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Processing of the Precursors for the Light-Harvesting Chlorophyll-Binding Proteins of Photosystem II and Photosystem I during Import and in an Organelle-Free Assay[1]

Steven E. Clark[2] and Gayle K. Lamppa*

Department of Molecular Genetics and Cell Biology, University of Chicago, 920 E. 58th Street, Chicago, Illinois 60637

ABSTRACT

We have investigated whether the precursors for the light-harvesting chlorophyll *a/b* binding proteins (LHCP) of photosystems II and I (PSII and PSI) are cleavable substrates in an organelle-free reaction, and have compared the products with those obtained during *in vitro* import into chloroplasts. Representatives from the tomato (*Lycopersicon esculentum*) LHCP family were analyzed. The precursor for LHCP type I of PSII (pLHCPII-1), encoded by the tomato gene *Cab3C*, was cleaved at only one site in the organelle-free assay, but two sites were recognized during import, analogous to our earlier results with a wheat precursor for LHCPII-1. The relative abundance of the two peptides produced was investigated during import of pLHCPII-1 into chloroplasts isolated from plants greened for 2 or 24 hours. In contrast to pLHCPII-1, the precursors for LHCP type II and III of PSI were cleaved in both assays, giving rise to a single peptide. The precursor for LHCP type I of PSI, encoded by gene *Cab6A*, yielded two peptides of 23.5 and 21.5 kilodaltons during import, whereas in the organelle-free assay only the 23.5 kilodalton peptide was found. N-terminal sequence analysis of this radiolabeled peptide has tentatively identified the site cleaved in the organelle-free assay between met40 and ser41 of the precursor.

We have previously demonstrated that the precursor for LHCP[3] type I of PSII (LHCPII-1) is cleaved at two sites—called the primary and secondary sites—during import into the chloroplast, yielding two peptides of approximately 26 and 25 kD. In contrast, in an organelle-free reaction containing a soluble enzyme, only the 25 kD peptide is produced due to secondary site cleavage (1, 8, 9, 19). This is the case whether the precursor of LHCPII-1 (pLHCPII-1) originates from *in vitro* translation of wheat or pea transcripts (10–13, 24), or is synthesized in *Escherichia coli* (2). In addition, pLHCPII-1 from tomato (27), tobacco (7), and *Lemna* (17) have been shown to be cleaved at multiple sites during import *in vitro*. Processing at the secondary site of wheat pLHCPII-1 removes the transit peptide and a basic hexapeptide previously thought to be present on all LHCPII-1 molecules (2). This domain also contains a threonine residue that is preferentially phosphorylated (21, 22). Using pLHCPII-1 from wheat and pea as substrates, we have shown that the determinants for cleavage at the primary and secondary sites are distinct: cleavage at the primary site requires an amino proximal basic residue, and the motif AKAK (residues 40–43 in wheat pLHCPII-1) promotes secondary site utilization (10). Thus, we have proposed that selective processing of pLHCPII-1 contributes to the heterogeneity of LHCP found *in vivo*.

A number of related questions remained unresolved in our earlier studies on LHCP maturation. First, were precursors for other members of the LHCP family, found in both PSII and PSI, cleavable in the organelle-free assay? Precursors for the small subunit of Rubisco, Rubisco activase, plastocyanin, acyl carrier protein, and heat shock protein 21 have been shown to be processed in this assay by a soluble enzyme with similar properties (2, 29). However, the primary site of pLHCPII-1 is not cleaved in this assay. Therefore, it occurred to us that if pLHCPII-1 was unique in having two cleavage sites, only one of which—the secondary site—was recognized in the organelle-free reaction, then the precursors for other members of the LHCP family might not be processed in this assay because of a missing or inactive component, even though normally, *i.e. in organello,* they would be substrates for processing by a general chloroplast-processing enzyme. To investigate this question, the tomato LHCP family was chosen because genes have been isolated and characterized that represent the diversity of LHCP found *in vivo* (28, for review see ref. 14). Second, we asked, were there developmental stages of the chloroplast that would promote cleavage at the primary or the secondary sites of pLHCPII-1? The LHCP population surrounding PSII is heterogeneous (25, 30) and its composition depends not only on the plant analyzed, but also changes under different light regimens and during development (3). This can in part be accounted for by the expression of different members of the multigene family (reviewed in ref. 14). To gain a better understanding of the variables that might influence LHCP maturation, chloroplasts were isolated from plants grown in the dark and then greened for 2 or 24 h and used in import experiments to examine processing.

