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Dual Signal Peptides Mediate the Signal Recognition Particle/Sec-independent Insertion of a Thylakoid Membrane Polyprotein, PsbY*

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Simon J. Thompson, Colin Robinson‡, and Alexandra Mant

From the Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, United Kingdom

The nuclear *psbY* gene (formerly *ycf32*) encodes two distinct single-spanning chloroplast thylakoid membrane proteins in *Arabidopsis thaliana*. After import into the chloroplast, the precursor protein is processed to a polyprotein in which each "mature" protein is preceded by an additional hydrophobic region; we show that these regions function as signal peptides that are cleaved after insertion into the thylakoid membrane. Inhibition of the first or second signal cleavage reaction by enlargement of the −1 residues leads in each case to the accumulation of a thylakoid-integrated intermediate containing three hydrophobic regions after import into chloroplasts; a double mutant is converted to a protein containing all four hydrophobic regions. We propose that the overall insertion process involves (i) insertion as a double-loop structure, (ii) two cleavages by the thylakoidal processing peptidase on the lumenal face of the membrane, and (iii) cleavage by an unknown peptidase on the stromal face on the membrane between the first mature protein and the second signal peptide. We also show that this polyprotein can insert into the thylakoid membrane in the absence of stromal factors, nucleoside triphosphates, or a functional Sec apparatus; this effectively shows for the first time that a multispanning protein can insert posttranslationally without the aid of signal recognition particle, SecA, or the membrane-bound Sec machinery.

The biogenesis of integral membrane proteins has attracted a great deal of experimental attention in recent years, in an effort to understand the mechanisms used to transfer hydrophobic regions from an aqueous milieu into the membrane bilayer and to achieve the correct final topology. Many of these studies have used bacterial proteins as model systems, and the emerging evidence points to the operation of two broad types of insertion mechanism, which can be classified as "assisted" and "spontaneous." Some proteins clearly rely on the membrane-bound elements of the Sec apparatus used for the export of proteins into the periplasm, although the extrinsic SecA ATPase appears to be used to a lesser extent unless large hydrophilic loops require translocation across the membrane (1–3). Signal recognition particle (SRP)[1] has also emerged as an important cytoplasmic factor that is required for the insertion of a range of membrane proteins (4–7). Cross-linking studies suggest that this ribonucleoprotein particle binds preferentially to highly hydrophobic regions, consistent with a role in membrane protein targeting (8). One such SRP-dependent protein has been shown to be inserted via the membrane-bound SecYEG complex, and it appears likely that most, if not all, SRP substrates will follow this route (9).

Other proteins are inserted by different means. The coat proteins of the M13 and pf3 phages have been shown to insert into the *Escherichia coli* plasma membrane by mechanisms that do not involve either SRP or the Sec machinery, and it has been proposed that these proteins insert spontaneously into the membrane (reviewed in Ref. 10). It should be noted though that other, as yet undefined membrane proteins could possibly assist in the insertion of this class of proteins (none of these proteins have been shown to insert with the correct topology into protein-free liposomes from an aqueous phase). A small number of other membrane proteins have similarly been proposed to follow either Sec- or SRP-independent insertion pathways (see *e.g.* Ref. 11), but in general, these studies have been carried out *in vivo* using Sec- or SRP-depleted cells, and the precise insertion requirements are difficult to monitor under these conditions. For example, one membrane protein previously designated "Sec-independent" using a SecY-deficient strain has recently been shown to be absolutely reliant on the Sec machinery in a SecE depletion strain that exhibits a stronger phenotype (9). Partly as a result of these problems, relatively few membrane proteins have been definitively shown to insert by Sec/SRP-independent mechanisms, and it has remained unclear whether this type of mechanism is widely used.

The chloroplast thylakoid membrane has emerged as an useful alternative system for this type of study because, although genetic analysis is more difficult, *in vitro* insertion assays have proved to be relatively facile (reviewed in Ref. 12). Again, two basic types of insertion mechanism have been characterized for the biogenesis of membrane proteins. The multispanning light-harvesting chlorophyll-binding protein (LHCP) of photosystem II is imported into the chloroplast by means of an envelope transit signal, after which it integrates into the thylakoid membrane by means of information contained in the mature protein (13, 14). This process requires stromal SRP (cpSRP54) and nucleoside triphosphates (NTPs), and hence the overall insertion process may well resemble bacterial SRP-dependent insertion events (15, 16). However, it should be noted that an RNA molecule has yet to be identified in chloroplast SRP, and certain aspects of the insertion process may therefore differ. Proteolysis of thylakoids destroys their ability to integrate LHCP (17), indicating that membrane-bound protein transport apparatus is required (probably the thylakoidal Sec machinery, although this remains to be confirmed).

A very different insertion process has been demonstrated for

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‡ To whom correspondence should be addressed. Tel.: 44-1203-523557; Fax: 44-1203-523701; E-mail: CG@dna.bio.warwick.ac.uk.

[1] The abbreviations used are: SRP, signal recognition particle; LHCP, light-harvesting chlorophyll-binding protein; NTP, nucleoside triphosphate; TPP, thylakoidal processing peptidase; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

a series of abundant single-spanning thylakoid membrane proteins: subunit II of the CF_o assembly of the ATP synthase (CF_oII) and the X and W subunits of photosystem II (PsbX and PsbW). In contrast to LHCP (and most other multispanning proteins), these proteins are synthesized with bipartite presequences in which the usual envelope transit peptide is followed by a cleavable signal peptide. Signal peptides usually specify an interaction with protein translocation systems, and most thylakoid lumen proteins are synthesized with this type of peptide and translocated by either a Sec- or ΔpH-dependent translocase in the thylakoid membrane (reviewed in Ref. 12). However, CF_oII, PsbW, and PsbX have all been shown to insert into thylakoids in the absence of SecA, SRP, or NTPs, and proteolysis of thylakoids has been shown to block Sec-dependent transport but have no effect on the insertion of the above proteins (17–21). These findings represent strong evidence that neither SRP nor the Sec apparatus is required, and it has been proposed that these proteins may insert spontaneously into the thylakoid membrane. In this insertion mechanism, the proposed role of the signal peptide is to provide an additional hydrophobic section, which, together with the corresponding region in the mature protein, is able to partition into the membrane and drive the transport of the hydrophilic region (the N terminus of the mature protein) into the lumen. Cleavage by the thylakoidal processing peptidase (TPP) on the *trans* side of the membrane then yields the mature protein. Inhibition of the processing step has been shown to generate such a loop intermediate (22) akin to that involved in the insertion of M13 procoat (10).

