Sec/SRP-independent insertion of two thylakoid membrane proteins bearing cleavable signal peptides

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Abstract Two imported thylakoid membrane proteins, PSII-X and PSII-W, are synthesised with cleavable N-terminal signal peptides that closely resemble those of Sec-dependent lumenal proteins. In this report we have reconstituted the insertion of pre-PSII-X and pre-PSII-W into isolated thylakoids. We show that insertion does not require either nucleoside triphosphates or stromal extracts, both of which are required for Sec- and signal recognition particle (SRP)-dependent targeting mechanisms. Insertion is furthermore unaffected by protease treatments that destroy the known protein translocation apparatus in the thylakoid membrane. We conclude that these membrane proteins are inserted by an unusual Sec/SRP-independent mechanism that probably resembles that used by CF_oII, and we discuss possible parallels with the biogenesis of phage M13 procoat.

Key words: Chloroplast; Membrane protein; Sec; Signal peptide; Thylakoid membrane

1. Introduction

A diverse array of pathways operate for the targeting of chloroplast thylakoid proteins. Most thylakoid proteins are imported after synthesis in the cytosol, and whereas a single mechanism appears to operate for their transport across the envelope membranes, four distinct pathways have been identified for their subsequent targeting into and across the thylakoid membrane (reviewed in [1]). A subset of thylakoid lumen proteins are imported by means of bipartite presequences which contain envelope transit and thylakoid signal peptides in tandem. After import into the stroma, the signal peptide directs transport across the thylakoid membrane by an ATP-dependent, Sec-related mechanism, which was probably inherited from the cyanobacterial progenitor of the chloroplast [2–5]. Another subset of lumenal proteins are likewise synthesised with bipartite presequences, but the signal-type peptides of these proteins (thylakoid transfer peptides) direct translocation by a very different transport mechanism that does not require either stromal factors or ATP, but which is totally dependent on the thylakoidal ΔpH [6–9]. After translocation, the stromal intermediates are processed to the mature forms by a thylakoidal processing peptidase (TPP). The targeting signals of these proteins are all similar in overall structure to bacterial signal peptides, but specify translocation by only one of the above pathways [10,11]. It is as yet unclear how the two translocation systems recognise only their cognate precursors, but signals for the ΔpH-driven system are known to contain a twin-arginine motif that is essential for transport [12].

Two further pathways appear to be used primarily by integral membrane proteins. The major light-harvesting chlorophyll-binding protein, LHCP, is synthesised with an envelope transit signal only, and hence integrates into the thylakoid membrane by means of information contained in the mature protein. The integration process requires GTP and a stromal homologue of the 54 kDa protein of signal recognition particles (SRPs) [13]. The insertion of this protein is probably similar in most respects to the insertion of many bacterial membrane proteins, since SRP has been identified as a key requirement for the biogenesis of a range of bacterial inner membrane proteins [14,15]. However, a fourth import pathway has been identified in chloroplasts for subunit II of the integral CF_o component of the thylakoidal ATP synthase (CF_oII), and this pathway has very unusual features. CF_oII is synthesised with a bipartite presequence yet the precursor protein integrates into thylakoid membranes in the absence of stromal factors, nucleoside triphosphates or a ΔpH [16]. Furthermore, whereas the Sec-, ΔpH- and SRP-dependent mechanisms all rely on protease-sensitive translocation apparatus to mediate transport across the thylakoid membrane, massive proteolysis of thylakoids has no effect on the integration of CF_oII [17]. It has therefore been proposed that this protein inserts spontaneously into the thylakoid membrane.

In this report we have analysed the integration of two integral thylakoid membrane proteins: subunits W and X of photosystem II (PSII-X and PSII-W). Both proteins contain a single transmembrane span and each is synthesised with a bipartite presequence in which a typical 'envelope transit' signal is followed by a signal-type peptide [18,19]. We show that these proteins integrate into thylakoids by a Sec- and SRP-independent mechanism that is apparently similar to that used by pre-CF_oII. The signal peptides of these membrane proteins thus execute a completely different function when compared with targeting by the Sec machinery, and the data suggest that this unusual, possibly spontaneous insertion process is a mainstream pathway for the insertion of single-span proteins.