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[2] Present address: Division of Biology, California Institute of Technology, Pasadena, CA 91125.

[3] Abbreviations: LHCP, Light-harvesting chlorophyll a/b binding protein; PVDF, polyvinylidene difluoride.

MATERIALS AND METHODS

Plant Growth and Chloroplast Isolation

Pea (*Pisum sativum,* Laxton's Progress) plants were grown and harvested as described previously (1, 19) to isolate chloroplasts for standard import reactions or to prepare chloroplast lysates for organelle-free processing. Procedures described by Bartlett *et al.* (4) were used to purify intact chloroplasts on Percoll gradients. To investigate whether the developmental stage of the chloroplast influenced import and processing, pea plants were grown in the dark for 7 d, and then the etiolated shoots were allowed to green for either 2 or 24 h before chloroplast isolation.

In vitro Transcription, Translation, Chloroplast Import, and Organelle-Free Processing

In vitro transcription reactions were carried out as recommended by the vendor with either T7 or SP6 polymerase (United States Biochemical Corp; Promega) using a wheat gene (18) or pea gene (6) coding for pLHCPII-1, or tomato genes coding for different members of the LHCP family (see summary of tomato genes in ref. 28). The pea and tomato constructs were kindly provided by Drs. Anthony Cashmore and Eran Pichersky, respectively. RNA was translated in a reticulocyte lysate (Bethesda Research Laboratories) to synthesize [^{35}S]methionine-labeled precursor polypeptides. The import and organelle-free processing reactions were performed as detailed previously (1, 19). Sizes of *in vitro*-synthesized precursors and the products of import and organelle-free processing were estimated based on gel migration adjacent to protein markers (Sigma MW-SDS-200).

N-Terminal Sequence Analysis

[^{35}S]methionine-labeled translation products for pLHCPI-1 encoded by the tomato gene *Cab6A* were incubated in an organelle-free processing reaction. The proteins were transferred to PVDF membrane (Immobilon, Millipore Corp.) after separation by SDS-PAGE (15% acrylamide). The Immobilon membrane was subjected to autoradiography, and the region corresponding to the 23.5 kD peptide was excised for Edman degradation N-terminal amino acid sequence analysis.

RESULTS

Organelle-Free Processing of Precursors for LHCP of Both PSII and PSI from Tomato

The genes *Cab3C* and *Cab4* from tomato (26, 27) coding for LHCP type I and type II of PSII, respectively, were transcribed *in vitro,* and transcripts were translated in the presence of [^{35}S]methionine in a reticulocyte lysate to obtain radiolabeled precursor polypeptides. The precursors were then incubated with intact pea chloroplasts in an import reaction, or with a soluble extract in an organelle-free assay. The precursor for LHCP type I (pLHCPII-1) gave rise to two mature peptides of approximately 26 and 25 kD that were in the membrane fraction isolated from chloroplasts treated with thermolysin before lysis (Fig. 1A). Only the 26 kD product