To date, only simple, single-span proteins have been definitively shown to insert into bacterial or thylakoid membranes by SRP/Sec-independent mechanisms, raising the possibility that these factors tend to be recruited for more complex proteins and that the biogenesis of multispanning proteins may thus be rather more involved in general terms. In this report, we describe the insertion pathway for a thylakoid membrane polyprotein. Mant and Robinson (23) characterized an unusual *Arabidopsis thaliana* cDNA encoding a protein that contains two separate regions bearing high homology to *ycf32* open reading frames encoded by several algal plastid genomes. Whereas *ycf32* genes encode small single-span proteins, the *Arabidopsis* protein was predicted to contain four hydrophobic regions, and it was proposed that this was in effect a polyprotein of two separate Ycf32-related proteins, each of which was preceded by a signal-type peptide. It has now been shown that the two proteins are indeed found in thylakoids, associated with photosystem II, and the gene has been designated *psbY* (24). The individual polypeptides are designated PsbY-A1 and PsbY-A2. Here we describe a complex insertion/maturation pathway for PsbY that involves the use of two separate cleavable signal peptides, and we show that the entire polyprotein can insert into the thylakoid membrane by an SRP/Sec-independent mechanism.

EXPERIMENTAL PROCEDURES

Constructs—Oligonucleotide-directed, site-specific mutagenesis was carried out on the *Arabidopsis* cDNA encoding pYcf32/pPsbY used in the study of Mant and Robinson (23), using the inverse polymerase chain reaction method (25). 35-Mer primers were used to alter the following Ala codons to Thr as follows: mutant PsbY/1 (see under "Results"), codon 66 from GCC → ACC; PsbY/1*, codon 78 from GCC → ACC; PsbY/2, codon 143 from GCT → ACT; PsbY/2*, codon 150 from GCT → ACT. In mutant PsbY/1/Leu, codon 66 was altered to CUC (Leu). The construct encoding pre-A2 was synthesized by polymerase chain reaction amplification of the *Arabidopsis* cDNA region encoding signal 2 and protein A2. The forward primer (5′-GAG AGT AAA CAT ATG GTT GTT GGT CTA GG-3′) introduced an *Nde*I restriction site at glycine 118, altering it to methionine. The reverse primer (5′-CGT AAG CTT GGA TCC TCT AGA GCG GC-3′) took advantage of the preexisting *Not*I linker restriction site in the cDNA template. The amplified region was cloned 5′ *Nde*I-*Not*I 3′ under control of the SP6 promoter of pGEM®5Zf (Promega) and completely sequenced before being used as a template for *in vitro* transcription and translation.

Import Assays—Precursor proteins were synthesized *in vitro* by transcription of cDNA clones followed by translation in a wheat germ lysate in the presence of [^{35}S]methionine or [^{3}H]leucine as detailed by Mant and Robinson (23) and Robinson *et al*. (17). Assays for the import of proteins by intact chloroplasts and isolated pea thylakoids were as in the same references; a more complete description of the thylakoid import assay is given in Brock *et al*. (26). Apyrase (Sigma, type VI) was used to deplete the import incubation of NTPs using a protocol adapted from (27). Twice-washed thylakoids (30 μg of chlorophyll for the experiment shown in Fig. 6; 20 μg of chlorophyll for the experiment shown in Fig. 7) resuspended in stromal extract were incubated for 10 min on ice with either 4 units of apyrase or 4 units of inactivated enzyme (100 °C for 10 min). Next, 10 μl of puromycin-treated translation mixture (see below) was added to the thylakoids on ice and incubated for a further 10 min before being transferred to the light bath for 30 min. The final volume of each incubation was 50 μl, and stromal extract was present at 1.3× the equivalent concentration of chlorophyll. Import buffer was 10 mM Hepes-KOH, pH 8.0, 5 mM $MgCl_2$ (HM buffer). Postincubation, the thylakoids were washed and analyzed directly (by mixing with protein sample buffer), after protease treatment with thermolysin at 0.2 mg ml^{-1} for 40 min on ice, or after washing the membranes with urea (see below).

Puromycin Treatment of Translation Mixtures—In order to avoid nonspecific association of precursor proteins with thylakoid membranes, *in vitro* translation mixtures were treated with 0.1 mg ml^{-1} puromycin (to dissociate ribosomes) for 2 min at room temperature, at the end of the translation incubation. The translation mixture was then centrifuged at 100,000 × *g* for 20 min at 4 °C to pellet any aggregated proteins. The resulting supernatant was used in thylakoid insertion assays.

Urea Washing of Thylakoid Membranes—These analyses were carried out essentially as described by Breyton *et al*. (28). Thylakoid membranes (10–20 μg of chlorophyll) were washed in ice-cold 20 mM Tricine-NaOH, pH 8.0, resuspended in a freshly prepared solution of 6.8 M urea/20 mM Tricine-NaOH, pH 8.0, and incubated for 10 min at room temperature (around 22 °C). The samples were then subjected to two cycles of freeze-thawing (dry ice-room temperature) and centrifuged at 120,000 × *g* for 15 min at 4 °C in a Beckman TL100 ultracentrifuge using a TLA100.3 rotor. Care was taken to remove the top 80 μl of supernatant without disturbing the membrane pellet. The remaining 20 μl of supernatant was discarded. The pellet was then resuspended once more in 100 μl of urea solution, and the whole process was repeated. The second extraction rarely removed any extra material from the thylakoid membranes. Samples of the final membrane pellet and the first supernatant (equivalent volumes loaded), along with the other assay samples, were analyzed by SDS-polyacrylamide gel electrophoresis followed by staining with Coomassie Brilliant Blue and fluorography.

