Small-GTPase-Associated Signaling by the Guanine Nucleotide Exchange Factors CpDock180 and CpCdc24, the GTPase Effector CpSte20, and the Scaffold Protein CpBem1 in Claviceps purpurea

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Monomeric GTPases of the Rho subfamily are important mediators of polar growth and NADPH (Nox) signaling in a variety of organisms. These pathways influence the ability of Claviceps purpurea to infect host plants. GTPase regulators contribute to the nucleotide loading cycle that is essential for proper functionality of the GTPases. Scaffold proteins gather GTPase complexes to facilitate proper function. The guanine nucleotide exchange factors (GEFs) CpCdc24 and CpDock180 activate GTPase signaling by triggering nucleotide exchange of the GTPases. Here we show that CpCdc24 harbors nucleotide exchange activity for both Rac and Cdc42 homologues. The GEFs partly share the cellular distribution of the GTPases and interact with the putative upstream GTPase CpRas1. Interaction studies show the formation of higher-order protein complexes, mediated by the scaffold protein CpBem1. Besides the GTPases and GEFs, these complexes also contain the GTPase effectors CpSte20 and CpCla4, as well as the regulatory protein CpNoxR. Functional characterizations suggest a role of CpCdc24 mainly in polarity, whereas CpDock180 is involved in stress tolerance mechanisms. These findings indicate the dynamic formation of small GTPase complexes and improve the model for GTPase-associated signaling in C. purpurea.

The ergot fungus Claviceps purpurea is a biotrophic plant pathogen that is able to infect more than 400 monocotyledonous host plants, among them important crops such as rye, wheat, and barley (1, 2). The infection occurs organ specifically, only on blooming ears. The first growth stage of C. purpurea in the host is characterized by a strict polarity, which changes into a branching growth pattern once the fungus reaches the basis of the ovary. The fungus then colonizes the whole ovarian tissue, developing a sclerotium (3–5). Infected plants do not show any obvious defense reactions against C. purpurea. In the first infection stage, the strict polarity of the hyphal growth partly resembles pollen tube growth, which, together with a possible secretion of effector proteins, might be instrumental for nonrecognition. However, the paths of pollen tubes and infection structures in the ovary differ slightly (5, 6).

Small GTPases are known to influence polarity in many organisms. They act as molecular switches that constantly cycle between an active, membrane-associated, GTP-bound state and an inactive, cytosolic, GDP-bound state. In fungi, highly conserved members of the Rho-GTPase family, Cdc42 and Rac1, are crucial for establishing and maintaining polarity during filament formation and budding (7–10). The importance of Rac homologues in comparison with Cdc42 homologues seems to increase with the developmental complexity of the fungal species (10–14). In C. purpurea, the two GTPases show overlapping and differing functions. CpCdc42 is a negative regulator of polarity and sporulation. Due to the defects in polarity during infection, the Δcdc42 strain is probably recognized as a pathogen by the plant (6). In contrast, CpRac is a positive mediator of polarity, sporulation, and general growth and is essential for infection (15). In comparison, Ras-GTPases are known to be involved in upstream Rho-GEF activation in various organisms, such as Saccharomyces cerevisiae, Schizosaccharomyces pombe, and Cryptococcus neoformans (16–18). No Ras homologue has been characterized in C. purpurea so far.

GTPase cycling is essential for proper cell growth and development, and guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs) are crucial for this process. Dissociation of GDP from the GTPases is triggered by GEFs, subsequently enabling GTP binding. The low intrinsic GTPase activity of Rho proteins is enhanced by GAPs, resulting in hydrolysis of the bound GTP. Inactive GTPases are removed from the membrane and sequestered in the cytosol by the activity of GDIs (19–22). Expression of dominant active or dominant negative Rho-GTPases affects mechanisms such as polarity, cell wall formation, branching, and sporulation in Penicillium marneffei, Aspergillus nidulans, and C. purpurea (6, 9, 23, 24). In fungi, two major families of Rho-GEFs are critical for activation of Rho-GTPases: GEFs of the Dbl homology (DH) family contain the catalytic DH domain in tandem with a pleckstrin homology (PH) domain that usually mediates membrane association (25, 26). Dock180-like GEFs of the CDM zim-imin homology (CZH) family possess a Dock180 homology region (DHR), which serves as a catalytic unit (27, 28). DH-GEFs have already been investigated in a variety of organisms. In S. cerevisiae, the DH-GEF Cdc24 is the sole and essential activator of Cdc42 (29, 30). Likewise, Cdc24 homologues are essential in many
other fungal species, including Ustilago maydis, Neurospora crassa, and Epichloe festucae, which is a close relative of C. purpurea. Le- thality is due mainly to an almost complete loss of cell polarity and severe impairment of filamentous growth, branching, and septa- tion (12, 31, 32). CZH-GEFs have not been studied in filamentous fungi so far. In mammals and the arthropod Drosophila melanogaster, homologues of one member, Dock180, act as Rac-specific GEFs (33–35). The only fungal homologue studied so far is Dck1 of Candida albicans, which is involved in invasive growth (35). Here we describe the first functional analysis of a Dock180 homology in a filamentous fungus.

