The protein Slr1143 is an active diguanylate cyclase in *Synechocystis* sp. PCC 6803 and interacts with the photoreceptor Cph2

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Abstract

Cyclic-di-GMP is an ubiquitous second messenger in bacteria. Several c-di-GMP receptor proteins have been identified to date, and downstream signalling pathways are often mediated through protein–protein interactions. The photoreceptor Cph2 from the cyanobacterium *Synechocystis* sp. PCC 6803 comprises three domains related to c-di-GMP metabolism: two GGDEF and one EAL domain. It has been shown that the C-terminal GGDEF domain acts as blue-light triggered c-di-GMP producer thereby inhibiting motility of the cells in blue light. The specific function of the other two c-di-GMP related domains remained unclear. In this study, we test knockout mutants of potential interaction partners of Cph2 for altered phototactic behaviour. Whereas wild-type cells are non-motile under high-intensity red light of 640 nm, the mutant Δslr1143 displays positive phototaxis. This phenotype can be complemented by overexpression of full-length Slr1143, which also results in an increased cellular c-di-GMP concentration. However, the non-motile phenotype of wild-type cells under high-intensity red light appears not to be due to an elevated cellular c-di-GMP content. Using co-precipitation and yeast two-hybrid assays, we demonstrate that the GGDEF domain of Slr1143 interacts with the EAL and the GGDEF domains of Cph2. However, under the test conditions, the interaction of the two proteins is not light-dependent. We conclude that Slr1143 is a new Cph2-interacting regulatory factor which modulates motility under red light and accordingly we propose Cip1 (Cph2-interacting protein 1) as a new designation for this gene product.

INTRODUCTION

The second messenger cyclic dimeric guanosine monophosphate (c-di-GMP) is ubiquitous in bacteria [1, 2]. It is synthesized from two molecules of GTP by diguanylate cyclases (DGCs), which possess the c-di-GMP synthesis GGDEF domain. c-di-GMP is catabolized by phosphodiesterases containing one of two known c-di-GMP hydrolysing domains, EAL and HD-GYP. Many cellular functions have been associated with c-di-GMP, including the production of exopolysaccharides [3], biofilm formation, virulence and cell differentiation [2, 4].

In the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (Synechocystis) c-di-GMP influences the decision between motility and sessility [5]. In blue light, wild-type cells are sessile while Δcph2 cells move towards the light source [6]. The product of the gene cph2 is a photosensory protein, Cph2, which consists of six domains with the architecture GAF-GAF-GGDEF-EAL-CBCR-GGDEF (GAF, cGMP phosphodiesterase/adenyllyl cyclase/FhlA; CBCR, cyanobacteriochrome; GGDEF, GAF, degenerate motif) (Fig. 1). The domains 1 (GAF) and 5 (CBCR) covalently bind phycocyanobilin (PCB) as a chromophore, allowing the absorbance of light in the visible spectrum [5, 7].

While the functions of Cph2 truncations have been reported, the behaviour of the full-length protein remains elusive. Biochemical studies using recombinant domains 1 and 2 (GAF1-GAF2) of Cph2 have revealed that these two domains are able to photoconvert between red and far-red-
light-absorbing states similar to that of classical phytochromes [7]. However, the domain organization of the red-/far-red-absorbing module of Cph2 with two GAF domains (GAF1–GAF2) differs from classical phytochromes, which consist of a PAS–GAF–PHY domain architecture (PAS, period/ARNT/single-minded; PHY, phytochrome). The second photosensory module of Cph2 comprises domains 5 and 6 (CBCR–GGDEF). Biochemical studies on this module revealed that domain 5 belongs to the group of CBCRs and can photoconvert between green- and blue-light-absorbing states [5]. Domain 6 (GGDEF) is a catalytically active DGC, the activity of which is controlled by the preceding CBCR domain 5. In blue light, the DGC is activated and shows in vitro a twofold higher c-di-GMP production rate in comparison to green light [5]. As mentioned earlier, wild-type cells are non-motile in blue-light illumination on phototaxis plates, whereas Δcph2 mutant cells move towards a blue-light source. This motile phenotype of Δcph2 can be abolished by expression of the C-terminal CBCR–GGDEF module of Cph2. Therefore, it has been postulated that a higher cellular c-di-GMP level in blue light, produced by Cph2, might inhibit the motility of *Synechocystis* cells [5]. In the same study, expression of the module Cph2(1–4) (containing the domains GAF–GAF–GGDEF*–EAL of Cph2) abolished the blue-light-dependent inhibition of motility, whereas a catalytically inactive mutant of the EAL domain (AAL) did not induce motility in blue light [5]. This indicates that the EAL domain of Cph2 may be catalytically active and possibly degrades c-di-GMP. However, it is as yet unknown how this EAL domain is regulated. For domain 3 (GGDEF*) of Cph2, the GGDEF motif is degenerate (HGDGF) and therefore is expected to be catalytically inactive.

