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Distinct Albino3-dependent and -independent pathways for thylakoid membrane protein insertion

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SUMMARY

The homologous proteins Oxa1, YidC and Alb3 mediate the insertion of membrane proteins in mitochondria, bacteria and chloroplast thylakoids, respectively. Depletion of YidC in *Escherichia coli* affects the integration of every membrane protein studied, and Alb3 has previously been shown to be required for the insertion of a signal recognition particle (SRP)-dependent protein, Lhcb1, in thylakoids. In this study we have analyzed the 'global' role of Alb3 in the insertion of thylakoid membrane proteins. We show that insertion of two chlorophyll-binding proteins, Lhcb4.1 and Lhcb5, is almost totally blocked by preincubation of thylakoids with anti-Alb3 antibodies, indicating a requirement for Alb3 in the insertion pathway. Insertion of the related PsbS protein, on the other hand, is unaffected by Alb3 antibodies, and insertion of a group of SRP-independent, signal peptide-bearing proteins, PsbX, PsbW and PsbY is likewise completely unaffected. Proteinase K is furthermore able to completely degrade Alb3 but this treatment does not affect the insertion of these proteins. Among the thylakoid proteins studied here, Alb3 requirement correlates strictly with a requirement for stromal factors and nucleoside triphosphates. However, the majority of proteins tested do not require Alb3 or any other known form of translocation apparatus.

INTRODUCTION

The post-translational insertion of proteins into their target membranes has attracted a great deal of experimental attention in recent years in an effort to determine how hydrophobic regions are transferred from an aqueous environment into the membrane bilayer, and how the correct topology is achieved during this process. In bacteria, a complex 'assisted' pathway (reviewed in [1,2]) has been characterized in which newly-synthesized membrane proteins interact with signal recognition particle (SRP), FtsY and membrane-bound components of the secretory (Sec) apparatus [3-8]. SRP appears to be involved in membrane protein biogenesis by virtue of its tendency to interact with particularly hydrophobic protein segments [6,9].

A broadly similar 'assisted' pathway operates in plant thylakoids for the targeting of the major light-harvesting chlorophyll-binding (LHC) protein, Lhcb1, after import of this protein from the cytosol. Insertion of Lhcb1 into thylakoids requires nucleoside triphosphates (NTPs), stromal SRP, FtsY, and a thylakoid translocase minimally comprised of Albino 3 (Alb3) [10-13]. Post-translational formation of a SRP/Lhcb1 targeting complex requires a hydrophobic domain along with a novel SRP binding element in Lhcb1, termed the L18 domain, which is found only in members of the LHC protein family [14,15]. These data along with studies on chloroplast-synthesized D1 [16] suggest that SRP is again used primarily to direct membrane proteins to the thylakoid membrane.

For many years it was believed that other membrane proteins, in both bacteria and chloroplasts, were targeted by unassisted or 'spontaneous' insertion pathways, in which the protein inserted directly into the bilayer without the aid of other protein factors. The best characterized example was M13 phage coat protein, which inserts into the *Escherichia coli*

plasma membrane. This protein is synthesized with an N-terminal signal peptide, which assists insertion into the bilayer after which it is cleaved by leader peptidase (reviewed in [17]). The precursor (procoat) does not interact with either the Sec machinery or SRP. A second protein, Pf3 coat protein, was also shown to insert by an SRP/Sec-independent mechanism that was presumed to be another example of spontaneous integration [18], although Pf3 coat, unlike M13 procoat, does not contain a cleavable signal peptide.

More recently the critical role of a novel family of targeting factor has come to light. The first member characterized was yeast Oxa1p which is located in the inner mitochondrial membrane, and which is important for the insertion of a range of membrane proteins from the matrix side [19,20]. A related protein, Alb3, is essential for the insertion of the SRP-dependent Lhcb1 protein in thylakoids [13] and the *E. coli* homologue, YidC has recently been shown to be equally important for the biogenesis of SRP substrates in this organism [21]. However, Samuelson *et al.* [21] made the important discovery that YidC is also essential for the insertion of M13 procoat, indicating a much wider role in membrane protein biogenesis. A possible model is that one population of SecYEG-bound YidC is used in the insertion of SRP substrates, while a separate pool acts as its own translocon for the insertion of SRP/Sec-independent substrates such as procoat.

M13 procoat and Pf3 coat protein are some of the few known SRP/Sec-independent membrane proteins in *E. coli*, but studies on thylakoids have revealed a surprising number of such proteins (reviewed in [1]). Several of these proteins, such as CF_oII, PsbW and PsbX, are single-span proteins that are synthesized with cleavable signal peptides but are nevertheless inserted by SRP/Sec-independent mechanisms [23-25]. PsbY is exceptional in containing two signal peptides which are cleaved several times after insertion to yield two single-span mature

proteins [26]. Other multi-spanning proteins (that are not synthesized with signal-type peptides) have also been shown to insert SRP/Sec-independently despite being related to the strictly SRP-dependent Lhcb1 protein [27]. The lack of requirement for SRP, Sec apparatus or any energy source raised the possibility of a spontaneous insertion mechanism for these proteins.

