Pathway specificity for a Δ pH-dependent precursor thylakoid lumen protein is governed by a 'Sec-avoidance' motif in the transfer peptide and a 'Sec-incompatible' mature protein

Erik Bogsch, Susanne Brink and Colin Robinson¹

Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK

¹Corresponding author

Cleavable N-terminal targeting signals direct the translocation of luminal proteins across the chloroplast thylakoid membrane by either a Sec-type or Δ pH-driven protein translocase. The targeting signals specify choice of translocation pathway, yet all resemble typical bacterial 'signal' peptides in possessing a charged N-terminus (N-domain), hydrophobic core region (H-domain) and more polar C-terminal region (C-domain). We have previously shown that a twin-arginine motif in the N-domain is essential for targeting by the Δ pH-dependent pathway, but it has remained unclear why targeting signals for this system (transfer peptides) are not recognized by the Sec apparatus. We show here that the conserved charge distribution around the H-domain in the 23K transfer peptide (twin-Arg in the N-domain, Lys in the C-domain) constitutes a 'Sec-avoidance' signal. The C-domain Lys, while not important for Δ pH-dependent targeting, is the only barrier to Sec-dependent translocation; its removal generates an apparently perfect signal peptide. Conversely, insertion of twin-Arg into the N-domain of a Sec substrate has little effect, as has insertion of a C-domain Lys, but the combined substitutions almost totally block transport. We also show that the 23K mature protein is incapable of being targeted by the Sec pathway, and it is proposed that the role of the Sec-avoidance motif in the transfer peptide is to prevent futile interactions with the Sec apparatus.

Keywords: chloroplast/protein transport/signal peptide/thylakoid membrane

Introduction

The assembly of the photosynthetic machinery requires the import of numerous proteins into the chloroplast and their subsequent targeting into or across the internal thylakoid membrane. The available data suggest that the initial steps in the import of these proteins, in which they are imported across the double-membrane envelope by an ATP-driven translocase, are common to all imported chloroplast proteins. However, the subsequent targeting of proteins into and across the thylakoid membrane is a complex issue involving the operation of at least four distinct pathways within the chloroplast (reviewed by Robinson and Klösgen, 1994). Two of the pathways are used by integral membrane proteins, and insertion

mechanisms have been characterized which rely either on a stromal homologue of signal recognition particle together with unknown import apparatus in the thylakoid membrane (Li *et al.*, 1995; Robinson *et al.*, 1996), or which appear to involve the spontaneous insertion of the protein into the thylakoid membrane (Michl *et al.*, 1994). The other pathways are utilized by luminal proteins, or proteins such as photosystem I subunit F (PSI-F) where the majority of the protein is localized in the lumen, and analysis of these pathways has unearthed some major surprises in recent years.

A subset of luminal proteins, including plastocyanin (PC), PSI-F and the 33 kDa photosystem II protein (33K) are synthesized with bipartite presequences that contain two signals in tandem. The first 'envelope transit' signal specifies translocation across the envelope and is usually removed by the stromal processing peptidase (SPP), after which the second signal directs translocation across the thylakoid membrane by a prokaryotic-type, Sec-dependent mechanism that relies on ATP and the presence of a stromal SecA molecule (Hulford *et al.*, 1994; Karnauchov *et al.*, 1994; Mant *et al.*, 1994; Nakai *et al.*, 1994; Yuan *et al.*, 1994). The second signal is then removed by a lumen-facing thylakoidal processing peptidase (TPP) that is mechanistically similar to bacterial signal peptidases (Halpin *et al.*, 1989). Other luminal proteins, including the 23 and 16 kDa photosystem II proteins (23K, 16K), photosystem II subunit T (PSII-T) and photosystem II subunit N (PSI-N) are similarly synthesized with bipartite presequences, but these proteins are translocated across the thylakoid membrane by a very different mechanism that does not require stromal factors or ATP, but which is totally reliant on the thylakoidal Δ pH (Mould and Robinson, 1991; Cline *et al.*, 1992; Henry *et al.*, 1994; Nielsen *et al.*, 1994). There is an intriguing correlation when one considers the evolution of the protein substrates involved. The Sec-dependent proteins mentioned above (PC, 33K and PSI-F) are also present in cyanobacteria, where they are synthesized with signals that very much resemble the thylakoid-targeting signals of their chloroplastic counterparts (e.g. Kuwabara *et al.*, 1987). This is entirely consistent with the widely held belief that chloroplasts arose from an endosymbiotic cyanobacterial-type prokaryote, and we assume that these proteins simply acquired envelope transit signals when the chloroplast genes were transferred to the nucleus after the initial endosymbiotic events. The actual thylakoid-transfer step is very likely to be basically similar in chloroplasts and bacteria. The origins of the Δ pH-dependent system, however, are presently unknown, but it is interesting that the four known substrates for the Δ pH-dependent system are all absent from cyanobacteria (at least those species that have been studied). There is thus a distinct possibility that this translocase arose relatively recently in phylogenetic terms.