The recruitment of active GTPases to the tip is mediated by scaffold proteins, which assemble complexes consisting of GEFs, GTPases, and effectors (36–38). In A. nidulans, the scaffold protein Bem1 is required for hyphal growth and conidium formation (39). In E. festucae, Bem1 links Rac1 not only to its GEF, Cdc24, but also to the NADPH oxidase complex via its regulatory protein NoxR. NADPH oxidase function has been shown to be important for symbiosis with the host plant (32).

Known downstream targets of small GTPases are p21-activated kinases (PAKs). In S. cerevisiae and U. maydis, the PAK Cla4 is part of the Bem1-Cdc24-Cdc42 complex and is probably involved in feedback regulations of GEFs and GTPases (36, 40, 41). The importance of Cla4 kinase signaling appears to be more important in higher fungi, while in yeasts the closely related PAK Ste20 fulfills a more crucial role (10, 42–46). In C. purpurea, Cla4 was previously shown to be the major downstream effector of Rac but not Cdc42 (15).

Small GTPase signaling in C. purpurea is strongly connected to NADPH oxidase (Nox) complex dynamics (47). Similarly, as in E. festucae (32), the regulatory protein CpNoxR is able to interact with CpRac in a loading-status-dependent manner, identifying it as a downstream factor of CpRac. Additionally, an involvement of the scaffold protein CpBem1 in this complex has been shown.

In this study, we investigated the composition and dynamics of small GT-Pase-associated complexes to gain further insight into signaling mechanisms during establishment and mainte- nance of cell polarity. We identified CpCdc24 and CpDock180 as Rho-GT-Pase-related GEFs. CpBem1 was identified as a connect- ing scaffold and CpSte20 as a downstream target of Cpc- Cdc42. Furthermore, CpRas1 affected GTPase signaling by in- teracting with the GEFs. By knockdown approaches, the hypothesis of a connection between GTPase signaling and Nox dynamics was strengthened.

MATERIALS AND METHODS

Strains, media, and growth conditions. The wild-type strain of C. purpurea (Fr.) Tul. used in this study was C. purpurea 20.1, a benomyl-treated putative haploid T5 derivative isolated from Secale cereale (48). Strains were incubated on BII agar for maintenance and DNA isolation and on Mantle agar for sporulation and growth tests (49, 50). Growth for transformation or RNA isolation was performed in liquid BII or Mantle medium (49). Plate assays were carried out on Mantle medium, either unsupplemented or supplemented with 0.4 M or 0.8 M sodium chloride, 0.1, 0.2, or 0.3 mM menadione, or 5 or 10 mM hydrogen perox- ide. Colony sizes were measured after 14 days. In all cases, incubation was done at 26.5°C in the dark. DNA cloning was performed in Escherichia coli TOP10 (Invitrogen, Darmstadt, Germany). Cells were grown on solid or in liquid LB medium with added antibiotics (51). Incubation took place at 37°C. Yeast recombinatorial cloning was done in Saccharomyces cerevisiae FY834 for knockout or reporter gene constructs and in strain SMY3 for yeast two-hybrid vectors (52–54). Yeast two-hybrid assays were performed in S. cerevisiae pJ69-4A (55). All strains were incubated at 30°C in yeast extract-peptone-dextrose (YPD), yeast extract-peptone-adenine- dextrose (YPAD), or synthetic dextrose (SD) medium lacking the selecting amino acids.

Nucleic acid analyses. Standard DNA recombinational methods were done as described before (56, 57). Genomic DNA was isolated from lyophilized mycelium of C. purpurea (58), and PCR was done using either Biotherm polymerase (GeneCraft GmbH, Lüdinghausen, Germany) or the proofreading Phusion polymerase (Biozym, Hessisch Oldendorf, Ger- many). Primer synthesis was done by Biolegio (Nijmegen, Netherlands). pGEM-T Easy (Promega, Madison, WI) was used as an intermediate clon- ing vector.

Generation of vectors. Exchange of a single base to create a dominant negative allele of cpcdc42 was performed by using a QuikChange II site- directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). A PCR on pGEM-CpCdc42 was performed according to the manufacturer’s in- structions, using DNcCdc42-mut-1 and DNcCdc42-mut-2 to insert the def- sired mutation. After DpnI digestion of the template, the mutated vector was transformed into E. coli strain XL1-Blue and isolated again for further cloning.

For generation of the expression vectors for GEF activity assays, cprac, cpcdc42, cprac1, cpcdc42DH, cpcdc42DHPH, cpdock180DOCK, and cpdock180DHR2 were amplified from cDNAs. The fragments were cloned into pGEM-T Easy, sequenced, and excised with BamHI and NotI or with Xhol and NotI, in the case of cpdock180. Then they were then ligated into the BamHI-NotI- digested gpx4ET1 expression vector, giving rise to pGEX-Cprac, pGEX-CpCdc42, pGEX-CpRas1, pGEX-Cdc24DH, pGEX-CpCdc42DHPH, pGEX- Cpdock180DOCK, and pGEX-Cpdock180DHR2. 3HA vectors for communiquepoxification were created by yeast recombinational cloning (59). For this purpose, the codon-modified 3HA tag, the gpd promoter, and a HindIII site were integrated into pRS426-Hph, giving rise to p3HA-GPD-Hyg. The open reading frames (ORFs) of cpdock180, cpcdc42, cpcdc24, and cprac20 were amplified from genomic DNA and integrated into HindIII-digested p3HA-GPD.

