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A chloroplast processing enzyme involved in precursor maturation shares a zinc-binding motif with a recently recognized family of metalloendopeptidases

(cDNA/primary structure/His-Xaa-Xaa-Glu-His motif/pitrilysins/expression)

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ABSTRACT **Nuclear-encoded proteins targeted to the chloroplast are typically synthesized with N-terminal transit peptides which are proteolytically removed upon import. Structurally related proteins of 145 and 143 kDa copurify with a soluble chloroplast processing enzyme (CPE) that cleaves the precursor for the major light-harvesting chlorophyll *a/b* binding protein and have been implicated in the maturation of the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase and acyl carrier protein. The 145- and 143-kDa proteins have not been found as a heterodimer and thus may represent functionally independent isoforms encoded by separate genes. Here we describe the primary structure of a 140-kDa polypeptide encoded by cDNAs isolated by using antibodies raised against the 145/143-kDa doublet. The 140-kDa polypeptide contains a transit peptide, and strikingly, a His-Xaa-Xaa-Glu-His zinc-binding motif that is conserved in a recently recognized family of metalloendopeptidases, which includes *Escherichia coli* protease III, insulin-degrading enzyme, and subunit β of the mitochondrial processing peptidase. Identity of 25–30%, concentrated near the N terminus of the 140-kDa polypeptide, is found with these proteases. Expression of CPE in leaves is not light dependent. Indeed, transcripts are present in dark-grown plants, and the 145/143-kDa doublet and proteolytic activity are both found in etioplasts, as well as in root plastids. Thus, CPE appears to be a necessary component of the import machinery in photosynthetic and nonphotosynthetic tissues, and it may function as a general stromal processing peptidase in plastids.**

Chloroplast biogenesis depends on the import of a large number of diverse proteins that are synthesized in the cytoplasm as precursors with N-terminal transit peptides. A transit peptide mediates precursor recognition by receptors on the chloroplast envelope (1, 2), and upon membrane translocation into the stroma, it is proteolytically removed, yielding the mature protein (3, 4). Studies have indicated that a general stromal processing peptidase (SPP) with the properties of a metalloprotease cleaves the transit peptides of proteins destined for different functional complexes (3, 4). Proteins targeted to the thylakoid lumen have a bipartite transit peptide that is cleaved first by SPP, then by a thylakoid protease (5–7). SPP thus plays a key role as part of the chloroplast import machinery.

We have identified a soluble chloroplast processing enzyme (CPE) with characteristics of SPP that cleaves the precursor for the major light-harvesting chlorophyll *a/b* binding protein (LHCP) (8, 9). Antigenically related proteins of 145 and 143 kDa copurify with this activity from pea. Immunodepletion experiments show that the 145/143-kDa doublet is indeed required for cleavage of the LHCP precursor (preLHCP) and indicate that these proteins are involved in the removal of the transit peptides of the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and the acyl carrier protein. The 145- and 143-kDa proteins appear not to form a stable heterodimer, and they may function separately during precursor cleavage (10). They either are products of different genes or arise through posttranslational modification of a single protein.

To understand the mechanism underlying precursor recognition and selective processing, we have begun to characterize genes coding for the 145- and 143-kDa proteins to describe their primary structure. In this study, cDNAs have been isolated,‡ initially employing antibodies to the 145/143-kDa doublet, that code for a 140-kDa polypeptide with a transit peptide. Antibodies raised against a recombinant protein corresponding to the C terminus of this polypeptide recognize only the 145/143-kDa doublet in a chloroplast extract. The 145/143-kDa proteins are expressed in both light- and dark-grown shoots and are also present in root plastids. Strikingly, the 140-kDa polypeptide contains a His-Xaa-Xaa-Glu-His (HXXEH) zinc-binding motif which is characteristic of a newly recognized family of metalloendopeptidases, the pitrilysins (11). This family includes *Escherichia coli* protease III (12), human (13) and *Drosophila* (14) insulin-degrading enzymes (IDEs), and subunit β of the mitochondrial processing peptidase (MPP) (15–17). Conservation extends beyond the HXXEH motif, which is required for catalysis (18–20), suggesting similar evolutionary origins of the CPE and these other metalloendopeptidases, although their substrate specificities have diverged.

