Iron deficiency induces a remodeling of the photosynthetic apparatus in *Chlamydomonas reinhardtii*. In this study we showed that a key mechanistic event in the remodeling process of photosystem I (PSI) and its associated light-harvesting proteins (LHCIs) is the N-terminal processing of Lhca3. N-terminal processing of Lhca3 is documented independently by two-dimensional gel electrophoresis and tandem mass spectrometry (MS/MS) analysis as well as by quantitative comparative MS/MS peptide profiling using isotopic labeling of proteins. Dynamic remodeling of the LHCI complex under iron deficiency is further exemplified by depletion of Lhca5 and up-regulation of Lhca4 and Lhca9 polypeptides in respect to photosystem I. Most importantly, the induction of N-terminal processing of Lhca3 by progression of iron deficiency correlates with the functional drop in excitation energy transfer efficiency between LHCI and PSI as assessed by low temperature fluorescence emission spectroscopy. Using an RNA interference (RNAi) strategy, we showed that the truncated form of Lhca3 is essential for the structural stability of LHCI. Depletion of Lhca3 by RNAi strongly impacted the efficiency of excitation energy transfer between PSI and LHCI, as is the case for iron deficiency. However, in contrast to iron deficiency, comparative MS/MS peptide profiling using isotopic labeling of proteins demonstrated that RNAi depletion of Lhca3 caused strong reduction of almost all Lhca proteins in isolated PSI particles.

The viability of a photosynthetic organism is dependent on its ability to regulate the structure and function of its photosynthetic apparatus in anticipation of or in response to external factors such as light quality and nutrient availability. Iron is a cofactor in many essential biological redox reactions, including respiration and photosynthesis, and its scarcity leads to chlorosis (chlorophyll deficiency) in photosynthetic organisms, which is connected to degradation and rearrangement of the photosynthetic machinery (1–4). The global impact of iron deficiency on photosynthetic productivity has been shown in vast ocean regions that are severely limited in iron (5). On the molecular level, photosystem (PS)1 is a prime target of iron deficiency, probably because of its high iron content (12 iron per PSI). In the green alga *Chlamydomonas reinhardtii* iron deficiency leads not only to a pronounced degradation of PSI but also to a remodeling of the PSI-associated light-harvesting antenna (LHCl), which proceeds severe iron deficiency (4). These structural changes decrease the functional efficiency of excitation energy transfer between LHCI and PSI and minimize photo-oxidative stress to the thylakoid membrane. Cyanobacteria also respond to iron deficiency by degradation of light-harvesting phycobilisomes (6). Additionally, cyanobacteria express the “iron stress-induced” gene isiA. The IsiA protein has significant sequence similarity with CP43, a chlorophyll a-binding protein of photosystem II (PSII (7, 8)) and forms a ring of 18 molecules around a PSI trimeric reaction center, as shown by electron microscopy (9, 10).

These results highlight the adaptive nature of the response to iron deficiency that is directed toward optimizing the photosynthetic architecture to the conditions in which iron is a limiting cofactor. In *Chlamydomonas*, neither the molecular mechanisms underlying the remodeling of LHCI nor its structural and functional characteristics have been investigated. It is of note that the LHCI composition of *Chlamydomonas* is complex and consists of nine distinct subunits (11). In this study we combined low temperature fluorescence spectroscopy, reverse genetics, and comparative quantitative proteomics to correlate the efficiency of excitation energy transfer with dynamic alterations of LHCI. We established an approach that is based on differential isotopic labeling of proteins in *Chlamydomonas* by isotopically labeled arginine and mass spectrometric quantitative analysis of labeled and unlabeled peptides.

Mass spectrometry has become a powerful tool for quantitative proteomics, because it allows sensitive, fast, and specific identification of proteins from complex mixtures (12–14). Comparative analysis of a protein between experimental and control samples can be used to monitor its relative changes in abundance under these two conditions. Comparing the relative amount of many proteins in parallel in this manner enables a quantitative overview of the dynamically altered proteome, in our case the remodeled photosynthetic apparatus. To distinguish proteins from control and experimental conditions, differential isotopic labeling strategies can be

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1 The abbreviations used are: PS, photosystem; LC-MS/MS, liquid chromatography and tandem mass spectrometry; RNAi, RNA interference; LHCI, light-harvesting complex; EST, expressed sequence tag.
employed. Tagging of protein mixtures is either achieved by
cross-linking proteins isolated from cells grown under differ-
ent conditions with isotopically labeled and unlabeled chem-
ical probes (15) or metabolic labeling with stable isotopes by
growing cells in isotopically enriched media (16, 17). After
tagging the protein or cells, pools are mixed, digested with
enzymes, and quantified by liquid chromatography and tan-
dem mass spectrometry (LC-MS/MS). A key assumption for
both processes is that isotopically labeled and native peptides
will behave similarly because the quantification is based on
intensities or peak areas of both forms of the same peptide as
measured in the mass spectrometer.

The green unicellular alga *C. reinhardtii* has emerged as a
eukaryotic model system for the investigation of fundamental
molecular processes in cell biology (18), and especially for the
study of chloroplast-based oxygenic photosynthesis (19, 20).
The availability of a draft genome sequence, including over
200,000 ESTs (21), allows application of genomic and pro-
teomic methodologies (22). Quantitative approaches that are
applicable on the proteomic scale, such as the one used in this
study, will aid in the characterization of dynamic changes
because of different physiological conditions or distinct ge-
etic backgrounds.