Most vectors for localization studies were created by yeast recombinational cloning (59). For cpdock180 and cpcdc42, ORF sequences were amplified from genomic DNA together with their endogenous promoters. For cpdock180, cpbem1, and cprac20, only the ORF sequences were amplified. cpdock180 was then recombined with NotI-digested pNAH-OGG, cpdock180 with Spel-Ncol-digested pNAH-OGG, cpbem1 with NotI-digested pNAH-OGG, cpcdc42 with Spel-Ncol-digested pNDH-OGG, and cprac20 with NotI-digested pNAH-OGG (59). The resulting vectors, pNAH- OGG-Cdc24, pNAH-OGG-Dock180, pNAH-OGG-Bem1, pNDH_ Pcd24-cdc24-DHG, and pNAH-OGG-Ste20, were selected on SD medium lacking uracil and expressed in E. coli TOP10. For generation of the vector pRac-P-Rac-GFP, the cprac promoter region was amplified from genomic DNA and then, after intermediate cloning into TOPO (Invitrogen) and excision with NotI and BamHI, ligated into pRS426. The ORF of cprac was amplified from cDNA and then, after intermediate cloning and excision by BamHI and EcoRI, ligated into pRS-Pcprac. Green fluorescent protein (GFP) was amplified and then, after excision with EcoRI and KpnI, ligated into pRS-Pcprac-cprac. The whole fragment, consisting of the promoter, the cprac CDNA, and GFP, was then excised with Clal and KpnI and inserted into pAN8_1UM. For generation of the vectors for bimolecular fluorescence complementation (BiFC), the ORFs of cpbem1 and cplcd4 were amplified from genomic DNA and integrated into NotI-digested pNAH-OGGCon (for cpbem1) or NotI-digested pNDB-AGNt (for cplcd4) by yeast recombinational cloning (39). After preparation, all vec- tors were used for transformation of C. purpurea.

Vectors for yeast two-hybrid assays were also created by yeast recombinational cloning. The ORFs of cpdock4, cpdock24DH, cpdock24DHPH, cpdock180, cpbem1, and cpDNcCdc42 were amplified using specific primers.
Eukaryotic Cell

Herrmann et al.

for every gene. The template for all fragments was C. purpurea cDNA, except for cpDndc42, which was amplified from pGEM-DNdc42. All fragments were cotransformed with EcoRI-Sall-digested pAD-Gal4-2.1 or pBD-Gal4 (Stratagene, Santa Clara, CA). Selection of yeast recombinants was done on SD medium lacking leucine (SD−Leu) for pAD vectors and SD medium lacking tryptophan (SD−Trp) for pBD vectors. The vectors were expressed in E. coli and, after preparation, used for yeast two-hybrid approaches.

For RNA interference (RNAi) vectors, sense and antisense fragments of cpldock180 were amplified using Dock-sense-Ncofor, Dock-sense-Salrev, Dock-anti-Notfor, and Dock-anti-Salrev. All fragments were cloned into pGEM-T Easy and sequenced. The sense sequences were then excised with Ncol and Sall, and antisense fragments with Sall and NotI, and then ligated into Ncol-NotI-digested pNDB-OGG (59).

Microscopic methods. Localization of fusion proteins was observed by growing strains for 3 days on slides covered by a layer of Gamborg’s B5 medium (Duchefa Biochemie BV, Netherlands). All observations were done with a Zeiss AxiosImager M1 microscope. GFP fusions were detected by use of filter set 38 (excitation filter, BP 470/40; beam splitter, FT 495; and emission filter, BP 525/50).

Yeast two-hybrid assay. Yeast two-hybrid assays were done as described before (60). Yeast strain p69-4A (55) was cotransformed with the described plasmid combinations by the lithium-acetate method (61) and selected on SD medium lacking leucine and tryptophan (SD−Leu−Trp) for 5 days. Cotransformed strains were inoculated into liquid SD−Leu−Trp medium and grown overnight. The optical density at 600 nm (OD600) was set at 1, and cells were starved in 1 M sorbitol for 5 h at 30°C. Serial dilutions were done, resulting in 1:10, 1:100, and 1:1,000 dilutions of the strains. Ten microliters of each dilution was spotted onto SD−Leu−Trp medium for growth control and SD−Leu−Trp−His medium supplemented with 80 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for interaction observation. Plates were incubated at 30°C until growth was visible.