The existence of parallel Sec-dependent and -independent translocation pathways is made even more intriguing when the properties of the targeting signals are taken into account, because all thylakoid lumen targeting signals are very similar to typical bacterial signal peptides that promote interaction with the Sec machinery. Like signal peptides, they can be divided into three domains: a charged amino-terminal region (N-domain), a hydrophobic core region (H-domain) and a more polar carboxy-terminal domain (C-domain) ending with short-chain residues at the -3 and -1 positions (usually Ala-Xaa-Ala). Yet the signals appear to be the sole arbiters of the targeting pathway followed, because exchange of presequences between the two groups leads to a re-routing of the attached protein, clearly indicating that the thylakoid-targeting signals contain the essential 'sorting' information that enables the two translocases to identify their cognate substrates (Henry *et al.*, 1994; Robinson *et al.*, 1994; Mant *et al.*, 1995). Lumen-targeting peptides have been previously referred to as 'transfer signals' but in view of the distinct types, and the prokaryotic origins of the Sec system, we propose to use the terms 'signal peptide' and 'transfer peptide' to describe the targeting signals for the Sec- and Δ pH-dependent thylakoidal protein translocases, respectively.

Only one concrete difference emerges when the two groups are compared: transfer peptides invariably contain a twin-Arg motif immediately prior to the H-domain, whereas thylakoid signal peptides usually contain Lys at this position. Chaddock *et al.* (1995) showed that substitution of either Arg residue in pre-23K (even by Lys) essentially blocked translocation, confirming that the twin-Arg is a crucial signal for targeting by the Δ pH-dependent pathway. The presence/absence of this motif is not, however, the means by which the two translocases identify their cognate substrates, because insertion of this motif in pre-PC did not divert PC from the Sec pathway. These findings have important implications for the sorting signals, because they imply the presence of both positive and negative signals. Firstly, the failure of this twin-Arg-containing PC mutant (PC/RR) to enter the Δ pH-dependent pathway indicates that an additional positive signal(s) must be required for targeting by the latter pathway. Secondly, the inability of the Sec system to translocate substrates for the Δ pH-dependent system, in spite of clear similarities between transfer and signal peptides, suggests the presence of a 'Sec-avoidance' motif in transfer peptides that must be subtle yet effective. In this report we have examined the characteristics of this Sec-avoidance signal within the wheat 23K transfer peptide.

Results

Structural characteristics of thylakoid-targeting signals

Figure 1 lists the signal and transfer peptides of known substrates for the Sec- and Δ pH-dependent thylakoidal protein translocases. The figure illustrates the point that transfer peptides contain a common twin-Arg motif immediately prior to the H-domain, whereas signal peptides usually contain a single lysine residue at this position (although the barley pre-PC signal peptide contains twin-Lys). On the basis of previous studies (Chaddock *et al.*,

Transfer peptides	
Sp 23K	AQKQDDNEANVLNSGVSRRLALTVLIGAAAVGSKVSPADA
Wh 23K	AQKNDEAASDAVVTSRRRAALSLLAGAAAIAVLVSPAAA
Wh 23K/RRhL	AQKNDEAASDAVVTSRRRAALSLLAGAAAIAVLVSPAAA
Sp 16K	--AQQVSAEAEETSRRAMIGFVAAGLASGSFVKAVLA
Ma 16K	--ASAEGDAVAQAGRRRAVIGLVATGIVGGALSQAARA
Ara 16K	--LVVRAQQSEETSRRSVIGLVAAAGLAGGSFVQAVLA
Ara 16K	--RAQQNVSPVSSRRSVIGLVAAAGLAGGSFVKAVFA
Bar PSI-N	--AAAKRVQVAPAKDRRSALLGLAAVFAATAASAGSARA
Ara PSI-N	--DGSNGRRSAMVFLAATLFTSTAASVASANA
Cot PSII-T	--VQMSGERKTEGNNRRREMFMAAAAAAICSVAGVATA
Ara PSII-T	--TPSLEVKEQSSTTMRDLMTAAAAAIVCSLAKVANA
Signal peptides	
Bar PC	ASLGKKAASAAVAMAGAMLLGGSAMA
Sp PC	ASLKNVGAADVATAAAGLLAGNAMA
Sp [Lys63] PC	ASLKNVGAADVATAAAGLLAGNAMA
Sp PC/KK	ASKKNVGAADVATAAAGLLAGNAMA
Sp PC/RR	ASRRNVGAADVATAAAGLLAGNAMA
Sp PC/RRhK	ASRRNVGAADVATAAAGLLAGNAMA
Sp PC/RRhL	ASRRNVGAADVATAAAGLLAGNAMA
Wh 33K	AFGVDAGARITCSLQSDIREVASKCADAAMKAGFALATSALLVSGATA
Sp 33K	AFGVESASSSGGRLSLSLQSDLELANCVDATKLAGLALATSALLVSGANA
Sp PSI-F	--QENDQQQPKKLELAKVGNAAAALSSVLLSSWSVAPDAAMA
Bar PSI-F	--SGDNNNSTATPSLSASIKTFSAALALSSVLLSSAATSPPPAAA
Fusion proteins	
23-PC	AQKNDEAASDAVVTSRRRAALSLLAGAAAIAVLVSPAAA-PC
23-PC/RRhL	AQKNDEAASDAVVTSRRRAALSLLAGAAAIAVLVSPAAA-PC
23-PC/KK	AQKNDEAASDAVVTSKKAALSLLAGAAAIAVLVSPAAA-PC
23-PC/KK	AQKNDEAASDAVVTSKKAALSLLAGAAAIAVLVSPAAA-PC

Fig. 1. Structural characteristics of thylakoid signal peptides and transfer peptides. Targeting signals are shown for lumenal proteins cloned from spinach (Sp), wheat (Wh), barley (Bar), cotton (Cot), maize (Ma) and *Arabidopsis* (Ar). The start sites of the wheat 23K, spinach PC and wheat 33K peptides have been delineated (Bassham *et al.*, 1991) but the remainder have yet to be identified. Also shown are the mutants described in this study, together with the structure of the [Lys63]PC mutant described in Brink *et al.* (1997). Charged residues are given in bold and the H-domains are underlined; residues introduced by mutagenesis are shown in italics.