It has been shown that GGDEF domains with degenerate motifs are present in c-di-GMP receptors [8]. Often the downstream signalling of c-di-GMP perception is dependent on interactions with other proteins [9, 10]. Therefore, we investigated potential interaction partners of Cph2. In a large-scale protein–protein interaction study of proteins from *Synechocystis* using yeast two-hybrid assays, Sato *et al.* [11] identified several interaction partners of Cph2. We analysed mutants of these potential interactors for effects on phototaxis. Our hypothesis was that disrupted interaction due to a missing interaction partner might alter the phototactic behaviour. Indeed, mutation of gene *slr1143* resulted in an aberrant phototactic behaviour in red light. Analysis of the c-di-GMP content in wild-type cells, Δslr1143 and Slr1143 overexpression mutant lines in different light qualities suggests that motility of *Synechocystis* cells can be affected by the cellular levels of c-di-GMP, but this appears to not be the exclusive reason for differences in motility. Furthermore, we have investigated the interaction of the Slr1143 protein with Cph2 both in *vitro* and in *vivo*, finding that the GGDEF domain of Slr1143 interacts with both the EAL domain and the GGDEF domains of Cph2.

**METHODS**

**Construction of plasmids**

*Escherichia coli* strain DH5α was used for cloning of plasmids and was cultured in LB medium, supplemented with antibiotics at standard concentrations. Cloning was performed using standard cloning techniques. All constructed plasmids were verified for correctness by sequencing. Primer sequences are listed in Table S1 (available in the online Supplementary Material). In brief, the coding sequence of gene *slr1143* was amplified from genomic DNA using primers P1 and P2 and ligated into the pJET1.2 vector (Fermentas). This plasmid served as template for all cloning steps of the gene *slr1143*. For complementation of the *slr1143* inactivation mutant, the *slr1143* gene was ligated into a modified pVZ321 vector carrying the petI promoter region [5]. For amplification of the *slr1143* gene the upstream primer P3 was used which includes an NdeI restriction site, while the downstream primer P4 contains an EcoRI restriction site followed by the sequence for a 3×FLAG tag and a subsequent BamHI restriction site. The pVZ321 vector and the PCR product were digested with NdeI and EcoRI and ligated using standard ligation techniques. For creation of plasmids for expression of *slr1143* in *E. coli*, primers were designed to enable ligation into the vector pET28a+ (Novagen), which was digested with Ncol and NotI. The upstream primer for the entire *slr1143* gene, and truncated versions, carried a PciI restriction site, and the downstream primer contained the sequence coding for a strep tag (for protein purification) followed by a PspOMI restriction site (primers P5–8). For yeast two-hybrid assays, the pCGADT7ah vector containing the GAL4 activation domain (AD) [12] and the D153 vector containing the GAL4 DNA-binding domain (BD) [13] were used. Full-length or truncated versions of *slr1143* were cloned into D153 with the coding sequence for BD downstream of *slr1143* leading to a C-terminal fusion protein, whereas full-length or truncated versions of *cph2* were cloned into pCGADT7ah also with AD as a C-terminal tag. Primers (P9–P20) for the amplification of the respective genes carried BamHI and NotI restriction sites for *slr1143*, and
BamHI and XhoI restriction sites for cph2. PCR–fragments were ligated into the respective vectors digested either with BamHI and NorI (D153) or with BamHI and XhoI (pCGADT7ah). Constructs carrying point mutations in the cph2 gene were amplified by PCR from appropriate templates and cloned as for the wild-type. Plasmids for disruption mutants of the potential Cph2 interaction partners were provided by Brita Fiedler (Humboldt University Berlin, Germany) and are described in Table S2. The plasmid for Cph2 expression in *E. coli* (using primers P21 and P22) was provided by Katrin Anders (Philippus University Marburg, Germany) and further modified by inserting the genes for PCB production *ho1* and *pcyA* from *Synechocystis* into the second multiple cloning site of the pCDF Duet-1 vector backbone (Novagen) using the restriction sites NdeI and XhoI (primers P23 and P24; for plasmid map see Fig. S1). Plasmids for the expression of Cph2 domains Cph2(3) and Cph2(4) were created using the pCDF Duet-1 vector backbone digested with Ncol/EcoRI and PCR-based inserts amplified with respective primer pairs (P51–P54, Table S1) and digested with BspHII/EcoRI. All cph2 constructs carry a C-terminal His6-tag.