**MATERIALS AND METHODS**

**Strains and Cultures**—The cell wall-less strain CC124 [CW15] or the
arginine auxotrophic strain CC424 was used for all experiments. For all
quantitative experiments using LC-MS/MS, strain CC424 was used.
This strain was kindly provided by the *Chlamydomonas* culture collec-
tion (for information see the website www.chlamy.org). Cells were
grown in the presence of either normal or isotopically labeled 13C6-L-
arginine (Cambridge Isotope Laboratories, Andover, MA). Arginine con-
centrations were 50 μg/ml (23). Cell culture and transfer of cells from
iron-sufficient to iron-deficient media were carried out essentially as
described (4).

**Plasmid Construction**—For generation of the inverted repeat Lhca3
construct, a short 216-bp fragment corresponding to the 3'-untrans-
lated region of Lhca3 was amplified with PCR by using the EST clone
AV387462 (www.kazusa.or.jp/en/plant/chlamy/EST/) (24) as template
and the primers LH-FORS-P (aaaactgcaggctccagtcagc, underlined PstI)
and LH-REV-E (atcgaattcggccgcagccacgac, underlined EcoRI) adding
EcoRI and PstI restriction sites. A longer 316-bp fragment additionally
contained 100 bp from the adjacent coding sequence that function as
spacer in the inverted repeat construct and was amplified by using the
primers LH-FORL-P (aaaactgcagtgtgatgaccggcaaag, underlined PstI)
and LH-REV-E (atcgaattcggccgcagccacgac, underlined EcoRI). Both
fragments were cloned in the antisense direction by using the vector
pBluescript SK. The resulting inverted repeat cassette was excised with
EcoRI and cloned in the unique EcoRI site of the *Mae7X* inverted
repeat construct that was kindly provided by H. Cerutti (see Ref. 25).

![Fig. 1. Induction of N-terminal processing of Lhca3 with the onset of iron deficiency correlates with the drop in efficiency in excitation energy transfer between LHCl and PSI.](http://www.jbc.org/)

A, analysis of thylakoid membranes isolated from *Chlamydomonas* cells grown for 0–5 days in iron deficiency revealed the induction of a new Lhca polypeptide, see arrow (adapted from Ref. 4). B, MS/MS analysis of this polypeptide identifies it as Lhca3. C, low temperature fluorescence emission spectroscopy of *Chlamydomonas* cells grown for 0–5 days in iron deficiency.
Iron Deficiency Induces N-terminal Processing of Lhca3

Nuclear Transformation and Selection—Nuclear transformation of Chlamydomonas was carried out by using a helium-driven PDS-1000/He particle gun (Bio-Rad) with 1100 pounds/square inch rupture discs (Bio-Rad). M10 tungsten particles (Bio-Rad) were coated with 2 μg of DNA of the IRMa7/IRLhca3 construct, washed three times with 500 μl of absolute ethanol, and resuspended in 25 μl of absolute ethanol; 7 μl of the suspension were used for one transformation. Wild-type cells were grown at 25 °C and 50 μE m⁻² s⁻¹ in liquid TAP medium, and 4 × 10⁷ cells were dispersed per TAP plate containing 1.5 mM t-tryptophan, 5 μg/ml paromomycin, and 5–10 μM 5-fluorouracil. The transformed cells were incubated at 25 °C and 3 μE m⁻² s⁻¹ for 1–2 weeks. The appearing transformants were transferred on fresh TAP plates containing t-tryptophan, paromomycin, and 5-fluorouracil and used for further investigations.

Isolation of Intact Chloroplasts—Cells were harvested by centrifugation (4 °C and 3,500 × g) and resuspended to a concentration of ~7 × 10⁹ cells/ml in buffer with 0.3 M sucrose, 25 mM HEPES, and 0.5 mM MgCl₂. Cells were disrupted in a self-made nebulizing chamber by nebulizing the cell suspension against a ceramic ball with N₂ gas at a pressure of ~15 pounds/square inch. Intact chloroplasts were harvested using Percoll cushions of 80, 60, and 40% (v/v). Thylakoid membranes were isolated as described previously (26) from broken chloroplasts.

Isolation of PSI—Enrichment of PSI from thylakoids solubilized in n-dodecyl β-maltoside using sucrose gradient centrifugation was done as described previously (27).

Protein Analysis—Protein concentrations were determined using the Bradford assay (28). Differentially labeled samples (¹³C and ¹²C) were combined on an equal protein basis. Proteins were separated on 13% gels by SDS-PAGE (29, 30).