1995; Brink *et al.*, 1997), we have concluded that a twin-Arg motif is an essential positive signal for the Δ pH-dependent system whereas any basic residue, or pair of residues, is tolerated by the Sec system. In this study we have examined the form and nature of the 'Sec-avoidance' directive within a precursor on the pH-dependent pathway.

Our initial studies centred on one of the few other features that differ to a notable extent in the two types of signal: the C-domain. The C-domains of transfer peptides are significantly more hydrophilic than those of the signal peptides due to the presence of either basic residues (the 23K, spinach 16K and barley PSI-N signals) or similarly hydrophilic Asn or Gln residues (maize 16K and *Arabidopsis* PSI-N signals). The only exception is cotton PSII-T, although in this case a Cys-Ser dipeptide towards the end of the H-domain also renders the carboxy-terminus of the signal fairly hydrophilic. In most cases these charged/hydrophilic residues have the effect of bringing the H-domain to an abrupt end, and the H-domains of 23K and 16K in particular are shorter than those of the thylakoid signal peptides. The C-domains in the PC, 33K and PSI-F signal peptides are mostly uncharged and more hydrophobic, in common with typical prokaryotic signal peptides (von Heijne, 1985).

The charge distribution within the 23K transfer peptide performs a 'Sec-avoidance' function

We investigated whether the C-domain Lys in the wheat 23K transfer peptide influences sorting by either promoting

Fig. 2. Substitution of the C-domain Lys in the 23K transfer peptide has no detectable effect on translocation or sorting of pre-23K. Pre-23K (left-hand panels) and 23K/RRhL (right-hand panels) were imported into chloroplasts in the absence or presence of 3 μ M pea pre-23K (upper and lower panels, respectively). After incubation, samples of the chloroplasts before and after protease-treatment (C, C+, respectively) and of the stromal (S) and thylakoid (T) fractions after lysis were analysed. Lane T+, protease-treated thylakoids. 'Pre' denotes precursor proteins; intermediate and mature forms are denoted i23K and 23K, respectively.

transport by the Δ pH-dependent system or repelling the Sec system. The mutants generated in this study are shown in Figure 1, and are designated according to the distribution of residues around the H-domain. Mutations that affect only the charged residues immediately *prior* to the H-domain are named according to the residues introduced (thus PC/RR contains twin-Arg in place of the usual Lys) whereas mutations that alter the residues *after* the H-domain (in this study, only the -7 residue is affected) are named according to the residue introduced, with the H-domain denoted as 'h'. The basic residues before the H-domain in these mutants are included for ease of reference, whether or not they have been mutated. Thus, 23K/RRhL contains the wild-type twin-Arg motif before the H-domain and a Leu residue afterwards, in place of the normal Lys residue at the -7 position. According to this system, the wild-type 23K and PC transfer/signal peptides could be regarded as 23K/RRhK and PC/KhL, although this is avoided in the text to prevent confusion with mutant presequences.

In a previous study (Brink *et al.*, 1997), we introduced a basic residue at the -7 position in spinach pre-PC, creating a mutant ([Lys63]PC) with a basic residue at the same position, relative to the TPP cleavage site, as that found in the C-domain of wheat pre-23K (see Figure 1). This mutant was transported efficiently by the Sec system, demonstrating that a C-terminal basic residue *per se* (and a relatively short H-domain) can be tolerated by the Sec system. In the present study we examined the importance of the C-domain basic residue for transport of pre-23K by the Δ pH-dependent pathway. The C-domain Lys was replaced with Leu, and the upper panel of Figure 2 shows the import characteristics of this mutant (23K/RRhL). Pre-23K and 23K/RRhL were synthesized by transcription-translation of cDNAs (lanes Tr) and incubated with intact pea chloroplasts. Both precursors are imported and processed to the mature form, which is found exclusively in the protease-treated thylakoid fraction (lanes T+, corresponding to the lumen contents). There is no evidence of the stromal intermediate form, indicating that intra-organellar targeting of the mutant precursor has occurred with high efficiency.

Further studies on this mutant have shown that translocat-

Fig. 3. A 23K-type distribution of basic charges blocks translocation of PC by the Sec pathway. Pre-PC, PC/RRhL and PC/RRhN were imported into pea chloroplasts, after which the organelles were fractionated as described in Figure 2. Arrows on the right-hand side denote three stromal intermediate forms generated during import of the mutant precursor proteins.