While SRP and the Sec apparatus are not involved in the insertion of most thylakoid protiens studied to date, the overall role of Alb3 has remained unclear because only Lhcb1 has been tested for Alb3 requirement [13]. Because YidC is required for the efficient insertion of every *E. coli* membrane protein tested [21], we have sought to examine the 'global' role of Alb3 in the insertion of distinct classes of thylakoid membrane protein, with particular emphasis on the PsbW-type proteins that so closely resemble M13 procoat. We have identified two further substrates for Alb3, both of which are LHC proteins, but we show that Alb3 is not required for the insertion of any SRP-independent protein analyzed to date, including several that are synthesized with cleavable signal peptides. These data indicate fundamental differences in the requirements for membrane protein insertion in *E. coli* and thylakoid membranes.

EXPERIMENTAL PROCEDURES

Preparation of radiolabeled precursors

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 (Lhcb1, 4.1, 5, PsbS) or [^{3}H]-Leucine (PsbW, X and Y) as detailed in [23-27].

Isolation of chloroplasts, thylakoids and stromal extract

Isolation of intact chloroplasts, isolated pea thylakoids and stromal extract was as described by Kim *et al.* [27]. All incubations were carried out in 10 mM Hepes-NaOH, pH 8.0, 5 mM $MgCl_2$ (HM buffer). Alb3 -antibodies were tested for their ability to inhibit protein insertion as described by Moore *et al*. [13], except that thylakoids were incubated in the presence of the antisera for 2 hours at 4°C, instead of 1 hour. Urea extractions were performed on PsbW, X, and Y samples according to [26,27], using a modification of the method described by Breyton *et al*. [28]. Protease treatment varied on the protein being imported. Lhcb1 samples were incubated with 0.2 mg/ml trypsin on ice for 30 minutes, and stopped by heating to 95°C. Lhcb4.1 and PsbS samples were incubated with 0.15 mg/ml proteinase K on ice for 40 minutes, and stopped by the addition of 2 mM phenyl methyl sulfonyl fluoride and subsequent heating as above. Lhcb5 samples were incubated with 0.2 mg/ml thermolysin on ice for 30 minutes, which was stopped by the addition of 50mM EDTA.

Analysis of samples: A portion of each thylakoid integration assay was analyzed by either Tricine SDS-polyacrylamide gel electrophoresis (PAGE) (PsbW, X and Y) or 17% SDS-PAGE (Lhcb1, 4.1, 5, PsbS) followed by fluorography. Quantification was carried out using Molecular Dynamics Image Quant Version 3.3

RESULTS

Alb3 is required for the insertion of Lhcb4.1 and Lhcb5, but not for the related PsbS protein

The aim in this study was to analyze the 'global' role of Alb3 in thylakoid protein insertion through an analysis of numerous different integral thylakoid membrane proteins. These proteins included proteins from the LHC family as well as proteins synthesized with cleavable signal peptides. The overall structures of the membrane proteins are summarized in Table 1.

Lhcb1 is the only protein tested for Alb3 requirement and the initial aim in this study was to test whether two other LHC proteins, Lhcb5 and Lhcb4.1, require this targeting factor. These proteins contain conserved first and third membrane-spanning domains but otherwise differ considerably in structure [29]. Lhcb5 has previously been shown to require stromal factors and NTPs for insertion into thylakoids [27] and in this work we found that the insertion of Lhcb4.1 displays identical requirements. Fig. 1A shows that after import of pLhcb4.1 into chloroplasts, the mature protein is found in the thylakoid membrane and that proteinase K generates a 16 kDa degradation product (DP) from the inserted Lhcb4.1. No fragments are detected when the translation product is incubated with proteinase K (not shown) and this fragment is thus diagnostic of correct insertion. Fig. 1B shows the insertion of pLhcb4.1 into isolated thylakoids under control conditions or after pretreatment of the assay mixture with apyrase, which hydrolyzes NTPs. After incubation, the 16 kDa degradation product is evident in the control assay, indicating insertion has occurred, but is completely absent from the apyrase-treated assay. These data show that insertion depends completely on NTP hydrolysis and other tests (not shown) demonstrated a complete dependence on stromal extract.

The role of Alb3 was tested by pre-incubating thylakoids with antibodies to Alb3 essentially as carried out in the studies on Lhcb1 insertion [13]. Thylakoids were preincubated with buffer, anti-Alb3 antibodies or pre-immune antibodies and then incubated with Lhcb1, Lhcb5 or Lhcb4.1 (Fig. 2). In the control assays (buffer-treated) insertion of all three proteins occurred, as demonstrated by the appearance of near-mature-size degradation products with Lhcb1 [13] and Lhcb5 [27] and the 16 kDa degradation product with Lhcb4.1 (lanes C). The pre-immune serum has very little effect on insertion (lanes PI) but the anti-Alb3 antibodies ('Alb' lanes) severely inhibit the insertion of all three proteins (down to 6%, 8% and 11% of the control insertion efficiencies for Lhcb1, Lhcb4.1 and Lhcb5, respectively). These data indicate that Alb3 is essential for the efficient insertion of all three proteins.

