NAB1 Is an RNA Binding Protein Involved in the Light-Regulated Differential Expression of the Light-Harvesting Antenna of Chlamydomonas reinhardtii

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Photosynthetic organisms respond to changes in ambient light by modulating the size and composition of their light-harvesting complexes, which in the case of the green alga Chlamydomonas reinhardtii consists of >15 members of a large extended family of chlorophyll binding subunits. How their expression is coordinated is unclear. Here, we describe the analysis of an insertion mutant, state transitions mutant 3 (stm3), which we show has increased levels of LHCBM subunits associated with the light-harvesting antenna of photosystem II. The mutated nuclear gene in stm3 encodes the RNA binding protein NAB1 (for putative nucleic acid binding protein). In vitro and in vivo RNA binding and protein expression studies have confirmed that NAB1 differentially binds to LHCBM mRNA in a subpolysomal high molecular weight RNA–protein complex. Binding of NAB1 stabilizes LHCBM mRNA at the preinitiation level via sequestration and thereby represses translation. The specificity and affinity of binding are determined by an RNA sequence motif similar to that used by the Xenopus laevis translation repressor FRGY2, which is conserved to varying degrees in the LHCBM gene family. We conclude from our results that NAB1 plays an important role in controlling the expression of the light-harvesting antenna of photosystem II at the posttranscriptional level. The similarity of NAB1 and FRGY2 of Xenopus implies the existence of similar RNA-masking systems in animals and plants.

INTRODUCTION

In oxygenic photosynthesis, light energy is converted to chemical energy through the cooperation of two photosystems, photosystem I (PSI) and photosystem II (PSII). To compensate for changes in light intensity or spectral quality, plants and green algae have developed several short-term and long-term mechanisms to regulate the amount of light that is captured by each photosystem (Allen, 1992; Finazzi et al., 1999, 2001). One long-term strategy that plants use to compensate for changes in light quality and quantity is to regulate the expression of the nucleic-encoded LHC gene family, which encodes the light-harvesting chlorophyll binding proteins (LHCII) of PSII (Escoubas et al., 1995; Durnford and Falkowski, 1997; Yang et al., 2001). For instance, the size of light antenna systems in algae and plants increases under low irradiance to enhance the capture of photons by PSI and PSII, but it is reduced under high irradiances to prevent overexcitation of the photosystems and so avoid potential photooxidative damage (Anderson and Kay, 1995).

Regulation of LHCII expression is known to occur at many levels, including transcription initiation (Maxwell et al., 1995; Millar et al., 1995) and posttranscriptionally (Flachmann and Kühlbrandt, 1995; Lindahl et al., 1995; Durnford et al., 2003). As yet, little is known about these processes at the molecular level, especially translational control.

The LHCII proteins in the green alga Chlamydomonas reinhardtii are homologous with those found in higher plants (Teramoto et al., 2002). EST sequence analyses have revealed the existence of nine expressed LHCII isoforms (denoted LHCBM1 to LHCBM6, LHCBM8, LHCBM9, and LHCBM11 [Elrad and Grossman, 2004]), of which eight have now been identified at the protein level (Stauber et al., 2003). Although the LHCII proteins are structurally very similar, it is likely that individual gene products might have specific functions (Elrad et al., 2002).

Recently, we (Kruse et al., 1999) and others (Fleischmann et al., 1999; Depege et al., 2003) have established rapid chlorophyll fluorescence-based plate assays to screen for mutants impaired in nonphotochemical and photochemical fluorescence...
quenching events, which include LHC state transitions (Bonaventura and Myers, 1969; Murata, 1969) and LHCII antenna size regulation. Here, we present the identification and characterization of the nuclear insertion mutant state transitions mutant3 (stm3) from C. reinhardtii, which was identified using such a screen. Our analyses of this mutant led to the identification of an RNA binding protein that regulates the expression of a specific set of LHCII subunits at the posttranscriptional level and have provided new insights into how C. reinhardtii is able to fine-tune the expression of its light-harvesting antenna.

RESULTS

Light Harvesting Is Perturbed in stm3

To identify nuclear genes involved in regulating light harvesting within C. reinhardtii, we screened a library of mutants generated after the random insertion of plasmid pSP124S, which contains the BLE gene conferring phleomycin resistance (Stevens et al., 1996; Lumbreras et al., 1998), into the nuclear genome. A plate-based fluorescence video-imaging screen, which involves the recording of chlorophyll fluorescence from individual colonies before and after changing the relative excitation of PSI and PSII (Kruse et al., 1999; Schönfeld et al., 2004). One such mutant, stm3, was subsequently confirmed to emit an unusually high level of chlorophyll fluorescence from the light-harvesting complexes (LHCII) associated with PSII (Figure 1A), despite the fact that PSII in stm3 was as active as in the wild type, as assessed by chlorophyll fluorescence parameters (maximum photochemical efficiency of PSII in the dark-adapted state $F_{v}/F_{m}$ = 0.79 in the wild type and 0.78 in stm3).

stm3 Contains a Single-Copy Insert and Its Phenotype Cosegregates with the BLE Cassette

DNA gel blot analysis confirmed that stm3 contained a single copy of the BLE gene inserted in the genome (Figure 1B). To test whether the BLE marker was linked with the stm3 phenotype, genetic crosses were performed between the wild type and stm3 and the progeny scored for phleomycin resistance and the inability to show normal fluorescence characteristics, as assessed by video imaging. All 53 examined phleomycin-sensitive progeny exhibited a wild-type fluorescence phenotype, whereas progeny showing phleomycin resistance also showed a high-fluorescence phenotype (data not shown). These data suggested that the high-fluorescence phenotype of stm3 was induced by the mutagenic event of plasmid DNA insertion.