Immunodetection and LC-MS/MS Analyses of Proteins—Immunodetection was carried out as described previously (31). Antibodies directed against PsaD were produced using the following two peptides for immunization of rabbits: peptide 1, EEAEAAPAAAKKAAEK; peptide 2, EAKKEQIFEMPFGA. Antibody production was managed by Eurogentec (Belgium). The antibodies were used in a 1:1000 dilution. The other antibodies used have been described before (4). In gel tryptic digestion was carried out essentially as described previously (31), with acetone as the organic phase. Antisera obtained from these antibody producing and chromatographing procedures were done essentially as described previously (11). The following changes were made. Samples were first injected onto a μ-Precolm (PepMap™ C18, 5 μm, 100 Å, 5 mm; LC-Packing, Sunnyvale, CA) and washed for 4 min at a flow rate of 0.25 μl/min with the aqueous phase by a nano-column switching device (Switchos, LC-Packing) that directed the flow to the analytical column. A fused silica needle with 8-μm aperture (FS360-75-8-N-5-C12; New Objective, Woburn, MA) was used for ionization of peptides. Mass spectra were measured with an LQD Deca XP Plus ion trap mass spectrometer (Thermo Electron, San Jose, CA). Acquisition of mass spectra and sequence identification using Sequest software (Thermo Electron, San Jose, California) were done as described previously (11). The location of the ¹³C-Arg label was taken into account (+6 Da). For quantitative analysis, doubly charged sister peptides were chosen. Tandem mass spectra were gathered within a m/z range so that both sister peptides would be fragmented concurrently. A wide enough range (7–10 m/z) was chosen so that all isotope peaks were acquired. This was important because we observed a broadening of the isotope profile for the ¹³C-labeled samples, which was probably because of metabolism of ¹³C-Arg and incorporation of ¹³C into other amino acids. Peak areas for the LC-MS/MS elution profiles of ¹³C- and ¹²C-labeled singly or doubly charged y ions were then determined using Qualbrowser software (Thermo Electron, San Jose, CA). The abundance ratios were calculated from the peak areas. Ratios were calculated for all identified y ion pairs, and the mean ± S.D. was calculated. Because ratios calculated from the LC-MS/MS data differed from those observed from immunoblot analyses, the peak areas from the LC-MS/MS analyses were normalized to the immunoblot signals for Lhca3, PsaD, and CF1.

Fluorescence Emission Analysis—Low temperature fluorescence emission spectra were recorded on whole cells with a Fluorolog-3 (Horiba, Jobin Yvon Inc., Edison, NJ) spectrophotometer. Data were normalized to the emission peak at 685 nm.

RESULTS

Identification of a New Lhca Protein by Two-dimensional Gel Electrophoresis and MS/MS—In a recent investigation, immuno blotting in combination with two-dimensional gel electrophoresis was used to detect a new light-harvesting complex protein that is specific for iron-deficient growth conditions (4).

The protein spot containing this novel Lhca protein had been shown to increase in abundance during the adaptation to iron-deficient growth conditions in thylakoid membrane preparations (Fig. 1A). Analysis of a tryptic digest of this spot by MS/MS identified the protein as Lhca3 based on the fragmentation pattern of the two doubly charged ions, m/z 701.8 and 1015.8, that were matched with the Lhca3 peptide sequences WILQYSEVIHAR and GSGDAAYPGPFFNLPGK, respectively, using Sequest software. The fragmentation pattern of the peptide with a m/z 701.8 is shown in Fig. 1B.

The New Lhca3 Protein Is a Result of N-terminal Processing—Immunodetection of higher molecular weight form of Lhca3 using an antibody specific for its N terminus (anti-14.1 (31)) showed that this form nearly completely disappeared in thylakoids isolated from iron-deficient growth conditions (4) (Fig. 2A). Because the lower molecular weight form of Lhca3 was no longer detected by the N-terminal specific antibody, it must be the product of a processing event at the N terminus of the protein. The protein spot corresponding to the processed form of Lhca3
has an isoelectric point (pI) of about 5.0 and is slightly more acidic as compared with the mature protein (pI 5.1) (4). Calculation of its molecular mass from SDS-PAGE analysis, using known masses for Lhca1, Lhca3, Lhca4, Lhca5, and PsaF yielded a value of about 23,800 Da. Molecular masses and pI of processed forms, assuming putative processing events within the N-terminal part, were calculated by using the computation pI/MW tool on the Expasy website (us.expasy.org/tools/pi_tool.html). This analysis revealed that processing between DR (closest to the N terminus, between positions 9 and 10) and DG (most distant from N terminus, positions 27 and 28) defined the region where processing occurs, because only processing in-between these sites would produce a product having a pI value more acidic as compared with the mature protein and an appropriate molecular mass in respect to the mass estimated for the processed form. By taking the molecular mass, the fact that the first 15 N-terminal amino acids were used for immunization (EEKSIAKVDRSKDQL), and the pI of the processed form into account, we suggested that the novel N terminus starts at amino acid 16 (YVGASQ . . . ) or amino acid 17 (VGASQ . . . ).

N-terminal Processing of Lhca3 Correlates with the Impaired Efficiency of Excitation Energy Transfer from LHCl to PSI—Adaptation of Chlamydomonas to iron deficiency leads to a decreased efficiency of excitation energy transfer between LHCl and PSI (4). To determine whether the impact on efficiency correlates with induction of the N-terminal processing of

**Fig. 3.** Comparative quantitative mass spectrometric peptide profiling using isotopic labeling of proteins demonstrates proteolytic processing of Lhca3. Thylakoids isolated from $^{13}$C$_6$-Arg-labeled cells (+Fe) and $^{13}$C$_7$-Arg cells (5 days –Fe) were mixed on equal protein basis and separated by SDS-PAGE. Protein bands corresponding to the position of the mature Lhca3 proteins and the putative processed form were excised, and the proteins were digested with trypsin and analyzed by LC-MS/MS. Pairs of $^{13}$C$_6$-Arg- and $^{13}$C$_7$-Arg-labeled sister peptide ions were measured in the same MS/MS spectrum by choosing a respective parent ion for collision-induced fragmentation and a mass window of ± 5 Da. Resolution of the $y_7$ H$^+$ ion derived from the MS/MS fragmentation of a doubly charged Lhca3 peptide ion WLQYSEVIHAR using the data filter 817.5–819.7 ($^{13}$C$_6$-Arg/Fe$^+$) and 811.5–813.7 ($^{13}$C$_7$-Arg/Fe$^+$) shows that the Fe$^+$ peptide is present in the higher molecular weight band in iron-sufficient conditions but absent from the lower molecular weight band, whereas the reverse is true for the Fe$^-$ peptide, demonstrating that only the processed form of Lhca3 is present under iron-deficient conditions. Resolution of the doubly charged $y_7$ ions of the D2 peptide NILLNEGIR shows nearly equal protein loading of the thylakoids isolated from iron-sufficient and -deficient conditions.
Iron Deficiency Induces N-terminal Processing of Lhca3