MATERIALS AND METHODS

cDNA Isolation and Sequencing. A λZapII (Stratagene) cDNA library (a gift from Neeraj Datta, University of Georgia, Athens), constructed with mRNA from dark-grown pea seedlings greened for 24 hr, was screened by standard methods (21) with polyclonal antiserum raised against the 145/143-kDa doublet (10). Digoxigenin-labeled DNA restriction enzyme fragments from the λZap clone were made by using the Genius kit (Boehringer Mannheim) and were used to screen a λgt11 library (Clontech) made by random priming and priming on the poly(A) tail of mRNA from 7-day-old dark-grown pea seedlings. The 5′ end of the cDNA was isolated by using 5′

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Abbreviations: CPE, chloroplast processing enzyme; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; LHCP, light-harvesting chlorophyll *a/b* binding protein; GST, glutathione *S*-transferase; IDE, insulin-degrading enzyme; MPP, mitochondrial processing peptidase; RACE, rapid amplification of cDNA ends; ORF, open reading frame.

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‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. U25111).

RACE (rapid amplification of cDNA ends) PCR essentially as described by the vendor (GIBCO/BRL) and using 10 μg of poly(A)$^+$ RNA from etiolated plants and the oligonucleotides as described in *Results*. Clones with serial deletions were prepared with exonuclease III and sequenced with the Sequenase Version 2.0 kit (United States Biochemical).

Preparation of Glutathione *S*-Transferase (GST)-Fusion Protein and Antiserum. A 2.1-kb *Xho* I fragment was ligated into the *Xho* I site of pGEX-KG to create an in-frame fusion of GST and the C terminus of CPE. The GST-fusion protein was expressed in *E. coli* strain BL21 at 25°C, affinity purified with glutathione-agarose (Sigma), and eluted with 10 mM glutathione (22). Rabbit polyclonal antiserum was generated by using ≈1 mg of GST-fusion protein.

RNA Isolation and Northern Blotting. Total RNA was isolated from leaf tissue of 7-day-old light-grown or 10-day-old dark-grown pea plants as previously described (23). Poly(A)$^+$ RNA was isolated by using poly(U)-Sephadex (GIBCO/BRL) as suggested by the manufacturer. Poly(A)$^+$ RNA (10 μg) was separated on a glyoxal/dimethyl sulfoxide gel and blotted to a nylon membrane (Boehringer Mannheim) as described (23), then UV cross-linked (Hoeffer UVC 1000). Hybridization at 65°C for 18 hr with digoxigenin-labeled DNA probe (20 ng of labeled probe per ml) and chemiluminescent detection were carried out as recommended (24).

Plastid Isolation. Chloroplasts and etioplasts were isolated (9) from pea (*Pisum sativum*, Laxton's Progress #9). For the greening experiment (Fig. 4 *B* and *C*) plants were grown for 9 days in the dark, then exposed to light before harvesting. Root plastids were prepared (25) from plants grown in Turface Regular (Applied Industrial Materials, Deerfield, IL) for 8 days.

Protein Analysis. Radiolabeled precursor synthesis, *in vitro* organelle-free processing reactions, SDS/PAGE analysis, and immunoblot detection of CPE were carried out as described (3, 10). For protein sequencing the 145/143-kDa proteins were isolated by preparative SDS/PAGE (10), and tryptic peptides were sequenced by Edman degradation at Rockefeller University.

RESULTS

Antibodies ("anti-145/143-kDa serum") raised against the 145- and 143-kDa proteins, originally purified from pea chloroplasts (10), were used to screen a λZap pea expression library made from poly(A)$^+$ RNA. A cDNA clone was isolated containing a 2.7-kb insert with a poly(A) tail, indicating that it corresponded to the 3′ end of the transcript. A 2.1-kb *Xho* I fragment containing an open reading frame (ORF) of 542 amino acids, equivalent to ≈65 kDa, was subcloned, and the ORF was overexpressed as a GST-fusion protein in *E. coli*. The GST-fusion protein was recovered in soluble extracts and purified by affinity chromatography. In immunoblot experiments, antibodies against the GST-fusion protein ("anti-GST-fusion serum") recognized a protein of ≈95 kDa from transformed *E. coli* lysates, as predicted from the size of GST and the insert ORF (Fig. 1). The anti-GST-fusion serum also recognized the 145- and 143-kDa proteins in a chloroplast soluble extract in a 1:1 ratio. No other chloroplast proteins were detected. In the reciprocal experiment, the anti-145/143-kDa serum recognized only the GST-fusion protein in the *E. coli* extract, as well as the 145/143-kDa doublet in the chloroplast extract used as a control (Fig. 1). These results establish that the cDNA codes for the C terminus of either the 145- or the 143-kDa protein and confirm that the correct ORF was expressed in *E. coli*. It was not possible to assign the cDNA to either the 145- or the 143-kDa protein because the anti-GST-fusion serum had an equal affinity for both proteins. It seems likely that the 145/143-kDa proteins either are isoforms