Figure 1. Identification of stm3 by Fluorescence Spectroscopy and of the Nucleus-Encoded NAB1 Gene in C. reinhardtii.

(A) PSI chlorophyll a fluorescence snapshot (55 μmol m⁻²s⁻¹, 620 nm of actinic light) of wild-type and stm3 colonies on Tris-acetate-phosphate (TAP) agar plates in a fluorescence video imager. Colonies were preilluminated for 20 min with 480 nm of blue light to fully gain state 2 conditions.

(B) DNA gel blot hybridizations with wild-type and stm3 genomic DNA. Hybridizations were performed with a pSP124S-specific probe. Gray arrows indicate unspecific hybridization, and the black arrow indicates specific hybridization. M, marker.

(C) Model of the NAB1 gene. DNA sequences were identified in the stm3 genome by LMS-PCR (Strauss et al., 2001) and subsequent sequence analysis. Black boxes represent exons, and the arrow marks the plasmid pSP124S insertion site.

(D) Model of the NAB1 protein with an N-terminal CSD and a C-terminal RRM, and comparison with the Xenopus protein FRGY2. B/A, basic/aromatic region.

(E) DNA gel blot hybridizations with wild-type and stm3 genomic DNA. Hybridizations were performed with a radioactively labeled 738-bp NAB1-specific probe.

(F) Amino acid sequence of the CSD of NAB1, and alignment with the Xenopus protein FRGY2. Boxes represent β-helix structures, and arrows represent functionally important Phe residues.
The Gene Disrupted in stm3 Encodes a Putative Cytosolic RNA Binding Protein

Ligation-mediated suppression (LMS)-PCR (Strauss et al., 2001) was performed to analyze the plasmid insertion site and to clone the DNA flanking the BLE cassette in stm3. A plasmid fragment of ~2500 nucleotides, including the intact coding region of the BLE gene, was inserted into a putative open reading frame of the stm3 genome. A total of 1900 nucleotides flanking the inserted vector were sequenced. Subsequent sequence analysis led to the identification of a nuclear gene with six exons and five introns (Figure 1C) encoding a putative 26.54-kD protein with two predicted nucleic acid binding domains: a cold-shock domain (CSD) at the N terminus and an RNA recognition motif (RRM) at the C terminus (Figure 1D). The gene and corresponding protein were designated NAB1 (for putative nucleic acid binding protein) and submitted to the databases. DNA gel blot analysis confirmed that the NAB1 gene was disrupted in stm3 (Figure 1E).

CSDs were originally identified in connection with cold-shock phenotypes (Jones et al., 1987). However, it is now established that this motif generally reflects a nucleic acid binding domain (Graumann and Marahiel, 1998). Detailed analysis of the Chlamydomonas genome databases suggests that NAB1 is the only protein that contains this CSD motif.

Overall, there were sequence similarities between NAB1 and several RRM domain–carrying Gly-rich proteins with different nucleotide binding functions, including GBP1, which is reported to bind single-stranded G-strand telomere DNA in C. reinhardtii (Petracek et al., 1994).

Another protein with high N-terminal sequence similarity to NAB1 is FRGY2 from C. reinhardtii. FRGY2 contains a N-terminal CSD motif (65% identity to NAB1 in the 68–amino acid domain) and, like NAB1, a second RNA binding domain at the C terminus (Matsumoto et al., 1996) (Figures 1D and 1F). Furthermore, analysis of NAB1 predicts structural similarities to FRGY2, including the presence of five β-helix structures (framed in Figure 1F) and functionally important Phe residues (arrows in Figure 1F) in the C-terminal RNA binding domain. FRGY2 is reported to mask maternal mRNAs in Xenopus oocytes and to control mRNA expression by repressing translation (Matsumoto et al., 1996; Manival et al., 2001). Initial RNA–protein binding via the CSD is followed by unspecific binding, coordinated by the second C-terminal RNA binding domain (Matsumoto et al., 1996).

RNA gel blot analysis confirmed the disruption of NAB1 gene transcription (Figure 2A). To confirm that this disruption caused the phenotype in stm3, the mutant was transformed with a plasmid-borne intact wild-type copy of NAB1. A cotransformation approach using this pNAB1 vector in combination with a second vector containing the CRY1 gene conferring emetine resistance as a dominant selectable marker (Nelson et al., 1994) was applied. Of ~150 emetine-resistant colonies assessed, 4 were found to contain the NAB1 gene.

Detailed analysis of one of them, designated nc1, showed that an intact NAB1 gene had been incorporated into the genome (Figure 2B) and was expressed to wild-type levels, as judged by RT-PCR (Figure 2C) and immunoblot analysis (Figure 2F). Fluorescence video imaging confirmed that, in contrast with stm3,
nc1 performed normal state 2 activities in actinic light (Figure 2D) and showed wild-type levels of chlorophyll fluorescence, as judged by induction kinetics (the so-called Kautsky curve; Figure 2E). From these data and the fact that we could not detect any spontaneous reversion of stm3 when vector pNAB1 was omitted from the transformation experiment, we conclude that the disruption of NAB1 is solely responsible for these defects in light harvesting.

