Communication

Chloroplast Import of Light-Harvesting Chlorophyll a/b-Proteins with Different Amino Termini and Transit Peptides

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ABSTRACT

We have previously isolated and sequenced two genes encoding light-harvesting chlorophyll a/b-proteins (LHCP) from *Lemna gibba*. One of these, AB30, encodes a protein that is highly homologous to LHCP sequences reported from other species, but the second, AB19, encodes a protein that has a transit peptide and first 12 amino-terminal residues of the mature protein that are substantially different. Despite these differences, we can demonstrate that AB19 encoded protein synthesized in vitro can be imported into isolated chloroplasts, and we provide evidence that at least some of the imported molecules are assembled into the light-harvesting complex of photosystem II. Thus, our results are consistent with the possibility that there are two functional forms of LHCP.

Most chloroplast proteins are encoded by the nuclear genome. After these polypeptides are synthesized as precursors on cytoplasmic ribosomes, they are imported into the chloroplast by an as yet undefined mechanism, and then compartmentalized within the organelle (4). At some point during or after uptake, an amino-terminal transit sequence is cleaved from each of the precursors (3). There is evidence that this transit sequence is necessary and sufficient to specify import into chloroplasts (12, 15).

We have studied the import of a nuclear-coded LHCP2 into *Lemna gibba* chloroplasts (9). Several size forms of LHCP are located in the thylakoid membranes and are members of a complex of protein, Chl and carotenoid that transfers absorbed light energy to photochemical reaction centers (5, 14). All higher plants studied to date have a family of genes encoding LHCP's, and it has been suggested that different members of the family have different functions (2, 7). *Lemna* LHCP is encoded by a gene family of approximately 12 members and 2 of these, AB19 and AB30, have been cloned and sequenced (7, 9). Both genes are transcribed, and they code for mature proteins whose sequences are 85% homologous to each other. The AB30 sequence is highly homologous to LHCP protein sequences reported for other species. However, the cleaved transit sequences and the first 12 to 16 amino-terminal residues of the predicted mature peptides are substantially different for AB30 and AB19 (Fig. 1).

The two transit peptides also differ extensively in their patterns of hydropathy (see Fig. 2) (cf. Ref. 10). Together, these differences suggested that at least two classes of LHCP are encoded in the *Lemna* nucleus, and raised the possibility that these two genes code for LHCPs that have distinct roles. Although both genes are transcribed in vivo (7, 9), it is not known whether both kinds of LHCP are translated or are functional in vivo.

Analysis of the transit sequences suggested that, despite the differences, both have features shared by known transit sequences for chloroplast proteins. The amino acids conserved in chloroplast transit sequences (8) are indicated by asterisks (*) in Figure 1. We have previously used an in vitro system to demonstrate that the protein encoded by the AB30 gene is capable of being imported into *Lemna* chloroplasts, inserted into thylakoid membranes, and incorporated into the green LHCII complex (9). In this work we report that the protein encoded by the AB19 gene (7) can also function similarly in this in vitro system. Although we have previously shown that a single LHCP gene can give rise to multiple size forms of the protein in LHCII, our results here support the possibility that the sequence heterogeneity within the nucleus could also account in part for the LHCP heterogeneity found in thylakoids.

MATERIALS AND METHODS

The cloning and in vitro expression of the genomic fragment AB30 has been described (9). The construct for the in vitro expression of the AB19 sequence was made by subcloning an XmaIII/HindIII 1.3 kb fragment from pLabAB19/H5c (7) into the SmaI/HindIII sites of pSP65 after treatment of the XmaIII site with the Klenow fragment of *Escherichia coli* DNA polymerase and four dNTPs. The templates were linearized with HindIII (which cleaves once downstream of the coding sequence), and capped LHCP message was synthesized using SP6 RNA polymerase (6) in a reaction that also included GppppG in a concentration of 250 μM. Capping increased the efficiency of translation 5- to 10-fold. AB30 and AB19 RNAs were then translated in a wheat germ system in the presence of [35S] methionine (9). The LHCP import into *Lemna* chloroplasts and the analysis of incorporated protein by gel electrophoresis were as described (9). Chl-protein complexes were analyzed by a modification of (9); LHCII was excited from the gel using a razor blade, denatured at 55°C for 20 min in denaturing buffer and then loaded directly onto a denaturing 12.5% polyacrylamide gel (11).

RESULTS

Synthesis of Precursor LHCPs. We used genomic DNA fragments that extend from the in vivo site of transcription initiation

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2 Abbreviations: LHCP, light-harvesting chlorophyll a/b-protein; pLHCP, precursor LHCP; LHCII, light-harvesting complex II.
Fig. 1. A comparison of the transit peptides and amino-terminal residues of the mature LHCP for AB19 and AB30. Amino acids are shown in the single letter code. AB19 is shown in its entirety, while only the amino acids of AB30 that are different from AB19 are shown. A (—) indicates a deletion at that position. (▼) indicates the proposed site of processing. Underlined amino acids of AB19 are repeated at the processing site and downstream of the intron (*) shows the residues common to known transit sequences (7). The position and predicted sequence of the AB19 intron are also shown.