The situation is very different with PsbS (Fig. 3). Kim *et al* [27] have shown that this protein inserts into thylakoids in the absence of stromal factors or NTPs; after insertion into isolated thylakoids, proteinase K treatment yields a close doublet of bands of 10-12 kDa. These degradation products (denoted by arrow) are evident in the protease-treated thylakoid sample (lane T+) in the chloroplast import experiment shown in the left hand panel, running just above smaller labeled fragments. The arrowed bands are only generated from thylakoid-associated PsbS and are absent when the translation product is incubated with the same concentration of protease (lane pre+). After import into thylakoids and protease-treatment of the membranes, these degradation products are again seen in the control lane of Fig. 3 and their intensity is essentially unaffected by preincubation of thylakoids with either pre-immune or anti-Alb3 antibodies. The Alb3 antibodies inhibit insertion slightly more than do the pre-immune antibodies but the effect is minor and we conclude that Alb3 is not important for the insertion of PsbS.

The SRP-independent, signal peptide-bearing proteins do not require Alb3 for insertion

The primary aim in this study was to determine whether Alb3 is required for the insertion of any of the SRP/Sec-independent proteins characterized to date, particularly those proteins synthesized with signal peptides which, in other systems, invariably specify interaction with either the Sec- or Tat-type protein translocases, with the exception of M13 procoat which requires only YidC [21]. CF_oII, PsbW and PsbX are all single-span proteins [23,24] whereas PsbY is translated with two signal peptides which are both cleaved to yield two single-span proteins [26,30,31]. The full precursor forms of these proteins are highly competent for insertion into isolated thylakoids.

Insertion of these precursor proteins into the thylakoid membrane involves the formation of a loop intermediate [32], which is rapidly followed by processing to the mature size by the thylakoidal processing peptidase (TPP). The TPP active site is exposed to the lumen, and processing to the mature size is therefore clear evidence that full insertion has taken place. A second criterion is that the inserted mature protein should be resistant to extraction by urea, since this extraction procedure is highly effective at removing extrinsic proteins [28]. We have found that single-span thylakoid proteins are partially extracted by this procedure [26] but the major portion of the mature protein is found in the urea-extracted thylakoids.

The question of Alb3 involvement was again addressed by pre-treating thylakoids with anti-Alb3 antibodies, and the upper panel of Fig. 4 shows assays for the insertion of the precursor form of PsbW (pPsbW). After the insertion reaction the thylakoids were analyzed directly ('total' panel) and the data show that mature-size PsbW is efficiently generated in the control assay (lane C) as found in [24]. Importantly, neither the pre-immune nor the anti-Alb3 antibodies have any adverse effect on insertion efficiency as judged by either the efficiency of

maturation or urea resistance of the mature protein. The mature-size PsbW is highly resistant to extraction by urea, since it is found primarily in the membrane pellet fraction rather than the wash supernatants, confirming that this protein is indeed inserted. An indication of the effectiveness of the urea extraction procedure is given in the lower panel, which shows the Coomassie-stained tricine gel of the insertion reaction. The urea-extracted thylakoid pellets contain the abundant membrane-spanning Lhcb1 protein and all of the chlorophyll (chl) but the urea quantitatively extracts the extrinsic proteins such as the α and β subunits of the ATP sythase (ATPase) and the lumenal 33 kDa component of the oxygen-evolving complex (33K). The presence of the antibodies does not affect the extraction procedure. These data reinforce the proposal that resistance to urea is an effective second criterion for insertion into the membrane, and the data thus demonstrate that Alb3 antibodies do not affect the insertion of pPsbW.

The insertion of PsbY and PsbX is analyzed in Fig. 5. Pre-PsbY (pPsbY) inserts with high efficiency in this type of assay [26] and the control assay shows the presence after insertion of A1 and A2 together with two larger intermediates. Again, the anti-Alb3 antibodies do not inhibit insertion to any significant extent (insertion efficiency is down to 72% of control value, but the pre-immune antibodies reduce insertion to 77%), indicating that Alb3 is not required for integration. In other experiments (data not shown) we have found that the pre-immune and anti-Alb3 antibodies had essentially identical effects on insertion.

PsbX inserts with the lowest efficiency in this type of assay and a typical result is shown in Fig. 5. Insertion does, however, occur with low-moderate efficiency, as shown by the appearance of mature-size PsbX which is highly resistant to extraction by urea. Again, the presence of the Alb3 antibodies does not inhibit insertion to any significant extent (to 72% of

the control value, whereas the pre-immune antibodies reduce insertion to 85%). We do not consider this difference to be significant since in other experiments (not shown) the pre-immune antibodies have had a slightly greater inhibitory effect.