tion into the thylakoid lumen is completely blocked by nigericin (not shown), suggesting that the Δ pH-pathway is utilized, and import into thylakoids does not require stromal extracts (another characteristic of the Δ pH-driven mechanism). However, these findings do not rule out the possibility that this mutant may also be translocated by the Sec pathway at a low rate, because nigericin also inhibits Sec-dependent transport across the thylakoid membrane to an appreciable extent, and in the absence of nigericin, Sec-dependent import into thylakoids would probably be masked by Δ pH-dependent import. We therefore carried out a further experiment to test this possibility, in which we assayed the ability of over-expressed pre-23K to compete with 23K/RRhL for translocation by the Δ pH-driven system. We reasoned that the *Escherichia coli*-expressed precursor would compete relatively poorly if the mutant pre-23K is able to use the Sec system as an alternative pathway. Cline *et al.* (1993) have shown that micromolar concentrations of *E. coli*-expressed pre-23K are able to inhibit the translocation of labelled, *in vitro*-synthesized precursor for translocation across the thylakoid membrane of intact chloroplasts. Complete inhibition can not be attained because the recombinant precursor saturates the envelope-based translocation system before totally saturating the Δ pH-driven thylakoidal system. The lower panels of Figure 2 show assays in which radiolabelled pre-23K and 23K/RRhL were imported into chloroplasts in the presence of 3 μ M unlabelled pea pre-23K. The presence of the over-expressed pre-23K markedly inhibits translocation across the thylakoid membrane, with the result that the stromal intermediate form predominates. Significantly, the over-expressed pre-23K inhibits translocation of pre-23K and 23K/RRhL to similar extents, providing strong evidence that the mutant is unable to utilize the Sec pathway when targeting by the Δ pH-dependent pathway is inhibited. These data indicate that either the mutant lacks targeting signals for the Sec pathway, or the 23K mature protein cannot be translocated by the Sec system.

The basic residue in the pre-23K C-domain is clearly not important for targeting by the Δ pH-dependent translocase, and the results of Brink *et al.* (1997) showed that a basic residue in the PC C-domain does not prevent targeting by the Sec system. To complete the analysis, however, we recreated a 23K-type charge distribution in a Sec signal (i.e. *two* basic residues before the H-domain and one afterwards). Figure 3 shows the import characteristics of two pre-PC mutants, PC/RRhK and PC/RRhN, which contain a twin-Arg motif prior to the H-domain together with a hydrophilic residue (Lys or Asn) at the

Fig. 4. A 23-PC fusion protein is targeted primarily by the Δ pH-dependent pathway. The 23-PC fusion protein comprising the wheat 23K transfer peptide linked to mature spinach PC was constructed as detailed in Materials and methods. The translation product (lane Tr) was imported into pea chloroplasts in the absence or presence of nigericin as indicated, under conditions also detailed in Materials and methods. Fractionation procedures and symbols as in Figure 2. i23-PC, stromal intermediate form.

–7 position. These mutants are efficiently imported into isolated chloroplasts, but sorting is now drastically affected; virtually no mature-size protein appears in the thylakoid fraction, with the vast majority of imported protein accumulating in the stroma as intermediate-size forms. The Sec system is thus completely tolerant of either a twin-Arg motif in the N-domain or Lys in the C-domain, but a combination of these features provides a remarkably efficient ‘Sec-avoidance’ function.

Removal of the C-domain lysine in the 23K transfer peptide enables the peptide to direct translocation by either pathway.

Although our data suggest that the C-domain Lys might prevent wild-type pre-23K from entering the Sec pathway, they predict that removal of the C-domain Lys from pre-23K would enable efficient Sec-dependent translocation to take place if the C-domain Lys is the *only* block. However, Figure 2 showed that the 23K/RRhL mutant is a very poor Sec substrate. Nevertheless, this may not reflect the characteristics of the targeting signal, because other data suggest that the 23K mature protein may be intrinsically difficult to translocate by the Sec pathway. Numerous constructs have been made between the presequences and mature proteins of Sec- and Δ pH-dependent proteins, and most mature proteins can be targeted by the alternative pathway when the appropriate type of presequence is provided (Claismeyer *et al.*, 1993; Henry *et al.*, 1994; Robinson *et al.*, 1994; Mant *et al.*, 1995). However, 23K is a notable exception in that the mature protein cannot be targeted into the lumen by the presequence of any Sec-dependent protein tested. In view of the implied inability of the Sec system to translocate 23K, we carried out further tests to characterize the putative Sec-avoidance signal in the 23K transfer signal, using a passenger protein known to be more amenable. We chose PC because it can be transported by either pathway; the presequence of spinach 23K has been shown to target spinach PC by the Δ pH-dependent pathway with very high efficiency (Robinson *et al.*, 1994). Because the initial mutagenesis tests in this study were conducted with wheat pre-23K, we used the same presequence for further analyses and constructed a similar chimera (23-PC) com-

Fig. 5. Removal of the C-domain Lys in 23-PC enables the signal to direct targeting by both pathways. Upper panel: the 23PC/RRhL mutant (lane Tr) was imported into isolated chloroplasts in the presence of nigericin or azide, and samples analysed as described in the legend to Figure 2. In the control incubation lacking inhibitors (Con) only samples of the chloroplasts and protease-treated chloroplasts were analysed. ‘int’ denotes stromal intermediate form evident in the nigericin and azide incubations. Lower panel: pre-23K was incubated with chloroplasts in the presence of nigericin and samples analysed as described above.

prising the wheat 23K presequence linked to mature spinach PC.