2. Materials and methods

2.1. Import assays

Precursor proteins were synthesised in vitro by transcription of cDNA clones followed by translation in a wheat-germ lysate in the presence of [^{3}H]leucine. Import assays involved the incubation of precursor proteins with intact pea chloroplasts as described in [6] or into isolated pea thylakoids essentially as described in [4] except that import of pre-PSII-W and pre-PSII-X into thylakoids involved the incubation of 10 μl translation mix with 40 μl thylakoids in 10 mM HEPES-KOH, pH 8.0, 5 mM $MgCl_2$ for 20 min at 25°C. Where applicable, preparation of stromal extract and apyrase treatments were as detailed in [4]. After incubation, thylakoids were treated

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with thermolysin (200 μg/ml for 40 min on ice). *Arabidopsis thaliana* pre-PSII-X was synthesised as described [19] and *Arabidopsis* pre-PSII-W was synthesised from a cDNA clone obtained from the *Arabidopsis* stock center (Ohio). The cDNA clone ID is 105K22T7 (accession number T22038). The predicted amino acid sequence is highly homologous to the spinach pre-PSII-W sequence [18] and identical to the predicted amino acid sequence of the *Arabidopsis* genomic pre-PSII-W sequence in the database (accession number X90769).

3. Results and discussion

Previous studies [18,19] have shown that the import and localisation of pre-PSII-X and pre-PSII-W into intact chloroplasts are unaffected by dissipation of the thylakoidal ΔpH, confirming that the ΔpH-dependent protein translocase is not involved in the membrane insertion processes. However, the possible involvement of the Sec- or SRP-dependent pathways remained open because effective inhibitors are not available to study these pathways in intact chloroplasts. An involvement of the stromal SecA component was effectively ruled out since azide, which partially inhibits this protein [11,20] had no effect on the import of PSII-W or PSII-X. However, the roles, if any, of the membrane-bound Sec machinery or of SRP could not be probed.

More detailed information concerning the integration process can be obtained using assays for the import of proteins into isolated thylakoids, and this type of assay has been used to define the requirements for Sec- and SRP-dependent translocation in some detail. The import into thylakoids of lumenal Sec substrates (e.g. PC and 33K) and the SRP substrate, LHCP, is stimulated by the addition of stromal extract (which contains the bulk of SecA and SRP), is absolutely dependent on nucleoside triphosphates and is sensitive to prior protease treatment of the thylakoids [4,7,13,17]. We used the same criteria to investigate the insertion of PSII-X and PSII-W. Fig. 1 shows thylakoid import assays using pre-PSII-W and pre-PSII-X, carried out in the presence or absence of stromal extract. The data show that incubation of the two precursor proteins with pea thylakoids leads to integration and processing to the mature size. The active site of the thylakoidal processing peptidase is on the lumenal face of the thylakoid membrane [21], and maturation therefore indicates that the N-terminus of the mature protein has been translocated across the membrane and the protein correctly integrated. We have also found (data not shown) that the mature PSII-X and PSII-W proteins are completely resistant to carbonate extraction of the membranes, which strips extrinsic proteins from the thylakoid membrane [16]. Mature-size PSII-W is converted to a smaller degradation product by protease treatment, identical in size to that observed in chloroplast import assays (see [18]), a further indication of correct integration. Incubation of pre-PSII-X with thylakoids leads to the generation of a mature-size protein that is fully protease-resistant; the protease resistance of the mature protein reflects the presence of only a very short C-terminal region following the predicted transmembrane span [19]. This particular precursor protein is unusually difficult to digest, and Fig. 1 also shows the presence of a degradation product between the precursor and mature proteins. Pre-PSII-W inserts with greater efficiency than pre-PSII-X, but the integration efficiency of pre-PSII-X is higher than is at first apparent because two thirds of the labeled leucine residues are lost when the presequence is removed. With both pre-PSII-X and pre-PSII-W, integration efficiency is es-