In Chlamydomonas, we measured low temperature fluorescence emission spectra from cells grown under iron-sufficient conditions and then shifted to iron-deficient growth conditions for 1–5 days (Fig. 1C). Fluorescence peaks at 685 and 711 nm obtained with whole cells grown under iron-sufficient conditions are characteristic of LHCII attached to PSII and LHCI attached to PSI, respectively (32). In the absence of PSI or in case of functional impairment of excitation energy transfer between LHCI and PSI, the LHCI fluorescence emission is shifted toward 700–705 nm (32, 33). Most interestingly, the low temperature fluorescence emission maximum from iron-replete cells at 711 nm changes to 704 nm after 2 days of iron deficiency (Fig. 1C). Concomitant with the “blue shift” is an increase of the fluorescence emission as normalized to emission at 685 nm, indicating that excitation energy transfer between LHCI and PSI was strongly impaired. In the subsequent days, the fluorescence emission maximum remained “blue-shifted,” and the extent of fluorescence decreased toward day 5. A significant portion of the processed product of Lhca3 was already detectable after 2 days (Fig. 1C) and the extent of fluorescence decreased toward day 5. A significant portion of the processed product of Lhca3 was already detectable after 2 days (Fig. 1C) and the extent of fluorescence decreased toward day 5.

TABLE I

Relative abundance of proteins from the LHCI and the PSI core complex, the LHCII and the PSII core complex, and proteins from the ATP synthase (Fig. 3) isolated from thylakoids from cells grown in iron-deficient and iron-sufficient conditions, respectively.

| Protein | Peptide | Band | Ratio Fe/Fe<sup>a</sup> | S.D. | n |
|---------|---------|------|------------------------|------|---|
| Lhca1   | FTESEVIHGR | 12   | 0.39                   | 0.08 | 7 |
| Lhca3   | WLQYSEVIHAR | 9a   | 0.02                   | 0.01 | 8 |
| Lhca3   | WLQYSEVIHAR | 10   | 0.17                   | 0.05 | 6 |
| Lhca3   | WLQYSEVIHAR | 10a  | 10.96                  | 5.26 | 6 |
| Lhca4   | WYAQAEMLNAR | 9    | 1.13                   | 0.26 | 4 |
| Lhca5   | QSELQHAR   | 9a   | 0.03                   | 0.03 | 3 |
| Lhca7   | FFDPMLGSLR | 11   | 0.31                   | 0.26 | 4 |
|         | WYVQAELVHGR | 11   | <0.01                  | <0.01| 3 |
| Lhca8   | WYQOAELHCR | 11   | 0.07                   | 0.03 | 2 |
| Lhca9   | GALAGDNGFDPLGLGQDEGR | 12 | 1.69 | 0.67 | 8 |
| PsaA    | DYDPTNNYNLLDR | 3  | 0.45                   | 0.14 | 4 |
| PsaB    | DKPVALSIVQAR | 3   | 0.34                   | 0.03 | 3 |
| PsaD    | EQFEMPTGGAAIMR | 13 | 0.55                   | 0.20 | 4 |
| PsaF    | EENITVSPR  | 13   | 0.38                   | 0.00 | 3 |
| Lhebm1–10 | ELELI  | 10   | 0.90                   | 0.19 | 9 |
| D1      | VLNVTWADIN | 6    | 0.83                   | 0.12 | 4 |
| CP43    | SPTGEIFGGETMR | 4  | 1.08                   | 0.21 | 4 |
| AtpA    | AFSQGLAQDPTTR | 4  | 0.76                   | 0.16 | 4 |
| AtpB    | DYNQKDVLFINDNIFR | 3c | 1.01 | 0.01 | 4 |

<sup>a</sup> Ratios were normalized with immunoblot data (Fig. 2).

<sup>b</sup> S.D. was calculated for different y ions, and for some peptides y-type ions from up to five independent measurements are presented.