**Bacterial strains and growth conditions**

The motile *Synechocystis* wild-type strain used in this study was originally obtained from S. Shestakov (Moscow State University, Russia), and was re-sequenced [14]. The kanamycin-resistant Δcph2 insertion mutant was described by Wilde et al. [6]. Transformation of wild-type cells to obtain knockout mutants was conducted as described earlier [15]. Complementation mutants were obtained by transferring the respective self-replicating plasmid to the knockout mutant using triparental conjugation [16]. Wild-type and mutant strains were propagated on BG11 agar plates and grown at 37°C until an OD$_{600}$ of 0.3. After decreasing the temperature to 18°C and further growth until an OD$_{600}$ of 0.5, expression was induced with 1 mM isopropyl β-D-thiogalactoside (IPTG). Following 22 h of induction, the cells were pelleted at 10 000 g for 20 min at 4°C. Cells were then resuspended in 15–20 ml PB buffer (50 mM K$_2$HPO$_4$, 300 mM NaCl, 10 mM imidazole, pH 8.0) per litre of cell culture and stored frozen at −80°C. After thawing, paraaminobenzamidine dihydrochloride (final concentration 4 mM) and 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (final concentration 1 mM) were added to the cell suspension and cells were lysed using a French press at 1000 p.s.i. Cell debris was pelleted at 21 000 g for 30 min at 4°C and the supernatant used directly for co-precipitation assays.

**Co-precipitation assays**

Slr1143 constructs were expressed in *E. coli* BL21 cells. Terrific Broth medium [21] supplemented with the respective antibiotics was inoculated 1:100 from an overnight culture and grown at 37°C until an OD$_{600}$ of 0.3. After decreasing the temperature to 18°C and further growth until an OD$_{600}$ of 0.5, expression was induced with 1 mM isopropyl β-D-thiogalactoside (IPTG). Following 22 h of induction, the cells were pelleted at 10 000 g for 20 min at 4°C. Cells were then resuspended in 15–20 ml PB buffer (50 mM K$_2$HPO$_4$, 300 mM NaCl, 10 mM imidazole, pH 8.0) per litre of cell culture and stored frozen at −80°C. After thawing, paraaminobenzamidine dihydrochloride (final concentration 4 mM) and 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (final concentration 1 mM) were added to the cell suspension and cells were lysed using a French press at 1000 p.s.i. Cell debris was pelleted at 21 000 g for 30 min at 4°C and the supernatant used directly for co-precipitation assays.

For expression of Cph2, a plasmid harbouring cph2 and *ho1/*pcyA genes (for plasmid map see Fig. S1) was co-transformed into *E. coli* BL21 with the plasmid pGro7 (TaKaRa) encoding the *E. coli* chaperone GroEL. Medium and culture conditions were as described for Slr1143 with the addition of 3 mg ml$^{-1}$ arabinose in the medium for induction of GroEL expression. Expression of cph2, *ho1* and *pcyA* was induced with 0.1 mM IPTG. Clarified cell lysate was added to a pre-equilibrated nickel affinity column [HisTrap FF crude, 5 ml (GE Healthcare); flow rate 1 ml min$^{-1}$] and
washed with three column volumes of PB buffer. Protein was eluted with 500 mM imidazole in PB buffer. The elution fraction was then used for co-precipitation assays. As Cph2 (3) (domain 3; GGDEF) and Cph2(4) (domain 4; EAL) of Cph2 do not bind PCB, these truncations were expressed in the absence of Ho1/PcyA, which are required for PCB synthesis.

For co-precipitation assays, clarified cell lysate of the respective Slr1143-expressing strain or PB buffer (as control) was added to 200 µl Strep-Tactin Sepharose (IBA), incubated for 15 min at 4°C with continuous agitation, and the supernatant was removed following centrifugation (1 min, 2000 g, 4°C). The sepharose pellet was then washed three times with 1 ml PB buffer before Cph2 protein solution or PB buffer (in case of control) was added. Samples were then incubated at room temperature (RT) for 1 h with continuous agitation and indicated illumination, using the LED light sources detailed above. Fluence rates are indicated for every experiment in Results. All the following steps were performed under these specific light conditions. After removal of unbound Cph2 (1 min, 2000 g, 4°C) the sepharose pellet was washed eight times with 1 ml PB buffer. Bound proteins were then eluted by incubation of the Strep-Tactin Sepharose in 200 µl elution buffer (50 mM K2HPO4, 300 mM NaCl, 5 mM b-biotin, pH 8.0) at RT for 10 min. Elution fractions were subjected to protein precipitation with sodium deoxycholate and trichloroacetic acid and then re-dissolved in ChuaA/ChuaB buffer (3:2, v/v) as previously described [22].