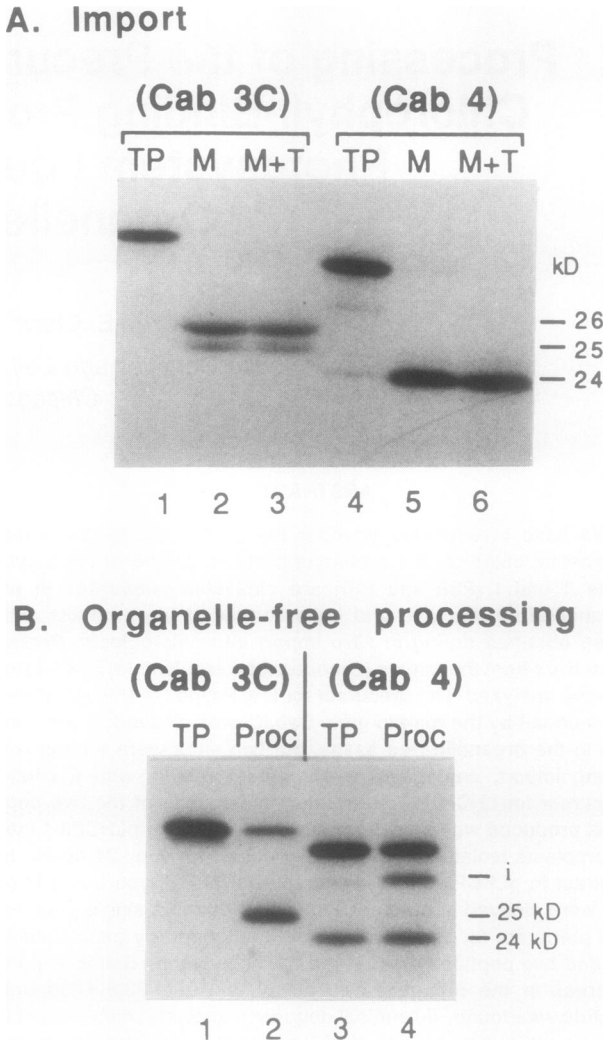

Figure 1. Import and organelle-free processing of pLHCPII-1 (type I) and pLHCPII-2 (type II) from tomato. A, Translation products (TP, lanes 1 and 4) generated from the *Cab3C* and *Cab4* genes encoding pLHCPII-1 and pLHCPII-2, respectively, were incubated with isolated pea chloroplasts and the membrane fractions of either thermolysin-untreated (M, lanes 2 and 5) or treated (M+T, lanes 3 and 6) chloroplasts were isolated. B, Translation products (TP, lanes 1 and 3) were incubated with a soluble chloroplast lysate in an organelle-free reaction for 1 h (Proc, lanes 2 and 4). Products in A and B were analyzed by SDS-PAGE, followed by autoradiography. Molecular mass estimates are given on the right side; the position of a processing intermediate is also indicated in B.

was observed in a previous study (27); however, this may have been due to the smaller amount of radiolabeled protein analyzed. In the organelle-free assay, in contrast, tomato pLHCPII-1 was cleaved to only the 25 kD form (Fig. 1B). Differential processing in these two assays has previously been observed using pLHCPII-1 synthesized from the wheat *Cab-1* gene (8, 9, 19). When the precursor for LHCP type II (pLHCPII-2) from tomato was used in an import reaction, a single peptide of 24 kD was found resistant to thermolysin (Fig. 1A). This form comigrated with a faint background