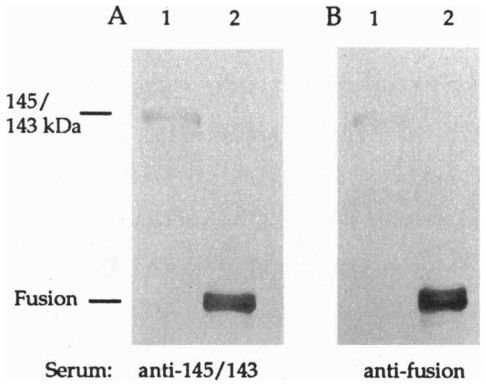

FIG. 1. Comparison of the proteins detected by antiserum raised to the 145/143-kDa doublet and to the GST-fusion protein. A soluble chloroplast extract (20 μg of protein, lanes 1) and the GST-fusion protein (200 μg of protein, lanes 2) were subjected to SDS/PAGE, transferred to nitrocellulose, and incubated with either rabbit anti-145/143-kDa serum (1:2500 dilution; *A*) or anti-GST-fusion serum (1:2500 dilution; *B*), followed by anti-rabbit IgGs conjugated with alkaline phosphatase and developed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

encoded by two related genes or represent posttranslational modification of a single gene product.

To obtain the 5′ end of the gene, a randomly primed cDNA library was screened by using labeled restriction enzyme fragments and two additional clones were identified. One contained a 1.7-kb insert that overlapped with the 5′ end of the 2.7-kb fragment by 131 bases. The other cDNA (0.96 kb) overlapped by 0.57 and 0.52 kb with the 2.7- and 1.7-kb fragments, respectively. The three cDNAs showed complete sequence identity in their overlapping regions, indicating that they originated from the same gene. To assess whether the full sequence of the transcript was encoded by these cDNAs, 5′ RACE PCR was carried out, using poly(A)$^+$ RNA and two nested oligonucleotides near the 5′ end of the 0.96-kb clone (see Fig. 2 for their positions). The major PCR product of ≈1.5 kb extended beyond the 5′ end of the 1.7-kb fragment by only 62 bases. Taking these results together, we conclude that the full-length cDNA is 4.3 kb. It codes for a polypeptide of 1259 amino acids, or 140 kDa, and contains presumptive 5′ and 3′ untranslated regions of 116 and 417 bases, respectively (Fig. 2). The ORF was also confirmed by microsequence analysis of a tryptic peptide released from the 145/143-kDa doublet (see Fig. 2).

The poly(A) tail at the 3′ end of the cDNA indicates that the 140-kDa polypeptide is encoded by the nuclear genome. Examination of the primary sequence of the 140-kDa polypeptide reveals that its N-terminal region has characteristics of a transit peptide (26)—i.e., it is rich in Ser and Thr residues (28 within the first 100 residues), and is basic with few acidic residues. Asp and Glu residues make up 12% of the remainder of the protein, which has a predicted pI of 5.8. The lack of a good consensus sequence, as well as the variable length of transit peptides of proteins targeted to the chloroplast, makes it difficult to identify a cleavage site. However, *in vitro* import experiments using a truncated form of the precursor, synthesized by *in vitro* transcription/translation, have confirmed the functional role of the transit peptide-like region and indicate that it is about 7 kDa (P.S.V. and G.K.L., unpublished results).