Highly specific antibodies raised against recombinant NAB1 protein were used in immunoblotting experiments (Figure 2F) to confirm the expression of NAB1 (predicted size of 26.5 kD) in both the wild type and the complemented strain nc1 but not in stm3. Immunogold labeling experiments (Figure 3) further indicated that NAB1 (visible in immunogold staining as black dots) was found in the cytosol (see zoomed sections b, c, and d) but not in the nucleus (section a), the mitochondria, or the chloroplast (section e). This result is consistent with the in silico analysis of the primary structure of NAB1, which suggested the absence of any N-terminal targeting sequence. Additionally, the location of NAB1 in the cytosol indicates that it is not involved in the binding of DNA, as described for a number of CSD proteins (Graumann and Marahiel, 1996).

**stm3 Cells Contain Highly Stacked Thylakoid Membranes and More Chlorophyll per Cell and Are Sensitive to High Light**

A striking feature of stm3 was a dark-green phenotype caused by an ∼50% greater total chlorophyll content per cell than in the wild type and nc1 cultures grown in standard white light (Figure 4A). The higher chlorophyll content in stm3 was accompanied by a decrease in the chlorophyll a/b ratio from 2.45 and 2.51 (wild type and nc1, respectively) to 2.21 (stm3). Both results were clear indications of an increased LHC antenna system in the mutant.

Because the size and structure of light-harvesting antennae have been shown to influence the formation of granal stacks in chloroplasts (Allen and Forsberg, 2001; Yang et al., 2001), electron microscopy was performed to assess whether the thylakoid membrane structure was altered in stm3. Images were taken from wild-type, nc1, and stm3 cells after cultivation to a log growth phase. The obtained images revealed remarkable differences in the arrangement of thylakoid membranes between the wild type, nc1, and mutant stm3, with the latter containing many more highly stacked granal regions (Figure 4A). This is in contrast with wild-type and nc1 cells, in which thylakoid membranes were generally loosely arranged with more free-floating stroma lamellae interrupted by a few pseudogranal structures (Figure 4A). stm3 also contained more starch, which is an indication of physiological stress (Grossman, 2000).

Growth experiments under moderate high light (400 μmol m⁻² s⁻¹) identified stm3 as a light-sensitive strain (Figure 4B). A 43% reduction in growth rate of stm3 cultures within 24 h of high-light incubation was accompanied by an 81% increase in cellular chlorophyll content, suggesting that an uncontrolled increase of the PSII–LHC antenna system in stm3 caused photoinhibitory effects.

Inefficient energy transduction from LHC proteins to chlorophyll P680 attributable to a bigger antenna system would also explain the high-fluorescence phenotype of stm3 and the observed decrease in FₚSII quantum yields (0.55 in the wild type compared with 0.42 in stm3).

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**Figure 3. Localization of NAB1 in the Cytosol by Immunogold Labeling.**

Electron micrographs of wild-type and stm3 cell sections showing anti-NAB1 immunogold-labeled NAB1 proteins as black dots located in the cytosol of the wild type (zoomed sections b, c, and d) but not in the nucleus (zoomed section a), mitochondria, or chloroplast (zoomed section e). Cp, chloroplast; Cyt, cytosol; M, mitochondrion; N, nucleus.
Loss of NAB1 Results in an Increase in Levels of LHCII Proteins in \textit{stm3}

The high-chlorophyll fluorescence phenotype, the increase in total chlorophyll per cell, the decrease in the ratio of chlorophyll \textit{a} to \textit{b} in \textit{stm3} compared with the wild type, the drastic changes in the chloroplast ultrastructure, and the sensitivity to moderate high light suggested that the disruption of \textit{NAB1} caused an increase in the size, or perhaps a change in the composition, of the LHCII antenna system.

To test this, immunoblotting experiments were performed using antibodies raised against higher plant LHCII proteins. The results showed that the levels of LHCII proteins per cell had increased in \textit{stm3} compared with the wild type (Figure 5A). Gel extraction of the two dominant immunoreactive bands followed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis suggested that these two bands consisted of LHCBM2/8 (lower band) and LHCBM4/6 (upper band) proteins.

To investigate this further, the LHCII proteins of the wild type and mutant were separated by two-dimensional gel electrophoresis. Immunoblotting was performed with antibodies raised specifically against the nearly identical N termini of LHCBM4 and LHCBM6 (Hippler et al., 2001). In agreement with previous work...
(Stauber et al., 2003), the antibodies recognized three spots in the wild type, \textit{stm3}, and \textit{nc1} (spots 22, 23, and 24) indicating that the increase of LHCBM4/6 protein levels in \textit{stm3} was not accompanied by the appearance of further modified LHCBM4/6-type proteins in the mutant (Figure 5B). Interestingly, however, the levels of spots 22 and 23 relative to spot 24 increased drastically in \textit{stm3} compared with the wild type and the complemented strain \textit{nc1} (Figure 5B).

Detailed analysis of all three spots by tandem mass spectrometry has so far revealed only one difference between spots 22/23 and spot 24 (Stauber et al., 2003). These data suggested that the deletion of NAB1 in \textit{stm3} caused a change in the LHCBM4/LHCBM6 protein expression ratio. Analysis of detergent-solubilized thylakoids by native gel electrophoresis confirmed that the altered LHCCI composition in \textit{stm3} did not prevent the formation of trimeric LHCCI complexes (Figure 5C).

