The signal recognition particle (SRP) and its receptor (FtsY in prokaryotes) are essential for cotranslational protein targeting to the endoplasmic reticulum in eukaryotes and the cytoplasmic membrane in prokaryotes. An SRP/FtsY-like protein targeting/integration pathway in chloroplasts mediates the posttranslational integration of the light-harvesting chlorophyll a/b-binding protein (LHCP) into thylakoid membranes. GTP, chloroplast SRP (cpSRP), and chloroplast FtsY (cpFtsY) are required for LHCP integration into thylakoid membranes. Here, we report the reconstitution of the LHCP integration reaction with purified recombinant proteins and salt-washed thylakoids. Our data demonstrate that cpSRP and cpFtsY are the only soluble protein components required for LHCP integration. In addition, our studies reveal that ATP, though not absolutely required, remarkably stimulates LHCP integration into salt-washed thylakoids. ATP stimulates LHCP integration by a mechanism independent of the thylakoidal pH gradient (∆pH) and exerts no detectable effect on the formation of the soluble LHCP-cpSRP-targeting complex. Taken together, our results indicate the participation of a thylakoid ATP-binding protein in LHCP integration.

Chloroplasts are structurally complex organelles with multiple membranes and aqueous compartments. The innermost chloroplast membrane, the thylakoid membrane, separates the stroma from the thylakoid lumen and is the site of light-driven photophosphorylation. Although chloroplasts possess their own genome and protein synthesis machinery, the majority of thylakoid proteins are encoded in the nucleus, synthesized in the cytoplasm, and imported into the stroma by a general protein translocase in the envelope. Imported precursor proteins are processed to pathway intermediates in the stroma and then enter one of four different targeting pathways for localization to the thylakoids (1, 2). The cpSRP-dependent pathway appears to specifically target the light-harvesting chlorophyll-binding proteins that use an Albino3-containing translocase for integration into the thylakoid membrane (3, 4).

The major light-harvesting chlorophyll-binding protein (LHCP)1 is an integral thylakoid membrane protein with three transmembrane helices. Following import into the stroma, LHCP binds cpSRP to form a soluble targeting complex (5–7). Whereas the 54-kDa subunit of cpSRP (cpSRP54) is thought to bind one of three hydrophobic domains, the 43-kDa subunit of cpSRP (cpSRP43) binds an 18-residue hydrophilic domain (L18) located between the second and the third transmembrane helices of LHCP (8, 9). Interaction between cpSRP43 and L18 is required for LHCP-targeting complex formation with cpSRP and confers cpSRP54 with its unique posttranslational binding activity. Both cpSRP and a chloroplast homologue of the bacterial SRP receptor FtsY (cpFtsY) are required for LHCP integration into thylakoid membranes (6, 7, 10, 11). As previous studies on reconstituting LHCP integration have never been conducted in the absence of wheat germ extract or reticulocyte lysate, it is not known whether additional soluble components are required for LHCP integration.

In the present study, we have reconstituted the LHCP integration reaction using purified recombinant substrate (His-cpSRP), purified recombinant soluble protein components (cpSRP54His, His-cpSRP43, Trx-cpFtsY), and salt-washed thylakoids. Our data demonstrate that GTP, cpSRP54, cpSRP43, and cpFtsY are the only soluble components required for LHCP integration into salt-washed thylakoids. Further, our studies reveal that ATP, though not essential, greatly stimulates LHCP integration into thylakoid membranes. ATP stimulates LHCP integration by a mechanism independent of the ∆pH previously shown to enhance LHCP integration into isolated thylakoids. As ATP shows no effect on the formation of the soluble LHCP-cpSRP-targeting complex, we propose that an ATP-binding protein at the membrane is involved in LHCP integration into thylakoid membranes.

EXPERIMENTAL PROCEDURES

Cloning of cpSRP43 and cpFtsY—First strand synthesis of the full-length cpSRP43 coding sequence was obtained using Moloney murine leukemia virus reverse transcriptase (Stratagene) according to the manufacturer’s instructions in a reaction containing total Arabidopsis RNA and a reverse primer complimentary to the stop codon and the last 25 bases of the cpSRP43 PCR amplification of the first strand was accomplished by adding a forward primer corresponding to the first 25 bases of the cpSRP43 precursor and containing an EcoRI site at the 5’ end. The resulting PCR product was digested with EcoRI and inserted into pGEM-4Z (Promega) at EcoRI and Smal sites to generate pGEM-cpSRP43. DNA sequencing analysis revealed the cpSRP43 coding sequence (GenBankTM accession no. AAD01509). The coding sequence for the precursor form of cpFtsY was amplified from Arabidopsis RNA by reverse transcription-PCR using ThermoScript reverse transcriptase (Invitrogen) and ligated into Smal-restricted pGEM-4Z in the SP6 direction to create cpFtsY4Z. The sequence was deposited to GenBank™ data base (accession no. AF120112).