The import characteristics of this 23-PC chimera are shown in Figure 4. In the absence of inhibitors, 23-PC is imported into chloroplasts and efficiently sorted; only the mature-size PC is evident. The presence of nigericin markedly inhibits translocation across the thylakoid membrane as expected, with the result that the stromal intermediate form is the major polypeptide. However, it is interesting that translocation across the thylakoid membrane is not completely inhibited; whereas wild-type pre-23K is converted almost entirely to the stromal intermediate form, a larger proportion of 23-PC is targeted into the lumen. Densitometric scanning of the bands in the ‘C+’ lanes reveals that the ratio of intermediate:mature size protein is 19.7 for 23K and 7.1 for 23-PC. These data indicate that the wheat 23K presequence has slightly different targeting properties, and further studies strongly suggest that it can direct low-level targeting by the Sec pathway (see below). Nevertheless, the chimera must be targeted predominantly by the Δ pH-dependent pathway, since nigericin has such a strong inhibitory effect and other experiments (data not shown) have shown that azide has no effect whatsoever on the import profile.

The important question is: does removal of the C-domain Lys in the 23K transfer peptide generate an efficient Sec signal? This issue was addressed using a 23-PC mutant (23PC/RRhL) in which the C-domain Lys is replaced by Leu, and the data are shown in Figure 5. Again, this mutant is efficiently imported and processed to the mature size in the control (Con) incubations, but this mutant differs dramatically from the parent fusion in that neither nigericin nor azide affect targeting to a significant extent. The mature-size protein is by far the major imported product in each case, whereas in the control import carried out with pre-23K, nigericin almost totally blocks transport across the thylakoid membrane. Quantitation of the data in the C+ lanes shows that the ratio of intermediate:mature

Fig. 6. Efficient Sec-dependent import of 23PC/RRhL by isolated thylakoids. **(A)** 23-PC was incubated with washed pea thylakoids under control conditions (C) or after preincubation of the import mixture with 1 unit of apyrase for 15 min on ice (lanes Ap). After further incubation for 20 min at 25°C, samples were analysed directly (C, Ap) or after thermolysin treatment (C+, Ap+). **(B)** 23PC/RRhL was incubated with isolated thylakoids in the presence of stromal extract and 6 μ M over-expressed pea pre-23K. Incubations were conducted under control conditions (C) or after preincubation in the presence of 1 unit apyrase (Ap) for 15 min on ice. Samples were analysed directly (C, Ap) or after thermolysin treatment of the thylakoids (C+, Ap+).

protein is 10.5 for 23K but only 0.075 for 23PC/RRhL. This result strongly suggests that 23PC/RRhL is efficiently translocated by both pathways.

Figure 6 provides final confirmation that 23PC/RRhL can be targeted by the Sec pathway. In this experiment we tested whether the mutant precursor can be imported by isolated thylakoids by the Sec pathway, taking advantage of the fact that this pathway has an absolute requirement for ATP (Hulford *et al.*, 1994) whereas the Δ pH-dependent pathway is totally ATP-independent (Cline *et al.*, 1992). Apyrase was used to hydrolyse all nucleoside triphosphates present in the incubation mixture. In the control experiments (Figure 6A), apyrase treatment has no effect on the import and processing of 23-PC in a standard thylakoid import assay carried out in the absence of stromal extract. This control experiment confirms that this chimera can be efficiently targeted by the Δ pH-dependent pathway, and shows that the apyrase has no non-specific effects on this translocation mechanism. In the experiment shown in Figure 6B, the 23PC/RRhL mutant was incubated with thylakoids in the presence of stromal extract, and 6 μ M unlabelled, over-expressed pea pre-23K was also included to suppress the Δ pH-dependent pathway. This concentration of pre-23K saturates the Δ pH-dependent pathway and completely blocks import of *in vitro*-synthesized precursor proteins by this pathway (Cline *et al.*, 1993). Import of *in vitro*-synthesized pre-23K was also totally blocked in our control experiments as expected (data not shown). In contrast, 23PC/RRhL is efficiently imported under these conditions, and translocation is completely abolished by apyrase treatment, demonstrating that the precursor is being translocated by the ATP-dependent Sec pathway.

The above data show quite clearly that the C-domain Lys prevents the 23K transfer peptide from engaging the Sec machinery to a large extent, but precisely how efficient a 'Sec-avoidance' directive is this Lys residue? In particular, we deemed it important to determine whether this is an all-or-nothing phenomenon, or whether the transfer peptide can direct low-efficiency targeting by the Sec pathway even when the Lys is present. These are critical questions because co-targeting by both pathways has not been demonstrated using wild-type precursors, yet it appears unlikely that such a simple signal could

Fig. 7. 23K transfer signals lacking the twin-Arg motif function as efficient signal peptides only when the C-domain Lys is removed. **(A)** 23-PC, 23PC/KK and 23PC/KKhL were imported into intact chloroplasts and samples analysed directly (lanes C) or after protease treatment of the chloroplasts (lanes C+). 'pre' denotes mobility of the precursor proteins; iPC denotes an intermediate-size form that accumulates during import of 23PC/KK. **(B)** 23PC/KKhL was imported into intact chloroplasts in the absence of inhibitors, or in the presence of azide or nigericin, as described in the legend to Figure 5. 'int' denotes stromal intermediate form.

quantitatively block recognition by the Sec machinery. The problem is that low-efficiency translocation by the Sec pathway is extremely difficult to measure against a background of efficient Δ pH-dependent targeting, and the most effective means of inhibiting the latter pathway (dissipation of the Δ pH) is also predicted to inhibit the Sec apparatus (see Discussion). We circumvented this problem by removing the twin-Arg motif known to be essential for targeting by the Δ pH-dependent system, reasoning that this mutation would not affect targeting by the Sec pathway. Other evidence (Brink *et al.*, 1997) has shown that twin-Arg and twin-Lys are identical in terms of their acceptability to the thylakoidal Sec system, and studies on bacterial signal peptides have shown that, while basic residues in the N-domain are important for translocation by the Sec machinery, Lys and Arg are equally effective in promoting translocation (Sasaki *et al.*, 1990). The twin-Arg motifs within 23-PC and 23PC/RRhL were therefore replaced with twin-Lys, generating 23PC/KK and 23PC/KKhL, respectively.