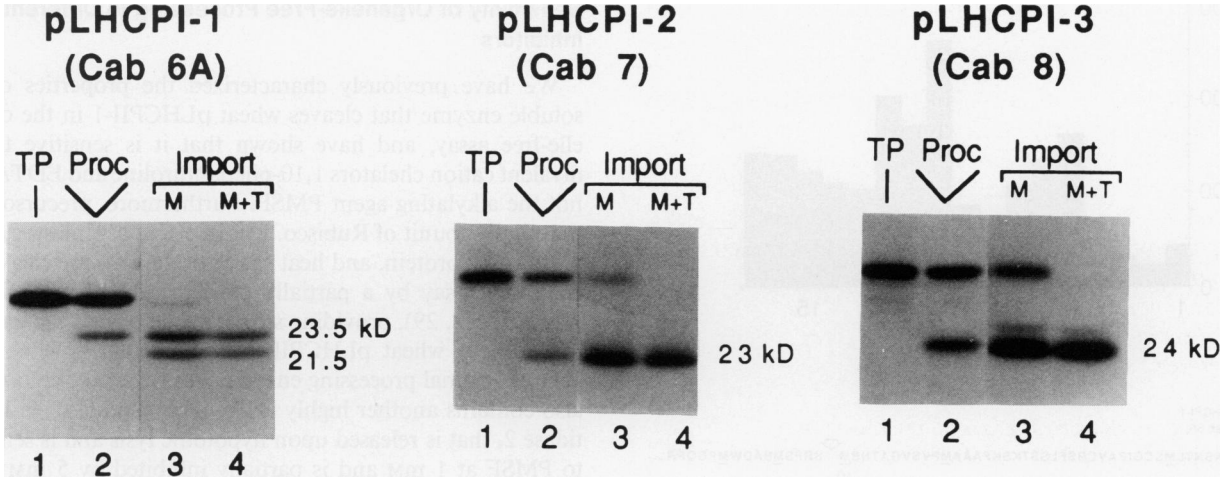

Figure 2. Import and organelle-free processing of pLHCPI-1 (type I), pLHCPI-2 (type II), and pLHCPI-3 (type III) from tomato. Translation products (TP, lanes 1) were incubated in an organelle-free reaction (Proc, lanes 2) or with isolated chloroplasts for import (Import). After an import reaction, chloroplasts were either not treated (M, lanes 3) or treated with thermolysin (M+T, lanes 4) before lysis, and the membranes isolated. Products were analyzed as described in Figure 1. To the right of each panel, estimated sizes are listed.

product in the translation reaction that is most likely the result of internal translation initiation at met37, numbered from the N-terminus of the precursor. This would place the start of the 24 kD peptide near that methionine, as earlier predicted by comparison with LHCP type I (27). In the organelle-free assay there was not an increase in the intensity of the 24 kD peptide above the background band (Fig. 1B, lane 4), nor has a lower molecular mass species been observed that would suggest cleavage at a "secondary site," although the identical extract efficiently processed pLHCPII-1 (Fig. 1B, lane 2). We conclude that pLHCII-2 does not contain a site near the transit peptide-mature protein junction that is efficiently recognized in the organelle-free assay. However, an intermediate-size protein usually appeared migrating slightly ahead of the precursor. We have previously observed intermediates when processing of pLHCPII-1 was inhibited by mutation (10).

Precursors for LHCP type I, type II, and type III of PSI, encoded by the tomato genes *Cab6A* (15), *Cab7,* and *Cab8* (28), respectively, were analyzed in the import and organelle-free processing assays. The LHCP type I precursor (pLHCPI-1) generated from transcripts of the *Cab6A* gene produced two mature forms of 23.5 and 21.5 kD upon translocation into the chloroplast (Fig. 2, left). To determine if the 23.5 kD peptide was eventually cleaved to the 21.5 kD form, time course experiments were performed (Fig. 3). Both peptides accumulated in parallel over a period of 25 min, suggesting that the 23.5 kD peptide is not an intermediate leading to the 21.5 kD form of LHCPI-1. The organelle-free processing reaction pLHCPI-1 produced only the 23.5 kD peptide (Fig. 2, left, lane 2). Thus, it appears that pLHCPI-1 contains two distinct processing sites, only one of which is recognized by a soluble enzyme independent of translocation into the chloroplast.

The precursor for LHCP type II (pLHCPI-2) of PSI was cleaved at only one site during import, giving rise to a 23 kD peptide (Fig. 2, middle). The precursor for LHCP type III

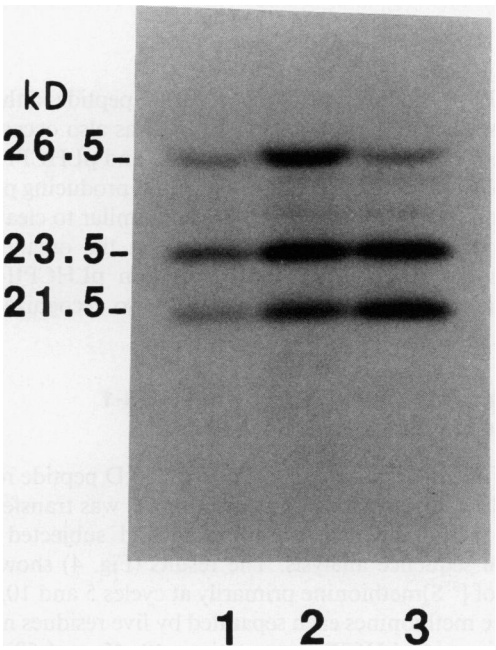

Figure 3. Time course of import of pLHCPI-1, encoded by *Cab6A*. The membrane fractions were isolated from chloroplasts after import reactions for 5 (lane 1), 10 (lane 2), and 25 (lane 3) min. Chloroplasts were not treated with thermolysin, and thus, the precursor migrating as a 26.5 kD species is observed. Estimated sizes of the imported products are given on the left.

