The High Light-inducible Polypeptides in *Synechocystis* PCC6803

EXPRESSION AND FUNCTION IN HIGH LIGHT*

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There are five *Synechocystis* PCC6803 genes encoding polypeptides with similarity to the Lhc polypeptides of plants. Four of the polypeptides, designated HliA–D (Dolganov, N. A. M., Bhaya, D., and Grossman, A. R. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 636–640) (corresponding to ScpC, ScpD, ScpB, and ScpE in Funk, C., and Vermaas, W. (1999) Biochemistry 38, 9397–9404) contain a single transmembrane domain. The fifth polypeptide (HemH) represents a fusion between a ferrochelatase and an Hli-like polypeptide. By using an epitope tag to identify specifically the different Hli polypeptides, the accumulation of each (excluding HemH) was examined under various environmental conditions. The levels of all of the Hli polypeptides were elevated in high light and during nitrogen limitation, whereas HliA, HliB, and HliC also accumulated to high levels following exposure to sulfur deprivation and low temperature. The temporal pattern of accumulation was significantly different among the different Hli polypeptides. HliC rapidly accumulated in high light, and its level remained high for at least 24 h. HliA and HliB also accumulated rapidly, but their levels began to decline 9–12 h following the imposition of high light. HliD was transiently expressed in high light and was not detected 24 h after the initiation of high light exposure. These results demonstrate that there is specificity to the accumulation of the Hli polypeptides under a diverse range of environmental conditions. Furthermore, mutants for the individual and combinations of the *hli* genes were evaluated for their fitness to grow in high light. Although all of the mutants grew as fast as wild-type cells in low light, strains inactivated for *hliA* or *hliC/hliD* were unable to compete with wild-type cells during co-cultivation in high light. A mutant lacking all four *hli* genes gradually lost its photosynthesis capacity and died in high light. Hence, the Hli polypeptides are critical for survival when *Synechocystis* PCC6803 is absorbing excess excitation energy and may allow the cells to cope more effectively with the production of reactive oxygen species.

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§ The abbreviations used are: HL, high light; NPQ, nonphotochemical quenching; ELIP, early light inducible proteins; TMH, transmembrane helices; LL, low light; TES, 2-{[(2-hydroxyethyl)methylamino]ethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; PCR, polymerase chain reaction; FPLC, fast protein liquid chromatography; MV, methyl viologen; PAGE, polyacrylamide gel electrophoresis; kb, kilobase; kbp, kilobase pairs.

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changes within the antennae complex (17, 26–28). Energy dissipation within the reaction center itself (29, 30) and cyclic electron flow around photosystem II that involves a low potential form of cytochrome b_{599} (31) may also contribute to photoprotection. Finally, reaction centers that are rendered nonfunctional via the absorption of excess excitation energy may continue to dissipate absorbed light energy as heat and serve a photoprotective role with respect to neighboring, functional photosystem II reaction centers (32). Other acclimation responses include the synthesis and recruitment of enzymes with antioxidant function such as superoxide dismutase (33), catalase (34–36), and ascorbate peroxidase (37, 38). Additionally, abundant soluble antioxidants in the chloroplast such as ascorbate and glutathione can act as quenchers of triplet chlorophyll and singlet oxygen (39).

One group of proteins that accumulates upon exposure of plants to HL is the ELIPs, or early light-inducible proteins. These were originally characterized as polypeptides that transiently accumulated in etiolated seedlings of pea and barley following HL treatment (40–44). This transient accumulation also occurred when plants were exposed to blue light, suggesting a role for the blue light photoreceptor in the induction process (45); other studies suggest that phytochrome may be involved in ELIP expression (46). In addition, ELIPs accumulate transiently under a variety of stress conditions (47–50) that would cause photoinhibition. This raises the possibility that the ELIPs function to protect plants from photooxidative damage and that expression of ELIP genes may be controlled by the redox state of the cell and/or the accumulation of reactive oxygen species.

