Involvement of G Proteins in the Mycelial Photoresponses of Phycymycetes

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ABSTRACT

Many responses of the zygomycete fungus Phycymycetes blakesleeanus are mediated by blue light, e.g. the stimulation of β-carotene synthesis (photocanthenogenesis) and the formation of fruiting bodies (photomorphogenesis). Even though both responses have been described in detail genetically and biophysically, the underlying molecular events remain unknown. Applying a pharmacological approach in developing mycelia, we investigated the possible involvement of heterotrimeric G proteins in the blue-light transduction chains of both responses. G protein agonists (guanosine triphosphate analogues, cholera toxin, pertussis toxin) mimicked in darkness the effect of blue light for both responses, except for cholera toxin, which was ineffective in increasing the β-carotene content of dark-grown mycelia. Experiments combining the two toxins indicated that photocarotenogenesis could involve an inhibitory G protein (Gi type), whereas photomorphogenesis may depend on a transducin (Gt type)–like heterotrimer. The determination of the carB (phytoene dehydrogenase) and chs1 (chitin synthase 1) gene expression and transcripts are discussed.

INTRODUCTION

Among common mechanisms in intracellular signaling is the use of guanosine triphosphate (GTP)–binding regulatory proteins (GBRP). These proteins participate in the regulation of a plethora of functions and share at least the common mechanistic feature to cycle between two interconvertible conformations, i.e. an active (GTP binding) conformation and an inactive (guanosine di-

phosphate [GDP] binding) one. The irreversibility of the GTP hydrolysis makes this guanosine triphosphatase (GTPase) cycle one-way, thus rendering GBRP as excellent molecular switches for signal propagation via an on–off mode. Besides this basic activity, a variety of upstream or downstream protein interactions with GBRP may determine the final downstream net effect (1–3). Major members of the GBRP superfamily are the large heterotrimeric G proteins that play a basic signaling role between plasma membrane–located receptors and various effector proteins (4–7). In asexual systems such as plants and fungi, the signaling role of heterotrimeric G proteins has just begun to be elucidated. Processes as diverse as auxin or gibberellic acid responses, regulation of plant cell division, brassinosteroid synthesis and signaling, stomatal opening, pollen tube growth or responses to light have been shown directly or indirectly to implicate G proteins in plants (8,9). In both ascomycetes and basidiomycetes, G proteins constitute an integral element of their pheromone-signaling cascades; furthermore, studies on various developmental–morphogenic processes in certain plant and human fungal pathogens showed that their virulence capacity is directly dependent on G protein regulation (10–12).

Light signaling studies in plants were the first to include the heterotrimeric G proteins in phytochrome transduction in the analogy of the light transduction mechanism of animal vision (13–18). Yet, the blue-light physiology did not remain indifferent to the G protein concept either. Detailed biochemical analyses in plasma membrane fractions from apical buds of etiolated peas revealed a blue-light–dependent GTPase activity that met many of the identification criteria for heterotrimeric G proteins. Among others, the relevant protein was thought to be highly likely to participate in the low blue-light fluence morphogenic system that is in operation in etiolated pea seedlings (19,20). Similar analyses in the eyespot apparatus of a flagellate green alga have suggested a role for G proteins in the phototactic rhodopsin-based signaling by blue-green light (21). A signaling role for G proteins was also considered possible during the blue-light–induced differentiation of the basidiomycete Coprinus (22). The wealth of information on G protein–based signaling modules in the blue-light–sensitive Neurospora is astonishing because the various G protein mutants of this organism show pleiotropic defects not only during their vegetative development but also during their sexual cycle–maturation (23,24). For example, Neurospora strains with activating gna-1 (coding one of the three Gz proteins of this organism) mutations produced longer, more abundant aerial hyphae and accumulated greater colony dry weights or less mycelial carotenoids than wild types in constant light (24), thus directly suggesting GNA-1 as a positive regulator of several...
morphogenic processes in this fungus (analogous to the ones of our study, although the Neurospora experimentation on G proteins has not been so far strictly photobiology orientated). Recently, it has been reported that transgenic Arabidopsis lines conditionally overexpressing the wild-type or activated allele of the Arabidopsis Gz showed enhanced seedling photosensitivity (short hypocotyls) in blue-light conditions, even in a cry (cryptochrome) 1 mutant background, indicating that the Gz signaling pathway may not be relevant to the blue-light receptor CRY1 pathway (25).