Construction of Expression Plasmids—The coding sequence for mature LHCP, starting with amino acid sequence MKSATT, was PCR-amplified from psAB80XD/4 (12) and cloned into pQE-80L (Qiagen) using BamH1 and Smal sites to produce pQE-LHCP. The coding sequence for mature cpSRP54, starting with amino acid sequence

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‡ The abbreviations used are: LHCP, light-harvesting chlorophyll a/b-binding protein; SRP, signal recognition particle; Chl, chlorophyll; IB, import buffer.
were verified by sequencing analysis.

**Expression and Purification of Recombinant Proteins**—Expression plasmids were transformed into *Escherichia coli* strain BL21 (Invitrogen), and cells were cultured in LB medium. Cells were induced with 0.5 mM isopropyl-1-thio-

**Miscellaneous Methods**—Chloroplasts and Thylakoids—Chloroplasts were isolated from 10- to 11-day-old pea seedlings (Laxton’s Progress) as described (15). Thylakoids were isolated from lysed chloroplasts by microcentrifugation. Preparative centrifugation of thylakoids resulted in an area-denatured HisLHCP (0.05 μg) to the reaction mixture containing cpSRP54his (4 μg), His-cpSRP43 (4 μg), 1 mM ATP, and 1 mM GTP or otherwise specified. The urea content in the reaction mixture was increased to 5 mM. Assays for LHCP Integration and Transit Complex Formation—Integration assays were carried out as described in Cline et al. (14). Assays (40 μl) for transit complex formation were performed according to Payan and Cline (5) with modifications. Complex formation was initiated by adding urea-denatured HisLHCP (0.05 μg) to the reaction mixture containing cpSRP54his (4 μg), His-cpSRP43 (4 μg), 1 mM ATP, and 1 mM GTP or otherwise specified. The urea content in the reaction mixture was increased to 5 mM. Assays were executed by incubating the reaction mixture at 25 °C for 30 min. Transit complex formation was analyzed by native gel electrophoresis and visualized by phosphorimaging.

**RESULTS AND DISCUSSION**

CpSRP54, cpSRP43, and cpFtsY Are the Only Soluble Protein Components Required for LHCP Integration—A minimal requirement for LHCP integration into isolated thylakoids has been shown previously to include GTP, cpSRP54, cpSRP43, and cpFtsY (11). However, the possibility that additional soluble protein(s) might have contributed was not ruled out because of the presence of a complex mixture of proteins in which thylakoid extract or reticulocyte lysate employed for protein synthesis in vitro. To address whether cpSRP54, cpSRP43, and cpFtsY were sufficient in supporting integration, we prepared purified recombinant proteins (Fig. 1A) and reconstituted the integration reaction using thylakoids washed with salt to remove residual cpFtsY and cpSRP from the membrane (Fig. 1B). Proper integration was demonstrated by the presence of a characteristic degradation product of LHCP (LHCP-DP). A smaller degradation product marked with a star (*) was shown previously to be a properly inserted but not fully assembled species of LHCP (17). As shown in Fig. 1B, LHCP integration occurred only when recombinant cpSRP54, cpSRP43, and cpFtsY were all included in the assay (lane 8). No integration of LHCP was obtained in the absence of any of the three proteins (lanes 1–7). Assays carried out in the presence of cpSRP and the Trx portion of the Trx-cpFtsY fusion protein did not support LHCP integration (data not shown), indicating that integration with Trx-cpFtsY resulted from the activity of cpFtsY. These results demonstrate that cpSRP54, cpSRP43, and cpFtsY are the only soluble protein components required for LHCP integration.