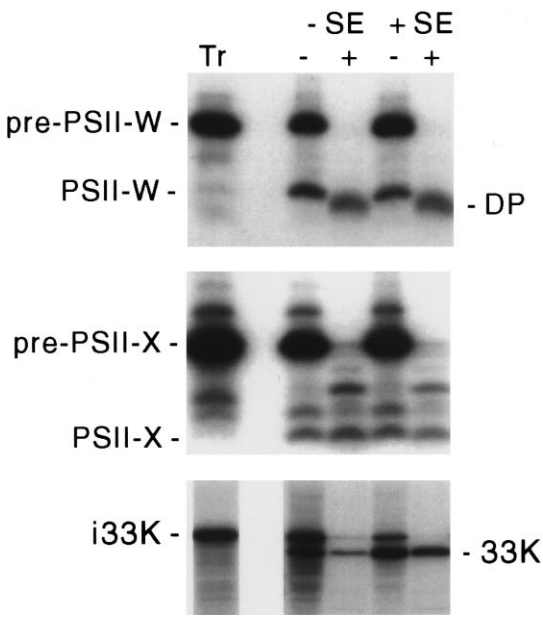

Fig. 1. Pre-PSII-W and pre-PSII-X integrate into isolated thylakoids in the absence of stromal factors. Pre-PSII-W and pre-PSII-X were incubated with isolated pea thylakoids in the presence or absence of stromal extract (SE) as indicated; the incubation conditions were as in Hulford et al. [4]. Further incubations with or without SE were carried out using i33K, an intermediate form of the lumenal 33K protein. After the import incubations, samples were analysed directly or after thermolysin treatment of the thylakoids (−, +, respectively).

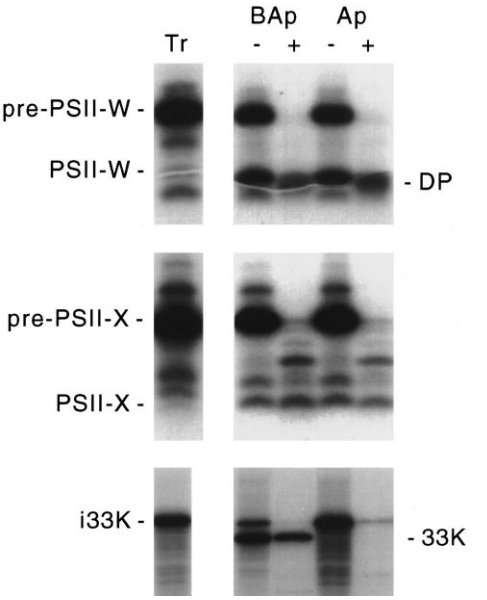

Fig. 2. Integration of pre-PSII-X and pre-PSII-W into thylakoids does not require NTPs. Pre-PSII-X and pre-PSII-W were incubated with isolated pea thylakoids in the absence of stromal extract. Incubations were preincubated at 4°C for 15 min in the presence of 1 unit apyrase (Ap) or an identical quantity of inactive (boiled) apyrase before the import incubation (BAp). Positive control for the apyrase experiment: an import mixture containing thylakoids, stromal extract and an intermediate-size form of 33K (i33K) was preincubated in the absence or presence of apyrase activity as above. After the import incubations, samples were analysed directly or after thermolysin treatment of the thylakoids (−, +, respectively).

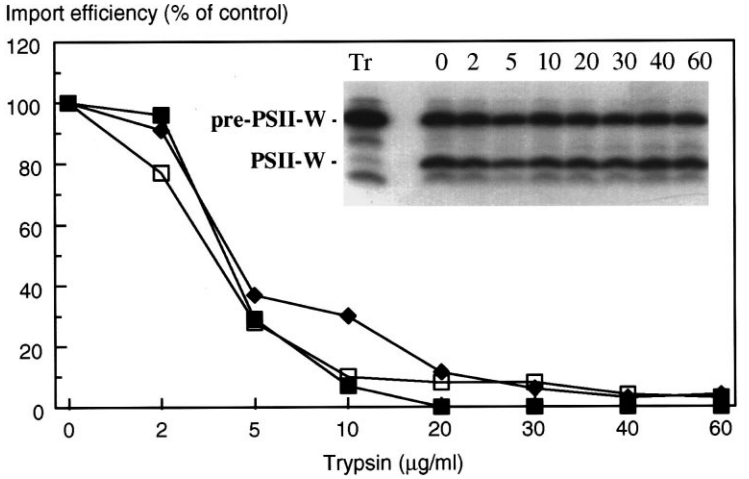

Fig. 3. Integration of pre-PSII-W is not affected by pretreatment of thylakoids with trypsin. Pea thylakoids were isolated as detailed in Section 2 and incubated with trypsin for 40 min on ice at concentrations (in μg/ml) indicated above the lanes. The thylakoids were then washed three times with HM buffer and incubated with pre-PSII-W as described in Fig. 2. Other incubations were conducted using pre-33K, pre-23K and pre-LHCP as substrates, as detailed in Robinson et al. [17]. After import, the import efficiencies were determined by laser densitometry, and are plotted graphically for 33K (diamonds), 23K (filled squares) and LHCP (open squares) relative to the control import efficiency (i.e. thylakoids preincubated with zero trypsin). The effects on pre-PSII-W integration are shown in the autoradiogram inset; densitometry confirmed that no loss of integration efficiency occurred after any of the trypsin incubations (data not shown). Other symbols as in Figs. 1 and 2.