For analysis, proteins were separated by SDS-PAGE [23] in running buffer supplemented with zinc acetate (1 mM final concentration) [24]. After zinc-induced UV-fluorescence detection of the chromatophore, either gels were stained with Coomassie blue or proteins were transferred to nitrocellulose membranes followed by immuno-blotting with specific antibodies [Strep-Tactin HRP (IBA), Penta-His HRP Conjugate (Qiagen), Monoclonal Anti-FLAG M2-Peroxidase (HRP) Clone M2 (Sigma)]. Signals were visualized using the CheLuminate-HRP PicoDetect Extended detection kit (AppliChem).

### Yeast two-hybrid assay

Yeast AH109 (Clontech) cells were transformed according to the manufacturer’s guidelines using the Frozen-EZ Yeast Transformation kit (Zymo Research) and selected on CSM –LT dropout medium (MP Biochemicals) at 30°C for 3–4 days. The yeast two-hybrid assays were performed as described previously [13]. The CSM dropout medium (–LT or –HLT) was supplemented with 40 µM PCB (purified as described by Kunkel et al. [25]) and different 3-amino-triazole (3-AT) concentrations (0, 10 mM, 20 and 100 mM). Emission spectra of light sources used for yeast two-hybrid assays are shown in Fig. S2b. The λmax values of used light sources were shown to be 740 nm for far-red, 645 nm for red, 525 nm for green and 440 nm for blue-light illumination. Fluence rates were measured as described above and are indicated for the specific experiments in Results.

### RESULTS

#### Phenotypic analysis of potential Cph2 interaction partner knockout mutants

Sato et al. [11] performed a large-scale protein–protein interaction analysis for *Synechocystis* using the yeast two-hybrid system. Based on this assay, several proteins have been postulated as potential interaction partners of Cph2 (Table S3). With the exception of ferrochelatase [26], all these proteins are hypothetical proteins. Two of these hypothetical proteins, Slr1143 and Slr1593, possess predicted domain architectures that implicate a possible role in c-di-GMP signalling. Slr1593 consists of a single EAL domain, whereas Slr1143 harbours a GAF and a GGDEF domain. Interactions were classified by Sato et al. [11] into categories A and B (multiple positive prey clones) and category C (single positive clone in interaction analysis). All six proteins from interaction categories A and B and, in addition, Slr1143 from interaction category C (see Table S3) were chosen for mutation analysis and mutants were created (Table S2).

As mutation of *cph2* leads to positive phototaxis in blue light [6], we tested motility of the knockout mutants in blue light (Fig. 2). All mutants behaved like the wild-type and did not move towards the blue-light source. However, when motility was tested under the other colours that can be absorbed by Cph2 (red, far-red, green) and white light, two mutations (Δsll0175 and Δsll0175) resulted in phototaxis towards relatively high-intensity red light of 40 µmol photons m⁻² s⁻¹ (640 nm) which is not observed in the wild-type (Figs 2 and S3a, b). For motility experiments, only the presence or absence of motility is considered. Differences in the distance covered by the strains on the agar plate are not reproducible and depend largely on the agar surface and the amount of moving cells.

The gene product Sll0175 has some similarity to annotated FAD-dependent dehydrogenases but harbours mostly

![Fig. 2. Phototactic behaviour of knockout mutants compared to wild-type under red and blue light. The mutant Δcph2 serves as a control in blue light. Dashed lines indicate the initial position of cells. The λmax of the specific light qualities are indicated in Methods. Fluence rates were measured to be 40 µmol photons m⁻² s⁻¹ for red, and 12.0 µmol photons m⁻² s⁻¹ for blue light. HI-red, high-intensity red light.](image-url)
undefined regions (see Table S3). In contrast, Slr1143 has a clearly defined domain architecture containing a GAF and a GGDEF domain. In addition, in vitro studies demonstrated c-di-GMP synthesis activity of the recombinant Slr1143 protein [27–29]. Therefore, Slr1143 was selected for further analysis.