Reports on the physical presence of heterotrimeric G proteins in the spores and the macro(sporangiophores) of the zygomycete Phycomyces (26,27) prompted us to test the possible signaling role of these proteins in two blue-light-regulated mycelial responses of this fungus, carotenogenesis and morphogenesis. Both responses have been described genetically and biophysically (28,29). Pharmacological and biochemical results have suggested various protein kinase–phosphatases as blue-light signaling components in both responses or a nitric oxide synthase in sporangiophore development (30,31). It was only recently that a pure molecular approach enabled the partial cloning of another blue-light–induced putative component, a heat shock protein, expressed at the onset of sporangiophore development (32).

In this contribution we present the results of monitoring the appropriate phenotypic variables for photocarotenogenesis and photomorphogenesis affected by various agents that act as agonists or antagonists of G proteins. Our study is also complemented by a molecular analysis of gene expression for a clearer conception of what really holds within Phycomyces mycelia regarding the object of this work.

MATERIALS AND METHODS

Organism and general handling. The standard wild-type strain NRRL 15555 (–) of Phycomyces blakesleeanus Burgeff was grown on minimal agar plates (8 cm diameter [33]). For convenience in handling, dialysis membranes (Visking C/110, Serva, Heidelberg, Germany) were cut into circles of the same diameter as that of the dishes and laid on the solidified content (25 mL) of the dishes. The membrane disks were autoclaved in water and before plate laying were rinsed first in sterile water and then in liquid minimal medium. The starting inoculum was always 10^7 heat-activated spores per flask. Culture flasks were double-wrapped with aluminum foil and agitated (200 rpm) at 22°C. All light fluence rates were measured by a photoradiometer (IL760/IL710/IL780; International Light, Newburyport, MA).

Treatments with nucleotide analogues. Stock solutions of the various nucleotide analogues (Sigma Chemical Co., St. Louis, MO) were made up in sterile polyoxymethylene sorbitan monooleate (Tween-80) solution (0.2 mL/L) (its pH adjusted to 7.0 with NaOH) and stored as aliquots at −30°C. The administered nucleotide concentrations (treatment solutions) were prepared before use by further dilution of any nucleotide stock aliquot of the appropriate volume in the same Tween-80 solution. Treatment solutions were administered 43 h after inoculation in a light-tight room. The light control exposed to a 2 min blue-light pulse (2 × 10^3 J/m^2) 48 h after inoculation (for details see Materials and Methods). β-Carotene extractions were applied in mycelia 72 h after the inoculation of dishes. The dry weight of macrophores and the microphore density were determined 96 h after the inoculation. Points and bars (means ± standard error) with common letters at their side define groups of statistical similarity among the corresponding treatments at α = 0.005 by Fisher’s multiple comparison procedure. The absolute mean values of dark controls that were set as 100% for each variable were β-carotene, 78.1 ± 3.2 μg/g of dry mycelial weight; macrophores dry weight, 124 ± 4.7 mg/plate; and microphores density, 44 ± 2 microphores/mm^2. The light (a 2 min blue-light pulse of 2 × 10^3 J/m^2) control averages for each variable were β-carotene, 133.3 ± 2.2 μg/g of dry mycelial weight; macrophores dry weight, 157 ± 6.1 mg/plate; and microphores density, 22 ± 1 microphores/mm^2.

Figure 1. Effects of Gpp(NH)p on morphogenesis and carotenogenesis of dark-grown Phycomyces surface cultures. Treatment solutions were administered 43 h after inoculation in a light-tight room. The light control exposed to a 2 min blue-light pulse (2 × 10^3 J/m^2) 48 h after inoculation (for details see Materials and Methods). β-Carotene extractions were applied in mycelia 72 h after the inoculation of dishes. The dry weight of macrophores and the microphore density were determined 96 h after the inoculation. Points and bars (means ± standard error) with common letters at their side define groups of statistical similarity among the corresponding treatments at α = 0.005 by Fisher’s multiple comparison procedure. The absolute mean values of dark controls that were set as 100% for each variable were β-carotene, 78.1 ± 3.2 μg/g of dry mycelial weight; macrophores dry weight, 124 ± 4.7 mg/plate; and microphores density, 44 ± 2 microphores/mm^2. The light (a 2 min blue-light pulse of 2 × 10^3 J/m^2) control averages for each variable were β-carotene, 133.3 ± 2.2 μg/g of dry mycelial weight; macrophores dry weight, 157 ± 6.1 mg/plate; and microphores density, 22 ± 1 microphores/mm^2.
RESULTS AND DISCUSSION