Proteolysis of thylakoids destroys Alb3 but does not inhibit the insertion of pPsbW

The data described above show that antibodies to Alb3 have essentially no effect on 'spontaneously' inserting thylakoid proteins such as PsbW, but we sought to use a second criterion for Alb3 involvement and found that a useful property of Alb3 is its extreme sensitivity to proteolysis. Previous studies [25,27] have shown that trypsin treatment of isolated thylakoids leads to a total block in import of SRP-, Tat- or Sec-dependent proteins, whereas the insertion of CF_oII or pPsbW is unaffected. We have also found that other proteases degrade Alb3 and these data are shown in Fig 6. Panel A shows an immunblot of thylakoids treated with thermolysin, trypsin and proteinase K, which demonstrates that thermolysin treatment (Th) generates a 29 kDa fragment. Since the antibody is raised against a stroma-exposed C-terminal epitope, this indicates degradation of an N-terminal region. Treatment with trypsin or proteinase K, on the other hand (Tr, PK) leads to a complete disappearance of the signal. To assess the extent to which the Alb3 protein as a whole is degraded, we imported [^{35}S]-labeled precursor to pea Alb3 [33] into intact chloroplasts. Fig 6B shows that the protein is imported, processed to the mature size and quantitatively inserted into the thylakoid membrane. This thylakoid-inserted Alb3 is again degraded to the 29 kDa fragment by thermolysin whereas trypsin treatment leads to a more substantial degradation with the appearance of several smaller fragments (denoted by asterisks). However, proteinase K is clearly an excellent tool for studying Alb3 involvement because treatment of thylakoids with this protease leads to a complete degradation of Alb3 (lane PK). Labeled methionine residues are evenly distributed throughout the pea Alb3 sequence [33] and the total absence of

even relatively small labeled fragments means that Alb3 is effectively destroyed by this treatment. After treatment with proteinase K under these conditions, the destruction of the Alb3 is predicted to lead to a block in the insertion of Lhcb1, and this is confirmed in Fig 6C (compare control and +PK panels in Fig 6C). On the other hand, Fig 6D shows that these proteinase K-treated thylakoids are nevertheless able to import pPsbW as efficiently as untreated thylakoids (compare control and +PK panels), and we conclude that Alb3 can not be required for this insertion event. Similar data were obtained for PsbX and PsbY (data not shown).

DISCUSSION

The depletion of YidC in *E. coli* is rapidly followed by dramatic adverse effects on the insertion of every membrane protein analyzed to date, including one considered for decades to insert spontaneously [21]. Disruption of the *oxa1* gene in yeast likewise leads to a severe inhibition of membrane protein insertion from the matrix side [19,20,34] and the only chloroplast protein studied in terms of Alb3-dependence, Lhcb1, was found to be very reliant on this factor for insertion into thylakoids [13]. These findings point to a major role for Oxa1-type proteins in these membrane systems, but we have sought to analyze the 'global' role of Alb3 in thylakoids by studying its role in the biogenesis of a variety of membrane protein types (including 'assisted' and 'spontaneous' substrates) under identical circumstances.
We have firstly shown that Alb3 antibodies almost totally block the insertion of Lhcb4.1 and Lhcb5, indicating a central role for Alb3 in the integration of these LHC proteins. An involvement of Alb3 could not be taken for granted because these proteins differ very significantly from Lhcb1 in structural respects.

Perhaps more surprising is the finding that the majority of thylakoid proteins tested in this study are completely unaffected by Alb3 antibodies, and are able to insert in the complete absence of a functional Alb3 protein. One of these substrates, PsbS, is related to the above-mentioned LHC proteins yet inserts by a completely different pathway in which SRP, GTP and FtsY are not required [27]. We have now shown that Alb3 is not required for the insertion of PsbS, indicating a much simpler insertion pathway. Even more significantly, Alb3 plays no role in the insertion of a series of proteins (PsbW, PsbX and PsbY) that bear signal peptides which are cleaved following insertion into thylakoids. These proteins are of particular interest because signal peptides almost invariably specify interaction with proteinaceous translocation

apparatus, yet these proteins do not require the thylakoidal Sec or Tat machinery and we have now ruled out an involvement of Alb3.

PsbW and PsbX are also of interest for a second reason, in that they closely resemble the well-characterized M13 procoat in terms of overall structure. They likewise contain a single transmembrane span in the mature protein, are synthesized with apparently similar signal peptides and even contain translocated loops of similar size (20-30 residues) and overall negative charge. However, we have found that these thylakoid proteins are completely unaffected by Alb3 antibodies or degradation, whereas procoat is almost totally dependent on YidC for insertion in *E. coli* [21]. These data indicate fundamental differences in insertion mechanisms for these simple proteins.