ELIP genes from a number of different organisms have been cloned and sequenced (43, 51–53). Sequence comparisons have revealed that they are members of the chlorophyll a/b-binding protein or Lhc superfamily of proteins (54). The ELIPs have three transmembrane helices (TMH I–III) that correspond to the TMHs of the Lhc polypeptides (55). Although pigment binding by ELIPs has not been directly demonstrated, all ELIPs contain conserved residues that could potentially bind chlorophyll a (55). Even though it has been suggested that the ELIPs function as “pigment-carrier” proteins involved in the turnover and/or redistribution of pigment molecules under conditions when photosystem II components are being rapidly degraded and repaired (47), the exact role of ELIPs under light stress conditions is not clear. Recently, the Cbr protein of Dunaliella was shown to be associated with light-harvesting antenna complexes II and preferentially associated with specific pigment-protein subcomplexes that contain high levels of lutein and other xanthophylls (56).

Members of the Lhc gene family have also been identified that encode proteins with one and two TMHs. In Arabidopsis, two ELIP-like genes that encode thylakoid membrane polypeptides with two TMHs (the proteins are called SepS, stress enhanced proteins) were isolated (57). Expression of Sep genes increased in HL but not during other stress conditions. An ELIP-like protein with a single TMH has also been isolated from Arabidopsis (58). These single TMH polypeptides, designated Hll or Scp (1, 2), were first discovered in cyanobacteria. The single TMH in these polypeptides resembles TMH 1 or III of the Lhc polypeptides. Expression of the genes is strikingly similar to that of ELIP genes, suggesting that they have similar functions. There are five monocistronic hli genes on the Synechocystis PCC6803 genome (59, 60) that compose an hli multigene family (Ref. 2, Cyanobase); one of these represents a fusion with the ferrochelatase gene.

We have examined accumulation of the four Hli proteins (the ferrochelatase was excluded) of Synechocystis PCC6803 under several conditions that would result in the absorption of excess excitation energy by the photosynthetic apparatus, and we have investigated the phenotypes of hli deletion mutants. Our results indicate that Hli polypeptides accumulate when cyanobacteria are exposed to HL or other stress conditions and that they may form distinct protein complexes in the thylakoid membranes. Furthermore, mutants that cannot synthesize Hli polypeptides show growth characteristics similar to that of wild-type cells in low light (LL) but are unable to compete with wild-type cells during exposure to HL. A strain deleted for all four of the hli genes gradually loses photosynthetic function and dies following exposure to HL.

**MATERIALS AND METHODS**

**Culture Conditions—**Synechocystis PCC6803 was cultivated in BG-11 medium (61) buffered with 10 mM TRIS, pH 8.2, at 30 °C. Cultures were bubbled with 3% CO₂ in air and illuminated with 40 μmol photon m⁻² s⁻¹ from incandescent bulbs. BG-11 medium lacking nitrogen (−N) or sulfur (−S) was prepared by replacing the NaNO₃ for −N medium and MgSO₄, ZnSO₄, and CuSO₄ for −S medium with equimolar amounts of the corresponding chloride salts (NaCl, MgCl₂, ZnCl₂, and CuCl₂, respectively). For nutrient starvation experiments, cells grown in BG-11 medium were pelleted by centrifugation (5,000 g, 5 min) and resuspended in −N or −S medium. This step was repeated prior to allowing cells to grow in −N or −S medium. Procedures for initiating nutrient deprivation have been described previously (62).

For HL treatments, cells in mid-logarithmic growth phase (OD₇₃₀ ~0.8) were diluted with fresh medium to an OD₇₃₀ of ~0.3. The cells (in 50-ml culture tubes) were then placed in a temperature-controlled glass chamber (maintained at 30 °C) and exposed to 500 μmol photon m⁻² s⁻¹ white light for various lengths of time, as indicated in the text. For cold treatment, cultures were diluted with BG-11 medium chilled to 4 °C and then allowed to incubate at 4 °C with constant shaking for 6 h.