Proteolysis of Thylakoid Membranes before Insertion Assays—The protocol was as described by Robinson *et al*. (17) with the following modifications. After treating the thylakoids with 60 μg ml^{-1} trypsin (Sigma, type XIII) for 10 min on ice, the digestion was stopped by the addition of 120 μg ml^{-1} trypsin inhibitor (Sigma, type I-S), and the thylakoids reisolated by centrifugation at 100,000 × *g* for 10 min at 4 °C. The thylakoids were washed twice in HM plus 60 μg ml^{-1} trypsin inhibitor (centrifuged at 100,000 × *g* for 5 min at 4 °C) and finally resuspended in stromal extract (for pPsbY and pre-A2) or HM buffer (p23K). Each sample contained thylakoids equivalent to 20 μg of chlorophyll, 0.5 mM MgATP, and 10 μl of translation mixture all buffered by HM (final volume, 50 μl). Stromal extract, when present, was at a concentration equivalent to 1.3× the chlorophyll concentration. The import/insertion incubation was carried out under a green safelight, for 30 min at 26 °C, and the prevailing ΔpH was measured by 9-aminoacridine fluorescence quenching as described (17); this was found to be invariably over 2 units.

Rapid Stopping of Chloroplast Import—A stock solution of 0.2 M $HgCl_2$ was prepared as described by Reed *et al*. (29). Aliquots of intact chloroplasts from an import assay (30 μl; 10 μg of chlorophyll) were mixed with 2 μl of $HgCl_2$ at each sampling time. Chloroplasts were pelleted by centrifugation at 2000 × *g* for 2 min at 4 °C, as soon as possible after mixing with $HgCl_2$. They were then gently resuspended in 1 ml of 50 mM Hepes-KOH, pH 8.0, 330 mM sorbitol, 10 mM EDTA, recentrifuged, and finally resuspended in the latter buffer plus protein sample buffer.

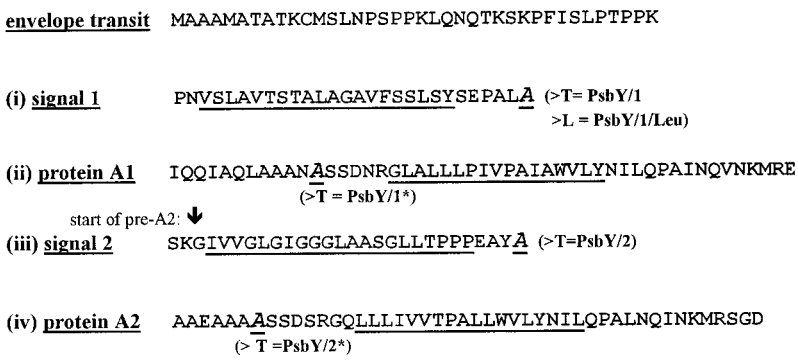

FIG. 1. **Primary structure and proposed domain organization of pPsbY.** The Fig. shows the full predicted sequence of *Arabidopsis* pPsbY (previously designated Ycf32 (23)). The N-terminal region contains an envelope transit peptide that is believed to be removed after import by the stromal processing peptidase (note that this processing site has not been identified). Proteins A1 and A2 are indicated (see text), each of which is preceded by an apparent signal peptide. The exact position of the junction between protein A1 and signal peptide 2 has not been determined. Hydrophobic regions in the signal peptides and mature proteins are *underlined*. Candidate −1 Ala residues of TPP cleavage sites are shown *italicized* and *underlined*; these were altered to Thr residues in four single mutants, the designations of which are shown. Construct pre-A2 was synthesized by amplification of the coding region for the C-terminal region of PsbY and the introduction of a start codon in place of the Gly indicated by an *arrow* (see under "Experimental Procedures").

RESULTS

The PsbY Translation Product Contains Two Signal Peptides—Our initial aim in this study was to determine whether the *psbY* gene product is indeed processed to two small single-span proteins through the use of dual signal peptides as proposed in Ref. 23. Fig. 1 shows the overall primary structure of the full precursor protein (which we term pPsbY) encoded by the *Arabidopsis psbY* cDNA protein, in which the structure is divided into five domains. The predicted sequence reveals an apparently typical stroma-targeting envelope transit that is basic, hydrophilic, and enriched in hydroxylated residues. This is followed by the initial "mature" protein (PsbY), which contains four hydrophobic regions: (i) a predicted signal peptide, (ii) a region that is closely homologous to algal *ycf32* open reading frames (protein A1), (iii) a second possible signal peptide, and (iv) a second region homologous to single-span Ycf32 proteins (protein A2). Thylakoid signal peptides are cleaved by a membrane-bound, lumen-facing TPP activity that cleaves after short-chain residues at the −3 and −1 positions in the substrate relative to the processing site; the presence of Ala at −1 is essential for efficient cleavage (30). Ala is also usually found at the −3 position. Hydrophobic regions i and iii above were considered to be possible signal peptides on the basis that these regions are followed by potential Ala-X-Ala TPP cleavage sites.

We first sought to clarify whether the internal hydrophobic region iii is in fact a cleavable signal peptide. The cDNA coding region for hydrophobic regions iii and iv was amplified using polymerase chain reaction and an ATG codon incorporated at the 5′-end in order to synthesize and import the second section of the PsbY polypeptide. This construct is termed pre-A2. The import of wild-type pPsbY into chloroplasts is shown as a time course analysis in Fig. 2*A*. In order to identify possible processing intermediates, the samples were rapidly treated with $HgCl_2$, which has been shown to inhibit import/processing events in studies on other chloroplast proteins (29, 31). Fig. 2*A* shows that the 23-kDa precursor protein, which migrates as 19 kDa in this gel system, is processed to two small polypeptides with mobilities of 6 and 7 kDa, as found by Mant and Robinson (23). However, other processing products are apparent, including the initial processed, imported form (PsbY), which migrates just below the precursor protein (see below).

The pre-A2 construct can not be imported into chloroplasts because it lacks an envelope transit peptide, and we therefore used assays for the import of proteins into isolated pea thylakoids. Fig. 2*B* shows that this construct is inserted and processed to a smaller product, providing strong evidence that the construct does indeed contain a cleavable signal peptide. This product has precisely the same mobility as the upper band from a chloroplast import reaction (Fig. 2*B, mark*), and we therefore assign this upper band to protein A2. These data provide further compelling evidence that pPsbY is processed to two individual single-span proteins.