The four Alb3-independent thylakoid proteins described in this study (PsbS, PsbX, PsbY and PsbW) are the first substantial group of membrane proteins reported to insert independently of Oxa1-type proteins in bacteria or thylakoids. We have also found recently that a fifth protein, PsaK, inserts as a 'horse-shoe' conformation in a mechanism that does not require Alb3 [35]. These data show that Alb3 is not required for the insertion of various types of thylakoid membrane protein and it will be of particular interest to determine whether YidC-independent membrane proteins are identified in bacteria. If such proteins do not emerge, or if the bulk of bacterial membrane proteins do transpire to be highly YidC-dependent, a possible explanation may hinge on the very different chemical properties of the two membrane types. The *E. coli* plasma membrane is composed primarily of phospholipids whereas galactolipids account for over 80% of thylakoid membrane lipid (reviewed in [36, 37]). The major species is the relatively unsaturated monogalactosyl diacylglycerol (MGDG), which has an intrinsic preference to form non-bilayer, hexagonal H_{II} structures when it is isolated from native

biological membranes. Thus, thylakoid lipids may provide a more fluid environment in which spontaneous (or at least, Alb3-independent) insertion is favoured.

It is notable that, among the seven membrane proteins studied here, Alb3 requirement correlates strictly with the 'assisted' pathway (by which we mean that insertion requires stromal factors, NTPs and protease-sensitive translocation machinery). If SRP is the stromal factor required for Lhcb4.1 and Lhcb5 insertion (but which has yet to be tested), this would suggest a mainstream pathway in which binding to SRP in the early stages is linked to Alb3-mediated integration at a later stage, with FtsY performing an important but so far poorly characterized function. A similar pathway operates in bacteria but one important point to note is that the Sec apparatus is also required for some bacterial membrane proteins [7] and a portion of YidC appears to be firmly bound to the *E. coli* Sec translocon [22]. It is so far unclear whether the chloroplastic SRP/Alb3 pathway involves the Sec translocon at any stage. Antibodies to thylakoid SecY block the translocation of SecA requiring proteins but do not inhibit Lhcb1 integration [13,38]. However, anti-SecY antibodies may act by inhibiting SecA binding to SecY rather than inhibiting SecY function *per se*. A second point is that M13 procoat and Pf3 coat require YidC, but not SRP or the Sec machinery, and it will be of interest to determine whether any thylakoid membrane proteins similarly require Alb3 but not SRP and *vice versa*.

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Table 1. Basic structures and topologies of thylakoid membrane proteins used in this study.

Protein	TM spans	Orientation
Lhcb1	3	Nin-Cout
Lhcb4.1	3	Nin-Cout
Lhcb5	3	Nin-Cout
PsbW	2*	Nin-Cin
PsbS	4	Nin-Cin
PsbY	4*	Nin-Cin
PsbX	2*	Nin-Cin

The Table illustrates the number of transmembrane (TM) spans and the overall orientations of the proteins used in this study. The orientations are described in terms of whether the N- or C-termini are 'in' (stroma-exposed) or 'out' (lumen-exposed).

*Shows the number of transmembrane spans present after insertion but prior to cleavage of the signal peptide(s) by the thylakoidal processing peptidase on the lumenal face of the membrane.

Figure legends

Figure 1. *Insertion of Lhcb4.1 requires nucleoside triphosphates*.

A. The precursor protein was synthesized by transcription-translation (lane pre) and incubated with intact chloroplasts as described in Experimental Procedures. After import, samples were analyzed of the chlorplasts (Lane C), chloroplasts after treatment with thermolysin (C+), stromal fraction (S), thylakoid fraction (T) and thylakoid fraction following treatment with 0.15 mg/ml proteinase K (T+). B: insertion of pLhcb4.1 into pea thylakoid membranes. pLhcb4.1 was incubated with pea thylakoids that had been pretreated with apyrase (1 unit, 15 min on ice) or an equivalent volume of import buffer as control, as indicated. After the insertion reaction, samples of the thylakoids were pelleted and analyzed (lanes T) or were treated with proteinase K as detailed in Experimental Procedures (lanes T+).

Figure 2. *Antibodies to Alb3 severely inhibit the insertion of Lhcb1, Lhcb4.1 and Lhcb5*.

Pea thylakoids were pre-incubated with anti-Alb3 antibodies (Alb), pre-immune (PI) antibodies or, as a control (lanes C), an equivalent volume of the buffer used to resuspend the antibodies. The thylakoids were then incubated with pLhcb1, pLhcb4.1 or pLhcb5. After insertion, the thylakoids were washed with insertion buffer and analyzed directly ('membranes' samples) or treated with protease as detailed in Experimental Procedures. DP, degradation product; pre, translated precursor. Insertion efficiencies are given below the lanes, expressed as a percentage of the control insertion efficiency.

Figure 3. *PsbS inserts by an Alb3-independent mechanism*.