Dose effect of 5′-guanylylimidodiphosphate

One of the established criteria that constitutes the first evidence of a GBRP regulatory involvement in a transmembrane signaling response is to evoke the response in the absence of the primary stimulus. This can be achieved by activating the transduction chain(s) one step beyond the responsible signal receptor(s) by way of nonhydrolyzable GTP analogues (11). 5′-Guanylimidodiphosphate (Gpp[NH]p) is such a GTP analogue for which dose–response curves were obtained (Fig. 1). Both morphogenesis (quantitated by the dry weight of macrophores and the number of microphores per culture plate) and carotenogenesis (β-carotene content per gram of dry mycelial mass) of dark-grown cultures were sensitive to the increasing concentrations of the administered Gpp[NH]p. This Gpp[NH]p sensitivity of the responses under study was confirmed by the ANOVA procedures (data not given) and the statistical similarity grouping of all administered doses, as this is shown for each response in Fig. 1.

At 2 μM, the aforementioned GTP analogue seemed to have maximal effect. The relative maximal increase of β-carotene was ~62% more than the dark control value, whereas the relative change of both morphogenic variables (increase of macrophores dry weight and decrease of microphores density) reached ~65% of the corresponding dark values. The apparent maximal effect of the 2 μM Gpp[NH]p, however, statistically was inferred only for the carotenogenic response because this treatment differed significantly from the other treatment concentrations and could not thus be grouped with any of them. Such a grouping holds true in the morphogenic variables for which the Fisher’s multiple comparison procedures of the administered doses led to different grouping patterns of statistical similarity as to their effectiveness. Up to ~2 μM of Gpp[NH]p, there was a marked increasing trend in both macrophorogenesis and carotenogenesis. This positive induction of each response is consistent with what is anticipated by the criterion. Thus, if, indeed, some GBRP are involved, then the ascending parts of the curves could reflect the activation of increasingly more GBRP molecules from those that are available in the developmental phase at which the experiment is carried out.

At exogenous Gpp[NH]p doses >2 μM, the kinetics of carotenogenesis and macrophorogenesis were differentiated. The
The effect of other nucleotide analogues (γ- and β-analouges)

The results with Gpp[NH]p led us to test at the same exogenous concentration range the effect of guanosine 5′-O-(3-thiotriphosphate) (GTPγS), another poorly hydrolyzable GTP analogue. All three variables showed the same induction pattern (Fig. 2) that was also observed in the Gpp[NH]p treatments, but with a less yield in microphorogenesis (an ~24% maximal decline as to the dark control level compared with the corresponding ~66% decline affected by Gpp[NH]p). The experimental use of GTPγS as a Li⁺-specific “attenuated” blue-light (a 2 min blue-light pulse of 2 × 10⁻² J/m²) signaling. Treatment solutions were administered 43 h after inoculation in a light-tight room. A 2 min blue-light pulse (2 × 10⁻² J/m²) was given 48 h after inoculation (for details see Materials and Methods). Points and bars (means ± standard error) with common lettering define groups with nonsignificant differences at the α = 0.05 level among the corresponding treatment means. All variables (A, B and C) were unaffected by adenosine 5′-O-(2-thiodiphosphate) because the ANOVA procedures could not detect significant deviations of any adenosine 5′-O-(2-thiodiphosphate) average from the respective light control average (ANOVA P values: β-carotene, 0.957; macrophores, 0.871; and microphores, 0.8124). Absolute light control averages set as 100% for each variable were β-carotene, 133.3 ± 2.2 μg/g of dry mycelial weight; macrophores dry weight, 157 ± 6.1 mg/plate; and microphores density, 22 ± 1 microphores/mm².