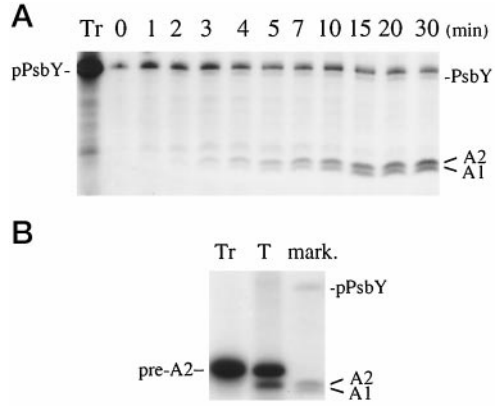

FIG. 2. **Protein A2 is preceded by a cleavable signal peptide.** *A,* [^{35}S]Methionine-labeled pPsbY was synthesized *in vitro* and incubated with intact pea chloroplasts. At time intervals indicated above the lanes (in min) samples were mixed with $HgCl_2$ and centrifuged briefly to pellet the chloroplasts. The chloroplasts were then washed with import buffer containing EDTA and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography (see under "Experimental Procedures" for further details). *B,* the pre-A2 translation mixture (*lane Tr*) was incubated with isolated pea thylakoids (*lane T*). After incubation, the sample was analyzed together with a marker sample (*mark.*) from the 30-min import of pPsbY shown in *panel A*. Proteins A1 and A2 are indicated together with pPsbY that is bound to the chloroplast envelopes in the marker lane.

PsbY Inserts into Thylakoids as a Double-loop Structure—The above data and sequence information strongly suggest that pPsbY contains a total of four hydrophobic regions, including two cleavable signal peptides. Because TPP is known to be active on the lumenal side of the thylakoid membrane, this protein offers attractive possibilities in terms of identifying the topology of the polypeptide chain during membrane insertion.

In order to address this topic more directly, we took advantage of the highly precise nature of the TPP reaction, by substituting Thr residues at possible −1 positions. This markedly inhibits the TPP processing reaction (22, 30), and we reasoned that this would lead to the identification of defined intermediates on the PsbY biogenesis pathway. Any observed inhibition

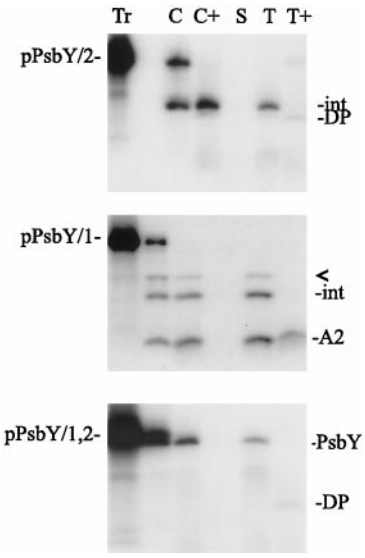

FIG. 3. **Accumulation of processing intermediates in the Ala → Thr mutants.** Mutants PsbY/1, PsbY/2, and the PsbY/1,2 double mutant (*lanes Tr*) were imported into intact chloroplasts and samples analyzed of the chloroplasts (*lane C*) and thermolysin-treated chloroplasts (*lane C+*). Other aliquots of chloroplasts were pelleted after protease treatment and lysed, after which, centrifugation yielded samples of stroma (*S*) and thylakoids (*T*). *Lane T+,* thermolysin-treated thylakoids. Processing intermediates (*int*) and degradation products (*DP*) are denoted. *Arrow* denotes larger cleavage product of unknown significance (see text).

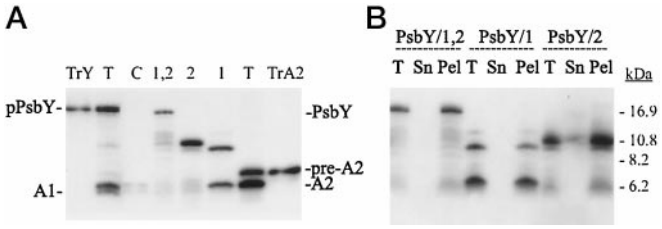

FIG. 4. **PsbY processing intermediates contain three hydrophobic regions and are stably inserted into the thylakoid membrane.** *A,* size comparison of the imported proteins. The figure shows the mobilities of the PsbY/1, PsbY/2, and PsbY/1,2 import products (*lanes 1, 2,* and *1,2,* respectively) together with a sample from a thylakoid import of pre-A2 and sample of pre-A2 translation product (*lanes T* and *TrA2* at the *right*) and a sample of the thylakoids from thylakoid and chloroplast imports of pPsbY (*lanes T* and *C,* respectively, at the *lefts*). Mobilities of molecular mass markers are indicated on the *right*. *B,* the three mutants described in *A* were imported into chloroplasts and the thylakoid fraction prepared after lysis (*T*). Samples of the membranes were then washed with urea and samples analyzed of the the supernatant fraction (*Sn*) and the pellet fraction (*Pel*) containing the membranes. *TrY*, pPsbY translation product.

would furthermore help to define these cleavable peptides as substrates for TPP because only minor, single substitutions were made at each site, and identical substitutions had no effect on the insertion of loop structures in pre-PsbW or pre-PsbX (22). Both terminal Ala residues were therefore altered to Thr by site-specific mutagenesis of the cDNA clone (the relevant residues are shown in Fig. 1, *italicized* and *underlined*). In addition, a mutant was made in which both sites were disrupted (PsbY/1,2). Two further potential TPP cleavage sites were also identified, and the −1 Ala residues mutated to Thr; these mutants were designated PsbY/1* and PsbY/2*, as shown in Fig. 1. Finally, we addressed the possibility that the presence of a Thr residue might affect the translocation of the intervening regions as well their removal by TPP. This was, in our view, extremely unlikely because Thr residues had no detectable effect on the rate of insertion insertion of PsbX or PsbW into the the thylakoid membrane or on the translocation of the hydrophilic section into the lumen (22). Nevertheless, we tested this possibility directly by altering the −1 Ala at the first predicted cleavage site to Leu on the basis that this would similarly prevent cleavage by TPP, while having no predicted effect on insertion (if anything, the more hydrophobic Leu residue should enhance insertion). This mutation is designated PsbY/1/Leu.