Data bases (Swiss-Prot, EMBL, GenBank, DDBJ) were searched to determine if the 140-kDa polypeptide is related to any known proteases. Beginning at Leu-222, the 140-kDa polypeptide shows strong similarity to a recently recognized family of metalloendopeptidases, the pitrilysins (11). This family is exemplified by *E. coli* protease III (12) and human (13) and *Drosophila* (14, 18) IDEs, which are ≈110 kDa and have related substrate specificities *in vitro*. A zinc-binding

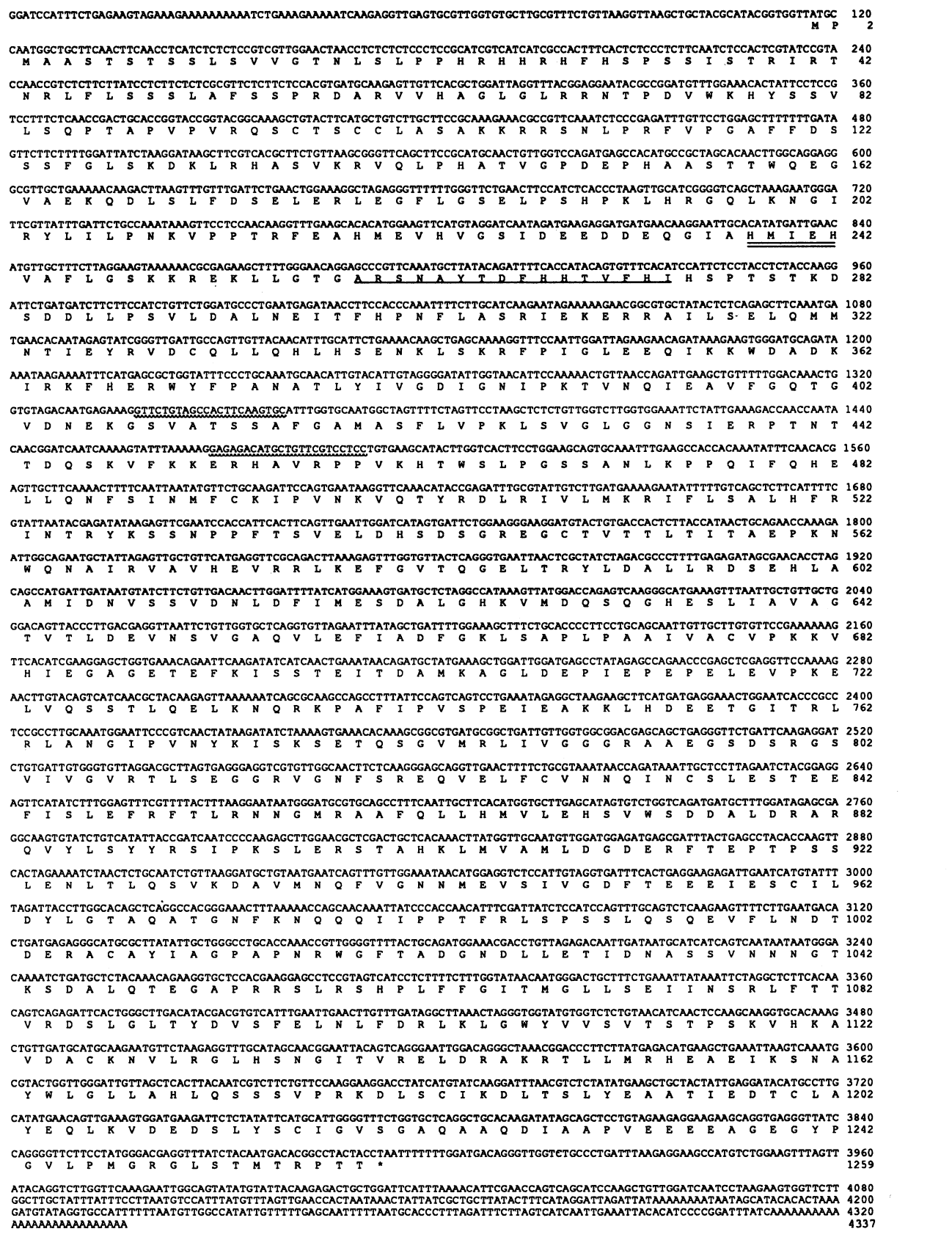

FIG. 2. Nucleotide sequence of a cDNA coding for CPE and its deduced amino acid sequence. Sequences complementary to primers used for 5′ RACE PCR are underlined with wavy lines. Amino acid sequence from tryptic peptide sequencing of the 145/143-kDa proteins is underlined with a solid line and the zinc-binding HXXEH motif is underlined twice.