The Lhca Proteins Are Affected to Varying Degrees by Iron Deficiency—We took advantage of arginine auxotrophic Chlamydomonas cells, which were grown in the presence of L-Arg in iron-replete conditions and transferred for 5 days to iron-deficient conditions. In parallel, the auxotrophic cells were grown under iron-sufficient conditions in the presence of isotopically labeled L-Arg, harboring six 13C atoms. Chloroplasts were isolated from both conditions and separated into thylakoid and envelope membranes and stroma proteins. Thylakoid samples were mixed on an equal protein basis and separated by SDS-PAGE (Fig. 3). Protein bands as indicated in Fig. 3 were excised, digested with trypsin, and analyzed by LC-MS/MS. In a first round of analyses, sequence identity and the presence of an Arg residue in the tryptic peptide was established by using the Sequest algorithm. In a second round of analyses, Arg-containing peptides were analyzed quantitatively. The selected peptide ion pairs ([13C<sub>6</sub>-Arg]<sup>+</sup>Fe) and [12C<sub>6</sub>-Arg]<sup>+</sup>Fe) were fragmented concurrently (Fig. 2B). In the MS/MS spectrum, singly charged y ions can be distinguished by a mass difference of 6 or 3 Da in the case of doubly charged ions. These different y ions originating from the sister peptide ions were used for quantification by comparing the total ion count peak area of respective labeled and unlabeled fragment ions (Table I). Quantification using fragment ions rather than parent ions reduces background signals from unrelated peptide ions significantly, and in most cases nearly completely. The abundance of the peptides was based on the peak areas for several fragment ions. We also performed quantitative immunoblot analysis of thylakoid membranes isolated from iron-sufficient and 5-day iron-deficient cells using anti-Lhca3, anti-PsaD, and anti-CF1 antibodies (Fig. 2A). These analyses showed that the mature forms of Lhca3 and PsaD were down to 5–10% and 20–50%, respectively, whereas the ATPase was unaffected as reported before.
More importantly, we used these immunoblot data to normalize the LC-MS/MS data for isotopically labeled peptides. By using the comparative quantitative assay, we investigated the N-terminal processing of Lhca3 (Fig. 3). Resolution of a singly charged y7 ion, derived from the MS/MS fragmentation of the Lhca3 peptide ion WLQYSEVIHAR[2H/H11001], using the m/z filters 817.5–819.7 (for the labeled ion) and 811.5–813.7 (for the unlabeled ion), showed that the labeled peptide was present in the higher molecular weight band in iron-sufficient conditions but was absent from the lower molecular weight band, whereas the reverse is true for the unlabeled peptide. These data demonstrated that the processed form of Lhca3 was dominant under iron-deficient conditions, whereas the mature form was largely diminished under the same conditions. Resolution of the doubly charged y6 fragment ion from the MS/MS fragmentation of the peptide NILLNEGIR[2H/H11001], originating from the PSII core subunit D2, showed nearly equal protein loading of the thylakoids isolated from iron-sufficient and -deficient conditions. Analysis of other y-type ions resulting from fragmentation of the Lhca3 or D2 peptide ions showed the same results (Table I). Consistent with the data for D2, analysis of CP43 peptides ions clearly indicated that PSII was not affected after 5 days of iron deficiency; however, the levels of D1 were slightly decreased with respect to iron-sufficient conditions. The ATPase subunits (AtpA and AtpB), as well as the major light-harvesting complex proteins Lhcbm (Table I) (all of which were used as loading controls), do not change significantly between iron-replete and -deficient conditions. Analysis of the PSI subunits PsaA, PsaB, PsaD, and PsaF showed that after 5 days in iron deficiency the PSI complex was down to about 45% with respect to iron-replete conditions (Table I). The impact of iron deficiency on LCHI was more dramatic. Besides the N-terminal processing of Lhca3, Lhca5 almost completely disappeared, and the amounts of Lhca1, Lhca7, and Lhca8 were reduced, whereas the Lhca4 and Lhca9 polypeptides were induced under iron-deficient conditions in respect to PSI (Table I). We confirmed the down-regulation of Lhca5 by immunoblotting using Lhca5-specific antibodies (31) (data not shown). We also detected peptides of Lhca2 and Lhca6 by MS/MS, but did not obtain adequate resolution of fragment ions for quantification.

The N-terminal Processed Form of Lhca3 Remains with the PSI Complex When Isolated from Iron-deficient Conditions—To evaluate whether the processed form of Lhca3 remained with PSI after the onset of iron deficiency and to define the LHCI composition under these conditions, we isolated PSI particles from arginine auxotrophic cells that were grown for 5 days.
under iron deficiency in the presence of $^{13}$C$_6$-Arg. In parallel, we isolated PSI particles from cells of the same strain that were grown under iron-sufficient conditions after sucrose density centrifugation together with PSI particles (confirmed by mass spectrometer analyses, see the presence of D1 and D2 PSI core subunits in iron-deficient PSI preparation in Fig. 4). PSI particles isolated from iron-deficient conditions had a higher chlorophyll $a/b$ ratio (9.3) as compared with iron-sufficient conditions (5.5). PSI particles from both fractions were mixed on an equal protein basis and fractionated by SDS-PAGE. Protein bands corresponding to the mature and processed form of Lhca3 were excised from the gel and digested in-gel with trypsin. The corresponding peptides were analyzed by LC-MS/MS. Resolution of the $y$$_2$ H$^+$ ion, from Lhca3 peptide ion WLQYSEVIHGR$^{2H^+}$, showed that the $^{13}$C$_6$-Arg/Fe$^+$/Fe$^-$ peptide is present in the higher molecular weight band in iron-sufficient conditions but absent from the lower molecular weight band, whereas the contrary was true for the $^{12}$C$_6$-Arg/Fe$^+$/Fe$^-$ peptide. These data demonstrated that the processed form of Lhca3 can be isolated with the PSI complex under iron-deficient conditions. In contrast the mature form of Lhca3 was significantly reduced under the same conditions as expected from the results obtained with thylakoids. The PSI core subunits isolated from iron-deficient conditions were slightly enriched compared with the PSI core complex isolated under iron-sufficient samples. Analysis of the PSI subunits Lhca4, Lhca5, and Lhca9 showed that the PSI core complex from iron-deficient conditions was about 1.6-fold more abundant than the one isolated from iron-sufficient conditions (Table II). The antenna proteins Lhca1, Lhca7, and Lhca8 were equally reduced with respect to iron-sufficient conditions. On the contrary, levels of Lhca4 and Lhca9 were significantly increased, which is consistent with the results obtained with whole thylakoids (Tables I and II). We did not obtain quantitative data for Lhca2 and Lhca6. When the relative abundance of Lhca polypeptides versus PSI subunits was calculated from ratios obtained from iron-deficient and iron-sufficient thylakoids and isolated PSI particles (Tables I and II), Lhca1 decreased from 0.86 in thylakoids to 0.38 in purified particles; the mature form of Lhca3 increased from 0.04 to 0.12; Lhca4 decreased from 2.53 to 0.79; Lhca5 remained low; Lhca7 slightly increased from 0.34 to 0.44; Lhca8 increased from 0.15–0.40, and Lhca9 decreased from 3.78 to 0.89 (Table III). The relative loss of Lhca1, Lhca4, and Lhca9 in PSI particles versus thylakoids could be explained by the fact that these proteins were lost during the purification procedure. Nevertheless, it is interesting to note that the relative abundance of Lhca1, Lhca7, and Lhca8 are the same and that Lhca4 and Lhca9 proteins are 2–3-fold higher as compared with the other three polypeptides per iron-deficient PSI. The same holds true for the processed form of Lhca3 that is significantly enriched in PSI particles isolated from iron-deficient conditions. The functional implication of the N-terminal processing of Lhca3 on the remodeling process of PSI-LHCI was further tested by deletion of Lhca3 using an RNAi strategy.