Analyses of several of the PsbY mutants are shown in Fig. 3. All of the mutant proteins are imported by isolated chloroplasts and found exclusively in the thylakoid fraction. The import and processing profiles of mutants PsbY/1* and PsbY2* were found to be exactly as those shown in Fig. 1 for the wild-type protein (data not shown), strongly indicating that these mutations do not lie at TPP cleavage sites. However, the remaining mutants exhibit severe defects in processing. PsbY/2 is imported and converted to a 10.8-kDa product, as shown in Fig. 3, *top panel*. Clearly, the Thr residue prevents cleavage at the second site by TPP, and a larger polypeptide accumulates. There is, however, good evidence that the first signal peptide has been cleaved because the mobility of the protein is consistent with a three-span protein rather than a protein containing all four hydrophobic regions (see below)

PsbY/1 is imported and converted to three polypeptides, one of apparently mature size (7 kDa) together with a processing intermediate (designated *int*), which migrates as 10.2 kDa. The 7-kDa protein co-migrates with protein A2 from chloroplast import assays with the wild-type protein or from thylakoid import assays using pre-A2 (see below), and we therefore conclude that the A2 protein is correctly removed from the PsbY/1 polyprotein upon insertion. Almost no A1 is formed, however, indicating that the presence of the Thr residue has a drastic effect on the release of this protein and providing very strong evidence that the first hydrophobic region is in fact a cleavable signal peptide that is recognized by TPP. Identical data were obtained for the PsbY/1/Leu mutant (not shown), indicating that the appearance of the PsbY/1 intermediate reflects an inhibition of processing rather than any difficulty of translocating the more hydrophilic Thr residue across the membrane.

A larger polypeptide was also apparent in this experiment (denoted by an *arrow*), but the identity of this band is unclear because it is usually present in lower quantities and is sometimes virtually absent (see, for example, the PsbY/1 import results in Fig. 4). In contrast, the 10.2- and 7-kDa proteins were always observed in approximately equal quantities.

The import experiment using the PsbY/1,2 double mutant is shown in Fig. 3, *bottom panel*. This protein was imported and cleaved to a larger product that migrates as a 17.5-kDa protein which is thus only marginally smaller than the full precursor protein. This product almost certainly results from removal of the envelope transit peptide (see below). Overall, these data strongly support the proposal that PsbY contains two signal peptides that are removed upon insertion by TPP. The results are, furthermore, consistent with data from the purified spinach A2 protein (24), the N-terminal sequence of which, ASEEIARGSDNRG, resembles the sequence following the second proposed TPP cleavage site motif that was mutated in our *Arabidopsis* mutant PsbY/2 (AAEAAAASSDSRG). However, the *first* cleavage site region within spinach PsbY is PAFAVQLADIAAEAGTSDNRG, and the N-terminal sequence of purified spinach A1 was deduced to be the AEAGTSDNRG sequence underlined above. This finding suggested that TPP cleaved after the sequence QLADIA, whereas we believe that TPP cleaves slightly upstream of this region (after PAFA in the spinach sequence, which corresponds to the PALA sequence targeted for mutation in our *Arabidopsis* sequence, as shown in Fig. 1). In our view, cleavage after DIA is highly unlikely

because charged −3 residues are unknown in thylakoid-targeting signals, and the corresponding region in the *Arabidopsis* PsbY sequence (QIAQLA) would contain Gln as the −3 residue. Side-chains of this length at the −3 position are not tolerated by TPP (for example, both Leu and Glu drastically inhibit processing (30)), and we propose instead that TPP cleaves after PALA in the *Arabidopsis* sequence (PAFA in the spinach sequence shown above), both of which are perfect TPP recognition sites. This would imply that a further cleavage by an unknown protease takes place to yield the mature A1 protein sequenced from spinach (24).

Further analyses of the processing mutants are shown in Fig. 4. *Panel A* shows a comparison of all of the intermediate forms together with appropriate marker proteins. The autoradiogram confirms that the imported PsbY/1,2 polypeptide is significantly larger than the intermediates generated during import of either PsbY/1 or PsbY/2 and that the latter intermediates are in turn significantly larger than the pre-A2 translation product containing two hydrophobic regions. This result strongly suggests that the two single mutants are imported and processed to polypeptides containing three hydrophobic regions. The imported PsbY/1,2 mutant is only marginally smaller than the full precursor and clearly contains all four hydrophobic regions. Fig. 4 also confirms the point made above, namely that the A2 protein is cleaved from mutant PsbY/1, because the smaller import product in *lane 1* co-migrates precisely with the A2 protein generated in a thylakoid import assay (adjacent *lane T*). We therefore conclude that neither of the single mutations prevents cleavage at the unmutated TPP cleavage site. The only difference in the import profiles of PsbY/1 and PsbY/2 is that a lower molecular mass cleaved product is visible in the former but not in the latter. This reflects the nature of the smaller cleaved species. Protein A2 is released from PsbY/1, and this protein is stable under these conditions, whereas cleavage of PsbY/2 at the first processing site leads to release of the signal peptide. We have in fact found that this signal peptide is undetectable even after over-exposure of the fluorographs and [^{3}H]leucine labeling (data not shown). After cleavage, these polypeptides are clearly turned over very rapidly indeed, and our attempts to visualize them have failed to date. Similar findings have been made with the single-span proteins PsbW and PsbX, which are also synthesized with cleavable signal peptides. Both proteins insert into thylakoids, yet the cleaved signal peptides are completely undetectable, despite being almost as large as the mature proteins (21).

All of the mutant forms are stably inserted into the thylakoid membrane because each is resistant to extraction by urea washing. There is now good evidence that this procedure effectively removes extrinsic membrane proteins from thylakoids (23, 28), and Fig. 4*B* shows that each of the intermediates is almost completely resistant to this extraction procedure. In all cases, the protein from the thylakoid fraction of a chloroplast import experiment (*T*) was almost totally recovered in the pellet fraction containing the urea-washed membranes (*Pel*), and very little protein was recovered in the supernatants (*Sn*). However, the precise topologies of the intermediates are difficult to determine. Studies on PsbW have shown that the precursor protein inserts as a loop intermediate prior to cleavage by TPP in the lumen (22), and the two signal peptides in PsbY probably form similar loops with their cognate mature proteins. It is, however, difficult to determine whether both loops have formed in the inserted PsbY/1 or PsbY/2 intermediate forms. In the case of PsbY/1, the second loop must have formed for protein A2 to be released, but it is notable that the remaining intermediate is highly sensitive to digestion by thermolysin

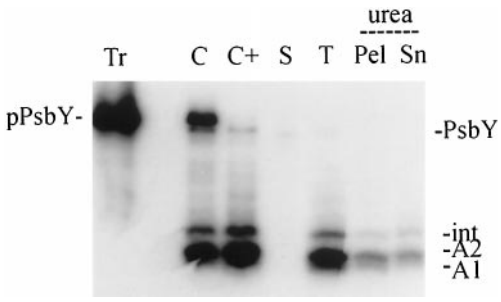

FIG. 5. **Urea resistance of thylakoid-integrated A1 and A2 subunits. pPsbY was imported into intact chloroplasts, and samples fractionated and analyzed as described in Fig. 3.** The thylakoid fraction (*lane T*) was then subjected to urea washing as detailed under "Experimental Procedures," and samples of the pellet (*Pel*) and supernatant (*Sn*) fractions were analyzed. Other symbols are as in Figs. 2–4.