Left hand panel: pPsbS was incubated with chloroplasts and the thylakoid fraction (T) was treated with 0.15 mg/ml proteinase K (T+). Right hand panel: pPsbS was incubated with pea

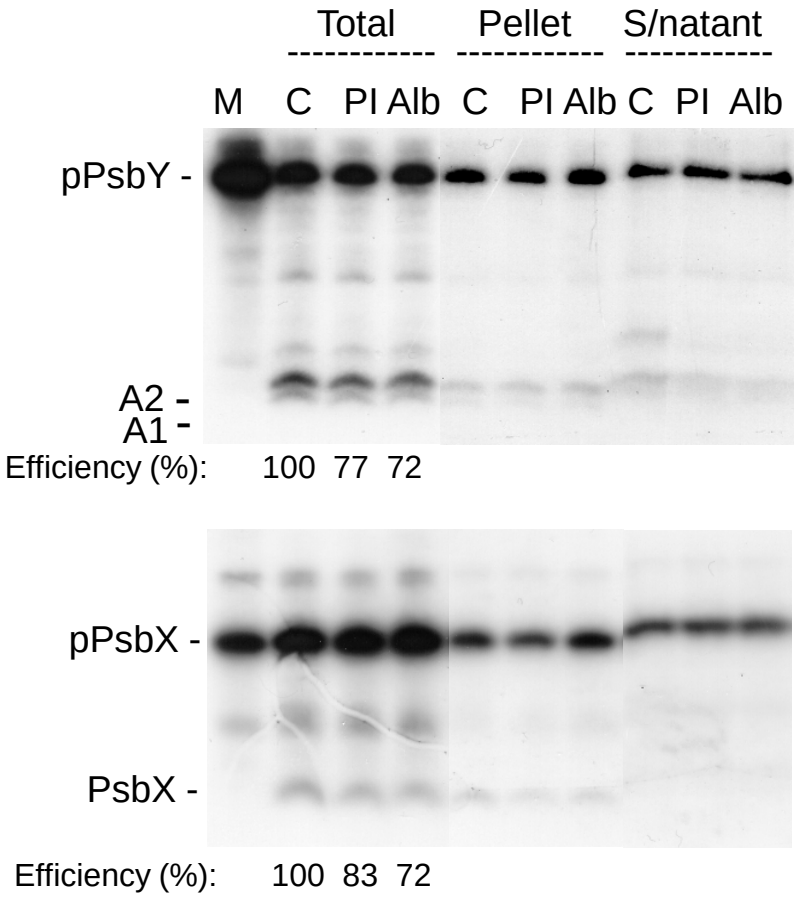
thylakoids that had been preincubated with buffer (control, lane C), pre-immune antibodies (PI) or anti-Alb3 antibodies as detailed in Fig. 2 and Experimental Procedures. After insertion, samples were analyzed of the pelleted thylakoids (membrane samples) or thylakoids treated with 0.15 mg/ml proteinase K as indicated. Insertion efficiencies are given under the lanes, expressed as a percentage of the control insertion efficiency. Arrow denotes fragments generated from degradation of inserted PsbS; asterisk denotes other degradation fragments.

Figure 4. *Insertion of pPsbW is not inhibited by anti-Alb3 antibodies*.
Upper panel: the precursor of *Arabidopsis* PsbW (pPsbW) was incubated with pea thylakoids that had been pre-incubated with buffer, anti-Alb3 antibodies or pre-immune antibodies as carried out with the LHC proteins described in Fig. 2. After the insertion reaction, samples of the thylakoids were pelleted and analyzed directly ('total' panel) or extracted twice with 6.8 M urea, after which samples of the membrane pellets or wash supernatants (s/natant) were analyzed. Insertion efficiencies are given under the lanes, relative to the control insertion efficiency. Lower panel: samples of the import reaction were run on an SDS-tricine gel and stained with Coomassie blue. Mobilities of the α and β subunits of the ATP synthase (ATPase), Lhcb1, the 33 kda protein of the oxygen-complex (33K) and free chlorophyll (chl) are indicated.

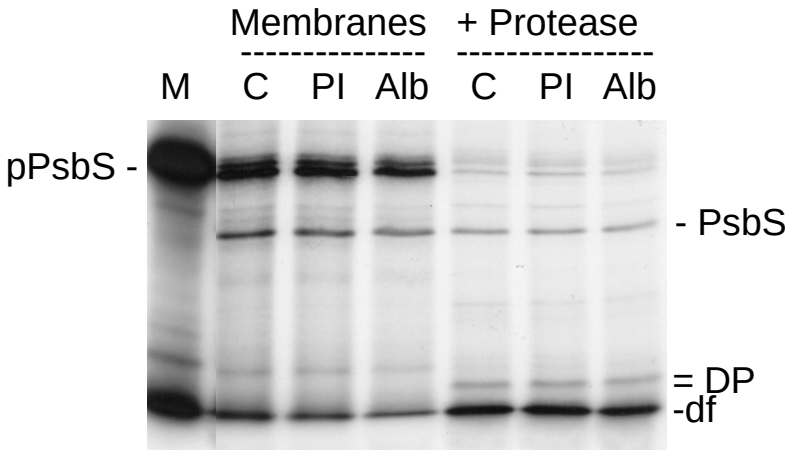
Figure 5. *Alb3 antibodies do not inhibit the insertion of pPsbY or pPsbX*.
Precursors of *Arabidopsis* PsbY or PsbX (pPsbY, pPsbX) were incubated with pea thylakoids that had been pre-incubated with buffer, anti-Alb3 antibodies or pre-immune antibodies. After the insertion reaction, samples were analyzed directly or after urea extraction of the thylakoids as described for PsbW in Fig. 4. Mobilities of mature PsbX and the two single-

span mature PsbY proteins (A1 and A2) are indicated, together with the insertion efficiencies (expressed as a percentage of the control panel insertion efficiency).