To assess whether the increased abundance of LHCCI proteins found in \textit{stm3} was attributable to an increased pool for LHCBM transcripts, RNA gel blot experiments were performed with probes specific for LHCBM4 and LHCBM6. Interestingly, these blots revealed that LHCBM6 transcript levels were reduced by nearly 70\% in standard light-cultivated \textit{stm3} cells compared with wild-type cells, whereas LHCBM4 transcript levels showed almost no differences between the mutant and the wild type (Figure 5D).

Recombinant NAB1 Binds LHCBM4 and LHCBM6 mRNA via Its N-Terminal CSD Motif in Vitro with Different Affinities

The results obtained so far suggested a model in which NAB1 might act bifunctionally at a posttranscriptional level to stabilize transcripts and to repress the translation of LHCBM mRNAs. In the absence of NAB1, expression of these genes would be upregulated. Based on the similarity to the CSD of FRGY2 from \textit{Xenopus}, in silico searches were performed to identify potential NAB1 binding sites within LHCBM mRNAs. Indeed, the mRNA consensus sequence motif (CSDCS), which is specifically recognized by the CSD of FRGY2 (GCCANACCAC/UCGC [Manival et al., 2001]), could be found with different degrees of sequence conservation in the coding regions of LHCBM1, -2, -3, -4, -5, -6, -8, and -9 cDNAs (Figure 6A). It should be noted that LHCBM7 and LHCBM10 were excluded from the original list of \textit{C. reinhardtii} LHCBM genes (Elrad et al., 2002) because, in agreement with recent results (Elrad and Grossman, 2004), we could not identify their products as independent isoforms, either in the nuclear genome or during intensive tandem mass spectrometry analyses of LHCBM proteins (Stauber et al., 2003).

To test the putative RNA binding activity of NAB1, UV cross-linking assays were performed in vitro. As shown in Figure 6B, recombinant NAB1 was able to bind to a radiolabeled RNA probe comprising the LHCBM6 CSDCS. In addition, competition experiments with various unlabeled RNA probes containing either the CSDCS region or sequences from the 5’ or 3’ untranslated region (UTR) of the LHCBM6 mRNA revealed that NAB1 bound with very high affinity to CSDCS and only weakly to the 5’ or 3’ UTR. By contrast, an unrelated AU-rich RNA probe from the chloroplast \textit{psbD} gene was not recognized at all by NAB1 (Figure 6B).

To further elucidate the role of the CSD recognition motif for specific LHCBM6 RNA binding, we changed a single nucleotide (A to T at position 4 of the recognition motif) in the LHCBM6 RNA sequence. This mutation resulted in a new sequence that now matched exactly with the CSDCS recognition motif of LHCBM4 mRNA. As a result of this single nucleotide exchange, the binding affinity of NAB1 decreased drastically (Figure 6B). This finding clearly showed that the putative CSD recognition motif CSDCS is functionally involved in NAB1 binding and suggested that the CSD of NAB1 plays an important role similar to FRGY2. Furthermore, the reduced binding of NAB1 to the LHCBM4-type recognition site motif showed that recombinant NAB1 discriminates between different LHCBM RNA isoforms.

By analogy with the function of FRGY2, the sequence specificity of NAB1 binding should be mediated via its CSD. To test this hypothesis, in vitro binding studies were performed with a 10-kD N-terminal NAB1 fragment that contains the complete CSD motif but lacks the C-terminal RRM domain (Figure 6C). In agreement with our hypothesis, the 10-kD CSD fragment of NAB1 showed a high and specific binding affinity to the CSDCS recognition motif of LHCBM6 RNA, similar to the results obtained with native NAB1 protein. Again, the binding affinity to the LHCBM4-type recognition site was reduced drastically (Figure 6C).

We conclude from the in vitro studies that NAB1 is indeed an RNA binding protein and that it binds via its CSD with different affinities to LHCBM mRNAs.

NAB1 Binds LHCBM6 RNA in Nontranslated Messenger Ribonucleoprotein Complexes

From our in vitro studies, NAB1 would represent a protein that binds and sequesters LHCBM mRNAs at a subpolysomal level, thus acting to fine-tune the expression of LHCBM6 proteins in \textit{C. reinhardtii}. To investigate this hypothesis, cytosolic mRNA was isolated from wild-type and \textit{stm3} cells in the presence of cycloheximide, separated into subpolysomal nontranslated messenger ribonucleoproteins (mRNPs) and monosomal and translated polysomal complexes by sucrose density gradient centrifugation (15 to 45\% sucrose) (Barkan, 1988; Shama and Meyuhas, 1996; Yohn et al., 1996), and further analyzed.

Combined LHCBM6 RNA slot blot (Figure 6D, middle panel) and anti-NAB1 immunoblot (Figure 6D, bottom panel) studies were performed in all 18 separated fractions derived from the sucrose gradients of the wild type and \textit{stm3}.

From the appearance of 18S RNA (representing the 40S subunit) and 25S rRNA (part of the 60S subunit) (Marco and Rochaix, 1980) in the separated fractions of the wild type (Figure 6D, top panel), we concluded that fractions 1 to 4 contain only nontranslated RNA in subpolysomal preinitiation complexes (mRNPs ± 40S subunits). By contrast, fractions 5 to 18 contain ribosomal complexes including 40S and 60S subunits (monosomes) and, from approximately fraction 8 onward, polysomes (Shama and Meyuhas, 1996). Protein and RNA gel blot studies with wild-type samples revealed that the vast majority of NAB1 cofractionates in fractions 2, 3, and 4 with preinitiation (mRNP – 40S) complexes (molecular mass of 400 to 900 kD). Most interestingly, in the wild type these fractions contained 40%
Figure 6. Function of NAB1 as an RNA Binding Translation Regulator.