Some protease-resistant HisLHCP together with some minor degradation products were observed to be associated with the membrane in assays lacking cpSRP54his or His-cpSRP43 (Fig. 1B, lanes 1 and 4). It has been demonstrated that denatured LHCP precursor protein interacts with thylakoids nonproductively in the absence of stromal proteins (15). The undigested HisLHCP may represent aggregates that are resistant to protease digestion. It is possible that the readiness of LHCP to adhere to thylakoids is a manifestation of its tendency to nonproductively insert and aggregate at the membrane. Before the identification of cpSRP, it was shown that one role of the stromal factor in LHCP integration was to keep LHCP soluble and translocation-competent (5). LHCP Integration Requires GTP and Is Stimulated by ATP—The nucleotide requirement for LHCP integration has been the subject of considerable study and debate. Early studies indicated that ATP is required for integration (12, 18, 19). Later, in light of the newly identified chloroplast homologue of SRP54 (13), a more careful examination of the nucleotide requirement implicated GTP as the only nucleotide required for integration (20). However, as ATP, CTP, and UTP all supported a low level...
of integration in the absence of GTP, a possible role of these nucleotides in LHCP integration was not ruled out (20). It has been suggested that the same enzyme that utilizes GTP also uses ATP, CTP, and UTP to a slight extent to support integration (20). An alternative explanation is that ATP, CTP, and UTP are used by kinases present in stromal extract and translation mixture to generate GTP by phosphorylating guanine-containing nucleotides retained in the stromal extract or the translation mixture.

To resolve this issue, we examined the nucleotide requirement for LHCP integration in a reconstituted integration assay with purified recombinant LHCP, cpSRP, cpFtsY, and salt-washed thylakoids (Fig. 2). GTP was absolutely required for LHCP integration; ATP (lane 2), CTP (lane 4), or UTP (lane 5) alone did not support appreciable integration. However, when ATP (lane 8) but not CTP (lane 9) or UTP (lane 10) was used in combination with GTP, integration of LHCP was enhanced more than 70%. Interestingly, AMP-PNP, a non-hydrolyzable analogue of ATP, also noticeably stimulated GTP-supported LHCP integration (lane 11). GMP-PNP (a non-hydrolyzable analogue of GTP) severely inhibited GTP-supported integration (lane 12), demonstrating that hydrolysis of GTP is required for LHCP integration.

In all experiments, we have consistently observed a 30–50% stimulation of LHCP integration by AMP-PNP in combination with GTP (data not shown). A similar level of stimulation in LHCP integration by AMP-PNP was also obtained by Jiang et al. (21). The fact that a non-hydrolyzable analogue of ATP stimulates LHCP integration indicates that hydrolysis is not a requirement for ATP to stimulate integration. Because ADP and AMP were found to have no effect on GTP-supported LHCP integration (data not shown), it appears that ATP is employed as a regulatory element and not used as an energy source in promoting LHCP integration. The fact that LHCP integration levels were the same using 1 mM GTP or 2 mM GTP, but nearly doubled by addition of 1 mM ATP to assays containing 1 mM GTP (data not shown) dem-
onstrates that the stimulation by ATP is not due to a dose effect of nucleotide or contaminating GTP in the ATP preparation.

ATP Stimulates LHCP Integration by a Mechanism Independent of the ΔpH—LHCP integration is stimulated by the ΔpH that forms during light-driven electron transport or through reverse proton pumping by the coupling factor in the presence of hydrolyzable ATP (12, 19, 22). To assess whether the ATP stimulation of LHCP integration is associated with the use of ATP to enhance the production of a ΔpH, we examined the effect of ATP on LHCP integration in the presence of nigericin, which dissipates the ΔpH. As shown in Fig. 3, nigericin was able to deplete the ΔpH and severely reduced the level of LHCP integration in light or in dark (compare lane 1 with lane 3; lane 2 with lane 4; lane 7 with lane 9; lane 8 with lane 10). Nevertheless, the ability of ATP to stimulate integration was not affected by the presence of nigericin. ATP stimulated LHCP integration both in the presence (compare lane 1 with lane 2; lane 5 with lane 6; lane 7 with lane 8; lane 11 with lane 12) and in the absence (comparing lane 3 with lane 4; lane 9 with lane 10) of the ΔpH, demonstrating that ATP is able to stimulate LHCP integration via a ΔpH-independent mechanism.