His-Xaa-Xaa-Glu-His (HXXEH) motif, which defines this family, is located at position 238–242 of the 140-kDa polypeptide. It has now been recognized in a growing list of metalloendopeptidases, including the MPP subunit β (15–17), an *N*-arginine dibasic convertase (27), a hypothetical protease YDDC within the glutamate decarboxylase operon of *E. coli*

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CPE      LKNGIRYLIL PNKVPPTRFE AHMEVHVGSI DEEDDEQGIA HMIEHVAFLG 247
ProtIII  LDNGMVVLLV SDPQ.AVKSL SALVVPVGSL EDPEAYQGLA HYLEHMSLMG  97
hIDE     LANGIKVLLM SDPT.TDKSS AALDVHIGSL SDPPNIAGLS HFCEHMLFLG 117
YDDC     LDNGLRYMIY PHAHPKDQVN LWLQIHTGSL QEEDNELGVA HFVEHMMFNG  85
Neu PEP  LKNGLT.VAS QYSPYAQTST VGMWIDAGSR AETDETNGTA HFLEHLAFKG  93
Rat PEP  LENGLR.VAS ENSGIS.TCT VGLWIDAGSR YENEKNNGTA HFLEHMAFKG 110

CPE      SKKR..EKLL GT........ GARSNAYTDF HHTVFHIHSP TSTKDSDDLL 287
ProtIII  SKKYPQADSL AEYLKM...H GGSHNASTAP YRTAFYLEVE ......NDAL 138
hIDE     TKKYPKENEY SQFLSE...H AGSSNAFTSG EHTNYYFDVS ......HEHL 158
YDDC     TKTWPGNKVI ETFESMGLRF GRDVNAYTSY DETVYQVSLP TTQKQN...L 132
Neu PEP  TTKRTQQQLE LEIENM.... GAHLNAYTSR ENTVYF.... ..AKALNEDV 133
Rat PEP  TKKRSQLDLE LEIENM.... GAHLNAYTSR EQTVYY.... ..AKAFSKDL 150

CPE      PSVLDALNEI TFHPNFLASR IEKERRAILS ELQMMNTIE 326
ProtIII  PGAVDRLADA IAEPLLDKKY AERERNAVNA ELTMARTRD 177
hIDE     EGALDRFAQF FLCPLFDESC KDREVNAVDS EHEKNVMND 197
YDDC     QQVMAIFSEW SNAATFEKLE VDAERGVITE EWRAHQDAK 171
Neu PEP  PKCVDILQDI LQNSKLEESA IERERDVILR ES...EEVE 169
Rat PEP  PRAVEILADI IQNSTLGEAE IERERGVILR EM...QEVE 186
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FIG. 3. Comparison of the region containing the HXXEH motif of CPE with representative members of the pitrilysin family. The alignment was created by using the programs PILEUP and PRETTY from the Genetics Computer Group. Residues that are identical to or conservatively changed (D and E; I, L, and V; R and K) compared with those in CPE are in boldface. Sequences aligned are Prot III (protease III; ref. 12); hIDE (human IDE; ref. 13); YDDC (Swiss-Prot no. P31828); and Neu PEP and Rat PEP [MPP β subunit from *Neurospora crassa* (16) and rat (17), respectively]. The numbers at right indicate the amino acid position in each protein.

(Swiss-Prot no. P31828), and a *Bacillus subtilis* ORF near the diaminopimelate operon (28). A comparison of the 140-kDa polypeptide with representatives of this family (Fig. 3) reveals 25–30% sequence identity, or 35–40% similarity, in an N-terminal region of 130 amino acids which continues beyond the HXXEH motif to residue 326; thereafter sequence similarity becomes more scattered. Overall, YDDC shows the most sequence relatedness to the 140-kDa polypeptide.