(A) Sequence alignment of LHCBM1 to LHCBM9 [(1) to (9)] cDNAs from *C. reinhardtii* with the consensus motif (CSDCS) recognized by FRGY2 from *Xenopus*. Identical positions are indicated by a black background, and sequence identity levels are given at right.

(B) In vitro RNA binding competition studies. Autoradiogram after UV cross-linking of recombinant NAB1 protein, radiolabeled LHCBM6-CSDCS RNA probe, and a 5-, 10-, and 50-fold molar excess of the indicated nonlabeled competitor RNAs. The exposure time was identical for all lanes. Competition with *PSBD* 5' UTR RNA was performed independently. Quantification of RNA binding intensities in relation to the NAB1 signal without competitor (0X value) from one representative experiment was estimated by signal densitometry and plotted as a diagram.

(C) In vitro RNA binding studies. Autoradiogram after UV cross-linking of recombinant N-terminal 83–amino acid NAB1 peptide fragment (carrying the complete CSD) and radiolabeled LHCBM6-CSDCS RNA probe (for details, see [B]). Indicated competitor RNAs were added in 5-, 10-, and 50-fold molar excess to the reactions.
higher levels of LHCBM6 mRNA compared with stm3 (Figure 6D). This is not the case with actively translating heavy polyosomes; both strains contained comparable levels of LHCBM6 RNA.

Partial digestion of RNA–protein complexes by RNase treatment of the wild-type sample before sucrose density fractionation resulted in the appearance of vast amounts of unbound NAB1 protein on top of the gradient (fraction 1), whereas NAB1–RNA high molecular weight complexes fully disappeared in fractions 4, 5, and 6.

To confirm that NAB1 does indeed bind to LHCBM6 RNA in vivo, we isolated native NAB1 protein by immunoprecipitation from soluble wild-type cell fractions using polyclonal NAB1 antibodies. Successful purification of native NAB1 was confirmed by immunoblotting (Figure 6E). Subsequent isolation of RNA from the purified NAB1 protein followed by slot-blot analysis and by quantitative real-time RT-PCR analysis confirmed highly abundant binding of LHCBM6 mRNA [cycle threshold, C(T), value of 16.9] to NAB1, whereas only very low concentrations of LHCBM4 mRNA were detected [C(T) value of 28.1] (Figure 6E).

In conclusion, these data show that NAB1 proteins are functionally located in subpolysomal nontranslated high molecular weight RNA–protein complexes of the cytosol, where they preferentially bind and sequestrate LHCBM6 mRNA and, to a very low level, LHCBM4 mRNA. Such a posttranscriptional control mechanism would enable the cell to acclimate to environmental changes by a fast expression of light-harvesting antenna proteins through the permanent pool of stored LHCBM6 mRNA.

**Figure 6.** (continued).

(D) Analysis of high molecular weight subpolysomal and polysomal RNA–protein fractions derived from wild-type and stm3 cell extracts after 15 to 45% sucrose gradient centrifugation. Isolated RNA from each fraction separated by gel electrophoresis (top panel), and LHCBM6 mRNA levels determined by RNA slot blotting (middle panel; standard errors are based on five independent measurements). Identification of fractions containing NAB1 proteins by anti-NAB1 immunoblotting of nontreated samples (− RNase) and samples pretreated with RNase before centrifugation and separation (+ RNase) (bottom panel).

(E) In vivo mRNA binding studies. Anti-NAB1 immunoblot showing purification of immunoprecipitated (IP) native NAB1 from cell extracts, and analysis of bound mRNA by slot-blot analysis and real-time RT-quantitative (Q)-PCR using probes and primers specific for LHCBM6, LHCBM4, and ACTIN. The agarose gel shows transcription abundance after 25 cycles. mRNAs from input cell extracts were used as a positive control. ACTIN, LHCBM4, and LHCBM6 transcripts became detectable after 14 to 16 cycles. Samples without reverse transcriptase (−RT) or template were used as negative controls. LHCBM6 transcripts in RT-Q-PCR studies, using immunoprecipitated NAB1-derived mRNA as a template, became detectable after 16 cycles, whereas LHCBM4 and ACTIN transcripts did not appear before cycles 28 and 39, respectively. Rates for relative transcript abundance are calculated from five independent measurements as 2−(C(T) transcript − C(T) actin), where C(T) = cycle values. The LHCBM6 values were set to 100%.
wild-type HA-LHCBM6 counterparts and exhibited a decrease of 37% in LHCBM6 abundance (Figure 7C).

From these results, we could assign 50% of the observed decrease in LHCBM6 protein level upon light treatment in the wild type to functional NAB1 binding with LHCBM6 mRNA, whereas the other 50% decrease, which occurs in both strains, must be assigned to additional posttranscriptional effects, such as protein degradation (Lindahl et al., 1995).

In summary, our results have clearly identified with NAB1 a novel cytosolic RNA binding protein that functionally binds LHCBM RNA through a LHCBM CSDCS recognition motif. NAB1 forms high molecular weight nontranslated mRNP complexes in the cytosol in which LHCBM6 RNA is sequestrated and thereby prevented from translation in polysomes.