Figure 3. GDPβS-specific “attenuated” blue-light (a 2 min blue-light pulse of 2 × 10⁻² J/m²) signaling. Treatment solutions were administered 43 h after inoculation in a light-tight room. A 2 min blue-light pulse (2 × 10⁻² J/m²) was given 48 h after inoculation (for details see Materials and Methods). Points and bars (means ± standard error) with common lettering define groups with nonsignificant differences at the α = 0.05 level among the corresponding treatment means. All variables (A, B and C) were unaffected by adenosine 5′-O-(2-thiodiphosphate) because the ANOVA procedures could not detect significant deviations of any adenosine 5′-O-(2-thiodiphosphate) average from the respective light control average (ANOVA P values: β-carotene, 0.957; macrophores, 0.871; and microphores, 0.8124). Absolute light control averages set as 100% for each variable were β-carotene, 133.3 ± 2.2 μg/g of dry mycelial weight; macrophores dry weight, 157 ± 6.1 mg/plate; and microphores density, 22 ± 1 microphores/mm².

The function of GBRP as a molecular switch is due to its GTPase activity, which alternates the protein molecule between the intracellularly active GTP-binding conformation and the inactive GDP-binding one. Based on this biochemical property of GBRP and because the results with the GTP analogues suggested the possible photoactivation of some GTPase, guanosine 5′-O-(2-thiodiphosphate) (GDPβS) (a GTP analogue) would be expected to interfere with this photoactivation by inhibiting as an antagonist the putative GTP binding induced by light. In our system, this would mean that a GDPβS inhibitory action in the presence of light could be manifested by a reduced morphogenic response and less β-carotene accumulation. The morphogenic effect of a blue-light pulse on dark-grown cultures pretreated with GDPβS appeared attenuated (Fig. 3). In cultures pretreated with 1.4 μM GDPβS, macrophorogenesis (an ~23% decline as to the light control level, Fig. 3B) and microphorogenesis (an ~46% increase, Fig. 3C) did not differ significantly from the corresponding dark control averages. A trend of attenuated signaling via GDPβS was also

doses of Gpp[NH]p did not appear positively effective at least for the β-carotene accumulation. Under these conditions, we could assume that part of the correspondingly higher, intracellularly active Gpp[NH]p quantity did not target only the putative GBRP. The action sites of other subcellular systems (translation elongation factors, tubulin, etc.), whose physiological molecular function depends mechanistically on GTPase activities, could equally well be targeted nonselectively by these higher intracellular concentrations of Gpp[NH]p and may cause distortions and apparent ineffectiveness. The Gpp[NH]p dose dependence of microphorogenesis in the reverse side compared with that of macrophorogenesis completes the aforementioned picture of the other responses. At low Gpp[NH]p doses, a typical decrease of microphones density and a corresponding increase of macrophones in the dark were observed, and this is a characteristic behavioral pattern after light action. Thus, the putative GBRP are suggested to constitute a common element normally activated by light within phototransduction chains regulating the developmental program of sporulation. The nonspecificity of higher Gpp[NH]p doses is also reflected in the relative decrease of macrosporangia amount and a corresponding increase of microsporangia numbers since microphorogenesis is, in general, favored under harsh growth conditions (34).
observed in carotenogenesis, but with a lower yield (an ∼21% maximal decrease of β-carotene as to the light control average, Fig. 3A).

In similar experiments where adenosine 5′-O-(2-thiodiphosphate) (an adenosine diphosphate [ADP] analogue) was administered, no significant effect of this analogue was observed on all three responses within the concentration range for which GDPβS appeared effective (Fig. 3). Thus, the nucleotide preference of the involved regulatory activity was again supported by the β-analogue experiments, in addition to the γ-analogue ones, which revealed this preference via the part of the biochemical activity cycle positively related with the final biological effect.