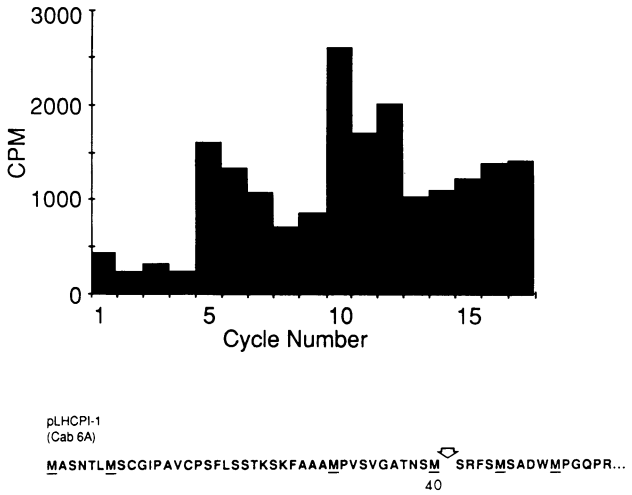

Figure 4. N-terminal sequence analysis of the 23.5 kD peptide produced in an organelle-free reaction from pLHCPI-1. The [^{35}S] methionine-labeled 23.5 kD peptide, transferred to PVDF membrane, was subjected to 17 cycles of Edman degradation, and the counts per minute (CPM) released at each cycle determined by scintillation counting. The N-terminal 55 amino acids of the precursor are given (single letter amino acid code) at the bottom with the methionines underlined. The arrow indicates the probable site of cleavage based on the radiolabeled peaks appearing at cycle 5 and 10. Other minor peaks are probably the result of a lag in the release of radiolabel from the PVDF filter and cannot be aligned with any methionine residues.

(pLHCPI-3) produced primarily a 24 kD peptide, although a minor species of approximately 25 kD was also occasionally observed (Fig. 2, right). Both pLHCPI-2 and pLHCPI-3 were cleavable in the organelle-free assay, again producing peptides of 23 and 24 kD (lanes 2), respectively, similar to cleavage in the import reaction. Thus, processing in the organelle-free assay is not restricted to the maturation pLHCPII-1, but precursors for the LHCPs of PSI are also recognized by a soluble chloroplast enzyme.

N-Terminal Sequence Analysis of LHCPI-1 Released in the Organelle-Free Assay

The [^{35}S]methionine-radiolabeled 23.5 kD peptide released from pLHCPI-1, encoded by gene *Cab6A,* was transferred to PVDF membrane by electroblotting and subjected to N-terminal sequence analysis. The results (Fig. 4) showed the release of [^{35}S]methionine primarily at cycles 5 and 10. There are three methionines each separated by five residues near the N-terminus of pLHCPI-1 at positions 40, 45, and 50 (Fig. 4, bottom). No other methionines show this spacing of five residues. However, the release of a [^{35}S]methionine was not evident at cycle 15, indicating that there are only two methionines near the N-terminus of the 23.5 kD peptide, rather than three. This places cleavage in the organelle-free assay immediately carboxy to methionine 40 in pLHCPI-1. Hence, the 23.5 kD peptide begins with the novel sequence SRFSMSADWMPGQPR before the start (at proline 56 in the precursor) of a highly conserved region that includes the first transmembrane domain of the protein.