(Fig. 3, *lane T+*). The lack of any defined protease digestion product means that we cannot be certain that the first loop has formed. With PsbY/2, a thermolysin degradation product is apparent that migrates as a 9-kDa protein (Fig. 3, *DP*), but further analyses are required before the topology of this protein can be determined.

Finally, the import data shown in Figs. 2–4 have another important implication for the overall insertion mechanism used by PsbY. Hydrophobic regions i and iii can now clearly be designated as cleavable signal peptides that are processed upon reaching the thylakoid lumen, and these peptides must therefore form loop structures with their partner A1 or A2 proteins. This means that an additional cleavage event must take place, between the A1 protein and the second signal peptide, and this event must furthermore take place on the stromal side of the membrane (see under "Discussion"). Our data indicate that this occurs relatively late in the maturation process because, given that each of the PsbY/1 and PsbY/2 processing intermediates shown in Fig. 3 contains three hydrophobic regions, neither can have undergone this cleavage step.

PsbY Inserts Primarily by an SRP/Sec-independent Mechanism—The single-span proteins CF$_o$II, PsbW, and PsbX are of interest because each is synthesized with a cleavable signal peptide, yet their insertion into thylakoids does not require nucleoside triphosphates or stromal factors, ruling out an involvement of SRP or SecA. Furthermore, extensive trypsin treatment of thylakoids blocks import by the Sec- and SRP-dependent pathways yet has no effect on the insertion of this group of proteins, strongly suggesting that the membrane-bound Sec apparatus is not involved (18–21). In this context, the insertion mechanism used by PsbY is of significant interest because this protein is far more complex in structural terms and is effectively a multispanning protein during the initial stages of the integration process. Similar tests were used to assess its mode of insertion into isolated thylakoids, and we used two criteria as evidence of correct insertion. Firstly, PsbY insertion should be accompanied by the appearance of the A1 and A2 subunits. This alone would indicate that insertion has taken place because TPP is active on the *trans* side of the membrane. Protease resistance is often also used as an alternative criterion for insertion, but this is unsatisfactory in the case of PsbY because proteases tend to cleave the precursor protein to a size similar to those of the mature A1 and A2 subunits (not shown). Instead, we used urea washing as a second criterion, as described above for the localization of the processing intermediates. However, control tests have shown that urea washing yields slightly less clear-cut results with small single-span proteins than with multispanning proteins, as illustrated in Fig. 5, which shows the urea resistance of authentic A1 and A2 proteins generated during a chloroplast

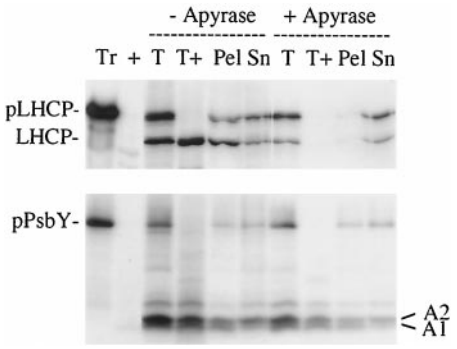

FIG. 6. **Insertion of pPsbY into thylakoids does not require nucleoside triphosphates.** Petunia pre-LHCP (*pLHCP*) and pPsbY were incubated with pea thylakoids in the presence of either 4 units of boiled apyrase (control conditions) or 4 units of active apyrase, on ice, as described under "Experimental Procedures." After incubation with thylakoids, samples were analyzed directly (*T*) or after treatment of the thylakoids with 0.2 mg/ml thermolysin for 40 min on ice (*T*+). Other samples of non-protease-treated thylakoids were washed with urea, and samples of the pellet (*Pel*) and supernatant (*Sn*) fractions were analyzed. *LHCP* denotes mature LHCP polypeptide.

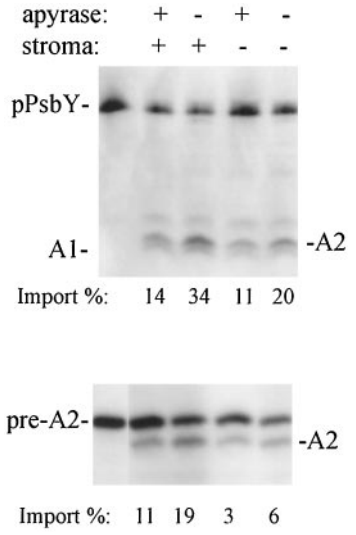

FIG. 7. **Stromal factors and NTPs stimulate but are not a prerequisite for the insertion of PsbY into thylakoids**. pPsbY and pre-A2 were incubated with pea thylakoids in the presence or absence of stromal extract as indicated; other incubations with (+) or without (−) stromal extract were preincubated with 4 units of apyrase as described in Fig. 6. Control incubations contained the same amount of boiled apyrase. Samples were analyzed of the thylakoids after incubation; symbols are as in Fig. 6.