**Effect of CTx and PTx on morphogenesis and carotenogenesis**

To gain the first sign of the nature of the GBRP revealed via the nucleotide analogues, we used two bacterial exotoxins, CTx and PTx. A certain protein subunit of these toxins exhibits an ADP ribosyl-transferase activity once found intracellularly (44,45). The mono-ADP-ribosylation directed by the toxin subunit at specific amino acid residues of certain heterotrimeric G proteins affects their signaling role with a distinct functional outcome. Thus, the modification of the “stimulatory” Gs subunits specifically via the CTx brings about their constitutive activation because their GTPase activity is inhibited (46). PTx, by its turn, modifies the “inhibitory” Gi subunits, offering a natural signaling barrier by causing an attenuated receptor activation of the inhibitory G protein (Gi) (47). Because the culture system of the nucleotide treatments would be inefficient to allow the biological action of the exogenous toxins upon the developing mycelia, the initial growth conditions started with a minimal liquid culture and a toxin challenge for 10 h. To check firsthand the possible competence of toxins in vivo, all cultures were kept in the dark (Fig. 4).

Both toxins appeared morphogenically effective, although at a different dosage range. The CTx-effected macrophore yield came near to the one effected by white light at more than 500 ng/mL. PTx was of the same macrophorogenic potency at about 100 ng/mL (increase of macrophores dry weight by ∼20% above dark control levels for both toxins, Fig. 4B). Similarly, in microphorogenesis, PTx reproduced the photonic action under dark conditions at 100 ng/mL (decrease of microphores density by ∼45%), whereas CTx reproduced at 1500 ng/mL (decrease by 42%, Fig. 4C). β-Carotene accumulation appeared insensitive to CTx, whereas the effect of PTx was statistically similar to the white-light treatment within 40–100 ng/mL (Fig. 4A). At first view, this notable differential sensitivity of the responses under study to the two toxins reveals a potentially distinct regulatory mechanism within the photosensory circuit sustaining morphogenesis and carotenogenesis. Based on the widely accepted notion that stimulatory G protein (Gs) may be discriminated from Gi considering their specific sensitivity to CTx and PTx, respectively, one could propose the following: carotenogenesis may activate Gt-type molecules during its photosignaling, whereas the final morphogenic pattern needs not only the putative Gi-like elements but also the Gs ones (48,49). It is also noteworthy from this analysis that PTx exhibits on balance a positive role in the absence of the primary stimulus (photon energy). This positive role of PTx is opposed to the main functional effect of this toxin, which is uncoupling of the responsible receptor from its downstream-regulated effector, thus leading to a final blocking of the effectordependent response.

We further investigated the positive role of PTx as well as the nature of the in vivo differential sensitivity of the two responses to the toxin challenge (Table 1). To this end, we used toxin doses with an expected half-maximal activity in vivo based on their dose–response analysis. Macrophorogenesis appeared statistically similar to the light control treatment when cultures were either toxin challenged in the dark for 10 h on end or given an extra blue-light pulse during this 10 h challenge (Treatments v, vi, ix and x vs treatment ii [middle column in Table 1]). This could mean that toxins may habituate the system so that its photoresponsiveness appears nonexistent. In experiments vii, viii, xi and xii instead of the blue-light pulse, the cultures were provided with the nonhydrolyzable GTP analogues, which by themselves were able to mimic the photonic effect (macrophorogenic averages in treatments iii and iv vs macrophorogenesis of treatment ii [middle
column of Table 1). Furthermore, the macrophorogenic average that resulted from the co-administration of the two toxins did not statistically differ from the light control and the rest treatment yields when toxins were given separately (treatment xiii vs treatments vi and x [Table 1]). If we considered that one targeted molecule shared as a substrate both CTx and PTx, then the aforementioned statistical similarity could be explained by a modifying activity of one toxin on the substrate molecule with the other toxin activity being excluded. This view relies on the trait proper of transducin (Gt) to become modified functionally by both PTx and CTx, thus leading to a positive outcome for the morphogenic responses.

### Heterotrimeric G proteins and gene expression

The culture system used in the toxin experiments showed that either the 10 h toxin challenge or the 5 min blue-light pulse potentially causes growth in liquid medium mycelium in a state that is capable of yielding a differential phenotypic pattern on a surface solid medium culture, even long after the removal of the exogenous stimulus. Because during this 10 h span (38–48 h of the liquid culture) actively growing mycelia constituted the only natural receivers of the exogenous factors (toxins and light energy), we decided to monitor indicatively gene expression variations effected by these factors along this developmental phase. Clones from the carB and chs1 genes (coding for phytene dehydrogenase and chitin synthase [CHS], respectively) enabled the detection and the relative quantitation of the corresponding mRNA, as these are presented in Fig. 5, under several conditions of exogenous challenge.