**Complementation of the Δslr1143 mutant**

To confirm that the phenotype of the Δslr1143 mutant is not due to a secondary mutation, a complementation strain was generated. A C-terminal FLAG tag was fused to Slr1143 not due to a secondary mutation, a complementation strain was generated. A C-terminal FLAG tag was fused to Slr1143 and the expression placed under control of the Cu²⁺ repressible petJ promoter. The complementation strain was then tested for phototactic behaviour under induced (0 µM copper) and repressed (2.5 µM copper) conditions (Figs 3a and S4a, b). Under non-inducing conditions, the complementation mutant moved towards the red-light source, resembling the Δslr1143 phenotype. However, under inducing conditions the complementation mutant Δslr1143/ slr1143+ behaved like the wild-type and was non-motile under red light. Therefore, ectopic FLAG-tagged Slr1143 can complement the Δslr1143 phenotype in red light. However, under inducing conditions the complementation strain displayed a non-motile phenotype at all light colours, while under non-inducing conditions it was motile (Fig. S4a). As Slr1143 harbours a GGDEF domain it is likely that overexpression of the protein leads to an accumulation of c-di-GMP that could inhibit motility irrespective of illumination. Furthermore, copper-dependent repression of slr1143 expression led to restoration of motility in the complementation strain Δslr1143/slr1143+ (Fig. 3b, c), presumably due to reduced cellular c-di-GMP content. Indeed, Ryjenkov et al. [27] have shown that the protein Slr1143 is a catalytically active DGC in vitro. The protein has also served as an efficient c-di-GMP producer in several other studies [28, 29]. Further, previous studies showed that Cph2 produces c-di-GMP specifically in blue light, postulating a higher cellular c-di-GMP content in blue light leading to inhibition of motility of *Synechocystis* cells under blue-light illumination [5]. Thus, we questioned whether inhibition of motility in wild-type cells under high-intensity red light is also the result of an elevated c-di-GMP content.

**Mass spectrometry quantification of cellular c-di-GMP concentration**

The cellular concentration of c-di-GMP was measured from cells grown on phototaxis plates (Fig. 4). As expected, under inducing conditions cells of the overexpression strain Δslr1143/slr1143+ exhibit a strongly enhanced c-di-GMP concentration that exceeds the c-di-GMP concentration in the wild-type and the Δslr1143 strain under all light qualities by 10- to 20-fold (Fig. 4). Under non-Slr1143-expressing conditions, the c-di-GMP content in the overexpression strain is comparable to wild-type levels. Thus, we conclude that lack of motility in the Δslr1143/slr1143+ complementation strain under inducing conditions at all light conditions is due to the elevated cellular c-di-GMP content generated by the DGC activity of Slr1143.

Interestingly, an approximately twofold higher c-di-GMP level was observed in all strains (except the induced overexpression strain) under blue light when compared to other light qualities. These data are consistent with previously published *in vitro* enzymatic measurements of the protein module Cph2(5-6), which demonstrated a twofold higher c-di-GMP synthesis rate under blue light in comparison to green-light illumination [5]. Obviously, this moderate blue-light-dependent increase in c-di-GMP synthesis is sufficient to inhibit motility, as no phototaxis is observed in blue light.

In contrast, inhibition of motility in high-intensity red light in the wild-type appears to be independent of elevated cellular c-di-GMP levels (Fig. 4). There is no significant difference (*P* = 0.75) between c-di-GMP concentrations in white and red-light treatments of the wild-type, yet wild-type cells are motile in white light, but sessile in high-intensity red light. However, there is a significant difference (*P* = 0.01) in the c-di-GMP content in red light between the wild-type (sessile) and the Δslr1143 mutant (motile). The c-di-GMP...
content between the Δslr1143 mutant and the Δslr1143/ slr1143+ strain under non-inducing conditions is also significantly different (P<0.01), yet both strains still are motile in red light. Furthermore, the c-di-GMP content of the Δslr1143/slr1143+ strain under non-inducing conditions in red light is comparable to wild-type cells, both in white light (wild-type is motile) and red light (wild-type is sessile), possibly due to basal expression of Slr1143 from the PpetJ promoter. The work by Ryjenkov et al. [27] characterized Slr1143 as a highly active DGC. Therefore, residual amounts of this enzyme in the cell might be sufficient to restore wild-type c-di-GMP levels. Though cellular levels of c-di-GMP affect motility in Synechocystis, the differences in motility between white light and high-intensity red light in wild-type and mutant cells occur independently of a change in the total c-di-GMP concentration. However, asymmetric distribution of c-di-GMP was observed in several bacteria [30]; therefore it is possible that localized c-di-GMP pools may exist also in Synechocystis, and that localized concentrations could be important for control of phototaxis.

**Protein–protein interaction of Slr1143 with Cph2**

As Sato et al. [11] discovered a possible interaction of Cph2 with Slr1143 we performed co-precipitation assays with chromophorylated Cph2 to confirm this interaction. Co-precipitation assays were performed with Slr1143 domains (for domain architecture of Slr1143 see Fig. 5a) captured on Strep/-Tactin Sepharose and His-tagged Cph2 purified from E. coli. Cph2 specifically bound both full-length Slr1143 and the GGDEF domain of Slr1143, but not the GAF domain (Fig. 5b). Thus, binding of Cph2 and Slr1143 is likely mediated through interaction of Cph2 with the GGDEF domain of Slr1143. Further, we can conclude that the interaction is not stoichiometric and an excess of Slr1143 is needed to co-purify sufficient Cph2 for detection. The controls show that there is no unspecific binding of Cph2 to Strep/-Tactin sepharose (Fig. 5b).