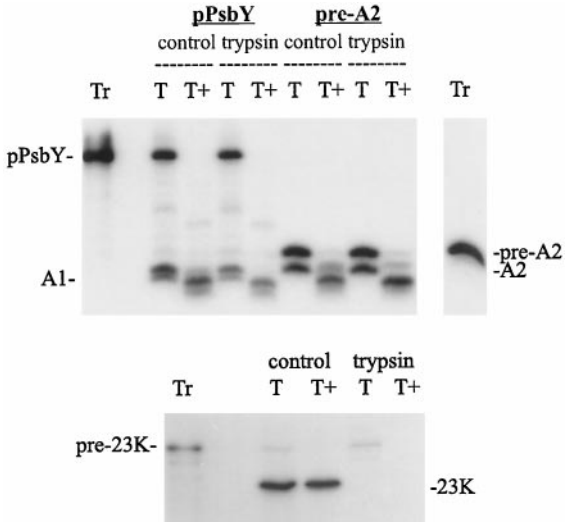

FIG. 8. **Protease treatment of thylakoid membranes does not block the insertion of pPsbY.** pPsbY, pre-A2 (*upper panel*), and pre-23K (*lower panel*) were incubated with thylakoids under control conditions or with thylakoids that had been pretreated with 60 μg/ml trypsin under conditions that retain a high thylakoidal ΔpH (see under "Experimental Procedures"). After the import incubations, samples were analyzed directly (*T*) or after thermolysin treatment of the thylakoids (*T*+). *23K,* mature 23K.

import experiment. A significant proportion of A1 and A2 is washed from the membrane and recovered in the supernatant fraction, apparently because the urea washing process is relatively harsh for single-span proteins (we have found that a similar proportion of mature CF_oII and PsbW is likewise extracted by urea; data not shown).

Fig. 6 shows assays for the insertion of PsbY into thylakoids, in which apyrase was used to deplete the system of all NTPs present. As a control, we analyzed the insertion of LHCP, which depends entirely on NTPs for SRP-dependent insertion (16, 32). After incubation of petunia pre-LHCP with thylakoids, the membranes were reisolated, and the *top panel* of Fig. 6 shows that this fraction (*lane T*) contains a mixture of precursor protein and processed, mature size LHCP (the result of cleavage by stromal processing peptidase present in the incubation). Treatment of these membranes with 0.2 mg/ml thermolysin (*lane T*+) yielded a significant amount of mature size protein, which represents inserted protein; previous studies (14, 17) have shown that this particular LHCP protein is completely resistant to further digestion under these conditions when correctly inserted. We also subjected the thylakoids to urea extraction, and the data show that a similar proportion of protein is found in the pellet fraction (Fig. 6, *Pel*), again indicative of correct insertion (it is notable that a proportion of both precursor and mature size protein is urea-resistant, indicating that insertion can precede proteolytic processing by the stromal processing peptidase). The apyrase-pretreated samples show a very different profile: essentially no protease-resistant protein was apparent, and the vast majority of protein was urea-extractable. These data agree with previous studies (16, 32) concerning the NTP dependence of LHCP insertion and show that urea resistance is a useful alternative criterion for insertion.

PsbY also inserts into isolated thylakoids and is efficiently processed to the A1 and A2 subunits in lane T of the control (minus apyrase) incubation. Urea washing of the membranes showed that about 50% of the protein is recovered in the pellet fraction (Fig. 6, *Pel*). Because this level of urea resistance is similar to that found for authentic A1 and A2 in chloroplast imports (Fig. 5), we concluded that the mature proteins were correctly inserted in the thylakoid import reactions. Significantly, apyrase treatment does not block import, because a high level of urea-resistant mature A1 and A2 was again generated. This indicates that NTPs are not required for insertion and hence that neither SecA nor SRP is required. Nevertheless, some inhibition was observed, and quantitation of the insertion reaction shows that apyrase treatment reduces insertion efficiency to 47% of the control value. In this respect the insertion of PsbY differs from that of the single-span proteins, such as PsbX or PsbW, where apyrase was found to have no effect whatsoever on insertion efficiency (21). This result suggests that some PsbY molecules may insert with the aid of SRP or SecA.

The same conclusion is reached in tests for dependence on stromal factors. LHCP insertion into thylakoids is almost totally dependent on the presence stromal extract, which contains the bulk of SRP (15, 32), and Fig. 7 shows the results of this type of analysis for PsbY. The data show that the presence of stromal extract enhances insertion efficiency (from 20 to 34%

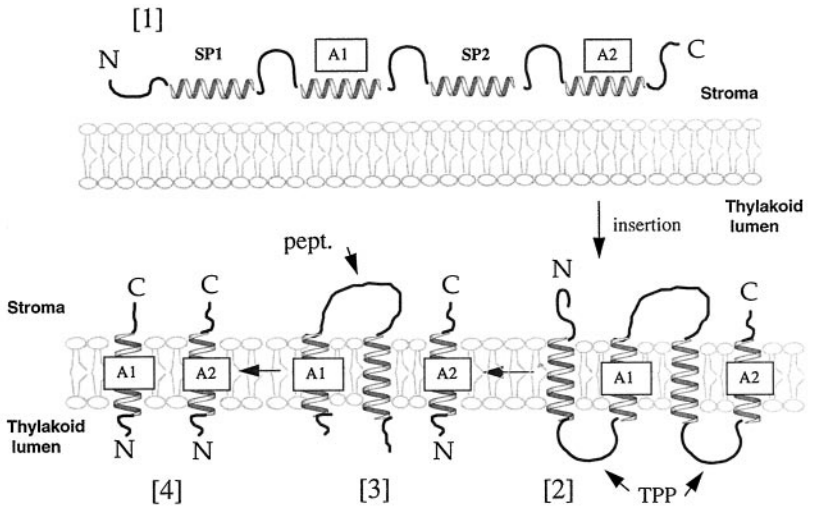

FIG. 9. **Model for the insertion of PsbY.** *Stage 1,* stromal PsbY contains four hydrophobic regions (shown as helices), which include proteins A1 and A2 together with associated signal peptides (*SP1* and *SP2*). This protein inserts as a double loop structure (*stage 2*) with the N terminus of the mature proteins located in the lumen. Cleavage at the TPP cleavage sites (*stage 3*) yields the mature A2 protein together with protein A2 and the attached signal peptide 2. Cleavage of the latter intermediate by an unknown peptidase (*pept.*) on the stromal face of the membrane generates the mature A1 protein (*stage 4*).

of available precursor), and it is notable that apyrase treatment largely abolishes this stimulatory effect, reducing insertion efficiency to 14% in the presence of stromal extract. This result is highly reproducible and points to a proportion of PsbY molecules being targeted by a pathway(s) that depend on both stromal factors and NTPs. Similar results were obtained for the pre-A2 construct, indicating that the partial NTP/stroma dependence is not simply due to the relative complexity of the PsbY polypeptide.