When plants are grown in the dark many nuclear-encoded proteins of the photosynthetic apparatus are not synthesized, in particular those involved in the light reactions such as LHCP (29). However, the plastid performs a number of other essential metabolic processes besides photosynthesis—e.g., fatty acid and amino acid synthesis—that are active in the dark (30). We therefore investigated whether expression of CPE is light dependent. We first compared the level of CPE poly(A)$^+$ mRNA from plants grown in either the dark or the light. The steady-state amounts of CPE mRNA (≈4.0 kb) relative to total poly(A)$^+$ mRNA were the same under the two growth conditions (Fig. 4*A*). Using immunoblots with the anti-GST fusion serum, we examined the amount of the 145/143-kDa doublet. Plastids were isolated from plants greened for 0, 6, or 24 hr, or grown under the normal light:dark cycle (16:8 hr), and equivalent amounts of soluble protein were analyzed. Examination of membrane and soluble protein by Coomassie blue-stained gels showed no LHCP and a low level of Rubisco at the 0 hr time point; both increased dramatically by 24 hr (T.M.B. and G.K.L., unpublished results). Nearly the same amount of the 145/143-kDa doublet was detected in the dark and over the greening period (Fig. 4*B*, lanes 1–4). Because no decline in the level of CPE was observed during a time when there is a large increase in the amount of Rubisco accumulating in the stroma, these results indicate that the absolute amount of CPE increased proportionally. The same extracts prepared from dark- and light-grown plants were tested for CPE activity by using preLHCP as a substrate in an organelle-free processing assay (3). PreLHCP was cleaved to approximately the same extent in each reaction (Fig. 4*C*, lanes 1–5), producing the expected 25-kDa mature form of LHCP (8). Taken together, these results show that CPE does not depend on light for expression in pea plants and suggest that increased synthesis accompanies the import of nuclear-encoded proteins which begin to rapidly accumulate upon exposure to light.

To determine if CPE is synthesized and active in organs not involved in photosynthesis, plastids were isolated from pea roots and soluble extracts were prepared. The anti-GST fusion serum recognized both the 145- and 143-kDa proteins, which

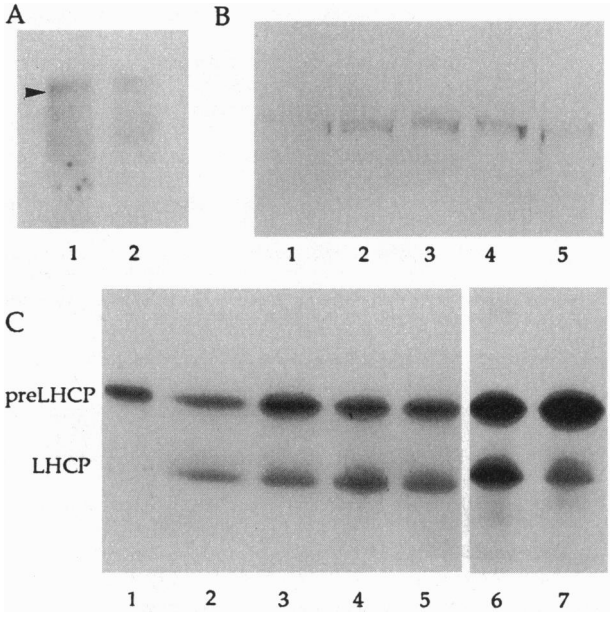

FIG. 4. Analysis of CPE expression in light- vs. dark-grown leaves, greening leaves, and roots. (*A*) CPE mRNA detected by Northern analysis. Poly(A)$^+$ RNA in 10-μg samples from light- (lane 1) or dark- (lane 2) grown leaves was subjected to denaturing gel electrophoresis (1% agarose), transferred to nylon membrane, and hybridized with a digoxigenin-labeled cDNA probe spanning nucleotides 1289–1790 as shown in Fig. 1. The arrowhead indicates the position of 25S ribosomal RNA (≈4.0 kb). (*B*) Protein immunoblot using the anti-GST fusion serum. Equivalent amounts of plastid protein (25 μg) from plants grown on a 16:8 hr light:dark cycle (lane 1), in the dark (lane 2), or greened for 6 hr (lane 3) or 24 hr (lane 4) were run on a 10% acrylamide gel, transferred to nitrocellulose, incubated with the anti-GST fusion serum, and detected as described in the legend of Fig. 1. Protein (75 μg) from isolated root plastids was also examined (lane 5). Lane 5 was developed until the reaction was completed. (*C*) Detection of CPE activity by using the organelle-free assay. Plastid extracts (25 μg of protein for lanes 2–6, 50 μg for lane 7) were incubated with [^{35}S]methionine-labeled preLHCP synthesized in *E. coli*. Lane 1 shows a mock reaction with no extract. Extracts were prepared from plants grown on the normal light:dark cycle (lane 2), in the dark (lane 3) or greened for 6 hr (lane 4) or 24 hr (lane 5). In a separate experiment, CPE activity was assayed from plants grown on a normal light:dark cycle (lane 6) and from roots of these plants (lane 7).