Purification of recombinant proteins. For isolation of proteins, the E. coli strain Arctic Express(DE3) (Stratagene, Santa Clara, CA) was used. Don1parp, Cdc24pH11002, and Rho-GTPases were expressed as glutathione S-transferase (GST) fusion proteins in E. coli by using the pGEX vector system (GE Healthcare). Purification was done as described before (62). dYF cultures were grown to an OD600 of around 0.5 and then induced with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). The cultures were grown at 18°C for 2 days and then harvested by centrifugation. After the addition of lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl2, 5 mM dithiothreitol [DTT], and 1 mg/ml lysozyme supplemented with EDTA-free protease inhibitor [Roche]), cells were lysed by sonication for coimmunoprecipitation assays and by French press for GEF assays and then spun down in an ultracentrifuge at 35,000 rpm for 1 h at 4°C. The supernatant was incubated with glutathione (GSH)agarose beads (Macherey-Nagel, Düren, Germany), and the GSH-bound GST-tagged proteins were then used for coimmunoprecipitation experiments or Western blotting. Alternatively, after washing of the beads three times with lysis buffer, the proteins were eluted from the beads by adding elution buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 10 mM MgCl2, 5 mM DTT, 10 mM reduced glutathione, pH 8.0). The proteins were then dialyzed against 20 mM Tris, pH 7.5, 50 mM NaCl, 10 mM MgCl2, 5 mM DTT, and 1% glycerol, concentrated to a final concentration of 50 μM, and stored at −80°C before use for in vitro GEF specificity assays.

In vitro GEF specificity assay. Guanine nucleotide exchange activity was measured by fluorometric determination of 2′-O-(N-methylanthraniloyl)guanosine 5′-diphosphate (MANT-GDP) incorporation as described previously (63), using a Tecxan Sapphire spectrophotometer at 25°C. Samples containing 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM MgCl2, 1% glycerol, 500 mM MANT-GDP, and 1 μM purified GTPase were incubated and equilibrated at 25°C for 5 min. After equilibration, recombinant GEF was added to a final concentration of 1 μM, and the fluorescence intensity (λex = 360 nm and λem = 440 nm) was measured for 60 min.

Coimmunoprecipitation. For coimmunoprecipitation, heterologously expressed and bead-bound GTases were loaded with either GDP (10 mM) or GTPγS (1 mM) in a solution with 20 mM EDTA. After addition of 25 mM MgCl2 and washing, the samples were incubated with whole-cell extracts of C. purpurea strains containing hemagglutinin (HA)-tagged proteins of interest. As a control, purified GST alone was also incubated with cell extract. Furthermore, a wild-type cell extract of C. purpurea without HA-tagged proteins was used as another control. After washing, the coimmunoprecipitation sample was denatured at 95°C, and the abundance of HA-tagged proteins was determined by Western blotting.

Western blotting. Lyophilized and ground mycelium of C. purpurea was lysed in protein lysis buffer (20 mM Tris-HCl, pH 8.0, 0.05% Triton X-100, 150 mM NaCl, 10 μl/ml [each] phosphatase and protease inhibitors). After shaking of the samples at 4°C for 15 min and subsequent centrifugation, the supernatant was transferred to a new tube, and the protein content was determined as described previously (64). SDS gel electrophoresis and Western blotting were performed as described before (15, 65). For coimmunoprecipitation assays, a magnetically activated cell sorting (MACS) anti-HA–horseradish peroxidase (HRP) antibody (Miltenyi, Bergisch Gladbach, Germany) was used at a dilution of 1:5,000. For detection of Sak1, a rabbit anti-Hog1 (Santa Cruz, Dallas, TX) or rabbit anti-phospho-p38 mitogen-activated protein kinase (anti-phospho-p38 MAPK) (Thr180/Tyr182) (Cell Signaling Technologies, Danvers, MA) primary antibody was used at a dilution of 1:1,000, and a donkey anti-rabbit–HRP secondary antibody (Santa Cruz, Dallas, TX) was used at a dilution of 1:10,000. Detection was done using enhanced chemiluminescence (ECL) detection (Bio-Rad, Munich, Germany).

Quantitative real-time PCR (qRT-PCR). RNA of lyophilized mycelium of C. purpurea was isolated using a total RNA isolation system (Promega, Madison, WI). For reverse transcription of the RNA template, Superscript II reverse transcriptase (Invitrogen, Darmstadt, Germany) was used.

Quantitative real-time PCRs were carried out with Bio-Rad iQ SYBR green supermix and an iCycler thermal cycler (Bio-Rad, CA). Programming, data collection, and analyses were performed with iCycler iQ real-time detection system software, version 3.0 (Bio-Rad, CA). Transcription of cpldock180 was detected by using the primers Dock-RT-for and Dock-RT-rev. Abundance levels of mRNAs were normalized by use of the housekeeping genes main (accession no. CCE34429.1), γ-actin (accession no. AEI72275.1), and glyceraldehyde-3-phosphate dehydrogenase (GPD) (accession no. X73282.1) (66), using the following primers: Tubuni and Tubrev, Actinuni and Actinrev, and Gpduni and Gpdrv, respectively. After performance of qRT-PCR, products were monitored by melting curve analyses and gel electrophoresis.

RESULTS

Identification and protein analyses of CpCdc24, CpDock180, CpBem1, CpSte20, and CpRas1. The Cdc24 homologue in C. purpurea was identified by similarities to the Cdc24 sequences of Magnaporthe grisea, Neurospora crassa, and Epichloe festucae. The ORF contains 3,326 bp in the genomic sequence (95) and is interrupted by two introns. The resulting protein consists of 1,020 amino acids and contains the following InterPro domains: a DH-PH tandem domain characteristic for DH-GEFs, an N-terminal calponin homology (CH) domain, and a C-terminal PB1 domain (Fig. 1). At the amino acid sequence level, the protein shares 78% identity with Cdc24 of F. festucae, 66% identity with Cdc24 of Beauveriana bassiana, and 55% identity with Cdc24 of Sordaria macrospora (see Fig. S1 in the supplemental material).