**Mutant Construction—**To construct cell lines in which each of the Hli polypeptides was tagged with the His₆ epitope, coding regions of individual hli genes were cloned in frame into the pQE expression vectors (Qiagen) (pQE-60 for sll16133 (hliC); pQE-70 for ssr2595 (hliB), ssr2596 (hliD), sll2542 (hliA)). Each hli promoter plus coding region (with the C-terminal His₆ tag) was ligated sequentially to the 5′ t-t p prokaryotic terminator, a drug-resistant cartridge, and the DNA sequences downstream of each of the corresponding hli genes. Fig. 1 shows a linear drawing of each plasmid containing an epitope-tagged chimeric hli gene, and the legend of the figure provides the sequences of the primers used to make these constructs. Each of the constructs was sequenced to ensure that no errors were generated during gene construction. The constructs were transformed into Synechocystis PCC6803; the wild-type hli sequence was replaced by the chimeric hli-His₆ sequence.

Plasmids containing the hliA and hliB genes interrupted by erythromycin and spectinomycin resistance cassettes, respectively, were gifts from Wim Vermaas (Arizona State University). The hliA gene was interrupted at a SacI site located 72 base pairs downstream of the translation start site. The hliB gene was interrupted at a SacI site located 12 base pairs downstream of its translation start site. These constructs were generated by Funk and Vermaas 2 and generously given to us. The gene disruptions were confirmed by PCR. The plasmids in which hliC and hliD were deleted (ΔhliC and ΔhliD) were generated by ligating a PCR fragment upstream of each gene (0.3 kb for hliC; 0.4 kb for hliD), a drug-resistant cartridge (kanamycin for hliC; chloramphenicol for hliD), and a PCR fragment generated to sequences downstream of each gene (0.4 kb for hliC; 0.5 kb for hliD), all in the proper orientation. Primers used for PCR amplifications, given in the legend of Fig. 1, incorporated different restriction endonuclease sites to facilitate cloning. A detailed representation of the constructs is depicted in Fig. 1.

The plasmids containing the interruptions/deletions were transformed into Synechocystis PCC6803, and transformants were selected on appropriate antibiotics. Single, double, and quadruple mutants (all of the hli genes were either disrupted or deleted) were constructed. Transformants were continuously subcultured until each mutant line contained homoplasmic interruptions of hli genes. Segregation of the deleted genes in each of the mutants was monitored by PCR of isolated

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2 C. Funk and W. Vermaas, unpublished data.

3 Q. He, N. Dolganov, O. Bjerkman, and A. R. Grossman, unpublished data.
genomic DNA using specific primers as follows: hliA, GATGCCTGTTGAGCAGTTCAT at position 701,127–701,162 and TTATCGAAGCTTGCCCAACTGGTCTC (701,375–701,395); hliB, CTCTTATGGCAGATGACTGCAG at position 839,188–839,206, and GAGCTTCATGTGGGTCGCA (849,355–849,381) 

Preparation of Thylakoid Membranes—Cyano bacterial cell pellets derived from cells grown to mid-logarithmic phase were resuspended in thylakoid buffer (1/100 of the original culture volume) containing 20 mM MES/NaOH, pH 6.4, 5 mM MgCl₂, 5 mM CaCl₂, 20% glycerol (v/v), 20 mM MES/NaOH, pH 6.4, 5 mM MgCl₂, 5 mM CaCl₂, 20% glycerol (v/v), and 5 mM dithiothreitol (DTT). The thylakoid membranes were pelleted at 4 °C by centrifugation (20 min, 40,000 rpm in a Ti 50.2 rotor). The membranes were washed once and resuspended in thylakoid buffer (1 ml of buffer to 200 ml of the original culture volume). Sucrose density gradient centrifugation (64) was used to purify thylakoid membranes. Purified membranes were resuspended in 0.1 M sodium phosphate buffer, pH 7.5, containing 0.3 M sucrose and 1 mM ethylenediaminetetraacetic acid (EDTA).