were equally abundant, but overall levels were 1/5 to 1/10 of those found in leaf extracts (Fig. 4*B*, lane 5). Significantly, preLHCP was cleaved in the organelle-free assay using the root extracts (Fig. 4*C*, lane 7) despite the fact that root plastids do not normally see preLHCP as a substrate. These results suggest that CPE is necessary in roots for the import and maturation of proteins targeted to the plastid for nonphotosynthetic functions.

DISCUSSION

In this report we describe the primary structure of a 140-kDa polypeptide deduced from cDNAs isolated by using antiserum raised against a 145/143-kDa doublet previously shown to be involved in proteolytic cleavage of chloroplast precursors (10). The 140-kDa polypeptide contains an N-terminal domain that is highly conserved in a recently recognized family of metalloendopeptidases (11). Interestingly, this domain contains a HXXEH zinc-binding motif that was demonstrated to be critical for proteolysis. Site-directed substitutions of the histidines or glutamic acid in the HXXEH motif of human IDE (19, 20) and its homolog from *E. coli*, protease III (18), block cleavage of insulin. The histidines are also essential for zinc

binding (18). Thus, it was concluded that the HXXEH motif constitutes a Zn^{2+}-coordinating catalytic site in these proteases. On the basis of the strong conservation with IDE and protease III, we suggest that the HXXEH motif near the N terminus of CPE may play a similar catalytic role in the cleavage of chloroplast precursors. Although it is unknown if CPE binds zinc, divalent cation chelators inhibit processing (3). Heterodimeric MPP of mitochondria may utilize a related mechanism for precursor cleavage, as its β subunit also contains the HXXEH signature (15–17), but it remains to be established which metal ion MPP requires.

From the diversity of substrates cleaved by members of this metalloendopeptidase family one would predict that the determinants for substrate recognition reside in novel regions of each enzyme, outside of the HXXEH-containing domain. It is relevant that mutations in the HXXEH sequence block proteolysis but not binding of IDE to insulin (20). Structural features must also exist in each substrate to facilitate specific cleavage. For processing by CPE, as well as MPP, the precursor N-terminal targeting signal and cleavage site *per se* are undoubtedly important (31, 32), but it is currently unclear how they participate in the reaction. With the cloning of CPE it should soon be possible to explore its interactions with precursors targeted to the plastid and to test by mutational analyses our predictions on the mechanism of precursor cleavage.

Our results demonstrate that CPE expression is not light dependent in pea. Not only are mRNA and the 145/143-kDa proteins present in the dark, but cleavage of preLHCP occurs with etioplast extracts. Mature LHCP, on the other hand, was not detectable in the etioplast membranes, in agreement with the fact that there is essentially no expression of the LHCP genes in the dark (29). Thus, the synthesis of CPE is regulated separately from LHCP, and it can precede the light-dependent accumulation of products needed for photosynthesis. Furthermore, CPE is active in root plastids, a finding that supports the conclusion that CPE is not preLHCP-specific, but rather has broader substrate specificity as a general stromal processing peptidase. Many metabolic pathways of the plastid—e.g., for fatty acid and amino acid biosynthesis—are functional in the dark and in other organs besides leaves (30) and depend on the import of numerous proteins from the cytosol. Indeed, immunodepletion experiments indicate that cleavage of the precursor of acyl carrier protein, a key protein involved in fatty acid synthesis, requires the 145/143-kDa proteins (10). It is likely, therefore, that CPE expression is activated by an endogenous developmental program that begins at an early stage of plastid biogenesis. Nevertheless, it appears that light can stimulate the synthesis of CPE in parallel with the large influx of nuclear-encoded proteins which occurs during greening and assembly of the photosynthetically competent organelle.

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