The dock180 gene of C. purpurea contains 5,640 bp and does not possess any introns. The derived CpDock180 protein contains
an N-terminal SH3 domain and two DHR domains: DHR1 and DHR2 (Fig. 1). BLAST analyses revealed 72% sequence identity with the Dock180 homologue of *Neurospora crassa*, 77% sequence identity with the Dock180 homologue from *Aspergillus niger*, and 80% sequence identity with Dock180 of *Fusarium oxysporum* (see Fig. S1 in the supplemental material).

CpBem1 was also identified by unrolling an ORF of 2,128 bp that is interrupted by two introns. The protein consists of 563 amino acids and contains two SH3 domains, a PX domain, and a C-terminal PB1 domain. It shows 76% sequence identity with BemA of *E. festucae* and 58% identity with *Fusarium oxysporum* Scd2 (47).

The p21-activated kinase (PAK) CpSte20 was identified by a direct genome search of *C. purpurea*. The ORF (CPUR_08010.1) consists of 2,950 bp and is interrupted by four introns, giving rise to an 874-amino-acid protein containing a Cdc42-Rac interaction binding (CRIB) domain, a kinase domain, and an IQ domain (Fig. 1). CpSte20 shows 74% identity with PakB of *E. festucae*, 65% identity with Mst20 of *F. oxysporum*, and 56% identity with Ste20 of *Magnaporthe oryzae* (see Fig. S1 in the supplemental material).

The Ras-GTPase CpRas1 was identified in *C. purpurea* by a direct genome search of *C. purpurea*. The ORF (CPUR_01564.1) has a size of 1,010 bp and is interrupted by four introns. The CpRas1 protein possesses 217 amino acids. It displays 70% identity with Ras1 of *Sordaria macrospora*, 81% identity with RasA of *Aspergillus fumigatus*, and 89% identity with Ras1 of *Trichoderma virens*.

CpCdc24 shows in *vitro* nucleotide exchange activity on both Rac and Cdc42 homologues. Rho-GEFs display different levels of specificity toward their GTPases, with some being very specific and others activating more than one GTPase. To find out more about the specificity of CpCdc24 and CpDock180, a fluorescence-based GEF in *vitro* activity assay was performed (63). CpRac and CpCdc42, as well as fragments of CpCdc24 and CpDock180, were heterologously expressed and purified from *E. coli*, and the nucleotide exchange activity of the GEFs was measured photometrically. Compared to the control reactions, the catalytic DH domain of CpCdc24 alone did not increase the fluorescence in samples with CpRac, CpCdc42, or either of the *U. maydis* GTPases (data not shown). When the CpCdc24 DH-PH tandem domain was tested, guanine nucleotide exchange activity toward CpRac was observed, but there was also weak but significant activity toward CpCdc42. The same activation pattern was observed if Um-Rac1 and UmCdc42 were used as substrates (Fig. 2; see Fig. S2A in the supplemental material). For the DOCK or DHR2 domain of CpDock180, no activity with CpRac or UmRac1 was observed. Also, CpCdc42 and UmCdc42 were not activated by the *C. purpurea* construct (data not shown). As a specificity control, the Cdc42-specific GEF Don1 of *U. maydis* was included in the assay mixture. Its specific activation of Cdc42 but not Rac was conserved with the GTPases from *C. purpurea* (see Fig. S2B).

CpCdc24, CpSte20, and CpCdc42 localize in crescent-like shapes at hyphal apexes, while CpRac and CpDock180 are cytosolic and partly membrane associated. To determine the subcellular localizations of CpCdc24, CpCdc24, CpDock180, CpSte20, CpRac, and CpBem1, GFP fusion proteins were expressed either under the control of their endogenous promoters (*cpdc24*, *cpdc42*, and *cpbem1*) or under the control of the strong constitutive oliC promoter (*cpdc24*, *cpbem1*, and *cpste20*). Since no knockout strains of *C. purpurea* were available and the Δcprac strain is morphologically too impaired for successful transformation, all fusion proteins were expressed in *Claviceps purpurea* wild-type strain 20.1. Thus, the functionality of the fusion proteins was not proven.

CpRac-GFP was present in the cytosol, sometimes concentrated in small vesicles. CpCdc42, in turn, showed a concentrated localization at the hyphal apex. Likewise, CpBem1 localized cytosolically but was concentrated in a crescent-like shape at hyphal tips. This localization was shared with CpSte20 and CpCdc42, which localized in an almost identical pattern, displaying an overall cytosolic localization with clear accumulation at the tip. CpDock180 showed a different localization along the plasma membrane and in small vesicle-like patterns along the hypha, resembling the CpRac-GFP localization (Fig. 3A). BiFC studies
showed that CpBem1 and CpCla4 interact at the hyphal tip (Fig. 3B).