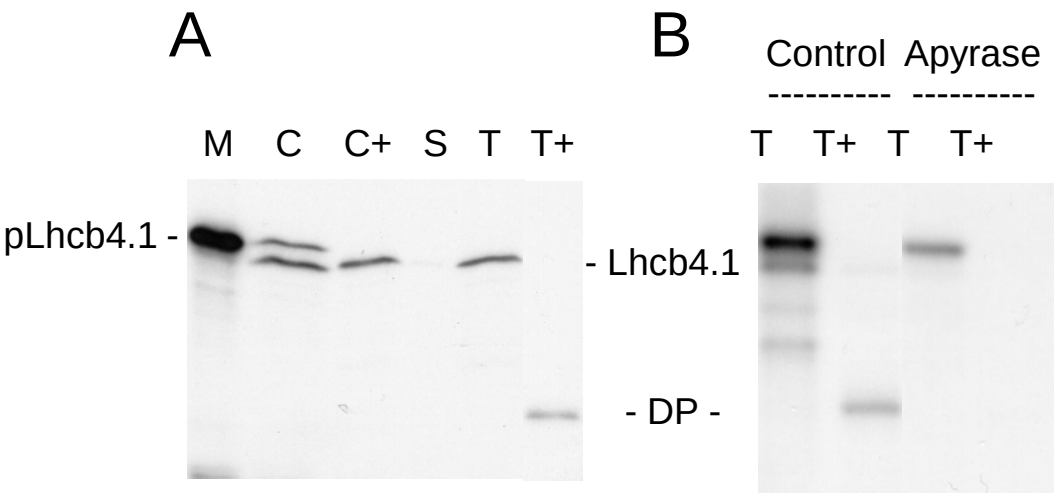
Figure 6. *Proteinase K completely degrades Alb3 but does not affect the subsequent insertion of PsbW.* A: immunoblot, using anti-Alb3 antibodies, of thylakoid membranes (lane Mb) and thylakoids that were treated with 200 μg/ml thermolysin for 40 mins on ice (Th), or with 60 μg/ml trypsin (Tr) or 100 μg/ml proteinase K (PK) for 20 min on ice. Mobility of Alb3 is indicated together with the mobilities of molecular weight markers (in kDa). Arrow denotes degradation product generated by thermolysin. B: Pea pAlb3 (lane pre) was imported into chloroplasts and samples were then analyzed of the chloroplasts (C), thermolysin-treated chloroplasts (C+), and the stromal (S) and thylakoid (T) fractions after lysis of thermolysin-treated chloroplasts. Further samples of the thylakoids were treated on ice with thermolysin (Th), trypsin (Tr) or proteinase K under the conditions used in A above. All samples were analyzed by gel electrophoresis and fluorography. df, dye front. C/D: pLhcb1 or pPsbW were incubated with thylakoids that had been incubated on ice with 100 μg/ml proteinase K and then washed three times in HM buffer (see Experimental Procedures) (PK panel). The control thylakoids were treated identically except that proteinase K was omitted. After incubation, thylakoids from the Lhcb1 insertion reactions were pelleted and analyzed directly (lanes T) or after trypsin treatment to generate the degradation product (DP) as described in Fig. 2 (lanes T+). Thylakoids from the pPsbW insertion reactions were analyzed directly (without pelleting, lane T) or after pelleting and two washes in HM buffer (lanes Tw).