Concentrations of soluble polypeptides and thylakoid membrane polypeptides solubilized by incubation in 2% SDS at 35 °C for 15 min were determined. Protein extracts were centrifuged at 16,000 × g for 2 min to remove insoluble debris; the supernatants were diluted 10-fold in water, and the protein concentrations were measured using the Bio-Rad protein assay reagents (Pierce) according to the manufacturer's instructions.

**Fluorescence Measurements**—The yield of chlorophyll fluorescence was continuously monitored using a pulse-amplitude-modulation chlorophyll fluorometer (Walz) with a pulse-amplitude-modulation 103 accessory, a water-jacketed cuvette, and a Schott KL 1500 lamp, which provided the actinic light. The cells were diluted to a chlorophyll concentration of 2 μg ml⁻¹ prior to analysis. The minimal fluorescence level (F₀) was monitored with red-modulated light (1.6 kHz) at 0.030 μmol photon m⁻² s⁻¹. The maximum fluorescence level of dark adapted (Fₘ) was determined by a 600 ms high intensity white pulse at 3400 μmol photon m⁻² s⁻¹. This light pulse transiently closes all of the photosystem II reaction centers (67). The maximal fluorescence level of a sample was determined in the presence of 20 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (68) and 25 μM norflurazon. The cultures were incubated in low or intermediate (200 μmol photon m⁻² s⁻¹) light, and OD₇₃₀ was used to determine the rate of growth of the cultures at various times following the addition of the herbicides.
Environmental conditions, we created four distinct Synechocystis PCC6803 cells following exposure to different marked by insertion of the His6 epitope. The sites of insertion of the Hli proteins were not analyzed, which is a fusion between the HemH protein, which is a fusion between the His6 epitope and an hli-like gene.

To measure the relative levels of the Hli polypeptides in Synechocystis PCC6803 cells following exposure to different environmental conditions, we created four distinct Synechocystis PCC6803 strains in which one of the four Hli proteins was marked by insertion of the His6 epitope. The sites of insertion of the epitope tags are shown in Fig. 1. Monospecific antibodies directed against the His6 epitope were used to examine the levels of the specific Hli polypeptides in total membranes following exposure of the epitope-tagged strains to different environmental conditions. We did not detect Hli polypeptides in cells grown in LL on complete medium, unless solubilized membrane proteins were enriched for Hli polypeptides by passage over a Ni2+ affinity column (which binds to the His6 epitope; not shown). Therefore, although the Hli polypeptides are present in cells maintained in complete medium under LL conditions, they only accumulate to very low levels.

As shown in Fig. 3, all four of the Hli polypeptides accumulated to high levels following exposure of cells to HL. We estimate that there is a better than 10-fold increase in the levels of HliA, HliB, and HliC polypeptides following 6 h of HL exposure. The Hli polypeptides also accumulated under other stress conditions. The levels of all of the Hli polypeptides increased upon nitrogen starvation. Starvation for sulfur or exposure to chilling temperatures led to the accumulation of HliA, HliB, and HliC; this accumulation was comparable to that observed in HL. Interestingly, following exposure of the cells to low temperature, two polypeptides that exhibited immunoreactivity with antibodies against the His6 epitope were not detected when cells were exposed to either N or low temperature conditions. The polypeptides were identified as HliC, which was MNNENS, and HliD, which was MNNENSF (where the X is ambiguous), respectively. Total membranes were isolated and analyzed for Hli polypeptides that were His6-tagged both before (-) and after (+) the cells were exposed to HL (HL, 6 h), low temperature (LT, 4 °C, 6 h), and nitrogen (-N) and sulfur (-S) deprivation for 12 and 30 h, respectively. Total membranes were isolated as described under “Materials and Methods” and membrane polypeptides fractionated by SDS-PAGE (12–16% polyacrylamide). The polypeptides were blotted onto nitrocellulose paper and probed with commercial antibodies specific for the His6 tag (Santa Cruz Biotechnology). Control samples (−) were maintained at standard growth conditions.
sequences. In contrast, the apparent molecular masses of HliC and HliD were considerably less and more, respectively, than the values predicted from Cyanobase information. The N-terminal sequence (see the legend of Fig. 2 for the sequence) of purified HliC polypeptide from Synechocystis PCC6803 cells exposed to HL revealed that this polypeptide initiates at a methionine that is 69 nucleotides downstream of the translation start site that had been predicted from the nucleotide sequence (Cyanobase). This smaller polypeptide was the only product detected by SDS-PAGE, although we cannot rule out the possibility that it resulted from a rapid and specific proteolysis that is not blocked by the suite of protease inhibitors used during the isolation of thylakoid membranes. The slow migration of HliD during SDS-PAGE may reflect altered binding of the anionic detergent.