DISCUSSION

Mutant stm3 Shows Aberrant Expression of the LHCII Antenna

The dark-green phenotype (Figure 4), the decrease in chlorophyll a/b ratio, the highly stacked grana, and the changes in LHCBM protein expression pattern (Figure 5) are clear indicators of an altered LHCII antenna system in stm3. Our data strongly suggest that absence of NAB1 causes the increased expression of certain LHCBM proteins, thus leading to an overall increase in the size of the LHCII antenna. The similarity of the chlorophyll fluorescence parameter (Fv/Fm) in the wild type and stm3 suggests that the additional antenna is still coupled to PSII reaction centers in stm3. If the additional antenna proteins were partially uncoupled, the initial (minimum) PSII fluorescence in the dark-adapted state (F0) would be expected to increase, leading to an overall decrease in the Fv/Fm ratio.

It should be noted that the observed phenotype of perturbed LHC state transitions in stm3 is most likely an indirect effect of the super-stacked grana organization caused by an altered antenna structure attributable to the disruption of the LHCBM expression profile.

The similarity of NAB1 to FRGY2 of Xenopus implies the existence of similar RNA-masking systems in animals and Chlamydomonas.

Complementation experiments have clearly confirmed that the effects on the LHCII antenna of stm3 are attributable to the inactivation of NAB1. NAB1 contains two RNA binding domains with a CSD motif at the N terminus and a RRM at the C terminus. The existence of two different RNA binding domains on one protein has been described previously (Graumann and Marahiel, 1998). However, we did not identify any other proteins with the
combination of CSD and RRM domains. Database searches have only identified 13 complete protein sequences with CSDs in plants (data not shown), five of them in Arabidopsis thaliana (AtGRP2, AtGRP2b, Atg17870, Atg436020, and Atg38680). Analysis of the Chlamydomonas genome indicates that NAB1 is the only protein in C. reinhardtii with a CSD. This result underlines the unique and special character of the RNA binding protein NAB1.

Temperature shock experiments (0 to 24 h at 4, 20, and 40°C; data not shown) did not reveal any obvious visual phenotypic difference between stm3 and the wild type, which suggests that NAB1 is not essential for temperature acclimation.

Interestingly, there are many striking similarities between NAB1 and the Xenopus RNA binding protein FRGY2. FRGY2 contains a CSD responsible for initial protein–RNA binding and a second RNA binding domain at its C terminus for effective RNA binding (Matsumoto et al., 1996; Manival et al., 2001). As soon as the initial binding has occurred at the CSD, more FRGY2 proteins bind to the target RNA with their unspecific C-terminal binding domain, leading to a repression of RNA translation.

Detailed analysis of the protein–RNA interaction site has led to the identification of an RNA binding consensus domain, which is needed for a specific CSD binding in FRGY2 (Manival et al., 2001). Database searches revealed that a similar 13-nucleotide sequence is found in LHCBM2, LHCBM6, LHCBM8, and LHCBM9 (85% identity). For the other LHCBM genes, the sequence is less conserved (69 to 77% identity).

**NAB1 Is a Cytosolic Translation Repressor and Essential for RNA Stabilization and Sequestration**

Immunogold localization studies have confirmed that NAB1 is indeed localized in the cytosol (Figure 3). This result excludes a bifunctional role for NAB1 as both an RNA and DNA binding protein. The analysis of nuclear protein localization by immunogold labeling is often affected by false-positive protein signals in the nucleus. In our case, however, the highly specific anti-NAB1 antibody showed no nuclear cross-reaction at all, which clearly demonstrated that NAB1 is not localized in the nucleus.

In vitro RNA binding studies with recombinant NAB1 protein, as well as with N-terminal CSD fragments of NAB1, have suggested that protein–RNA interaction is mediated by the specific binding of the CSD to a CSDCS RNA consensus domain in LHCBM6 (Figures 6B and 6C). In vivo binding studies confirmed the role of NAB1 as a functional RNA binding protein that preferentially binds LHCBM6 RNA (Figure 6E). The obtained drastic difference in NAB1–RNA binding capacity between LHCBM4 and LHCBM6 in conjunction with the clear differences in the abundance of LHCBM4 and LHCBM6 proteins in the wild type and stm3 support a role for NAB1 in the differential expression of LHCBM subunits in C. reinhardtii. However, it should be noted that several other predicted mRNAs contain potential CSDCS-like binding domains, which makes it possible that the RNA binding activity of NAB1 is not limited to LHCBM mRNAs.

Immunoblot and RNA slot-blot analyses of subpolysomal and polysomal RNA fractions from sucrose gradients (Figure 6D), LHCBM4/6 RNA gel blot analyses (Figure 5D), in vivo RNA binding studies by immunoprecipitation and real-time RT-PCR (Figure 6E), in vitro RNA binding studies (Figures 6B and 6C), and protein expression studies with LHCBM4/6 transcription control–insensitive clones of the wild type and stm3 (Figure 7) support a model in which NAB1 functionally occurs in subribosomal high molecular weight protein–RNA complexes in which it binds to LHCBM mRNAs with different affinities, sequesters them, and represses translation. The clear difference in NAB1 binding affinity between LHCBM4 and LHCBM6 mRNA explains why in its absence in stm3, LHCBM6 expression is much more affected than LHCBM4 expression (Figure 5B).