Figure 7A shows chloroplast import data for these proteins, together with a control import using the 23-PC from which they were derived. In the control import, 23-PC is again imported, efficiently sorted and processed to the mature size as shown above in Figure 4. 23PC/KK, on the other hand, is imported into the organelles but further transport across the thylakoid membrane is inhibited to a large degree, with the result that most of the protein is found as the intermediate form; this polypeptide is located in the stroma (data not shown). The absence of a twin-Arg motif in this mutant precludes targeting by the Δ pH-dependent pathway, and this result therefore implies that low-efficiency translocation by the Sec pathway does occur. However, when the C-domain Lys is replaced by Leu, the resulting 23PC/KKhL mutant is

Fig. 8. Transport of the 23PC/KK mutant by the Sec pathway is largely dependent on the thylakoidal Δ pH. 23PC/KK was incubated with intact chloroplasts in the presence of nigericin or azide and samples of the chloroplasts, protease-treated chloroplasts, stroma, thylakoids and protease-treated thylakoids were analysed. In the control incubation lacking inhibitors, only the chloroplast and protease-treated chloroplast fractions were analysed. 'int' denotes stromal intermediate form.

targeted much more efficiently and only the mature form is detected; other data (not shown) have confirmed that the mature form is located in the thylakoid lumen. These data are fully consistent with the data shown above and once again point to the Sec-avoidance characteristic of the C-domain Lys in the 23K transfer signal. Additional experiments have confirmed that the import of 23PC/KK_hL exhibits a classical Sec-type sensitivity to inhibitors, in that targeting is nigericin-insensitive but partially azide-sensitive (Henry *et al.*, 1994; Knott and Robinson, 1994). Figure 7B shows that nigericin has a very minor effect on the import of 23PC/KK_hL into the lumen (a very low level of stromal intermediate is apparent in lane S), and import is partially inhibited by azide.

Although the C-domain Lys in the 23PC/KK mutant prevents efficient translocation by the Sec pathway, it is interesting that translocation occurred at all; the finding that nigericin blocks translocation of spinach and pea 23-PC was taken as strong evidence that targeting occurred exclusively by the Δ pH-dependent pathway (Henry *et al.*, 1994; Robinson *et al.*, 1994). However, it was pointed out above that the transport of the 23-PC chimera used in the present study (containing the wheat 23K transfer peptide) is not totally blocked by nigericin. These findings suggest that the chimeras are being transported by the Sec system at an appreciable rate, and we therefore examined the effects of nigericin and azide on the transport of 23PC/KK across the thylakoid membrane. The control import in Figure 8 again shows the presence of both intermediate- and mature-size protein; azide is able to completely inhibit transport across the thylakoid membrane, with the result that only the stromal intermediate can be detected. Clearly, this mutant is targeted exclusively by the Sec pathway. Nigericin also inhibits translocation though to a lesser extent, strongly suggesting that low-efficiency translocation by the Sec pathway is highly dependent on the Δ pH. Assuming that the Sec system is equally tolerant of twin-Arg and twin-Lys prior to the H-domain, we propose that these data represent evidence that the wild-type wheat 23K transfer peptide is capable of directing low-level transport by the Sec pathway, providing that the passenger protein can be handled by the Sec apparatus.

Discussion

Studies on known lumenal proteins have strongly suggested that a given protein is targeted primarily, if not exclusively, by a single pathway (Cline *et al.*, 1993; Henry

et al., 1994; Robinson *et al.*, 1994; Mant *et al.*, 1995; Voelker and Barkan, 1995). These findings were at first surprising because all of the targeting signals resemble signal peptides, and two questions in particular have dominated discussions of the underlying sorting mechanisms: (i) why are transfer signals for the Δ pH-dependent system not recognized by the Sec machinery; and (ii) what is required for targeting by the Δ pH-dependent pathway, in addition to a twin-Arg motif? The latter question awaits clarification, but we have shown in this report that the failure of the 23K transfer peptide to direct targeting by the Sec pathway can be attributed to the C-domain Lys. Removal of this Lys generates a seemingly perfect signal peptide.

We wish to emphasize that a C-domain Lys *per se* is not an obstacle to the Sec machinery, because the introduction of Lys at precisely the same position in the PC signal peptide has negligible effects on translocation across the thylakoid membrane (Brink *et al.*, 1997). Rather, it is the combination of N-domain twin-Arg motif and C-domain Lys that is so effective in prohibiting translocation by the Sec pathway. Accordingly, we propose that the C-domain Lys—within the context of the 23K transfer peptide—constitutes a 'Sec-avoidance' signal. In this context it is interesting to note that bacterial signal peptides often contain twin basic residues in the N-domain and very rarely contain basic residues in the C-domain (von Heijne, 1985; Izard and Kendall, 1994), raising the possibility that a C-domain basic amino acid could give rise to a similar effect in bacterial signal peptides. How the pre-23K Lys actually repels the Sec apparatus is presently unknown, and it will be of interest to determine the point along the Sec pathway at which targeting is aborted. It would seem logical to avoid interaction with the first element of the pathway (probably SecA or SecB), in order to prevent non-productive use of the Sec machinery. Nevertheless, we reiterate that further work is required to determine the point at which transfer peptides are refused entry onto the Sec pathway.