Suppression of Lhca3 by RNAi Technology Impairs Excitation Energy Transfer between LHCI and PSI and Abolishes Binding between LHCI and PSI—Using a tandem inverted repeat system for selection of effective transgenic RNAi strains in Chlamydomonas (25), we generated mutant strains with significantly reduced levels of Lhca3 protein expression. For construction of the RNAi expression cassette, we used 216 bp of the 3’-untranslated region of the lha3 gene. Low temperature fluorescence emission spectroscopy (Fig. 5A) using whole cells of a strain that contained less than 10% of Lhca3, as assessed by immunoblot analysis (see Fig. 5B), showed a blue-shifted fluorescence emission maximum at 704 nm and exhibited a higher level of maximal fluorescence as compared with wild type. These observed changes in the low temperature fluorescence spectrum between the Lhca3-depleted mutant and wild type were very similar to spectral changes obtained with iron-deficient wild-type (Fig. 1), indicating that in terms of excitation energy transfer, LHCI and PSI are largely disconnected in the absence of Lhca3. Solubilization of Lhca3-depleted thylakoid membranes with detergent and fractionation by sucrose density centrifugation resulted, in contrast to wild type, in PSI particles that contained different amounts of Lhca3 with respect to the PSI core (as assessed by PsAD immunoblot analysis, see Fig. 5C). The lower sucrose density

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**Table II**

| Protein | Peptide          | Band   | Ratio Fe$^+$/Fe$^-$ | S.D. | n  |
|---------|------------------|--------|---------------------|------|----|
| Lhca3   | WLQYSEVIHGR      | 26     | 0.62                | 0.04 | 3  |
| Lhca5   | QSELQHAR         | 21     | 0.13                | 0.07 | 6  |
| Lhca7   | WYYQAEILVHGR     | 23     | 0.72                | 0.15 | 8  |
| Lhca8   | WYYQAEILHCR      | 23     | 0.66                | 0.34 | 12 |
| Lhca9   | GALADNGFDPLGLGQDEGR | 25,26 | 1.45                | 0.45 | 22 |
| PsaA    | DTYQNTNYYNLDR    | 6      | 1.30                | 0.81 | 4  |
| PsaB    | DKPVALSIVQR      | 5,6    | 1.33                | 0.65 | 8  |
| PsaD    | EQIFEMPTGGAAIMR  | 28     | 1.73                | 0.45 | 19 |
| PsaF    | LAWQAGGWLAAVQELQR| 28     | 1.38                | 0.33 | 8  |

**Table III**

| Protein | Ratio subunit/PSI core |
|---------|------------------------|
|         | Thylakoids      | Purified PSI particles |
| Lhca1   | 0.9                | 0.40                   |
| Lhca3   | 0.04               | 0.10                   |
| Lhca3 N-terminal processed | 2.50 | 5.00 |
| Lhca4   | 0.80               |                        |
| Lhca5   | 0.07               | 0.08                   |
| Lhca7   | 0.34               | 0.45                   |
| Lhca8   | 0.15               | 0.40                   |
| Lhca9   | 3.8                | 0.90                   |
fraction 1 contained about 40% and the lighter sucrose fraction 2 less than 10% of the wild-type Lhca3 levels (Fig. 5C). Most interestingly, PSI particles that are strongly depleted in Lhca3 (fraction 2) were also depleted in PsaK (Fig. 5D). To assess the LHCI polypeptide composition of the Lhca3-depleted cells with respect to wild type, we isolated thylakoids and PSI particles. The isolated membranes and particles were mixed on an equal protein concentration with corresponding material isolated from wild-type cells grown in the presence of isotopically labeled arginine and separated by SDS-PAGE. Protein bands containing LHCI and PSI polypeptides were excised, digested with trypsin, and analyzed by LC-MS/MS for quantitative peptide profiling. By using this technique, we were able to generate comparative quantitative data for PsaA and PsaB as well as Lhca1, Lhca3, Lhca4, Lhca5, Lhca7, Lhca8, and Lhca9 polypeptides (Tables IV and V). In thylakoid membranes, the Lhca subunit to PSI core ratios were about 1 and 0.9 for Lhca7 and Lhca5, respectively, slightly decreased for Lhca1 and Lhca4, increased for Lhca9, and strongly diminished for Lhca3, as expected from the immunoblot results (Fig. 5B). These data showed that stable accumulation of Lhca1, Lhca4, Lhca5, Lhca7, Lhca8, and Lhca9 polypeptides was not severely affected by the absence of Lhca3. The relative Lhca3 polypeptide to PSI core content in isolated Lhca3-depleted versus wild-type...
Iron Deficiency Induces N-terminal Processing of Lhca3