This is further supported by the drastic differences obtained for LHCBM4 and LHCBM6 expression by moderate high-light treatment. HA tagging allowed quantitative estimation of protein levels by immunoblotting and revealed that LHCBM4 RNA translation is not influenced by light, whereas LHCBM6 RNA translation clearly is (Figure 7).

The observed reduced level of LHCBM6 transcripts in stm3 compared with the wild type (Figure 5D) could be explained by the absence of LHCBM6 RNA sequestration in the mutant, leading to an increase of RNA instability and degradation, similar to the function described for FRGY2 (Matsumoto et al., 2003) and YB-1, another CSD protein involved in mRNA translation control (Evdokimova et al., 2001). From our studies, it is feasible to suggest the existence of similar RNA-masking systems in animals and Chlamydomonas. As with FRGY2 and YB-1, NAB1 contains two RNA binding domains, with one of them a CSD at the N terminus for specific RNA binding.

**LHCII Antenna Size in C. reinhardtii Is Controlled at Many Levels**

It is well established that the expression of LHCII proteins is regulated at the level of gene transcription (Shepherd et al., 1983; Escoubas et al., 1995; Maxwell et al., 1995; Teramoto et al., 2002). Under low-light conditions, LHCBM mRNA levels increase, whereas levels decrease under increasing light conditions (Erad and Grossman, 2004). A specific role of the redox state of the plastoquinone pool as a sensor of the imbalances in photosynthetic electron transport has been proposed (Escoubas et al., 1995).

In addition, expression of some photosynthesis genes is also controlled at the translational level (Danon and Mayfield, 1994; Danon, 1997; Petracek et al., 1997; Drapier et al., 2002). This can be of particular importance for light-regulated chloroplast proteins that are encoded in the nucleus. In this case, a steady state pool of mRNA in the cytosol would enable the cell to respond faster to events in the chloroplast. However, the existence of ready-to-use mRNA requires a stress-dependent regulation system that operates at the posttranscriptional level. To date, there has only been indirect evidence to indicate the existence of posttranscriptional control of LHC protein expression (Flachmann and Kühlbrandt, 1995; Tang et al., 2003). However, recent results (Durnford et al., 2003) have revealed that posttranscriptional control becomes more important than redox-regulated transcription regulation under long-term stress conditions. The work described here on the identification of NAB1 gives new insights into how posttranscriptional control of light harvesting is achieved in C. reinhardtii. It complements the picture emerging through other recent work about eukaryotic gene expression and
shows that posttranscriptional control is an elaborate way in which the cell regulates and adjusts adequate protein synthesis (Moore, 2005).

METHODS

Strains and Culture Conditions

Liquid cultures of Chlamydomonas reinhardtii were grown in continuous white light (40 μmol·m⁻²·s⁻¹). TAP and high-salt media were prepared as described (Harris, 1989). 1.5% Select Agar (Gibco BRL) was added to prepare solid plates, and 13 μM phleomycin (Sigma-Alrich) or 100 μM emetine diahydrochloride (Sigma-Aldrich) was added for screening of pSP124S or p613 transformant, respectively. TAP 1:10 N was prepared by reducing the amount of NH₄Cl to 1:10.

Mutant Construction and Genetic Analysis

Mutant stm3 was generated in the background of Chlamydomonas strain CC849 (Duke University) by transformation with plasmid pSP124S (Stevens et al., 1996; Lumbreras et al., 1998) as described previously (Kindle, 1990) and grown on TAP medium containing phleomycin to test for resistance to the drug. Generation of gametes, matings, and zygote analysis were performed as described (Harris, 1989).

Isolation of Nucleic Acids, and Hybridizations

Manipulations of nucleic acids were performed according to standard methods (Sambrook et al., 1989). DNA for gel blot analysis, DNA of the wild type and stm3 was restricted with PvuII, HincII, or Smal enzymes and probed with labeled PCR products synthesized with primer pairs wild type and mutant sequences. The resulting DNA fragment of apparently 2 kb was gel-extracted, cloned into pGEM-T Easy vector (Promega), and sequenced.

LMS-PCR

Genomic DNA flanking the inserted vector in stm3 was identified by LMS-PCR (Strauss et al., 2001). LMS adapters were ligated, and suppression PCR was performed with vector-specific primer and adapter-specific primer. The PCR product was used as a template in a second, nested LMS-PCR. The resulting DNA fragment of apparently 2 kb was gel-extracted, cloned into pGEM-T Easy vector (Promega), and sequenced.

Chlorophyll Fluorescence Measurements

Room-temperature fluorescence video imaging to detect mutants with perturbed state transitions was performed as described (Kruse et al., 1999). Kautsky fluorescence induction was recorded by illuminating dark-adapted cells for 2 min with 55 μmol·m⁻²·s⁻¹ red light (620 nm). Fv/Fm was recorded from dark-adapted cells with actinic light (620 nm, 55 μmol·m⁻²·s⁻¹) and calculated by (Fm – F0)/Fm (Maxwell and Johnson, 2000), where Fm is steady state fluorescence yield.