As green light was used during experimental manipulations, we then investigated the light dependency of the Cph2–Slr1143 interaction. Cph2 can absorb light from four parts of the visible spectrum: red, far-red, green and blue. As shown in Fig. 5(c), interactions were observed in all these light qualities. Slight differences between light qualities seen in Fig. 5(c) were not reproducible; therefore, we conclude that the interaction between Cph2 and Slr1143 is light-independent, at least in vitro.

To verify this protein–protein interaction in vivo, we performed yeast two-hybrid assays (Figs 6 and S5). Different fragments of slr1143 and cph2 were expressed in yeast cells grown on medium supplemented with PCB to ensure chromophorylation and correct folding of Cph2, and grown in green, blue, red and far-red light. For every light condition, cells were spotted on a non-selective (−LT medium, 0 mM 3-AT), a selective (−HLT medium, 10 or 20 mM 3-AT) and an outcompeting plate (−HLT medium, 100 mM 3-AT) (Figs 6 and S5). Yeast cells expressing the full-length Slr1143-GAL4-DNA-binding domain fusion combined with the activation domain fusion of Cph2 were unable to rescue growth on selective His-lacking medium, and thus full-length proteins cannot be used in the assays. Interactions of heterologous proteins can be inhibited in yeast by both poor expression and alternative post-translational modifications compared to their organism of origin [31]. However, the GGDEF domain of Slr1143 [Slr1143 (GGDEF)] was observed to interact with Cph2 truncations. This result is in agreement with the co-precipitation assays, where Slr1143(GGDEF) interacted with Cph2, but not the GAF domain of Slr1143 [Slr1143(GAF)]. Using Slr1143(GGDEF) we demonstrate that Slr1143(GGDEF) interacts with various constructs of Cph2, Cph2(1–4), Cph2(1–4) W389A, Cph2(3–4) and Cph2(4) but not with Cph2(1–2) or Cph2(3). This indicates that domain 4 of Cph2, corresponding to the EAL domain (see Fig. 1), is responsible for interaction with Slr1143, which is in accordance with Sato et al. [11]. Additionally an interaction with the Cph2(5–6) protein was detected, which corresponds to the CBCR and GGDEF domains of Cph2. As GGDEF domains tend to dimerize [32], we suspect that the two GGDEF domains interact here.

Further, we tested whether these interactions are light-dependent in a yeast two-hybrid assay. Although yeast cells were supplemented with chromophore, no light-dependency of the Cph2–Slr1143 interaction was observed (Figs 6 and S5). Furthermore, we used Cph2 mutant proteins with a defect in the photoswitch. Cph2 variants carrying the mutation W389A do not reach the P photostate [33, 34] while the Cph2 mutant C994A lacks the ability to switch between green and blue-light-absorbing photostates [5]. None of these mutant
variants showed a different result in yeast two-hybrid analysis compared to wild-type proteins. To demonstrate that yeast cells supplemented with PCB are competent for investigating light-dependent interactions of phytochromes, the plant phytochrome phyA from *Arabidopsis thaliana* with its interaction partner FHY1 was used [35]. Only in red light, when PhyA is in the Pfr photostate, but not in far-red, can the photoreceptor interact with FHY1. Prolonged illumination with green light can establish a photoequilibrium of 50% Pfr of phyA [36, 37] and therefore the interaction of phyA with FHY1 could also be detected in green light. The constitutively active PhyA (Y242H) mutant can interact with FHY1 at all light conditions [12]. These controls prove that the experimental conditions are competent for testing light-dependency; therefore, we can conclude that the interaction of the GGDEF domain of Slr1143 with domain 4 (EAL) and domain 6 (GGDEF) of Cph2 is not light-dependent, at least for the truncations and conditions used.