TABLE IV
Relative abundance of proteins from the LHCI and the PSI core complex isolated from thylakoids and isolated PSI particles from wild type cells (13C6-Arg-labeled cells) and cells of an Lhca3-depleted RNAi strain (strain B, Fig. 5, 12C6-Arg cells)

| Protein | Peptide | Ratio IRLhca3/WT | S.D. | n |
|---------|---------|------------------|------|---|
| Lhca1   | FTSEVHHR | 0.59             | 0.14 | 9 |
| Lhca3   | WLYSEVHHR | 0.14             | 0.03 | 7 |
| Lhca4   | WYAQELMNAR | 0.58             | 0.26 | 4 |
| Lhca5   | QSELQRHAR | 0.91             | 0.38 | 3 |
| Lhca7   | FFDPMGLSR | 1.37             | 0.64 | 4 |
| Lhca9   | WYQAELVHHR | 0.62             | 0.44 | 14 |
| PsaA    | EIPLHDLLLNR | 1.95             | 0.48 | 5 |
| PsaB    | FSQGLAQDPTTR | 0.83             | 0.25 | 8 |

TABLE V
Differential ratios for LHCI proteins and the PSI core derived from thylakoids and isolated PSI particles from wild type cells and cells of an Lhca3-depleted RNAi strain (strain B, Fig. 5)

| Protein | Peptide | Ratio subunit/PSI core | PSI particles, fraction 1 | PSI particles, fraction 2 |
|---------|---------|------------------------|--------------------------|--------------------------|
| Lhca1   | FTSEVHHR | 0.33                   | 0.12                     | 16 |
| Lhca3   | WLYSEVHHR | 0.15                   | 0.04                     | 13 |
| Lhca4   | WYAQELMNAR | 0.39                   | 0.16                     | 3 |
| Lhca5   | QSELQRHAR | 0.60                   | 0.09                     | 3 |
| Lhca7   | WYQAELVHHR | 0.20                   | 0.10                     | 8 |
| Lhca8   | WYQAELHHR | 0.57                   | 0.27                     | 3 |
| Lhca9   | GALAGDFDPLGLGQDEGR | 1.22                | 0.36                     | 10 |
| PsaA    | EIPLHDLLLNR | 1.60                   | 0.21                     | 3 |
| PsaB    | FSQGLAQDPTTR | 2.73                   | 0.95                     | 5 |

PSI declined to 0.3 and 0.07 in sucrose gradient fractions 1 and 2 (Fig. 5C), respectively. Strong depletion of Lhca3 (fraction 2) suppressed the accumulation of Lhca1, Lhca4, Lhca5, Lhca7, and Lhca8 with PSI below 30% in respect to wild type, suggesting that their binding to PSI was destabilized in the absence of Lhca3 (Fig. 6). In contrast, the ratio of Lhca9 to PSI appeared to be less affected by Lhca3 protein levels, indicating that Lhca9 binding to PSI might be more independent of Lhca3.

DISCUSSION

Adaptation to iron deficiency requires remodeling of the photosynthetic apparatus in Chlamydomonas (4). Our study reveals that a key mechanistic event of the remodeling process that affects PSI and its associated LHCI complex is the N-terminal processing of Lhca3. The induction of N-terminal processing of Lhca3 by progression of iron deficiency has been shown independently by 2-DE and mass spectrometric analysis and quantitative MS/MS peptide profiling using isotopic labeling of proteins. We further demonstrate that the remodeling of the LHCI complex under iron deficiency involves up-regulation of Lhca4 and Lhca9 polypeptides and down-regulation of Lhca5 in respect to PSI, which implies specific functional roles for these proteins. More importantly, the induction of N-terminal processing of Lhca3 by progression of iron deficiency correlates with the functional drop of excitation energy transfer efficiency between LHCI and PSI as assessed by low temperature fluorescence emission spectroscopy (Fig. 1). Most interestingly, depletion of Lhca3 by an RNAi approach also strongly impacts the efficiency of excitation energy transfer between PSI and LHCI (Fig. 5). However, in contrast to iron deficiency, depletion of Lhca3 abolishes stable binding of most Lhca proteins to PSI.

The recent crystal structure of PSI to 4.4 Å resolution from Pisum sativum (34) indicates that LHCI of higher plants forms a crescent structure that associates with the reaction center primarily through interactions between Lhca1 and the PsaG pole of PSI as well as through weaker interactions (mostly on the stromal side) between Lhca3 and the PsaK pole. The partners participating in the weaker of the interactions (PsaK/Lhca3) are primarily affected during iron deficiency in Chlamydomonas (4). N-terminal processing of Lhca3 could initiate the remodeling process and may also readily impair the excitation energy transfer between the antenna and the reaction center. In vascular plants as well as in Chlamydomonas, the major contributor to far red fluorescence is the LHCI complex, which contains chlorophylls that absorb at 710 nm and emit at 730 nm in vascular plants (~710 nm in Chlamydomonas (32, 33, 35)). These low energy forms have a large impact on energy transfer, and most of the excitation energy passes through them, via thermal activation to bulk chlorophylls, on the way to P700 (36, 37). Thermal transfer of energy may occur at the Lhca3/PsaK pole through the densely packed chlorophylls that are visible there in the crystal structure. The structural
changes that occur may hamper this energy transfer. In vascular plants, Lhca3 contributes significantly to the most far red emissions (38, 39). In *Chlamydomonas* depletion of Lhca3 also causes a blue shift of the maximal low temperature fluorescence emission. However, in contrast to vascular plants (40), we show here that Lhca3 appears to be required for stable binding of Lhca1, Lhca4, Lhca5, Lhca7, and Lhca8 to PSI. Therefore, the fluorescence emission in the Lhca3-depleted strain may originate mainly from unassembled LHCI as observed in a PSI-deficient mutant (33). In light of the pea PSI crystal structure (34), it is surprising that depletion of Lhca3 has such a strong impact on binding of Lhca polypeptides to PSI. However, in contrast to vascular plants, LHCI can be isolated in the absence of PSI as a stable oligomeric complex from *Chlamydomonas* (33). Most interestingly Lhca2, Lhca3, and Lhca9 are not required for the stable oligomeric structure of the LHCI complex (33). It was suggested that association of these polypeptides with the LHCI complex is stabilized by the presence of the PSI core complex and/or promote formation of the PSI-LHCI complex. In line with this, we propose that Lhca3 functions as a linker to support formation of the PSI-LHCI supercomplex in *Chlamydomonas*.