Sensitivity of Organelle-Free Processing to Different Inhibitors

We have previously characterized the properties of the soluble enzyme that cleaves wheat pLHCPII-1 in the organelle-free assay, and have shown that it is sensitive to the divalent cation chelators 1,10-phenanthroline and EDTA, but not the alkylating agent PMSF. Furthermore, precursors for the small subunit of Rubisco, Rubisco activase, plastocyanin, acyl carrier protein, and heat shock protein 21 are cleaved in the same assay by a partially purifed enzyme with similar properties (2, 29), providing strong evidence that the enzyme that cleaves wheat pLHCPII-1 at its secondary site is the general stromal processing enzyme. However, the chloroplast also contains another highly active endopeptidase, endopeptidase 2, that is released upon hypotonic lysis and is sensitive to PMSF at 1 mM and is partially inhibited by 5 mM 1,10-phenanthroline and 10 mM EDTA (23). We examined the processing of pLHCPII-1 and pLHCPI-1, encoded by the tomato genes *Cab3C* and *Cab6A,* in the presence of 2 mM PMSF, 1 mM 1,10-phenanthroline, and 5 mM EDTA. Processing of both was strongly inhibited by the latter two reagents (Fig. 5, lanes 4 and 5), but relatively insensitive to the addition of PMSF (Fig. 5, lanes 3), supporting the conclusion that it is most likely the general stromal processing enzyme, and not endopeptidase 2, which cleaves these substrates in the organelle-free reaction.

Import of pLHCPII-1 into Chloroplasts from Dark-Grown Plants Greened for 2 and 24 h

The heterogeneous LHCPII population changes during chloroplast development and in response to different light regimens for growth (3). We examined whether selective processing of pLHCPII-1 at the primary and secondary sites was sensitive to the developmental/physiological stage of the chloroplast used in the import reaction, which would be reflected in the ratio of the 26 and 25 kD peptides. At the same time, we asked whether this would depend on the precursor substrate imported, because not all forms of

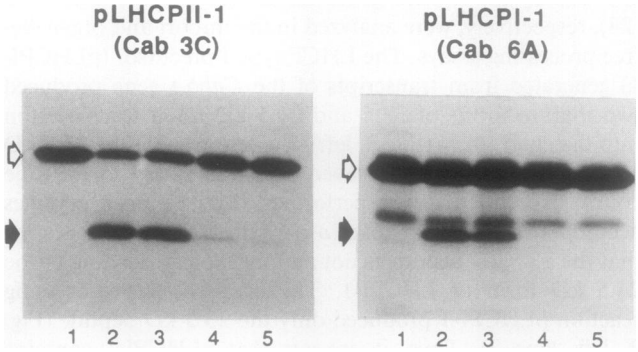

Figure 5. Processing in the organelle-free reaction in the presence of PMSF, 1,10-phenanthroline, and EDTA. Translation products (lanes 1) for pLHCPII-1 and pLHCPI-1 encoded by the *Cab3C* and *Cab6A* genes, respectively, were introduced into a standard organelle-free reactions (lanes 2) or with 2 mM PMSF (lanes 3), 1 mM 1,10-phenanthroline (lanes 4), or 5 mM EDTA (lanes 5).

pLHCPII-1 have shown the same levels of processing at the primary and secondary sites during import into chloroplasts isolated from light-grown plants (9, 10). Four substrates were used to investigate the relative abundance of the 26 and 25 kD peptides: pLHCPII-1 from tomato (gene *Cab3C*), wheat (gene *Cab1*), and pea (gene *CabAB80*), and in addition a modified form of the pea precursor with the substitution AKA for TTK at position 42–44. This substitution was shown previously to enhance cleavage of the pea precursor at the secondary site during import and in the organelle-free reaction (10). Plants were initially grown in the dark and then transferred to light for either 2 or 24 h before chloroplast isolation. Membrane fractions were isolated after import reactions and analyzed by SDS-PAGE (Fig. 6). After autoradiography, gel slices corresponding to the 26 and 25 kD peptide bands were excised and subjected to scintillation counting. The 26/25 kD peptide ratios from two separate greening experiments were averaged. All substrates showed a reduction in the 26/25 kD peptide ratios between 2 and 24 h of chloroplast greening, an indication of enhanced secondary site cleavage (Fig. 6). The ratio for pea:pLHCPII-1 was 7:1 at 2 h, and 4.8:1 at 24 h. Thus, chloroplasts greened for 24 h produced 38% more of the 25 kD peptide when compared with the products of import using chloroplasts greened for 2 h. The ratio for the tomato substrate showed a reduction from 2.6:1 to 1.5:1, or a 43% increase in the amount of the 25 kD peptide produced during import. The changes for the wheat precursor and the modified pea substrate with the AKA substitution were not as significant, equal to increases of 10 and 17% in the amount of the 25 kD peptide, respectively.