CpCdc24 and CpDock180 show interactions with the scaffold protein CpBem1, the GTPase CpRas1, and the GTPase effector CpNoxR but not with the putative targets CpRac and CpCdc42. To investigate the connections between the GEFs and GTPases, physical interactions of CpCdc24 and CpDock180 with CpRac or CpCdc42 were tested in yeast. For this purpose, both GEFs were fused to either the DNA-binding domain or the activation domain of the Gal4 transcription factor and tested with fusion proteins of CpRac and CpCdc42. The GTP-locked (G14V) and GDP-locked (T22N) forms of both GTPases were included in the assay, as the loading state has been shown to be important for interaction patterns of GTPases of C. purpurea with the p21-activated kinase CpCla4 and the NADPH oxidase regulator CpNoxR (15, 47). All cotransformed yeast strains were able to grow on control SD medium lacking leucine and tryptophan, whereas no growth could be detected on SD medium lacking histidine, selecting for interaction. GTPase forms lacking the C-terminal CAAX motif responsible for membrane binding did not interact with any GEF either. To rule out possible autoinhibitory effects of the N-terminal domain of GEFs, an N-terminally truncated version of CpCdc24 lacking the CH domain was created and tested with all GTPase derivatives. Furthermore, the CpCdc24 construct used for the GEF assay, containing only the DH-PH domain, was used for another interaction assay with all GTPase forms (Fig. 4B). In none of these approaches was any growth on interaction-selective medium detected (data not shown). In contrast to the lack of interactions between the GTPases and the GEFs, strains containing CpCdc24 and the polarity scaffold protein CpBem1 showed growth on SD medium lacking histidine. This interaction was also present when the ΔCH construct was used but was completely abolished when only the DH-PH fragment lacking the PB1 domain was cotransformed (Fig. 4A). Likewise, CpDock180 was also able to interact with CpBem1. To further characterize the role of CpBem1 as a GTPase complex scaffold, interactions of the GTPases CpRac and CpCdc42 with CpBem1 were tested. CpRac showed an interaction with the scaffold protein CpBem1, which was significantly enhanced when the dominant active form CpRacG14V was used. No interaction of CpBem1 was observed with the small GTPase CpCdc42, independent of its loading status (Fig. 4A).

The small GTPase Ras1 acts as an upstream activator of Cdc24 in S. pombe and C. neoformans (18, 67). In order to check a possible connection between the C. purpurea Ras1 homolog and CpCdc24 as well as CDPDock180, the GEFs were tested for physical interactions with CpRas1. Since no direct interaction between CpCdc24 or CDPDock180 and the target GTPases CpRac and CpCdc42 could be shown, this approach was also used to evaluate the general ability of the yeast two-hybrid system to detect GEF-GTPase interactions. CpRas1 and CpCdc24 showed a clear interaction that was also present with the ΔCH fragment but was not observed with the DH-PH construct (Fig. 4A). Coimmunoprecipitation assays revealed a preferred binding of CpCdc24 to GTP-loaded CpRas1, corroborating its potential function as a GEF activator (Fig. 4C). A weaker interaction of CpRas1 with CDPDock180 could also be observed in yeast two-hybrid assays. Apart from that, CpRas1 did not interact with any GTPase, PAK, scaffold, or effector connected to the C. purpurea GTPase complex.

The CpRac complex is linked to the NADPH oxidase pathway by the regulatory protein CpNoxR and its interaction with CpRac...
and CpBem1 (46). To check for additional links between the Nox complex and the small GTPase complexes, yeast two-hybrid assays of CpNoxR with the GEFs CpCdc24 and CpDock180 were performed. Strains containing CpCdc24 and CpNoxR showed significant growth on selective medium. This interaction was also present when an N-terminally truncated version of Cdc24 lacking the CH domain was used. Equivalently to CpCla4, CpSte20 interacted with CpCdc24 and GTP-locked CpCdc42G14V but hardly interacted with GDP-locked CpCdc42T22N. No interaction could be observed with CpRac or CpRacT22N (Fig. 4A). This nucleotide-dependent interaction of CpCdc42 and CpSte20 was confirmed by communoprecipitation (Fig. 4C) and represents the equivalent of the interaction pattern determined for CpCla4 and CpRac. To include the PAKs in the GTPase complex model, interactions of CpCla4 and CpSte20 with CpBem1 were tested. Yeast two-hybrid analyses demonstrated an interaction between CpCla4 and CpSte20 with CpBem1. In addition, CpCla4 and CpSte20 were able to form homo- and heterodimers (Fig. 4A). This was only possible when at least one of the interaction partners was present in the shortened version (see Fig. S3 in the supplemental material). After identifying CpSte20 and CpCla4 as downstream targets of CpCdc42 and CpRac, respectively, their interaction patterns with the GEFs CpCdc24 and CpDock180 were tested as well. CpSte20 was able to interact with CpCdc24 only, whereas CpCla4 interacted with CpDock180 and, weakly, with CpCdc24 (Fig. 4A).

CpCdc24 and CpDock180 fulfill crucial cellular functions. To characterize the functions of CpCdc24 and CpDock180 of C. purpurea, knockout mutants of the two GEFs were created and confirmed by Southern blotting (see Fig. S4 in the supplemental material). However, wild-type signals were still present and could not be eliminated after several rounds of single-spore isolation. The heterokaryon of the Δcpcdc24 strain already showed a strongly altered morphology, displaying compact and corraline-like growth and extremely reduced sporulation; the few spores we could detect were swollen and malformed. In contrast, the heterokaryon of the Δcpdock180 strain showed wild-type-like growth but produced hardly any spores, and these were also unable to germinate (Fig. 5).