**Kinetics of Hli Polypeptide Accumulation upon High Light Exposure**—To define the kinetics of accumulation of the different Hli polypeptides, we isolated total cellular membranes at various times following transfer of different epitope-tagged strains to HL, and we evaluated the levels of the specific Hli polypeptides using antibodies against the His6 epitope tag. Western blot analyses of total membrane proteins are shown in Fig. 4. The accumulation of the HliA and HliB reached a maximum level within 1 h of transfer to HL. This level was maintained for up to 6 h following the initial transfer, after which the levels of these polypeptides gradually declined. HliC exhibited a slightly slower rate of accumulation, reaching maximum abundance at 3 h; this maximal level was maintained over the entire 24-h period tested. The level of the HliD polypeptide was lower than that of the other polypeptides. This polypeptide peaked in abundance at 6–9 h following the onset of HL and rapidly declined thereafter. These results suggest that all of the Hli polypeptides play a role in the acclimation of *Synechocystis* PCC6803 to HL. Temporal differences in polypeptide levels that are observed may reflect the different requirements of cells as they develop long term strategies for surviving HL conditions.

**Reduction in Hli Levels following Transfer of Cells to Low Light**—The ELIPs are rapidly degraded during recovery of cells from excess excitation (69). Many of the characteristics of ELIPs are similar to those of the Hli polypeptides. To investigate the stability of the Hli polypeptides in LL, we transferred *Synechocystis* PCC6803 hli-targeted strains that had been exposed to HL for 6 h to LL and immunologically monitored Hli polypeptide levels. Aliquots of cells at different times following a return to LL growth conditions were used for thylakoid membrane isolation. As shown in the Western blots of Fig. 5, the HliA and HliB polypeptides were extremely unstable, and there is a loss of more than 80% of these polypeptides within 1 h of transfer of cells to LL. The HliC and HliD polypeptides are stable for the initial 3 h, after which they are rapidly degraded. This delay in the reduction in HliC and HliD levels coincides with a delay in the recovery of cell division and accumulation of phycocyanin and chlorophyll, suggesting that HliC and HliD may be important during this “latent” recovery stage. Interestingly, only when the Hli polypeptides were barely detectable did cell division and pigment accumulation proceed.

**FPLC Fractionation of Hli Complexes**—The Hli polypeptides were demonstrated to be exclusively in the thylakoid membranes (data not shown). To determine if they were constituents of multisubunit membrane complexes or were functional as monomers, we isolated thylakoids from the His6-tagged cell lines grown in HL for 6 h, solubilized the membranes with non-ionic detergents, fractionated membrane-protein complexes by FPLC, and tracked His6-tagged Hli polypeptides using the epitope-specific antibodies. As shown in Fig. 6, HliA and HliB polypeptides co-eluted in the ~100-kDa fraction, whereas HliC and HliD co-eluted in the ~50-kDa fraction. These data suggest that the Hli polypeptides function as complexes in the thylakoid membranes and that pairs of the Hli polypeptides may be associated with each other.

**High Light Sensitivity of hli Mutants**—We constructed *Synechocystis* PCC6803 strains in which the *hli* genes were inactivated by insertion of a drug-resistant marker gene; single, double, and quadruple mutants were constructed (see Fig. 1 and under “Materials and Methods”). The *hliA, hliC,* and *hliD* genes are monocistronic (2); therefore, interruption or deletion of these genes should not have a polar effect on downstream sequences. The *hliB* gene is co-transcribed with the open reading frame slr1544 that encodes a hypothetical protein of 103 amino acids. Interruption of the *hliB* gene may have a polar effect; however, interruption of the *hliB* gene does not have any observable phenotype under the conditions tested. The relationship of the co-transcribed sequence to the *hli* genes and its potential role in acclimation needs further analysis.