It is interesting to note that binding of Lhca9 to PSI is less dependent on Lhca3. Lhca9 even seems to be induced by the absence of Lhca3, a behavior that has been also observed for Lhca5 in the absence of Lhca4 in *Arabidopsis* (41). As Lhca5 from *Arabidopsis*, Lhca9 from *Chlamydomonas* is, with respect to the other Lhca polypeptides, a rather low abundant protein (11). In contrast to Lhca3-depleted PSI particles, the levels of Lhca1, Lhca4, Lhca7, and Lhca8 are higher in the iron-deficient particles and do not correlate with levels of mature Lhca3. This indicates that the presence of the processed form of Lhca3 is sufficient to promote more stable binding between these Lhca polypeptides and PSI, which seems to be particularly important for the elevated accumulation of Lhca4 under iron deficiency. On the contrary, the level of Lhca5 to PSI ratio is lower under iron deficiency as compared with the ratio in Lhca3-depleted PSI particles, pointing to additional regulatory mechanisms that affect levels of Lhca5 accumulation under iron deficiency.

The fact that the processed form of Lhca3 remains with PSI is an important finding, because it strongly suggests that the processing of Lhca3 occurs at the level of the functionally assembled LHCI/PSI complex, which in turn implicates that the N-terminal processing of Lhca3 is a regulatory process that is catalyzed by a site-specific protease. It was shown recently (42) that de-epoxidation of violaxanthin to zeaxanthin is specifically associated with Lhca3. It was postulated that this conversion may function in scavenging reactive oxygen species. Therefore, the processed form of Lhca3 might stay with the PSI complex to fulfill this function.

From the crystal structure, the helix nearest the N terminus (helix B) of Lhca3 appears to project into the stroma and away from any other proteins present in the structure. Sequence alignments indicate that this unusually long helix B has been conserved throughout evolution (not shown). The excised N-terminal epitope contains eight charged amino acids, which likely serves as an electrostatic protein-protein interaction module for additional Lhca present in the *Chlamydomonas* PSI-LHCI supercomplex. Removal of this N-terminal domain could likely alter protein-protein interactions within LHCI, which thereby facilitates remodeling of the complex.

Besides, N-terminal processing of Lhca3, the down-regulation of Lhca5 and the up-regulation of Lhca4 and Lhca9 on protein levels in respect to PSI, are also major changes induced by iron deficiency. Lhca4 and Lhca5 are close homologues (11). It is tempting to speculate that the increase in Lhca4 and/or Lhca9 compensates for the loss of Lhca5 under iron deficiency. This may implicate that Lhca4 and Lhca9 are different in function with respect to Lhca5. Both proteins could be more efficient in energy dissipation than in light-harvesting, a hypothesis that can be tested in the future. It is interesting to note that both Lhca4 and Lhca9, but not Lhca5, possess an asparagine rather than a histidine residue at chlorophyll position a5. The presence of an asparagine residue at this position has been shown to be responsible for far red fluorescence in higher plants (39). Providing that there are no bulk chlorophylls connecting Lhca4 and Lhca9 and PSI, these proteins may act as sinks for dissipation of light energy.

In summary, we suggest that the N-terminal processing of Lhca3 induces a conformational change that triggers (i) the remodeling of LHCI and (ii) a decrease in the efficiency of excitation energy transfer from the antenna to the PSI reaction center. By comparing iron-deficient cells with those depleted in Lhca3 by using RNAi, we demonstrate here that the truncated form of Lhca3 must act as a keystone to stabilize the interaction of LHCI with PSI because lack of Lhca3 abolishes the interactions of other Lhca with PSI. Under this scenario, truncation of Lhca3 alters the structure of LHCI, and concurrently modulates the functional interaction of LHCI with PSI but does not abolish the physical link between LHCI and PSI. We also speculate that the loss of Psak because of the onset of iron deficiency (4, 43) may be responsible for the enhanced susceptibility of Lhca3 to proteolytic processing, which in turn initiates the entire process.

The comparative quantitative proteomic approach using isotopic labeling of proteins by labeled arginine has proven to be a very valuable tool. It is foreseeable that this approach will be attractive for studies in *Chlamydomonas* where physiological changes are related to dynamic comparative alterations in the proteome.
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N-terminal Processing of Lhca3 Is a Key Step in Remodeling of the Photosystem I-Light-harvesting Complex Under Iron Deficiency in *Chlamydomonas reinhardtii*  
Bianca Naumann, Einar J. Stauber, Andreas Busch, Frederik Sommer and Michael Hippler

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