta \)parA \( \Delta \)pabA double-deletion strain (ZGB09) showed more severe growth defects, with very tiny and needle-like colonies, but did not have the synthetic lethality phenotype; this suggests that ParA and PabA may function synergistically during colony formation (Fig. 7A). A microscopic study showed that both single and double \( \Delta \)parA and \( \Delta \)pabA mutants were significantly impaired in the formation of the single axis of hyphal polarity, resulting in more branched hyphae than those seen in the wild type, which had organized, parallel, and defined hyphal filaments. Most surprisingly, when the \( \Delta \)parA \( \Delta \)pabA double-deletion mutant (ZGB09) was cultured in liquid medium, gernilms and mature hyphal cells showed almost normal septum formation, suggesting that parA deletion was capable of suppressing the septation defect of the pabA mutant (Fig. 7B; see Fig. S5 in the supplemental material). This also indicates that parA deletion can bypass the requirement for pabA during septation. Next, we wondered how the overexpression of parA or pabA affects septation and conidiation. In two conditional strains, ZGB03 and ZGB04 (that had been induced on threonine-containing medium [MMGTPR] for 3 days at 37°C) (Fig. 7D), expression of both parA and pabA was increased to some extent as shown by quantitative PCR analysis, indicating that the overexpression of parA and pabA did indeed happen. Next, we checked the colony and septum phenotypes of these strains cultured on the above-mentioned overexpression medium. Consequently, at the point of conidiation, either deletion or overexpression of parA caused a fluffy colony phenotype. However, toward the end of septation, in contrast to the case for parA deletion, overexpression of parA almost completely abolished septum formation in gernilms or mycelia, suggesting that parA may work as a negative regulator of septation. To further confirm this phenomenon, we constructed another expression vector, pAL5, in
which the entire parA open reading frame was inserted into the alcA promoter vector, and then integrated this vector into the wild-type strain TNO2A7 to obtain a new strain, ZGB10, in which there was an extra copy of parA in addition to the original parA gene. Consistent with the above-described data, ZGB10 also showed that overexpression of parA was capable of inactivating septation, resulting in abolished septum formation in hyphal cells. In contrast, under the same culture conditions, overexpression of pabA in strain ZGB04 was unable to induce any detectable abnormal phenotype compared to the wild-type strain (Fig. 7A).

Genomic information analysis indicated that both the parA and pabA genes encode homologs of PP2A regulatory subunits, which implied that parA and pabA may cause similar defective phenotypes. However, as discussed above, not only did the parA mutation induce a phenotype different from that induced by pabA mutation, but also the localization patterns of ParA and PabA were markedly different. In order to understand why the loss or overexpression of function of these two putative PP2A regulatory subunits would induce different phenotypes, we tested how much of PP2A function would be affected in two different mutants by analyzing PP2A protein phosphatase activity. As described in previous studies for specific detection of PP2A activity (48), an EGTA buffer was utilized to chelate Ca$^{2+}$ in reaction buffer to remove PP2B (calcineurin) activity, because calcineurin function depends on Ca$^{2+}$ activation. The results showed that loss of either parA, pabA, or both caused a sharp decrease in PP2A activity, such that only 12% of PP2A protein phosphatase activity was left in the parA, pabA, and parA/pabA mutants, respectively, compared with the parent control strain, WJA01 (100%) (Fig. 7C). Moreover, overexpression of parA caused a 38% increase of PP2A protein phosphatase activity, but the PP2A activity was almost unchanged in the mutant with overexpressed pabA (Fig. 7D). To verify the method, there were no significant differences among the above-described samples if the substrate peptide was omitted; this indicated that the background phosphoric acid transferase activity was very low and almost the same for extraction buffers from different strains. These data suggest that both ParA and PabA are essential...
for normal PP2A protein phosphatase activity and that overexpression of ParA, but not PabA, cannot enhance PP2A protein phosphatase activity.

**DISCUSSION**

**Homologs of the B’ and B subunits of PP2A.** PP2A is a heterotrimERIC complex that contains a catalytic subunit (C) and a structural subunit (A) associated with a third, hypothetically competitive and variable regulatory subunit (B, B’, or B”). There appears to be a limited quantity of protein serine/threonine phosphatase catalytic subunits, with substrate specificity determined by association with a variety of regulatory and targeted subunits (15). As indicated using comparative genomic analyses of the *Homo sapiens* PP2A-B and PP2A-B’ subunits via BLAST searches, we reported that *A. nidulans* has only one ortholog each of the B and B’ subunits. As shown in Fig. 1A, conserved domain analysis indicated that the PP2A-B’ subunit, ParA, contains a B56 domain, which in humans exists as an ~56-kDa protein family (56), whereas the PP2A-B subunit, PabA, possesses seven repeats of the WD40 domain, ending with Arg-Trp, which mediates protein interaction (57). Meanwhile, the available genome survey revealed that regulatory subunits of PP2A proteins are ubiquitous and relatively conserved in higher eukaryotes, such as single-cell yeasts, filamentous fungi, and mammals. Except for variable fragments in the N and C termini of ParA orthologs, the identities of the B56 domain are very high (ranging from 53% to 96%) among the selected species in this study. Similarly, the seven repeats of the WD40 domain also show very high identities, from 58% to 98%. This indicates that the conserved domains of both ParA and PabA orthologs show a highly conserved characterization.