How widespread is the C-domain Sec-avoidance signal within transfer peptides, and what forms can it take? A total of six pre-23Ks have been cloned from both mono- and dicotyledonous plants and, although the transfer peptides vary in sequence, it is notable that all contain Lys at the -7 position (not shown), suggesting that this feature is functionally significant. Pre-16K is also targeted primarily, if not exclusively, by the Δ pH-dependent pathway, and the transfer peptides of spinach and maize pre-16K likewise contain basic C-domain residues, although in the latter case the residue is further towards the cleavage point at the -2 position (see Figure 1). Unlike the 23K mature protein, mature 16K *can* be targeted by the Sec pathway if a Sec-targeting signal is provided (either the 33K or PC signal peptides; Mant *et al.*, 1995), suggesting that the 16K transfer peptide must contain a Sec-avoidance signal. This point is reaffirmed in a study by Voelker and Barkan (1995) who have characterized maize mutants that are defective in either the Sec- or Δ pH-dependent targeting pathways. Pulse-chase labelling studies in the latter mutant showed no accumulation whatsoever of mature 16K over a 70-min period, emphasizing the point that pre-16K is an extremely poor substrate for the Sec apparatus and implying the existence of a powerful Sec-avoidance signal.

In the light of our data on pre-23K, it is extremely likely that the C-domain Lys residues in these pre-16Ks carry out this function. Similarly, barley pre-PSI-N and *Arabidopsis* pre-PSII-T both contain basic residues in the C-domain. However, a basic residue is not found in all C-domains, and a notable exception is cotton pre-PSII-T which has been shown by Henry *et al.* (1994) to be targeted by the Δ pH-dependent pathway. It is also the case that one of the *Arabidopsis* pre-16Ks contains no C-domain positive charge. One possibility is that strongly hydrophilic residues can perform a similar function to basic residues, and this would explain why Lys is found at the -5 position in two of the pre-16Ks whereas Gln is present in the other two. This would also be consistent with our observation that a C-domain Asn blocks translocation of PC/RR as effectively as Lys.

Although the precise form of this type of Sec-avoidance signal in other substrates remains to be determined, it seems clear that this form of signal differs fundamentally from that involved in the Sec-independent insertion of some thylakoid membrane proteins, such as CF₀II. This protein is also synthesized with a bipartite presequence, but membrane insertion is entirely independent of the Sec apparatus (Michl *et al.*, 1994). In this case the presequence does not contain a 23K-type distribution of basic residues around the H-domain, and it appears more likely that the Sec-independent insertion stems from the fact that only a very short section is translocated across the membrane.

Although the C-domain Lys in the 23K transfer peptide drastically inhibits recognition by the Sec machinery, our data suggest for the first time that some transfer peptides may in fact direct translocation by this pathway to a small but significant extent. Replacement of the 23K twin-Arg with twin-Lys creates a peptide that is capable of low-level Sec-dependent translocation and, assuming that twin-Arg and twin-Lys are equally acceptable to the Sec system, this finding strongly suggests that the wild-type 23K transfer peptide can function as a weak signal peptide. Other transfer peptides may well have similar abilities, particularly those without basic residues in the C-domain. In the case of pre-23K, Sec-dependent targeting is precluded by the inability of the Sec system to translocate the 23K *mature* protein, but other mature proteins may be more amenable.

Our results also suggest that such low-level targeting by the Sec pathway may be very difficult to measure unless the twin-Arg motif is knocked out, because there is no foolproof method to measure weak Sec-dependent transport against a background of efficient Δ pH-dependent transport. Compounds that dissipate the thylakoidal Δ pH are clearly unsuitable as tools because, although the Δ pH-dependent translocase is blocked, there is increasing evidence that low-efficiency translocation by the Sec machinery is equally affected. This can be observed with normal Sec substrates such as pre-33K and pre-PC, whose import into isolated thylakoids becomes increasingly dependent on the Δ pH as the ATP concentration is lowered (Mant *et al.*, 1995). Other experiments showed that Sec-dependent targeting of mature 16K by the 33K or PC presequences was totally dependent on the Δ pH, possibly because the translocation was relatively inefficient. The data in the present study are fully consistent with these reports: we have shown that the weak Sec-dependent

targeting of the 23PC/KK mutant is highly dependent on the Δ pH. The only alternative approach to determine pathway specificity is the use of competition assays (Cline *et al.*, 1993). Here, the inclusion of unlabelled, over-expressed pre-16K or pre-23K leads to accumulation of the stromal intermediate form of the co-imported, labelled protein if it is likewise targeted by the Δ pH-dependent pathway, due to competition for the thylakoidal protein translocase. However, complete saturation of the translocase cannot be achieved because import into the chloroplast becomes saturated beforehand; hence competition is manifested by the appearance of a mixture of intermediate and mature forms. Under these conditions, co-targeting by the Sec pathway should theoretically reduce the accumulation of intermediate form, but low-level targeting may not have a sufficiently marked effect for this approach to be viable.