We also examined the effect of ATP on LHCP integration in the presence of tentoxin, which binds to coupling factor 1 of the coupling factor and prevents ATP from being used to generate a ΔpH or add to an existing ΔpH (19). As light provides energy for making the ΔpH, tentoxin showed no effect on LHCP integration in light (compare lane 1 with lane 5; lane 2 with lane 6), which also serves as evidence for tentoxin not having any effect on the protein targeting/integration machinery. In dark, tentoxin had little influence on LHCP integration in the absence of ATP (compare lane 7 with lane 11) but severely inhibited LHCP integration in the presence of ATP (compare lane 8 with lane 12), demonstrating that tentoxin is able to prevent an ATP contribution to the ΔpH and exerts its effect on integration through preventing ATP hydrolysis by the coupling factor. Yet, in light or in dark relative to GTP alone, ATP was able to stimulate LHCP integration in the presence of tentoxin (compare lane 5 with lane 6; lane 11 with lane 12), which further establishes that one role of ATP is to promote the ΔpH-independent integration of LHCP.

ATP Has No Effect on the Formation of the Soluble LHCP Targeting Complex—The targeting of LHCP to the thylakoid membrane is a multi-step process. The first major step in this process is the formation of a soluble targeting complex termed “transit complex” previously shown to contain LHCP, cpSRP54, and cpSRP43 (6–9). Although there is evidence that purified precursor LHCP interacts with purified cpSRP, a transit complex made of only the three proteins has not been demonstrated directly (7). As cpFtsY is required for LHCP integration and the majority of cpFtsY is present in the stroma (11), it is possible that cpFtsY is involved in transit complex formation. To resolve this issue, the requirements for forming the soluble LHCP targeting complex were examined with purified components. As shown in Fig. 4A, in the absence of soluble proteins all of the radiolabeled His-LHCP remained in the sample well (lane 1). When individual cpSRP54his (lane 2), HiscpSRP43 (lane 3), or Trx-cpFtsY (lane 4) was added to the assay, a varying, small amount of labeled HisLHCP migrated into the gel (lanes 2–4), suggesting some interaction between HisLHCP and the individual proteins. However, the interaction between HisLHCP and the individual protein components is very weak compared with the ability of cpSRP (a complex of cpSRP54his and HiscpSRP43) to promote formation of the transit complex (lane 5). Neither a combination of cpSRP54his and Trx-cpFtsY (lane 6) nor a combination of HiscpSRP43 and Trx-cpFtsY (lane 7) promoted transit complex formation. In fact, Trx-cpFtsY reduced the level of cpSRP-mediated transit complex formation (lane 8), suggesting that Trx-cpFtsY may interact with the transit complex to promote HisLHCP release when the targeting complex reaches the membrane. Regardless, our results have conclusively demonstrated that cpSRP54 and cpSRP43 are the only protein components required for forming the soluble LHCP targeting complex.

Although there is no evidence for a nucleotide requirement in LHCP transit complex formation, we assessed the possibility that the ΔpH-independent stimulation of integration by ATP might be due to an increase in transit complex formation. We examined the effect of various nucleotides on the formation of the transit complex with purified HisLHCP, HiscpSRP43, and cpSRP54his. As shown in Fig. 4B, no nucleotide is required for the formation of the transit complex (lane 1). The addition of ATP (lane 2), AMP-PNP (lane 3), CTP (lane 7), UTP (lane 8), a combination of ATP and GTP (lane 9), or a combination of AMP-PNP and GTP (lane 10) showed no detectable effect on the amount of transit complex formed. GTP (lane 4), GDP (lane 5), GMP-PNP (lane 6), GTP plus GMP-PNP (lane 11), GTP plus CTP (lane 12), or GTP plus UTP (lane 13) had only a very minor inhibitory effect on the formation of the transit complex. At the present, we do not know whether the minor inhibitory effect caused by guanine nucleotides has any biological significance. It is attractive to hypothesize that GTP or GDP binding to cpSRP54 weakens the interaction between LHCP and cpSRP and causes release of LHCP from cpSRP. Empty site forms of SRP54 and SRP receptor a-subunit GTPases are believed to mediate targeting of ribosome-nascent chain complexes to the endoplasmic reticulum (23). Regardless, our results have clearly shown that the ATP-stimulated integration reported in this study is not due to an effect of ATP on the formation of the soluble LHCP targeting complex. The fact that LHCP integration occurs in the absence of the chloroplast SecA (cpSecA) suggests that the targeting of LHCP to the thylakoid membrane is not mediated by a signal sequence.

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ATP in SRP/FtsY-supported Protein Integration

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