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**Fig. 5.** Interaction analysis of Cph2 and Slr1143 using co-precipitation assays. (a) Domain organization of Slr1143. (b) Co-precipitation assays of different constructs of the Slr1143 protein including the full-length construct [Slr1143(full)], GAF domain only [Slr1143(GAF)] and GGDEF domain only [Slr1143(GGDEF)] with Cph2. In weak green light (2.5 µmol photons m⁻² s⁻¹), Slr1143 was immobilized through an added Strep-tag, and co-purified His-tagged Cph2 detected by immunoblot and zinc-induced fluorescence of Cph2-bound chromophore. Estimated molecular weights: Slr1143(full), 40 kDa; Slr1143(GAF), 22 kDa; Slr1143(GGDEF), 20 kDa. Slr1143(GGDEF) migrates faster than the expected molecular weight. (c) Co-precipitation assay of Slr1143 and Cph2 in far-red (720 nm, 9.5 µmol photons m⁻² s⁻¹), red (640 nm, 100 µmol photons m⁻² s⁻¹), green (525 nm, 5 µmol photons m⁻² s⁻¹) or blue light (440 nm, 12 µmol photons m⁻² s⁻¹). +, Protein was added to the batch; −, buffer was added (as control reaction).
To the best of our knowledge, this is the first instance of an interaction between a GGDEF domain and an EAL domain. In order to confirm this interaction we performed co-precipitation assays with these individual domains. Domain 4 (EAL) of Cph2 was fused to a C-terminal His-tag, expressed in *E. coli* cells and purified. Slr1143 was immobilized on Strep-Tactin sepharose, incubated with the Cph2 domains, washed and proteins were eluted from the sepharose material (Fig. 7). Here, the interaction of Slr1143 (GGDEF) with the EAL domain of Cph2 (domain 4) detected in the yeast two-hybrid assay was verified. Given the abundance of data on the tendency of GGDEF domains to dimerize [8, 32, 38], it was surprising that there was no interaction between the inactive GGDEF domain 3 of Cph2 and Slr1143 GGDEF detected in yeast-two hybrid analysis. For that reason we tested also this interaction in pull-down assays (Fig. 7). As Cph2(3) has the same molecular weight as Slr1143(GGDEF), we used the full-length Slr1143 to interact with Cph2(3). These data show that the predicted catalytically inactive GGDEF domain 3 of Cph2 was co-purified with full-length Slr1143.

**DISCUSSION**

Savakis *et al*. [5] showed that *Synechocystis* cells are non-motile in blue light when cells expressed the module Cph2 (5–6) or the full-length Cph2. They further demonstrated that in an *in vitro* enzymatic assay, the synthesis of c-di-GMP by Cph2(5–6) is enhanced in blue light [5]. Agostoni *et al*. [39] revealed that *Synechocystis* wild-type cells cultivated in liquid culture show a two-to threefold higher cellular c-di-GMP level in blue light compared to cells cultivated under white light. This fits well to the data presented in this study, where cellular c-di-GMP levels were determined using cells moving on agar surfaces. Wild-type and Δslr1143
cells showed a twofold higher c-di-GMP level in blue light compared to all other light conditions tested. The absolute values of cellular c-di-GMP concentration differ between this study and the data published by Agostoni et al. [39]. These differences may reflect the different growth mode (liquid versus solid), mixotrophic versus autotrophic growth or different procedures for normalization to soluble or whole cell protein content used in the two studies. However, based on these findings it can be stated that c-di-GMP production by Cph2 is induced upon blue-light illumination resulting in an increase of the cellular c-di-GMP content by two fold. This comparably small increase in total c-di-GMP concentration seems to be sufficient to inhibit motility.

The fact that c-di-GMP influences the switch between sessility and motility has been shown for many bacteria [2]. However, the mechanism of action has only been elucidated in a few species. In the case of E. coli it has been shown that the swimming speed is regulated by the protein YcgR, which interacts directly with flagellar motor components upon binding c-di-GMP and functions as a 'brake' [40, 41]. For Pseudomonas aeruginosa it is known that the GGDEF-EAL domain protein FimX is required for type IV pilus biogenesis and subsequently affects Pseudomonas twitching motility [42]. c-di-GMP is detected by the degenerate EAL domain of FimX but the exact molecular mechanism influencing the type IV pilus biogenesis and twitching motility remains elusive [43, 44]. In Synechocystis, the downstream c-di-GMP-dependent signalling pathway involved in motility is as yet unknown. Increased cellular c-di-GMP levels inhibit motility, but it is not clear whether exopolysaccharide secretion, which was shown to be important for motility [45], or functioning of the pilus apparatus is impaired at high c-di-GMP contents.