Ten minutes after the 5 min blue-light pulse, the abundance of the carB transcript (representative for the photocarotenogenesis) was measured ~70% above dark control levels (Fig. 5B). According to the detailed blue-light–effected time course analysis of the carB mRNA given by Ruiz-Hidalgo et al. (35) or Blasco et al. (51), this positive average percentage effected by blue light corresponds to a decrease of the carB transcript abundance after high values of strong transient transcript accumulation. In our analysis, these high, transient percentages of the carB transcript photoinduction are absent in the time interval 0–15 min. The reason we chose the first 15 min as the first time point in our time course analysis was that, in yeasts, CTx could induce a greater course analysis was that, in yeasts, CTx could induce a greater
min was described by Ruiz-Hidalgo et al. (35) too, but under continuous light conditions. The variance of the time spans (75 vs 25 min) between these measurements could be ascribed to both the different culture conditions and the kind of photonic mycelial stimulation used. The observed qualitative (it is not easy to compare really quantitatively the effects of the chemicals with those of the blue light) similarity between the blue-light pulse action and the PTx treatment until the first 90 min of mycelial sampling confirms the specific positive correlation of carotenogenesis with Gi as a PTx target that was alluded to above (Table 1, Fig. 4). Thus, the PTx target very likely forms an integral mediating component of the system regulating the prompt photoresponses. Until the first hour of sampling, the continuous presence of CTx did not change the carB mRNA steady-state levels. Therefore, the CTx-induced increase of the carB transcript (∼82%) at 90 min may be correlated with a CTx action on regulatory targets that may not belong to the prompt photocontrol circuits of carotenogenesis. The combined CTx + PTx challenge was particularly potent to increase the carB mRNA levels cumulatively equivalent to the average effect of each toxin separately at the 1.5 h time point (Fig. 5B). It is likely for the modifying action of PTx to have been exerted on target molecules distinct from those targeted by CTx. During the same time interval, by illuminating the mycelia continuously, the carB transcript levels were kept almost steady in low abundance (decline below dark control). This low representation of the carB transcript, due to the continuous illumination, contrasts with the positive contribution to the transcript abundance caused by the continuous action of the

Figure 5. The relative mRNA abundance of the carB (B) and chs1 (C) genes under various treatments: BL, minimal liquid culture that was illuminated with a 5 min blue-light pulse of $2 \times 10^{-2} \text{J/m}^2$ at the 38th hour of the culture; CTx, minimal liquid culture treated with CTx (200 ng/mL) for 10 h in the dark; PTx, minimal liquid culture challenged by PTx (50 ng/mL) for 10 h in the dark; CTx + PTx, minimal liquid culture challenged by both toxins (at the concentrations given above) for 10 h in the dark; CL, minimal liquid culture illuminated continuously with white light of spectral fluence rate (370–520 nm) 0.1 J/m² s. Representative autoradiograms and the corresponding ribosomal images of the same filters are given in (A), where D refers to a dark control culture. The densitometric values of all autoradiographic intensities were normalized to the corresponding ones of both ribosomal bands (here only the 18S bands are shown) developed by reversible methylene blue staining of blot filters before their isotopic hybridizations. In (B) and (C) the plotted normalized averages (± standard error) from three independent RNA isolations and hybridizations are expressed as to the corresponding dark control mean levels (set as 1) per time point. The numbers in the x-axis correspond to the mycelia sampling time points selected after the 38th hour (time point 0) of the culture when the exogenous challenge was applied.
CTx + PTx combination at the same time point. This could mean that physiologically the developing mycelia, under constant photon stimulation, can inactivate via an adaptation–habituation mechanism the circuit(s) that depend on the signaling action of heterotrimeric G proteins. The photoadaptation property was reported not only for the carB mRNA levels in Phycomyces but also for the carB gene in Mucor or the homologous al-1 gene of Neurospora (35,51,53). Apart from this, Phycomyces was one of the first model systems for the approach of aneural sensory adaptation via the study of its specific photosensory (54). At the third hour within the 10 h sampling period, the carB transcript abundance appeared decreased compared with the dark control one, for each treatment type (Fig. 5B). Either a transcriptional activation or a variation in the transcript stability could be responsible for this rise–decline of the carB transcript levels between a high and low limit value of abundance, irrespective of the inducing agent, as was discussed before (35). In the next 4 h of white-light stimulation, the mycelial adaptive capacity was potentially completed because the carB messenger abundance coincided with the dark control one. During this time interval, the same behavior was exhibited in the dark by the CTx treatment, a fact that uncovers the active participation of the CTx-targeted G proteins in the phase of completing the adaptation process. The dynamic action of the CTx + PTx combination was also shown by the new increase of the carB transcript abundance at the 7 h time point. Yet, smaller positive variations of the average carB transcript levels were effected either by PTx at the 5 h time point or by the 5 min blue-light pulse at the 7 h time point (Fig. 5B). At the final sampling time point, a new decline of the carB mRNA representation to the dark control levels (by the CTx + PTx combinatorial treatment) or lower than that (by the rest treatments) was resumed.