sentially identical in the absence or presence of stromal extract; quantitation of the data shows that the import efficiencies for pre-PSII-X (in terms of the percentage of available precursor molecules integrated) are 12% and 11%, respectively, in the absence and presence of stroma. The corresponding figures for pre-PSII-W are 18% and 21%. These data strongly suggest that neither SecA nor SRP is involved because the import into thylakoids of both Sec- and SRP-dependent substrates is markedly enhanced by the presence of stromal extract [4,7]. The lower panel of Fig. 1 shows a control assay using 33K, a Sec-dependent lumenal protein, in which import is clearly stimulated by the addition of stromal extract.

The non-involvement of SecA or SRP is confirmed by the experiment shown in Fig. 2, in which import incubation mixtures were pretreated with apyrase to hydrolyse all nucleoside triphosphates present. Integration of both pre-PSII-X and pre-PSII-W is essentially unaffected, confirming that NTPs are not required, whereas the import of 33K in the positive control is totally blocked as found by Hulford et al. [4]. The targeting of a multi-spanning thylakoid membrane protein, LHCP, by the SRP-dependent mechanism is also absolutely dependent on NTPs [7,22], and an involvement of SRP in the targeting of PSII-W or PSII-X can thus also be ruled out. We conclude that the two precursor proteins integrate in the absence of SecA, SRP, or NTPs.

Although PSII-X and PSII-W are clearly not targeted by the 'standard' Sec-, SRP- or ΔpH-dependent mechanisms, the possibility remains that they might utilise membrane-associated translocation machinery while dispensing with soluble, NTP-hydrolysing elements. We have previously shown that all three of the above pathways require trypsin-sensitive translocation components in the thylakoid membrane [17] and we tested whether pre-PSII-W or pre-PSII-X rely on these components for integration. Fig. 3 shows the results of an experiment in which isolated thylakoids were treated with up to 60 μg/ml trypsin prior to incubation with pre-PSII-W, pre-23K (ΔpH-dependent), pre-33K (Sec-dependent) or pre-LHCP (SRP-dependent). A high ΔpH was maintained by driving the thylakoidal ATP synthase in reverse using the procedure described in [17]; the ATP synthase is unusually resistant to proteolysis and is able to generate a high ΔpH in the dark even after treatment of thylakoids with 60 μg/ml trypsin. The control tests in Fig. 3 confirm that the import of ΔpH-, Sec- and SRP-dependent substrates is either abolished or drastically inhibited, as found by Robinson et al. [17]. In contrast, the integration and processing of pre-PSII-W are entirely unaffected (inset in Fig. 3). Clearly, PSII-W does not utilise the membrane-bound transport apparatus used in these pathways. Similar tests have shown that the integration of pre-PSII-X is likewise totally unaffected by incubation of thylakoids with 60 μg/ml trypsin (data not shown).

In summary, the insertion of PSII-X and PSII-W takes place by a Sec/SRP-independent mechanism that is probably similar to that used by CF_oII. It is now clear that this is a mainstream insertion pathway for thylakoid membrane proteins and the data raise the possibility of a spontaneous insertion mechanism, although the possible involvement of uncharacterised thylakoid proteins cannot yet be ruled out. Whatever the mechanistic details, this type of insertion process is highly unusual and this is indeed the only known mainstream targeting pathway in which membrane proteins are synthesised with cleavable signal peptides but integrated by a Sec/SRP-independent mechanism. Only one protein has been shown to be inserted by such a process: the single-spanning coat protein of phage M13, which is synthesised with an apparently perfect signal peptide but inserted into the *Escherichia coli* plasma membrane by a Sec/SRP-independent mechanism (reviewed in [23]). The insertion of this precursor protein has been elegantly dissected and the signal peptide shown to function primarily by providing a second hydrophobic domain which, in concert with the hydrophobic sequence in the mature protein, partitions into the membrane forming a loop intermediate. Cleavage by signal peptidase then yields a transmembrane mature protein. Our data suggest a broadly similar type of insertion mechanism for this group of thylakoid pro-

teins and further work should provide a more detailed comparison with that of procoat.

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