**ParA and PabA are involved in asexual and sexual development.** The model filamentous fungus *A. nidulans* develops both sexual and asexual spores through complicated regulatory mechanisms. During these reproductive processes, reversible protein phosphorylation is an important regulatory procedure in which protein phosphatases counteract the activities of protein kinases. In this study, we found that the mutants of *parA* and *pabA* or the conditionally deleted strains showed fluffy, nearly aconidial phenotypes compared to the wild-type strain, which exhibited robustly developed conidia under the same conditions. These data suggest that both putative regulatory subunits (ParA and PabA) are required for the developmental process of conidiation in *A. nidulans*. Previous studies indicated that in *S. pombe*, the *pab1* mutant of the B regulatory subunit was unable to sporulate normally and also showed defective cell wall synthesis and cytoskeletal organization (26). In comparison, inactivation of *rgb-1*, encoding the *Neurospora crassa* B regulatory subunit of PP2A, resulted in a low hyphal growth rate and abnormal morphology, but the *rgb-1* point mutant was female sterile and could produce abundant amounts of arthroconidia (58). This indicates that mutation of the gene that encodes the B regulatory subunit may not affect all sexual processes. Interestingly, as shown in Fig. 3, our data indicated that deletion of the B’ subunit (*parA*) or the C subunit (*pabA*) almost completely abolished self-fertilization, but not heterothallic sexual development, in *A. nidulans*. This phenomenon raises the question of whether there is a common signaling pathway between asexual and sexual development activation. Several lines of evidence suggest that transcription factors such as SteA, a homeodomain protein carrying two tandem C2H2 zinc finger domains, and NsdD, a putative GATA-type transcription factor carrying a type IVb C2H2 zinc finger, are able to positively regulate both sexual and asexual development (59, 60). Moreover, it has been verified that proteins highly localized on septum and conidiophores or nuclei, such as AnAXL2 (localized in conidiophores), PLKA (localized in the spindle pole body [SPB] and the nucleus), and BudA (localized in septa), may play important roles during asexual and sexual development. Consequently, defects of these proteins produce abnormal nuclear division or septum formation (61–64). Thus, we hypothesize that defects caused by *parA* and *pabA* may be due to the reasons for the defects in mitosis and cytokinesis. As shown in Fig. 3C and 4, deletion of *parA* caused a hyperactivated septation phenotype, especially in the stalk, accompanied by a corresponding delay of nuclear division, which is somewhat similar to the irregular morphologies and distribution of nuclei of the *pphA* mutant (37). In contrast, *pabA* deletion induced abnormal division of nuclei, accompanied by delayed septation (Fig. 4). Most interestingly, *parA* deletion was capable of suppressing the septation defect of the *pabA* mutant (Fig. 7B), suggesting that ParA counteracts PabA during septation. In contrast, the double mutant of *parA* and *pabA* led to a synthetic defect of colony growth, indicating that ParA functions synthetically with PabA during hyphal growth (Fig. 7A).