As for the chsl gene (representative for photomorphogenesis), although to date there has been no report on the possible photoregulation of its expression, we observed a transient abrupt increase of its chsl mRNA steady-state levels above the dark control ones 10 min after the termination of the 5 min blue-light pulse (Fig. 5C). This increase was not noted in the rest toxin treatments, whereas the continuous illumination effected, on the contrary, a steep decrease (below dark control levels) of the chsl transcript representation at the same time point. It is noteworthy that an abrupt decline of the total CHS activity (basal plus zymogenic) was also effected in developing macrophores by continuous white illumination during the first 5 min, although afterward the measured CHS activity had positive values peaking at 20 min of continuous photon stimulation (55). In our case, an analogous increase was not noted for the mycelial chsl transcript levels but only their restoring to dark control values at the 30 min time point. Until the first hour in the 5 min blue-light pulse treatment, the chsl messenger levels reached a valley value about which they varied for the next 2 h, as opposed to the second positive peak of the carB transcript abundance at the 1.5 h time point (Fig. 5C). The toxin action, as an induction of low chsl transcript levels, began to be seen after the first half hour in contrast with the toxin-effected carB mRNA variation that manifested itself from the first 15 min; CTx was the most effective until the first hour of incubation. The first sudden decrease of the chsl transcript levels (time point, 15 min) by the continuous illumination treatment was restored to dark control levels 15 min later (time point, 30 min); whereas from the first hour onward there appeared a new trend of their slow decline that continued until the third hour (Fig. 5C). The low chsl mRNA abundances first induced by all toxin treatments between 30 min and 1 h time points were also restored up to positive mean percentages (20–83% above dark
control) at the 1.5 h of mycelial incubations. These positive representation values of the chsl transcript are in contrast with the corresponding low average values affected by the light treatments at the 1.5 h time point; this could imply that, in this developmental phase, the mycelial photobehavior for the chsl transcript would not need the activity of heterotrimeric G proteins. After 1.5 h (time point, 3 h), the chsl messenger abundance affected by each toxin treatment appeared decreased in comparison with that of dark control. It is characteristic that the CTx + PTx combination effect coincided with that brought about either by the continuous illumination or by the blue-light pulse treatments. Particularly, during the rest sampling time interval (time points, 3–10 h), the fluctuations of the average chsl mRNA levels caused by the CTx + PTx combination followed the corresponding pattern of changes affected by the continuous illumination. Namely, it took 3 h of constant incubations with both toxins in absolute dark for the developing mycelium to behave as it did by the continuous photon stimulation, a fact that corroborates the view of a photoadapation mechanism functionally relied upon an intracellular circuit of G protein targets modifiable by both toxins. The increased yield of the steady-state chsl transcript levels at the fifth hour of either toxin incubation convinces that either toxin action deregulated the chsl, since at the proper time point the CTx + PTx combination, on the contrary, mimicked the corresponding white-light–affected yield that was adapted to the dark control levels. Specifically, the deregulatory action of PTx remained in operation for the next 5 h, up to the end of the 10 h sampling period, whereas CTx, during the same developmental phase, induced the restoration of the previously (time point, 3 h) increased chsl transcript levels up to the dark control values (Fig. 5C). This distinctive behavior of the two toxins did not appear to be reflected in the long-range morphogenic final output, and we cannot hypothesize what this would mean intracellularly. In darkness, the toxin–affected posttranslational modification of target trimeric G proteins might influence differentially the CHS1 activity, and this, in turn, could signal in the nucleus the need for an increase or a decrease of the chsl messenger levels, considering the developmental requirements of the mycelial population, too. A functional connection of the regulatory nature between a G protein activity and a CHS activity is challenging and may not be excluded because both proteins in question are membrane localized. As for the coupling of CHS activity with chsl transcript level variations, studies in yeast showed that CHS are regulated at both the transcriptional and posttranslational stages, the former ensuring enzyme availability when needed and the latter a spatial regulation (56).