To determine the fitness of the *hli* deletion strains to compete with wild-type cells in HL, we performed competition
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experiments in which the wild-type and mutant strains were mixed, placed in HL, and samples of the culture taken at various times following HL exposure to determine the wild-type:mutant ratio in the cultures. When single mutants, double mutants, and the quadruple mutant in the hli genes were mixed with wild-type cells, the ratio of mutant to wild-type cells remained constant during growth in LL for at least 4–6 days. Furthermore, the hliB, hliC, and hliD single mutants appeared to have none or little competitive disadvantage in HL relative to wild-type cells (data not shown). In contrast, when either the hliA or the hliA/hliB and the hliC/hliD double mutants were mixed with wild-type cells and exposed to HL, the ratio of wild-type to mutant cells rapidly increased. Four days following the initiation of HL exposure, the cultures contained 10% or less of the mutant cells (Fig. 7). The quadruple mutant was very sensitive to HL; its photosynthetic capacity was reduced to a very low level within 12 h of the onset of HL (Fig. 8A). After 2 days in HL this mutant stopped dividing and gradually died (Fig. 8B). These results clearly establish that the Hli polypeptide family is required for the acclimation of Synechocystis PCC6803 to HL, and also suggests that there is some redundancy in function of the Hli polypeptides. Although all of the single and double hli mutants can grow in HL, some cannot grow as well as wild-type cells. In contrast, when all of the hli genes are disrupted, HL becomes lethal, although the strain grows at a rate comparable to that of wild-type cells in LL.

Oxidative Stress Induction—To test the sensitivity of the hli quadruple mutant to artificially generated reactive oxygen species, the growth of wild-type cells and the quadruple mutant was monitored at a light intensity of 200 \( \mu \text{mol photon m}^{-2} \text{s}^{-1} \) in the presence and absence of 0.5 \( \mu \text{M} \) MV or 25 \( \mu \text{M} \) norflurazon; these doses of the herbicide were sublethal for Synechocystis PCC6803. In photosynthetic organisms, MV catalyzes the formation of \( \text{O}_2^* \) primarily at the acceptor side of photosystem I. In contrast, the herbicide norflurazon promotes the accumulation of \( \text{O}_2^* \) within thylakoid membrane as it inhibits biosynthesis of carotenoids, which are the dominant quenchers of \( \text{O}_2^* \) generated by the antenna pigments. As shown in Fig. 9, an intensity of 200 \( \mu \text{mol photon m}^{-2} \text{s}^{-1} \) does not markedly inhibit the growth of the quadruple mutant, and MV at a sublethal concentration of 0.5 \( \mu \text{M} \) inhibits growth of both strains to the same extent. These results suggest that the activities of detoxification enzymes such as superoxide dismutase, peroxidases, and catalases are not significantly affected in hli mutants. In contrast, norflurazon was shown to inhibit consistently the growth of the quadruple mutant to a greater extent than that of wild-type cells, suggesting that the Hli polypeptides may play a role, either directly or indirectly, in detoxification of \( \text{O}_2^* \) generated within thylakoid membranes. The sublethal concentration of norflurazon used to retard growth of Synechocystis PCC6803 was 25 \( \mu \text{M} \), which is substantially higher than the dose (0.5–5 \( \mu \text{M} \)) reported to kill Synechococcus PCC7942 (70, 71).
Cells were incubated in 200 m\textsuperscript{experiments. Errors were within 5% for all points. generated by averaging the data obtained from three representative measured as a change in optical density at 730 nm. Curves were even under LL conditions. However, the accumulation of the results demonstrated the presence of all four Hli polypeptides sion patterns of the Hli polypeptides. Although this procedure about 25% of peak levels.