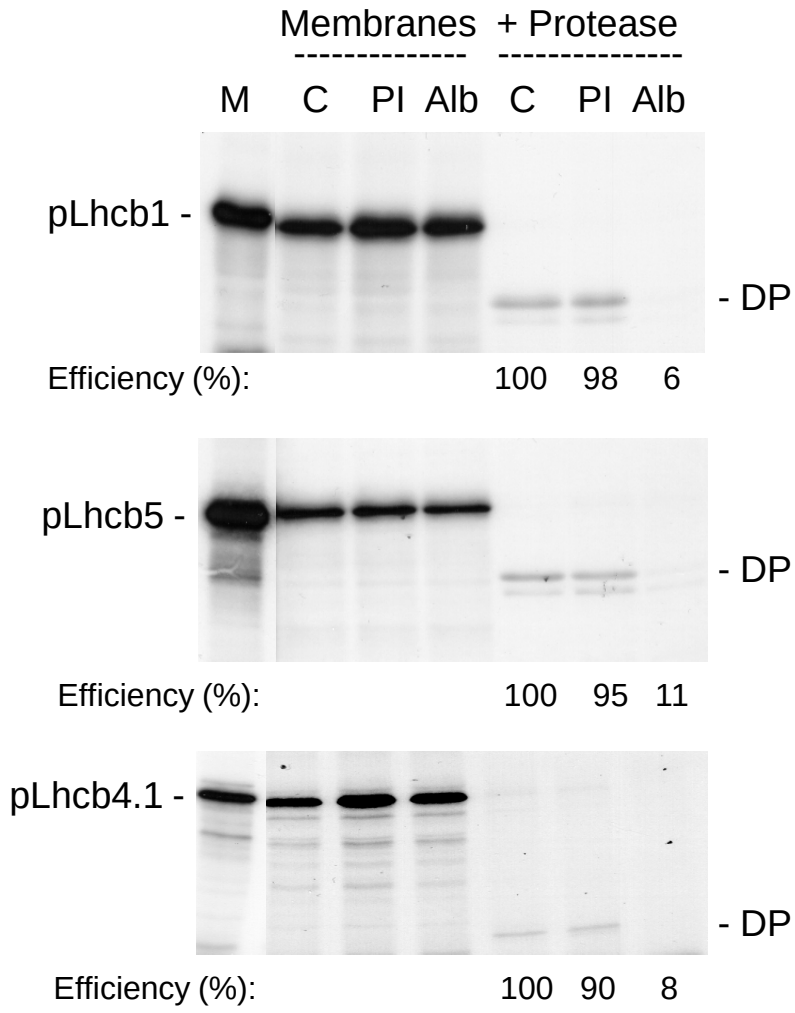
Woolhead *et al*., Fig. 6



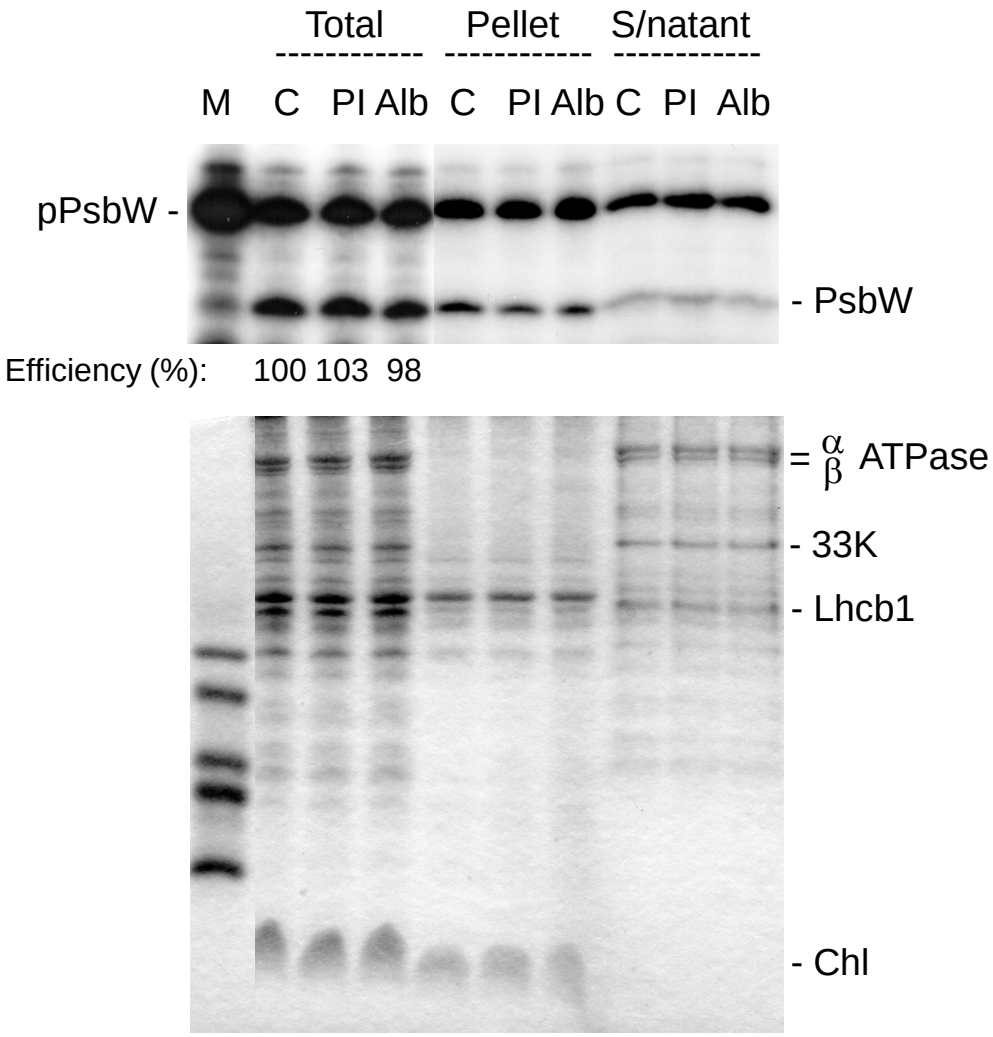
Woolhead *et al*. Figure 4



Woolhead *et al*., Fig. 2



Woolhead *et al*., Fig. 3



Woolhead *et al.*, Fig. 5