Although several studies have shown, both *in vitro* and *in vivo*, that the known luminal proteins are tightly restricted to a single targeting pathway inside the chloroplast, it has remained unclear why this system is maintained. Our data suggest one possible reason, relating to the mature proteins. We have shown quite clearly that some property of the 23K mature protein renders it incapable of being targeted by the Sec pathway; the 23K/RRhL mutant lacking the Sec-avoidance signal contains an extremely efficient signal peptide, yet is wholly incapable of utilizing the Sec pathway. The 33K and PC signal peptides are likewise completely incapable of targeting mature 23K into the lumen (Clausmeyer *et al.*, 1993). Even the wild-type 23K transfer peptide should be capable of low-level Sec targeting, but this appears not to occur (Voelker and Barkan, 1995). Given that the 23K mature protein contains its own intrinsic Sec-avoidance capability, we suggest that the highly conserved Sec-avoidance motif in the transfer signal must play an important role, and one possible role is to avoid potentially unproductive engagement of the Sec machinery. There is a strong possibility that the Sec apparatus could otherwise recognize the transfer peptide and initiate translocation, but be forced to abort translocation at a later stage, thereby reducing the overall efficiency of the Sec apparatus. Consistent with this idea, mature 16K is targeted rather inefficiently by the Sec pathway when a signal peptide is attached (Clausmeyer *et al.*, 1993), and we have found that removal of the C-domain Lys from spinach pre-16K (which should generate a functional signal peptide analogous to that in the 23PC/RRhL mutant) does not enable the protein to be transported by the Sec pathway to any significant extent (data not shown). These findings suggest that mature 16K, like 23K, can not be translocated by the Sec pathway. In turn, these observations provide a plausible explanation for the emergence of the Δ pH-dependent protein translocase—its appearance may have been vital for the targeting of these Sec-incompatible photosynthetic proteins. It will be interesting to determine whether other Δ pH-dependent substrates are easier to target by the Sec pathway, in which case such proteins may well turn out to be targeted by both pathways (if the above idea is correct, any Sec-avoidance characteristics should be lost due to a lack of selective pressure).

While the present study has focused on the Sec-avoidance aspect of transfer signals and not the positive

signals required for targeting by the Δ pH-dependent system, our data do have important implications concerning the latter signals. We have previously argued that the presence of the twin-Arg motif was not sufficient to divert PC onto the Δ pH-dependent pathway (Chaddock *et al.*, 1995) but this proposal was based on the observation that azide affected translocation of pre-PC and PC/RR to a similar extent. Low-efficiency translocation by the Δ pH-dependent pathway could not have been detected against a background of efficient Sec-dependent targeting. However, the introduction of Lys at the -7 position in the PC/RR mutant means that the precursor created (PC/RRhK) has a charge distribution identical to that of the 23K transfer peptide. If anything, this would be predicted to enhance translocation by the Δ pH-dependent system, yet this protein is essentially blocked in thylakoid translocation. Thus, even when the creation of the Sec-avoidance signal eliminates translocation by the Sec pathway, the twin-Arg motif alone is unable to support translocation by the Δ pH-dependent pathway to any detectable extent. Transfer peptides must therefore contain additional information that is not merely important, but essential. In summary, the critical features of the 23K transfer peptide can be defined at present as a twin-Arg motif, a Sec-avoidance signal and at least one further essential positive signal, all of which are superimposed on a typical signal peptide template.

Materials and methods

Import experiments

Precursor proteins were synthesized by transcription-translation of cDNA clones in the presence of [³⁵S]methionine and imported into isolated pea chloroplasts essentially as described by Mould and Robinson (1991). Incubations involving the use of nigericin or azide were as detailed by Mould and Robinson (1991) or Knott and Robinson (1994), respectively. Thylakoid import experiments were carried out as described in Hulford *et al.* (1994) and Knott and Robinson (1994).

Mutagenesis

DNA encoding spinach pre-PC (Rother *et al.*, 1986) or wheat pre-23K (James and Robinson, 1991) were subcloned into M13 mp 19 and oligonucleotide-directed mutagenesis was carried out as described by Kunkel (1985). Mutations were verified by sequencing the entire DNA insert and the inserts were then ligated into pGEM4z (Promega Biotech) and transcribed using T7 RNA polymerase. A construct encoding the pre-sequence of wheat 23K and mature PC was generated by amplifying the coding region for the wheat 23K presequence (from the SP6 promoter site in the pGEM4z vector to the TPP cleavage site) using PCR, after which the amplified product was blunt-ended and ligated in frame with the coding sequence for mature size spinach plastocyanin (Clausmeyer *et al.*, 1993) in pGEM4z (Promega Biotech).

Sequence data

The targeting signals shown in Figure 1 were taken from the following sources: spinach pre-23K and pre-16K (Jansen *et al.*, 1987); wheat pre-23K (James and Robinson, 1991); barley pre-PSI-N (Knoetzel and Simpson, 1993); cotton pre-PSII-T (Kapazoglou *et al.*, 1995); maize pre-16K (note that this cDNA was erroneously believed to be from pea when the sequence was published; Ettinger and Theg, 1992); barley pre-PC (Nielsen and Gausing, 1987); spinach pre-PC (Rother *et al.*, 1986); wheat pre-33K (Meadows *et al.*, 1991); spinach pre-33K (Tyagi *et al.*, 1987); spinach pre-PSI-F (Munch *et al.*, 1988); and barley pre-PSI-F (Scott *et al.*, 1994). Expressed Sequence Tag sequences were taken from the database; accession numbers were: H36850 and T76472 (*Arabidopsis* pre-16Ks), and R64829 (*Arabidopsis* pre-PSII-T).

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