Fig. 2. Hydrophobicity plots for the transit sequences of AB19 and AB30 LHCP. Mean hydrophobicities using the scale of Kyte and Doolittle (10) are plotted for successive 9-residue segments of the transit polypeptide. (——) shows AB19; (· · ·) shows AB30. (———) indicates a midpoint line in the relative scale shown on the ordinate (10).

to several hundred base pairs beyond the transcription termination sites as templates for the two precursor LHCPs (pLHCPs) (7, 9). AB19 contains an intron within the amino-terminal coding region which is in-frame with the translation code. The 28 additional amino acids encoded by the intron and the 7 fewer amino acids in the coding region of AB19 results in an in vitro synthesized protein that should be 21 amino acids larger than AB30 protein (Fig. 1). Figure 3 shows a comparison of the translation products of AB19 and AB30 RNAs and their uptake and incorporation into thylakoid membranes. Translation of AB30 RNA (lane a) produced the expected 32 kD protein. AB19 RNA served as template for the predicted 34 kD polypeptide (lane c).

Import of AB30 and AB19 Proteins into Chloroplasts. Figure 3, lane b, is a display of denatured thylakoid membrane proteins from protease-treated intact chloroplasts reisolated after uptake of AB30 pLHCP. AB30 is processed into two mature sized LHCPs corresponding to the sizes of the endogenous LHCPs (cf. Ref. 9). In different experiments the number of newly incorporated bands varies from two to three and the relative intensities of these band changes. We have not been able to determine the cause of the variation. Figure 3, lane d, shows denatured thylakoid membranes isolated from chloroplasts that have been incubated with AB19 pLHCP. Many labeled polypeptides are found in the thylakoid, only one of which corresponds in size to that expected of a correctly processed AB19 LHCP (top arrow, lane d, Fig. 3). Neither AB30 nor AB19 protein are detected in the stroma (data not shown).

Both AB30 and AB19 Can Be Incorporated into LHChII. Figure 4 shows that both AB19 and AB30 LHCPs can be incorporated into LHChII. After chloroplasts were incubated with either AB30
or AB19 pLHCP, the thylakoid membranes were partially denatured and the Chl-protein complexes were resolved (Fig. 4a). The Chl-containing LHClII band was excited (arrow, lane a), denatured and run into a fully denaturing gel (11). Lane c shows labeled denatured proteins of LHClII isolated after the import of AB30 pLHCP, two correctly processed forms are observed (cf. Ref 9). LHClII isolated from chloroplasts incubated with AB19 pLHCP (lane b) contains two radioactive bands; a 31 kD band that corresponds in size to a processed form expected of mature AB19 LHCP (upper arrow, Fig. 3d) and another that is significantly smaller than that expected (lower arrow, Fig. 3d).

**DISCUSSION**

The *Lemna* nuclear genome encodes at least two distinct LHCPs. Two isolated coding sequences, AB30 and AB19, predict smaller mature polypeptides that differ extensively in their amino termini. The transit peptides have few amino acid residues in common. We show here that both of these distinct pLHCPs can be imported into isolated *Lemna* chloroplasts and assembled into LHClII.

AB30 is processed into several forms that differ by several hundred daltons in size (9). These sizes are identical to native LHCP visualized by either Coomasie blue or monoclonal antibody staining, but we do not know what is involved in the formation of these multiple forms or whether they represent the forms found in *vitro*. It is likely that the modification is associated with the amino terminus since a tryptic digestion of LHCP removes both the amino terminus and reduces the number of bands to just one (cf. Refs. 1, 9, 13).

AB19 protein can also be imported into *Lemna* chloroplasts. While many labeled proteins are seen in the thylakoid, only a correctly processed and one smaller form can be found in LHClII. The size of the smaller processed AB19 band of LHClII is consistent with a site of processing approximately 20 amino acids on the carboxy side of the intron site. Upon inspection of the sequence, we found four amino acids (underlined in Fig. 1) that occur both 10 to 17 amino acids away from the intron site and at the presumed site of processing. The smaller AB19 form could be produced if this putative cryptic cleavage sequence becomes accessible in the protein containing the translated intron.

Both AB19 proteins in LHClII are represented in the total thylakoid preparation (arrows, Fig. 3), yet unlike AB30 protein, only a small amount of these are assembled into the complex: lane 4b is exposed 5 times longer than lanes 3d, 3b, and 4c. Thus, the insertion of 28 residues within the amino terminus of LHCP does not block assembly into LHClII. It is not known whether AB19 LHCP itself or the additional translated intron sequences cause reductions in both the efficiency of assembly and the fidelity of processing. We have not determined whether the multiple forms found in the thylakoid but not in LHClII (Fig. 3d) are the result of incorrect processing or degradation. We presume that these bands represent LHCPs which lack sufficient structural information to direct assembly into LHClII. We have noted previously (9) that internal deletions of LHCP block LHClII assembly but do allow insertion into the membrane. Moreover, deletion mutations that remove portions of the LHCP amino terminus can disrupt normal processing (BD Kohorn, unpublished data).

The isolation of an additional genomic fragment that is homologous to AB19 but that does not contain an intron (7) and other LHCP coding sequences should help to further characterize the LHClII classes. We have shown here that two distinct LHCPs that differ mainly in their transit sequence and mature amino termini can be imported into isolated chloroplasts and assembled into LHClII. It will be of interest to determine whether the existence of two types of LHCP is of any developmental or functional significance. We have compared the ability of chloroplasts of different maturities to incorporate preferentially either AB19 or AB30 *in vitro*, but have seen no difference (data not shown). It is noteworthy however, that there is a petunia gene highly homologous to AB19 (M. Stayton, Advanced Sciences, Oakland, CA, personal communication), and this fact may indicate that the presence of these two LHCP classes is of general significance.

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**LITERATURE CITED**