readily detected after 24 h in HL, whereas HliA and HliB fall to between 6 and 9 h, after which they begin to fall, except for HliD. The levels of all of the Hli polypeptides peak the only Hli polypeptide that cannot be detected after 1 h of exposure to HL. The levels of all of the Hli polypeptides peak at approximately 6 h in HL. The initial accumulation of Hli polypeptides occurs during the phase of acclimation in which the cells are unable to divide. Once the cells reach a new physiological steady state that accommodates the new light conditions, they begin to divide and the levels of Hli polypeptides fall. This decline may reflect a modification of the polypeptide and lipid composition of the photosynthetic machinary that enables the cells to balance more efficiently the utilization and dissipation of absorbed light energy, which allows for continued growth. These modifications also alter the need for Hli polypeptides. The results also suggest that HliA, HliB, and HliC may be important for sustained growth in HL and that these polypeptides may have some overlapping function. This possibility is supported by the finding that whereas the quadruple hli mutant dies upon exposure to HL, none of the single or double mutants die (although a number of them do not grow as fast as wild-type cells following exposure to HL).

Some results suggest that HliA/HliB and HliC/HliD may form complexes in the photosynthetic membranes. However, these results are only based on co-migration of polypeptides following solubilization of thylakoid membranes. The possibility of a complex between HliA/HliB has some support with the findings that these polypeptides increase and decrease with exactly the same kinetics following exposure to HL. Although the kinetics of accumulation of HliC and HliD differ following the transfer of cells from LL to HL, they decrease with the same kinetics following transfer from HL to LL. In addition, the wild-type HliD polypeptide was found to be present in sucrose gradient fractions containing His\textsubscript{6}-tagged HliC. Hence, the Hli polypeptides are likely to exist in multimeric structures, as suggested by the migration of the solubilized polypeptides during gel filtration (Fig. 6) and their sedimentation in sucrose gradients. However, the nature of these complexes and the structural relationships among the different Hli polypeptides remain to be established.

A number of polypeptides have been identified that increase the ability of photosynthetic organisms to survive HL exposure. Some of these polypeptides such as superoxide dismutase and ascorbate peroxidase may be involved in rapidly eliminating potentially toxic, reactive oxygen species that form following HL exposure. Others such as PsbS (21, 73) and IsiA (74) may be involved in quenching singlet excited chlorophyll molecules, which would prevent the accumulation of toxic oxygen species. The specific role of the Hli polypeptides in photoprotection is still not clear, although it has been proposed to function in the dissipation of excess absorbed excitation energy (1, 58) or serve as a chlorophyll carrier (2).

A recent report by Funk and Vermaas (2) suggests that the hli genes of Synechocystis PCC6803 are not significantly induced when the cells are transferred from moderate light (50
μmol photon m⁻² s⁻¹) to HL. (250 μmol photon m⁻² s⁻¹).

However, high level hli expression was observed in glucose-grown cells lacking photosystem I or lacking both photosystems I and II (2). The authors suggest that the Hli polypeptides function to bind free chlorophyll under such conditions and that they may not be responsive to the redox conditions of the cell. The binding of chlorophyll and/or chlorophyll intermediates could protect the cyanobacterium from the potentially phototoxic effect of these free pigments. Some aspects of these data are difficult to interpret. The HL intensities used may not have been sufficiently high to induce the hli genes (especially if the signal for their induction relates to the accumulation of reactive oxygen species) and/or the treatment times may have been suboptimal for detecting hli transcripts (1). Furthermore, mutants devoid of the photosystems may be aberrant in membrane structure/organization because of the absence of major complexes within the membranes. It would be difficult to predict the redox state of such cells or their tendency for generating reactive oxygen species; either direct or indirect methods would be required to quantify the levels of such species.