**Relationship between PabA/ParA and the SIN complex.** In fission yeast, the B subunit of phosphatase 2A is a component of the SIN complex, and the loss of *pab1* function rescues the lethality of mutants of the *etd1*, *mob1*, *sid1*, and *cdc11* genes in the SIN cascade (24). Similarly, the PP2A-B’ regulatory subunit Par1 is also a suppressor of the SIN; loss of *par1* rescues defects of *cdc11*, *cdc7*, and *spg1* mutants but is unable to rescue other SIN mutants (17, 22). PP2A-Pab1- or PP2A-Par1-mediated dephosphorylation likely inhibits the SIN activity, whereas *Cdc11*, *Cdc7*, and *Spg1* act as protein kinases and the GTPase, activate the SIN by phosphorylation, such that they may function antagonistically on the same substrate (65, 66). *Cdc11*, *Cdc7*, and *Spg1* are positive regulators that activate the SIN during septation and cytokinesis, whereas PP2A-Pab1 and PP2A-Par1 are negative regulators that deactivate the SIN (67). Thus, both scenarios coordinate cell division and nuclear division. However, our genetic analyses showed that the roles played by ParA and PabA in *A. nidulans* are distinctly different from those played by the other known B’ or B regulatory subunits in yeasts. Our data indicated that the *ΔparA sepH1* double mutant had a reduced colony size, with synthetic defects, compared to the parental strains, whereas the *ΔpabA sepH1* mutant was similar to the *sepH1* mutant cultured at the restrictive temperature of 42°C (Fig. 6B). These data suggest that loss of *parA* or *pabA* function is unable to suppress defects of the PHK kinase in the filamentous fungus *A. nidulans*. There are two potential explanations for this hypothesis. First, the PP2A complex in *A. nidulans* may have substrates different from SEPH, such that depletion of PP2A could not suppress dysfunction of the SEPH kinase. Second, aside from being a positive regulator of the SIN, PP2A may have multiple essential roles during morphogenesis. As shown in our data in Fig. 7C, deletion of *parA*, *pabA*, or *parA* and *pabA* sharply decreased PP2A protein phosphatase activity, resulting in only 12% ± 1%, 9% ± 8%, and 13% ± 2% of the PP2A protein phosphatase activity, respectively, remaining in cells of the control strain, WJA01 (100%). Thus, a proper PP2A activity level is required for cell growth. In addition, in *S. pombe*, almost all *par1* or *pab1* mutants, which rescue defects of mutants in *cdc11*, *cdc7*, and *spg1* or of other SIN mutants, are temperature-
sensitive mutants, not whole-gene deletions. To further confirm the relationship between PabA/ParA and the SIN during septation and cytokinesis in *A. nidulans*, we may have to carry out the above approaches, using site-mutated mutants, temperature-sensitive mutants, or conditional strains, to test their function in the near future.

**Locations of ParA and PabB and the functions of these proteins.** In higher eukaryotic cells, the B subunits of heterotrimeric PP2A enzymes determine the substrate specificity of the complex in different areas. Therefore, in different organs, there are different modes of phosphatase regulation, enabling it to perform different functions. For example, in mammals, four different families of B subunits have been identified: the B, B', B", and B‴ families (16, 17). Each B subunit contains different genes, which are expressed in a tissue-specific manner. For example, the B family is composed of four members of PR55 (α, β, γ, and δ). Among them, PR55α and PR55β have a widespread tissue distribution, whereas PR55β and PR55γ are highly enriched only in the brain (18–20). In this study, by using GFP-tagged live-cell imaging, we found GFP-ParA located at the center of septa in germinals but extended to whole septum sites in mature hyphal cells. In all eu- karyotic organisms, mitotic exit and cell division must be integrated spatially and temporally to facilitate equal division of genetic material between daughter cells (53, 68). In the filamentous fungus *A. nidulans*, multicellular hyphae are compartmentalized by the formation of septa where assembly of the actomyosin-based cytokinetic ring (CR) and CR constriction occurred (64). The CR protein complex includes structural proteins, molecular motors, and signaling enzymes. The location of ParA in the center of the hyphal septum (probably around the pore) indicates that ParA may be involved in the function of the CR site signaling enzyme to convey activating or inhibitory cues to their targets via posttransla- tional modifications, such as dephosphorylation (33). In comparison, GFP-PabA fusion fluorescence always accumulates densely as spots with nuclei along mature hyphal cells, suggesting that pabA may play an important role in phosphatase regulation in nuclei, possibly during mitosis in *A. nidulans*. Although GFP-PabA was not localized to the septum, instead showing a clear nuclear localization, the ΔpabA mutation caused a clear delayed-septation defect. It is likely that PabA mediates dephosphorylation of the SIN activity, while other protein kinases and the GTase activate the SIN by phosphorylation, such that both scenarios coordinate cell division and nuclear division. Thus, even PabA is not directly localized in the septum but indirectly affects septation. In regard to the localization artifacts due to overexpression, we re- checked the localization pattern under inducing and derepressing conditions. We found that inducing conditions (threonine) caused much stronger signals for GFP-ParA and GFP-PabA than did derepressing conditions (glycerol); however, both proteins consistently displayed similar localization patterns.

In addition, based on the comparative genomic analysis by BLAST searching shown in Fig. 1, there was only one each of the PP2A regulatory subunit (B and B′) orthologs in *A. nidulans*. Thus, PabA or ParA may have an important comprehensive function as a partner of holoenzyme complexes of PP2A, both spatially and temporally. Furthermore, we found that deletion of either parA or pabA caused a sharp decline in PP2A enzyme activity, suggesting that both ParA and PabA are essential for PP2A protein phosphatase activity and even that, during specific periods, they may have their own specific regulatory functions. The nonessen- tial function of the case for the essential PP2A catalytic subunit (36).

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