DISCUSSION

We have extended our earlier studies (1, 2, 8–10, 19) on the maturation of the major light-harvesting chlorophyll binding protein of PSII, encoded by the wheat *Cab-1* gene, to members of the LHCP family from tomato. pLHCPII-1, encoded by the tomato gene *Cab3C,* yields two peptides of 26 and 25 kD during import, but is cleaved to only the 25 kD form in an organelle-free assay, as we predicted from its sequence relatedness to the wheat precursor at the transit peptide-mature protein junction. The sequence RKTAAKAK is found immediately carboxy to the primary site in the wheat precursor (where the residues at the secondary cleavage site are underlined) and, in the corresponding position, RKTATKAK is found in the tomato substrate. On the other hand, pLHCPII-2, encoded by the gene *Cab4,* was not processed in the organelle-free assay, perhaps due to the absence of three

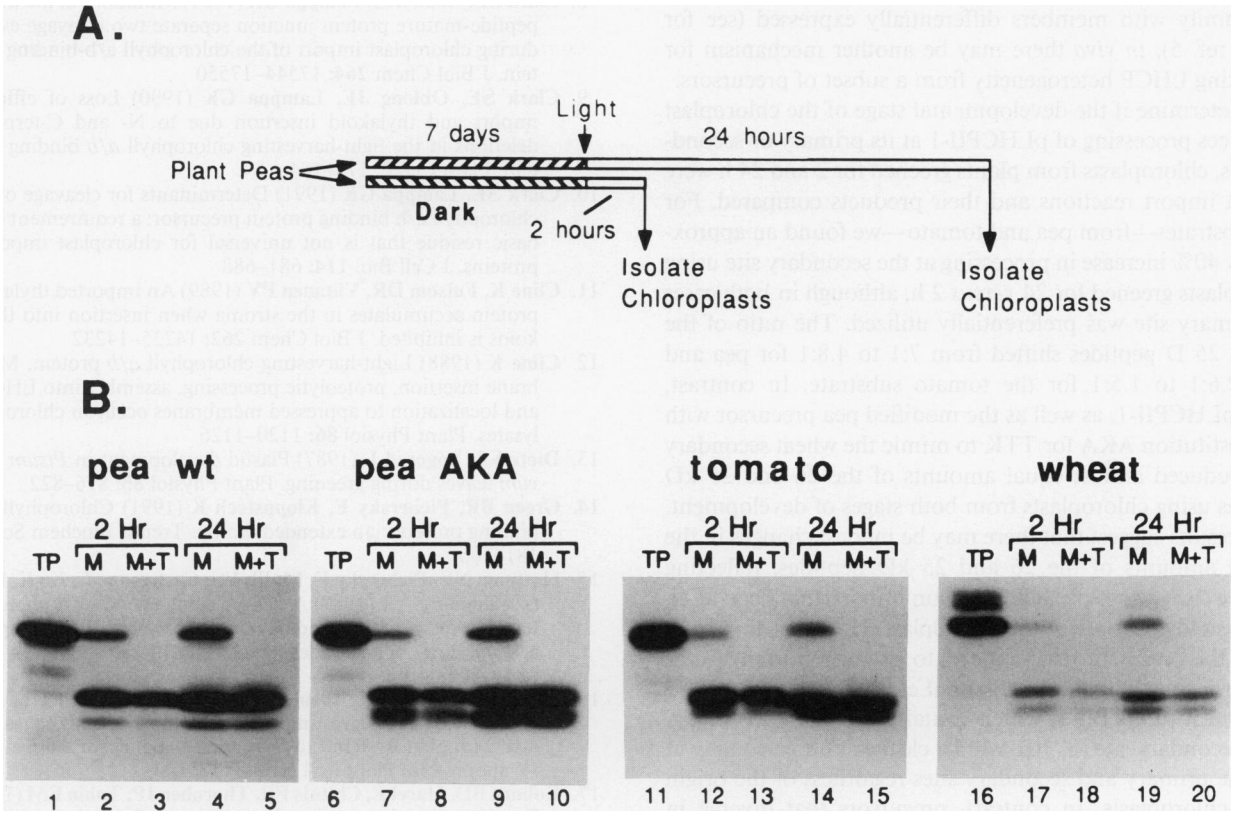

Figure 6. pLHCPII-1 import using chloroplasts greened for 2 and 24 h. A, Plants were grown in the dark and then transferred to light for either 2 or 24 h before harvesting as indicated. B, Import reactions using the translation products (TP, lanes 1) for the wild-type pea precursor (pea wt, gene *AB80*), the modified pea precursor (pea AKA), the tomato precursor (gene *3C*), or the wheat precursor (gene *Cab1*). The total membrane fraction before (lanes 2, 4, 7, 9, 12, 14, 17, and 19) and after (lanes 3, 5, 8, 10, 13, 15, 18, and 20) treatment of chloroplasts with thermolysin were analyzed.

amino acids in this region (27), which includes the AKA motif at the secondary cleavage site if the sequences of pLHCPII-1 and pLHCPII-2 are aligned for maximum homology (20).

In this work, we have also demonstrated that the precursors for the LHCPs of PSI are cleaved in the organelle-free assay by a soluble chloroplast enzyme. Three substrates were employed representing the three types of LHCPs associated with PSI originally deduced from an analysis of the tomato multigene family encoding these proteins (15, 26–28). The precursors for type II and type III were cleaved similarly in the organelle-free reaction and during import, in each case producing a single peptide. This suggests that LHCPI-2 and LHCPI-3 maturation does not require thylakoid membrane insertion, and that processing occurs before this step *in vivo*. Interestingly, the precursor for the type I protein, encoded by the *Cab6A* gene, is cleaved at two sites during import, producing peptides of 23.5 and 21.5 kD, but only one site is recognized in the organelle-free assay, producing the 23.5 kD species. Our N-terminal sequence data indicate that the 23.5 kD peptide begins at met40, whereas Ikeuchi *et al.