Finally, we tested the effects of pretreating the thylakoids with trypsin because this treatment has been shown to destroy their ability to import substrates on the ΔpH-, Sec-, or SRP-dependent pathways (17). The data (Fig. 8) show that both pPsbY and pre-A2 are imported and processed after this treatment, although a slight inhibition was apparent in the case of PsbY, which again points to an assisted pathway being used by a subset of molecules. The import of pre-23K into the lumen is completely blocked by the trypsin treatment, and similar effects were observed for LHCP and 33K, a Sec substrate (not shown). We therefore conclude that PsbY can insert into thylakoids in the absence of NTPs, a ΔpH, SecA, SRP, or a functional Sec complex in the thylakoid membrane.

DISCUSSION

Our data indicate that the insertion and maturation of the PsbY A1 and A2 proteins takes place by an unusual pathway involving multiple proteolytic processing steps. After import, the removal of the first, envelope transit peptide is presumably carried out by the stromal processing peptidase, as with all other import stromal/thylakoid proteins (12). Thereafter, we propose that two distinct cleavable signal peptides are used to assist the insertion of the A1 and A2 proteins. The evidence is overwhelmingly in favor of this premise: these sequences are certainly removed and then degraded, the sequences bear the typical hallmarks of thylakoid signal peptides (a hydrophobic region followed by a helix-breaking Pro or Gly residue and then an Ala-*X*-Ala motif) and the substitution of the −1 alanine residues results in the almost complete inhibition of TPP activity as found in other studies using thylakoid signal peptides (22, 30). The *psbY* gene therefore encodes the first chloroplast-targeted polyprotein to be characterized in higher plants.

The precise topologies of the mature A1 and A2 proteins have yet to be established, but because TPP is active in the thylakoid lumen, it appears inevitable that the N termini of the mature proteins are located in this compartment and that the signal peptides therefore function in a manner analogous to those of PsbW and PsbX. With these single-span proteins, the role of the signal peptide appears to be to assist integration by providing an additional hydrophobic region, and pre-PsbW has been shown to form such a loop intermediate prior to cleavage by TPP (22). Interestingly, this type of insertion process appears to be used only when the protein is first imported into the chloroplast. Genes encoding PsbX and CF_oII have been identified in cyanobacteria and the plastid genomes of several eukaryotic algae, and in no case is the protein preceded by a signal-type peptide (21, 33). This raises the possibility that the signal peptides have been acquired only after the transfer of the genes to the nucleus because the initial endosymbiotic events involving a cyanobacterial-type organism, and that the more complex import pathway necessitates the presence of the second hydrophobic region, for unknown reasons. A basically similar situation applies to the *Arabidopsis psbY* gene: the homologous proteins encoded by open reading frames in the cyanobacterium *Synechocystis* PCC6803 and in several plastid genomes are devoid of signal peptides (23), suggesting that both of the PsbY signal peptides were acquired after transfer of the gene to the nucleus. All known cyanobacterial/plastid-encoded *ycf32* genes encode single-span proteins, indicating that the *Arabidopsis* gene arose by gene duplication, together with the acquisition of the two signal peptides. A working model for the insertion of PsbY is shown in Fig. 9. In this model, the A1 and A2 proteins each insert as loop structures together with their associated signal peptide. Because inhibition of cleavage at either site has no apparent effect on cleavage at the nonmutated site, we believe that the entire PsbY protein first inserts as a double-loop structure, after which cleavage takes place at the two TPP cleavage sites. A different, as yet unidentified peptidase is believed to cleave between the A1 protein and the second signal peptide, and this step must take place at the end the insertion process because no smaller cleaved products are apparent when TPP cleavage is inhibited in the PsbY/1,2 double mutant. According to the above model, this step has to take place on the stromal face of the thylakoid membrane; any other scenario would require transmembrane segments to reverse orientation, and this would be unusual in the extreme. One interesting possibility is that this cleavage site is tightly constrained in the PsbY polyprotein (perhaps as a tight loop) but that it becomes accessible when the two TPP cleavage events release a smaller, more flexible structure.

Although the maturation of PsbY is unique among known chloroplast proteins, our studies on the insertion requirements have more general implications because PsbY is clearly able to insert in the absence of either SRP or a

functional Sec apparatus. No NTP hydrolysis is required, and it was shown (23) that the thylakoid protonmotive force is likewise not required for efficient insertion. Thus, the absence of any identifiable essential insertion factor suggests that this protein may insert spontaneously into the thylakoid membrane, a possibility also proposed for CF_oII, PsbX, and PsbW (17–21). However, as in these previous studies, we would caution that other, as yet unidentified proteins could conceivably assist in the insertion process and that further studies are therefore required to confirm or refute this proposal. Nevertheless, the data are of general relevance because, although PsbY may resemble the above single-span proteins in some respects, it is far more complex in structural terms and is effectively a multispanning protein at the point of insertion. SRP has been implicated in the insertion mechanisms for a range of bacterial proteins and one thylakoid membrane protein (4–7, 15), and one bacterial SRP substrate has now been shown to use the Sec apparatus (9). However, our data demonstrate quite clearly that a complex multispanning membrane protein can insert with high efficiency in the absence of either SRP or Sec machinery, and there is in our opinion a high probability that the insertion process is indeed spontaneous. These findings have important implications for the mechanism used by SRP. Cross-linking studies on both the bacterial and chloroplast SRP have revealed a marked preference for binding to highly hydrophobic regions (8, 34), and the chloroplast SRP was not observed to bind to the less hydrophobic signal peptides of Sec- and ΔpH-dependent lumenal proteins (34). This observation is certainly consistent with the known substrate specificity of SRP, and it was suggested that the binding sites for SRP are the more highly hydrophobic transmembrane spans of integral membrane proteins. However, hydropathy analysis of PsbY and the LHCP that has been shown to insert by the SRP-dependent pathway (15), using, for example, the dense alignment surface (DAS) or TopPred II prediction methods (35, 36), suggests that the transmembrane segments of PsbY are at least as hydrophobic as those of LHCP (not shown). This raises the possibility that SRP may recognize a rather complex determinant that includes features other than a particularly hydrophobic region. In the case of PsbY, however, it is also of interest that insertion does not only occur by the stromal factor/NTP-independent route. Insertion is clearly stimulated by the presence of stroma and NTPs, and we speculate that a proportion of the PsbY molecules may in fact be targeted by the SRP pathway. Further studies should help to reveal why this highly hydrophobic molecule can insert with high efficiency by either of two very different mechanisms when LHCP is so completely dependent on both SRP and NTPs.

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