Though no PilZ domain protein has been identified in Synechocystis, several new candidates for putative c-di-GMP receptor proteins have been postulated. MshE, the ATPase responsible for pilus assembly in Vibrio cholerae has been reported as a new type of c-di-GMP receptor protein [46, 47]. The MshE N-terminal domain from Vibrio cholerae was shown to carry a unique sequence pattern for c-di-GMP binding [48]. It consists of two highly conserved binding motifs arranged in a tandem array. A broad search for these two motifs in sequence databases revealed that more than 10,000 proteins from different bacterial lineages were found to carry these motifs. In Synechocystis, four proteins with the MshE-type c-di-GMP-binding motif were identified, among them the type IV pilus assembly ATPases PilB1 and PilB2 [48]. In addition, the same authors predicted that Synechocystis PilB1, but not PilB2, most probably binds c-di-GMP. Direct binding of c-di-GMP to PilB2 of Clostridium perfringens supports the idea that type IV pilus assembly is controlled by cellular c-di-GMP levels [49]. Further research is required to elucidate if PilB1 is the c-di-GMP receptor connecting c-di-GMP with the motility response in Synechocystis.

Interestingly, blue light does not always trigger the same responses in different bacteria. In Synechocystis, blue light leads to an enhanced c-di-GMP level whereas in the closely related cyanobacterium Synechococcus elongatus blue light triggers the degradation of c-di-GMP by a photoreceptor protein consisting of a light, oxygen, voltage (LOV) and an EAL domain [50]. Enomoto et al. [51] demonstrated conclusively that in two Thermosynechococcus strains there is a network of three CBCRs fused to GGDEF and EAL domains which ensure elevated c-di-GMP levels in the cell under blue-light illumination. In Thermosynechococcus vulcanus blue-light-dependent increase of c-di-GMP levels leads to cell aggregation which is the result of activation of a cellulose synthase harbouring a c-di-GMP-binding PilZ domain [52]. In E. coli, the blue light using FAD (BLUF)-EAL protein YcgF does not act as a phosphodiesterase but as a blue-light-triggered direct anti-repressor [53].

In this work we show that motility can be inhibited by red light without influencing the total pool of cellular c-di-GMP. Wild-type Synechocystis cells are sessile under high-intensity red light, but Δslr1143/slrl143+ cells at non-inducing

![Fig. 7. Interaction analysis of Cph2(3) and Cph2(4) with Slr1143 using a co-precipitation assay. In weak green light (2.5 µmol photons m⁻² s⁻¹), Slr1143 was immobilized through an added Strep-tag, and captured His-tagged Cph2 detected by immunoblot. Estimated molecular weights: Slr1143(full), 40 kDa; Slr1143(GGDEF), 20 kDa; Cph2(3), 20 kDa; Cph2(4), 42 kDa. Both Slr1143(GGDEF) and Cph2(3) migrate faster than their expected molecular weights.](image-url)
conditions under red light, as well as wild-type cells under white or far-red light, are motile while having similar cellular c-di-GMP levels (Fig. 4). A possible explanation could be a locally increased c-di-GMP pool (e.g. at the front side of the cell with PilB1 patches; [19]) which does not influence the overall cellular c-di-GMP concentration. Moreover, local excitation of photoreceptors based on the focusing effect of cyanobacterial cells [54] could lead to asymmetric c-di-GMP distribution in the cell. Alternatively, the c-di-GMP threshold level for activation of motility might depend on a protein–protein interaction, such as Slr1143 and Cph2, which may depend on other input signals such as light, phosphorylation or ligand association. In Xanthomonas campestris, only a complex of the HD-GYP domain protein RfpG with two GGDEF domain proteins, but not either of the three proteins alone, recruits an adaptor PilZ domain protein which then interacts with the type IV pilus ATPases, thereby reducing pilus-dependent motility [55–57]. Thus, the physical interaction between RfpG and GGDEF domain proteins is crucial for motility. In X. campestris and maybe also in Synechocystis, the regulation of motility seems to be a sensible interplay of several factors.

In this work we were able to reveal a function of the Cph2-interacting protein Slr1143 in inhibition of motility towards high-intensity red light of a Cph2-specific wavelength. We verify that Slr1143 is a highly active DGC also in vivo in Synechocystis cells and that over-expression of this DGC leads to a high cellular c-di-GMP concentration resulting in non-motile cells. Light perception is unlikely to be mediated by Slr1143 itself as the GAF domain of Slr1143 has been postulated to be non-photoactive [58]. It should also be noted that the protein Slr1143 is transcribed in an operon together with Slr1142 (Table S2, [59]) which consists of a single GAF domain. It can be speculated that Slr1142 might be necessary for the formation of a photochemically active protein complex together with Slr1143 and Cph2. Clearly, more work is needed to uncover this complex decision-making of Synechocystis cells in response to different light qualities and quantities and the mechanisms behind it. On the basis of the interaction analysis we propose to rename the slr1143 gene as trip1 (Cph2-interacting protein 1).

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Conflicts of interest

The authors declare that there are no conflicts of interest.

References


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