* (16) have recently reported that the N-terminus of a smaller peptide (estimated to be 20.5 kD) encoded by the *Cab6A* gene begins at ser45. We propose that pLHCPI-1, analogous to pLHCPII-1, contains two cleavage sites, with distinct determinants for recognition, that give rise to two mature forms with different N-termini. Hence, in addition to being encoded by a multigene family with members differentially expressed (see for review ref. 5), *in vivo* there may be another mechanism for generating LHCP heterogeneity from a subset of precursors.

To determine if the developmental stage of the chloroplast influences processing of pLHCPII-1 at its primary or secondary sites, chloroplasts from plants greened for 2 and 24 h were used in import reactions and their products compared. For two substrates—from pea and tomato—we found an approximately 40% increase in processing at the secondary site using chloroplasts greened for 24 *versus* 2 h, although in both cases the primary site was preferentially utilized. The ratio of the 26 and 25 D peptides shifted from 7:1 to 4.8:1 for pea and from 2.6:1 to 1.5:1 for the tomato substrate. In contrast, wheat pLHCPII-1, as well as the modified pea precursor with the substitution AKA for TTK to mimic the wheat secondary site, produced almost equal amounts of the 26 and 25 kD peptides using chloroplasts from both stages of development. These results suggest that there may be modest changes in the relative amounts of the 26 and 25 kD peptides, reflecting selective cleavage of pLHCPII-1 upon import, that depend on the physiological state of the chloroplast. However, the structure of the precursor itself appears to be an important factor in determining whether preferential cleavage can occur, and thus, wheat pLHCPII-1, which contains an efficiently recognized secondary site (8, 10), will be cleaved almost equally at both the primary and secondary sites regardless of the origin of the chloroplasts. In contrast, precursors that diverge in sequence from the wheat substrate at the transit peptide-mature protein junction may require special conditions to promote secondary site recognition. Import reactions employing chloroplasts representing additional developmental stages may help resolve this question.

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