There are several lines of evidence to support the proposal that the Hli polypeptides are required for survival and acclimation of cells to the absorption of excess light energy and that they are probably not major chlorophyll carriers in the cell (although they may be adapted to bind and store free chlorophyll specifically when cells are absorbing excess excitation). First, accumulation of Hli polypeptides is triggered whenever Synnechocystis PCC6803 is absorbing excess excitation energy. Second, some of the single (hliA) and double (hliC/hliD; hliA/hliB) mutants cannot compete with wild-type cells during exposure to exposure excitation energy; their growth rate is equal to that of wild-type cells in LL (doubling time of approximately 8 h). A mutant defective for all four hli genes dies upon exposure to HL. When wild-type cells are exposed to HL, cell growth stops and only proceeds after approximately 6 h of acclimation. The cells then begin to rapidly divide. Although the quadruple mutant grows at a similar rate to wild-type cells even at light intensities up to 200 μmol photon m⁻² s⁻¹ (Fig. 9), it only grows to a small extent following the transfer to 500 μmol photon m⁻² s⁻¹. After the mutant experiences the 6-h HL acclimation period, it exhibits slow growth that ceases after about 30 h, at which time the cells are nearly all dead. Furthermore, during the first 6–10 h of acclimation, wild-type cells lose approximately 50% of their capacity for photosystem 2 activity (the variable fluorescence declines by 50%); the remaining activity is sustained during HL growth. In contrast, photosystem 2 activity in the quadruple mutant declines to nearly zero following 10 h in HL, suggesting the destruction of the photosynthetic machinery in the mutant strain. If the Hli polypeptides served as major chlorophyll carriers, the quadruple mutant would be expected to be impaired in growth in LL and moderate light since they would likely be required as the cells are synthesizing high levels of chlorophyll and chlorophyll-protein complexes under such conditions. Furthermore, bleached, nitrogen-starved cells also synthesize high levels of the Hli polypeptides. When nitrogen is provided to the starved cultures, the cells regain their pigmentation and the Hli proteins disappear. However, the disappearance of these proteins precedes re-greening of the cell; these kinetic features are not so easy to reconcile with a major chlorophyll carrier function.

The biggest questions that still remain are as follows. 1) How widespread are the Hli polypeptides in photosynthetic organisms? 2) How are they organized in the photosynthetic apparatus? 3) What are their specific functions? 4) How do they perform these functions? 5) What are the different specificities among the different members of this polypeptide family in cyanobacteria? 6) What features of the different polypeptides confer this specificity? Genes encoding Hli proteins have been identified in a number of cyanobacteria (1, 60, 75) and red algae (76–78). Recently, an hli cDNA was also identified from Arabidopsis (58). Characterization of the Arabidopsis hli gene suggests that the vascular plant Hli polypeptide is imported into chloroplasts and, similar to observations made with cyanobacteria, the level of the hli transcript increases following exposure of Arabidopsis to HL (58). These authors suggest that Hli polypeptides function in the dissipation of excess excitation energy.

The work presented here demonstrates the accumulation of the different Hli polypeptides in the thylakoid membranes and their requirement for survival during exposure to HL. Furthermore, HL completely destroys photosystem II function in the hli quadruple mutant, suggesting that these polypeptides are involved in protecting/stabilizing the photosynthetic apparatus, and perhaps other aspects of the metabolic machinery of the cell, from photodestruction. Although the precise mechanism for protection is still not clear, it is likely to involve either suppressed generation or elevated rates of quenching of reactive oxygen species by pigment-protein complexes containing Hli polypeptides. This possibility is suggested by preliminary experiments in which cyanobacterial cells were exposed to norflurazon. This inhibitor of carotenoid synthesis facilitates the accumulation of singlet oxygen (79) and leads to the induction of the Hli polypeptides in wild-type Synnechocystis PCC6803. Interestingly, the hli quadruple mutant is significantly more sensitive to the administration of sublethal doses of norflurazon than wild-type cells. The finding suggests that the mutant strain has a reduced capacity for detoxification of singlet oxygen. A more detailed biochemical analysis of the wild-type and mutant strains should clearly establish the role of the Hli polypeptides in maintaining photosynthetic activity and viability of the cells in HL.

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