Complementation Experiments

For complementation of stm3, the CRY1 gene, conferring resistance to emetine (plasmid p613) (Nelson et al., 1994), was used as a dominant selectable marker together with plasmid pNAB1 containing the complete nuclear NAB1 gene cloned into pBluescript II SK– (Stratagene). stm3 cells were transformed with 2 μg of each plasmid by the glass bead method (Kindle, 1990). Transformed cells were incubated in TAP 1:10 N for 4 d, resuspended in TAP medium for 8 h, and spread onto TAP/Emetine plates. Colonies appeared after 12 d and were screened for the integration of pNAB1 by PCR. RNA was isolated (RNeasy mini kit; QiaGen) from PCR-positive clones and reverse-transcribed (GeneScript reverse transcriptase; Genecraft), and RT-PCR was performed with NAB1-specific primers.

Overexpression of NAB1 and Fragmentation

NAB1 cDNA was cloned into a pQE80L vector (Qiagen) and transformed into Escherichia coli strain M15. Overexpression and purification of the N-terminally 6×-His-tagged NAB1 protein were performed according to the manufacturer’s instructions (Qiagen). An N-terminal 83–amino acid peptide fragment of recombinant NAB1 was purified by nickel–nitrilotriacetic acid agarose column chromatography after thrombin digestion.

Cloning of HA-Tagged LHCBM Genes

An HA epitope–tagged form of the LHCBM6 gene was cloned into the pGEM-T Easy vector (Promega) and modified by in vitro PCR mutagenesis (QuickChange; Stratagene) to replace adenine at position 730 by thymidine in LHCBM6, which resulted in a nucleotide sequence identical to the CSDCS recognition motif (Matsumoto et al., 1996) of LHCBM4. Genes were ligated into the PSAD promoter–containing expression vector pGenD (Fischer and Rochaix, 2001) and cotransformed using the CRY1 marker gene as described above. Potential cotransformants were screened by immunoblotting using a HA-specific monoclonal antibody (Roche). For moderate high-light treatment, cells were illuminated with white light (180 μmol·m⁻²·s⁻¹) for 22 h.

Gel Electrophoresis and Immunoblotting

Proteins were separated by SDS-gel electrophoresis and subsequently electrophoblotted onto nitrocellulose membranes (Amersham). Sample preparation for two-dimensional gel electrophoresis is described elsewhere (Hippler et al., 2001). Native green gels were prepared as described (Allen and Staehelin, 1991). His-tagged NAB1 was used for synthesis of the anti-NAB1 antibody (SeqLab). Antisera were used in conjunction with the Amersham enhanced chemiluminescence system. LHCBM4/6-specific antibodies were raised against the synthetically produced 25 N-terminal amino acids of the LHCBM6 precursor protein (Hippler et al., 2001). Signals were quantified using the programs Quantity One (version 4.1.1; Bio-Rad) and GelScan 2.0.

MALDI-TOF and Electrospray Ionization Tandem Mass Spectrometry

MALDI-TOF mass spectrometry (Hillenkamp and Karas, 1990) was performed using a Bruker Biflex III apparatus and Xmass 5.0 software. For sample preparation, the Coomassie Brilliant Blue G 250–stained protein band of interest was cut out and treated according to the description at http://info.med.yale.edu/wmkeck/prochem/geldig3.htm. Acquisition of electrospray ionization tandem mass spectrometry data was achieved as described previously (Stauber et al., 2003).

Electron Microscopy

For transmission electron microscopy, cells were grown in TAP medium to an OD₇₅₀ of 0.6 and prepared as described (Engels et al., 1997).
In Vitro Synthesis of RNA and UV Cross-Linking of 
RNA with Proteins

Templates for the in vitro synthesis of the LHCBM6 and LHCBM4 cDNA probes were generated by PCR amplification using gene-specific oligo-nucleotides derived from the 5’ and 3’ UTRs.

Each template contained the T7 promoter sequence fused to the 5’ end of the appropriate fragment. In vitro transcription of RNA, UV cross-linking of RNAs with proteins, and quantification of binding signals were performed as described (Ossenbühl and Nickelsen, 2000). For competition experiments, radiolabeled RNA and nonlabeled competitor RNA were mixed before the addition of the proteins.

Subpolysome and Polysome Complex Fractionation

Untranslated RNA in mRNPs and polysomes were fractionated as described (Barkan, 1988; Yohn et al., 1996). A cell lysate was layered onto a 15 to 45% sucrose gradient (Shama and Meyuhas, 1996). If desired, samples were pretreated with 500 μg/mL RNase A (Boehringer) for 5 min at 20°C. Collected fractions were supplemented with 0.5% (w/v) SDS and 20 mM EDTA before RNA extraction by phenol/chloroform extraction and isopropanol precipitation. Precipitated RNA was dissolved in 20 mM EDTA before RNA extraction by phenol/chloroform extraction and isopropanol precipitation. Precipitated RNA was dissolved in 20 μL of dimethylcarboxylate-treated water, and 1 μL of each sample was separated on a 1.5% agarose-formaldehyde denaturing gel.

Immunoprecipitation of NAB1 and in Vivo RNA Binding Studies

A wild-type cell lysate was incubated with anti-NAB1 antiserum coupled to protein A-Sepharose (Seize classic immunoprecipitation kit; Pierce). Immunocomplexes were eluted according to the manufacturer’s instructions, and RNA was isolated in the presence of 20 μg/mL RNase A (Boehringer) for 5 min at 20°C. Coprecipitated RNA was subjected to real-time RT-PCR analysis with the manufacturer’s kit.

Accession Number

The accession number for NAB1 is AY157846.

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REFERENCES


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