A knockdown approach for cpdock180 resulted in a mutant with a significantly reduced transcript level of cpdock180 (Fig. 6A). The mutant displayed the same sporulation and germination defect as the heterokaryon and was more sensitive to osmotic stress and menadione-induced oxidative stress (Fig. 6B and D). To follow up the mechanisms behind this stress sensitivity, the abundance and phosphorylation status of stress-activated kinase 1 (Sak1; CPUR_05040.1) were tested using an anti-Hog1 antibody to detect the abundance of the CpSak1 protein and an anti-p38 antibody to detect the phosphorylation state by Western blotting. Both antibodies gave rise to a defined single band, indicating specific detection. Whereas the general translation of the protein was identical in the wild type and the CpDock180-RNAi strain, the phosphorylation of CpSak1 was decreased in CpDock180-RNAi (Fig. 6C; see Fig. S5 in the supplemental material).
GTPase complex composition and dynamics contribute essentially to cellular processes. Thereby, different levels of regulation apply for the transduction of signals by GTPase cascades. Regulators of GTPases contribute to the specificity of the signal. The number of different Rho-GEFs exceeds that of Rho-GTPases by far, indicating that, in general, GTPases can be activated by more than one GEF (21). Therefore, choosing a certain GEF for activation of a particular GTPase already can determine the subsequent signaling pathway. The GEFs investigated in this study illustrate this assumption: for CpCdc24, we observed activation of both CpRac and CpCdc42 in vitro, indicating a dual specificity of this protein. In addition, the activation patterns of CpCdc24 were identical with UmRac1 and UmCdc42 of U. maydis as substrates, suggesting a conserved role of this GEF. A similar dual-nucleotide exchange activity has also been observed for Cdc24 homologues of N. crassa and U. maydis (12; B. A. M. Tillmann and M. Böker, unpublished data). In contrast, Dock180 homologues have been described as Rac-GEFs in mammals, Drosophila melanogaster, and Caenorhabditis elegans (28, 33, 34, 67). In this context, the missing

**FIG 4** Interaction studies of C. purpurea GTPase complex components. (A) Yeast two-hybrid assays. Growth controls on SD−Leu−Trp medium (SD−L−W) are shown in the respective upper panels, and interaction tests on SD−Leu−Trp−His medium (SD−L−W−H) are shown at the bottom. Usually, growth at a dilution of 1:10 is depicted. In all assays shown, Ste20 was used and is labeled “Ste20” only. (B) Protein domain structure of Cdc24. The fragment used as the ΔCH construct is boxed in black, and the DH-PH fragment is marked with a red box. (C) Coimmunoprecipitation assays of GTPases. The Ponceau dye of the filter serves as a loading control of the GST fusion bait proteins. Signals with the anti-HA antibody indicate the abundance of the HA-tagged prey protein in the complex.

**DISCUSSION**

GTPase complex composition and dynamics contribute essentially to cellular processes. Thereby, different levels of regulation apply for the transduction of signals by GTPase cascades. Regulators of GTPases contribute to the specificity of the signal. The number of different Rho-GEFs exceeds that of Rho-GTPases by far, indicating that, in general, GTPases can be activated by more than one GEF (21). Therefore, choosing a certain GEF for activation of a particular GTPase already can determine the subsequent signaling pathway. The GEFs investigated in this study illustrate this assumption: for CpCdc24, we observed activation of both CpRac and CpCdc42 in vitro, indicating a dual specificity of this protein. In addition, the activation patterns of CpCdc24 were identical with UmRac1 and UmCdc42 of U. maydis as substrates, suggesting a conserved role of this GEF. A similar dual-nucleotide exchange activity has also been observed for Cdc24 homologues of N. crassa and U. maydis (12; B. A. M. Tillmann and M. Böker, unpublished data). In contrast, Dock180 homologues have been described as Rac-GEFs in mammals, Drosophila melanogaster, and Caenorhabditis elegans (28, 33, 34, 67). In this context, the missing
Small GTPase Signaling in Claviceps purpurea

The role of CpCla4 as an effector of active CpRac has been shown before (15) and has now been confirmed by coimmunoprecipitation. The association of both CpRac and CpCla4 with CpBem1 probably contributes to this effect. The interaction of CpCla4 and CpCdc24 is in parallel with the described CptSte20-CpCdc24 association and could represent a feedback effect equivalent to the negative-feedback system described for S. cerevisiae or U. maydis (36, 40, 41). The described formation of homo- and heterodimers of CptSte20 and CpCla4 is in accordance with PAK dimerizations reported for different organisms, including mammals, S. cerevisiae, and S. pombe, mostly resulting in autoinhibition due to the covering of the catalytic kinase domain by the CRIB domain (79–83). However, dimerization of a dimerization segment within the CRIB domain has also been observed in mammalian cells (84). Since dimerization of the CRIB domains alone was shown in this study, the latter mechanism is likely and possibly represents an autoinhibition mechanism as well.