An overall impression of all the above is that the exogenous toxin challenges were able to impose on developing mycelia fluctuations of the relative steady-state abundances of the carB and chsl messengers. At certain time points, these abundances could coincide with or be counter to the corresponding yields affected either by the blue-light pulse or by the continuous illumination. Typical of these fluctuations was that between two time points of a minimal transcript representation a trend of its increase, usually above the corresponding dark control levels, was observed. Moreover, restoration of the steady-state expression to the levels of the dark growth mode could constitute part of the light adaptation mechanism of mycelia, and G proteins seem to have a role in this process, too.

According to the data presented here, the physiological activation of the responsible photoreceptor(s) for photocarotenogenesis and photomorphogenesis is very likely coupled with some GBRP activity. This activity could potentially be brought into action quite early in the photosensory circuit of morphogenesis and carotenogenesis because both responses appeared specifically sensitive to the administration of agonists–antagonists (Gpp(NH)p, GTPγS–GDPβS). Yet, an agonist effect on the marker responses of that study was also brought on by the bacterial toxins CTx and PTx under dark growth conditions. The specific PTx-stimulated accumulation of β-carotene in the absence of photontic stimulus urges us to support prima facie that a heterotrimeric Gα-type molecule is likely to intervene somewhere between the photoactivation step of the responsible light receptor(s) up to the final output product (β-carotene). The sensitivity, however, of morphogenesis to both toxins does not predispose for an analogous hypothesis as easily as with carotenogenesis. It was evident from the co-administration experiments of both toxins that either toxin somehow appeared to act by excluding the modifying interference of the other. This was reported to be true for the α subunit of Gα for which, under different conformations in vitro, successive ADP ribosylations by the two toxins were mutually inhibited (50). Because Gα behaves as a model substrate for both CTx and PTx, we would tenably argue that the Phycomyces morphogenic pattern of behavior depends on a photosensory pathway that may involve minimally one Gα-type molecule (57), a common substrate target of both toxins. All the above could well be integrated into a simple scheme that also embodies elements from our previously proposed model (Fig. 6).

The Northern analyses of this study, however, showed that the molecular events coupled with light signaling are not as simple intracellularly as they are insinuated to be by the simple measurements of the β-carotene levels or the morphogenic variables. The variations in the carB messenger abundance under the various challenging conditions of this study suggested that the blue-light signaling cascade behind the regulation of the steady-state carB mRNA levels could involve a PTx–sensitive Gβ-type molecule. This Gβ did not appear to participate when the photoadaptation of the carB mRNA was in operation. Conversely, the CTx-targeted G protein activity seemed to play a role during this photoadaptation process (note the light-mimicking effect of the CTx treatment from 3 to 10 h time points in Fig. 5B). Furthermore, the photoadaptation mechanism of the chsl messenger levels appeared to depend on G protein targets modifiable by both toxins (see in Fig. 5C the similar transcript variation by continuously illuminated culture (CL) and CTx + PTx from 3 to 10 h time points). The above observations on the chsl messenger variations plus the fact that the prolonged action of either toxin finally deregulated the chsl expression prove that the molecular photo-physiology of the chsl expression must be more complex than that of the carB. This study engages and urges to further explore in Phycomyces other possible photosignaling mediators acting as downstream effectors targets of G proteins, such as cyclic nucleotides, phospholipids or Ca2+. Applying the same experimental rationale in a number of Phycomyces mutants will allow whatever biochemical entities implicated in photosignaling to be mapped hierarchically as to the various gene products that are required in the photosensors of this fungus.

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