Contrasting the putative dual function of CpCdc24, the findings for CptDock180 interactions and localization so far only suggest a connection of this protein to CpRac signaling. Presenting data on a dock180 homologue in filamentous fungi for the first time, we showed an interaction of CptDock180 with CptBem1, which indicates a role of CptBem1 as a scaffold mediating the activation of CpRac by its GEFs. Additionally, the known CpRac effector CptCla4, but not the CptCdc42 effector CptSte20, interacts with CptDock180, possibly representing a feedback effect equivalent to the above-described CptCla4-CpCdc24 system and further
underlining the connection between CpDock180 and CpRac. In this context, CpBem1 could be a mediator of GTPase-GEF-effector complex assembly at the hyphal tip, as illustrated by the interaction of CpBem1 and CpCla4 at the apical hyphal region by BiFC. This might be important to facilitate and specify signaling events, as shown for mammalian scaffold proteins (85–87). The cytosolic and vesicle-like localization of CpRac differs from the pattern observed for Rac of *N. crassa*, which is present at the hyphal tip.
FIG 7 GTPase complex model. The schematic model shows putative small GTPase complexes in C. purpurea based on interaction, localization, and activation data. Light arrows represent proved activation, and dark arrows illustrate interactions with CpNoxR. See the text for further details.

underneath the apical Cdc42 crescent (12). However, the vesicle-like distribution is shared by CpDock180, further strengthening the connection between these two proteins. In addition, the Cdc10 localization at the plasma membrane corresponds to the predicted association of active GTPases within the plasma membrane and could represent the recruitment and subsequent activation of GTPases at the cell periphery. A similar vesicle localization has been observed for CpNoxR and NoxR of B. cinerea (88; D. Buttermann and P. Tudzynski, unpublished data), further strengthening the close connection between Rac and Nox signaling in C. purpurea (47). Another hint for the cross talk between CpRac and Nox signaling is the interaction of the regulatory Nox protein CpNoxR with Cdc24 and Cdc10 (47). The interaction of NoxR and Cdc24 occurs similarly in E. fuscace (32) and is mediated by the type I PB1 domain in Cdc24 and the type I/II PB1 domain in CpNoxR. We recently showed that CpNoxR is able to interact with Rac, in a loading-status-dependent manner, and binds to Cbpem1 (47). All these characteristics indicate a role of CpNoxR as another type of downstream effector of CpRac. In accordance with this, Cdc24 could also perform a feedback loop on both Cdc24 and Cdc10 (Fig. 7). This connection could also be responsible for the enhanced sensitivity of a cdpk1018-silenced mutant against menadione-mediated oxidative stress. Deletion mutants of the catalytic Nox genes cpnox1 and cpnox2 have been shown to display elevated sensitivity to menadione as well (47). In addition, deletion of cprac or cplc4 is shown to negatively affect expression and activity of the reactive oxygen species (ROS)-scavenging genes cep1, cat1, and gsh1 and the MAP kinase Cpmk2, which in turn negatively regulates ROS-scavenging enzymes (15, 89). Direct activation of CpNoxR by Cdpk1018/CpRac signaling could thus be crucial for proper ROS-scavenging responses. The elevated sensitivity of a cdpk1018-silenced mutant against oxidative and osmotic stress suggests an involvement of this GEF in different stress response pathways. However, Nox signaling is not involved in osmotic stress pathways. The stress-activated kinase 1 (Sak1) of C. purpurea is a homologue of S. cerevisiae Hog1, which is closely connected to osmoterolance, in concert with Cdc42 (90, 91). Similarly to SakA of A. nidulans, it positively influences osmotic stress responses (92, 93; Buttermann and Tudzynski, unpublished data). The decreased phosphorylation of CpSak1 in the cdpk1018-silenced strain indicates that Cdpk1018 influences CpSak1 signaling and, hence, osmoterolance.

Ras-GTPases represent another level of GEF regulation and have been shown to activate Cdc24 homologues in S. cerevisiae, S. pombe, C. neoformans, and mammals (16–18, 94). In contrast, no data exist on a nucleotide exchange function of Cdc24 or Dock180 homologues on Ras homologues. Therefore, the interaction of Cpras1 with Cpdock180 and Cdpk1018 could represent an upstream activation mechanism.

In this study, we showed different mechanisms of Rho-GTPase regulation and downstream signaling by GEFs and effectors. Thereby, we assume a role of Dock180-Rac-Cla4 signaling mainly in stress responses, whereas the Cdc24-Cdc42-Ste20 and Cdc24-Rac-Cla4 pathways are preferably active at the hyphal tip and possibly trigger mainly polarity and general growth. The known model of GTPase signaling could be extended by the putative upstream activation of Cpdock180 and Cdpk1018 by Cpras1 and the close connection between Cprac and Nox signaling. This analysis of GTPase signaling and the first characterization of a Dock180 homologue in a filamentous fungus illustrate the specificity of GEF-GTPase-PAK signaling cascades in C. purpurea. The exact fine-tuning and regulation of these processes will be the subjects of further studies and will provide deeper insight into the highly regulated GTPase signaling cascades.

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April 2014 Volume 13 Number 4
ec.asm.org 479
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