Targeting of thylakoid proteins by the ΔpH-driven twin-arginine translocation pathway requires a specific signal in the hydrophobic domain in conjunction with the twin-arginine motif

Susanne Brink, Erik G. Bogsch, Wayne R. Edwards, Peter J. Hynds, Colin Robinson*

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

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Abstract Superficially similar cleavable targeting signals specify whether lumenal proteins are transported across the thylakoid membrane by a Sec- or ΔpH-dependent pathway. A twin-arginine motif is essential but not sufficient to direct ΔpH-dependent targeting; here we show that a second determinant is located in the hydrophobic region. A highly hydrophobic amino acid is found either two or three residues C-terminal to the twin-arginine in all known transfer peptides for the ΔpH-dependent system, and substitution of this residue in the 23-kDa (23K) peptide markedly inhibits translocation. Further, whereas the insertion of twin-arginine in a Sec-dependent precursor does not permit efficient ΔpH-dependent targeting, the simultaneous presence of a leucine at the +3 position (relative to the RR) enables the peptide to function as efficiently as an authentic transfer peptide. RRNVL, RRAAL and RRALA within a Sec targeting signal all support efficient ΔpH-dependent targeting, RRNVA is less effective and RRNAA/RRNAG are totally ineffective. We conclude that the core signal for this pathway is a twin-arginine together with an adjacent hydrophobic determinant.

Key words: Chloroplast; Protein transport; Targeting signal; Thylakoid; Sec-independent

1. Introduction

The translocation of proteins across biological membranes involves, in most cases, the 'threading' of a partially folded protein through a complex protein translocase and the subsequent refolding of the protein on the *trans* side of the membrane (reviewed in [1]). However, there is now compelling evidence for the existence of a novel type of protein translocase in a range of organisms, which serves to transport fully-folded proteins across even tightly coupled membranes. This type of translocase was first discovered in chloroplasts during studies on the targeting of lumenal proteins across the thylakoid membrane (reviewed in [2]). Although a subset of proteins are transported by an ATP-dependent, Sec-type system [3–5], others are transported by a mechanism that does not require nucleoside triphosphates or soluble factors, but which is totally dependent on the thylakoidal ΔpH [6–10]. The cloning of the first component of the translocase, termed Hcf106 [11], revealed the presence of homologues in numerous prokaryotes encoded by previously unassigned reading frames. Recent work has confirmed the operation of a Sec-independent protein export system in *Escherichia coli* involving two Hcf106 homologues together with at least two further components encoded by linked genes [12–15]. There are also clear indications that this system is involved primarily in the export of periplasmic proteins that bind any of a range of redox cofactors in the cytosol, thereby necessitating the transport of a fully-folded protein [16]. While this premise remains to be confirmed experimentally, the available evidence certainly suggests a novel type of mechanism for this system.

In both chloroplasts and bacteria, Sec-independent targeting is specified by cleavable targeting signals (transfer peptides) that very much resemble typical Sec-type 'signal' peptides in overall structure. Mutagenesis studies have shown that a key feature in the thylakoid system is the presence of an essential twin-arginine motif immediately prior to the hydrophobic region [17] and predicted substrates for the bacterial systems likewise contain this characteristic motif [16]. However, in the above mutagenesis study [17] it was found that the insertion of twin-arginine into the corresponding position of a Sec substrate, pre-plastocyanin (pre-PC) did not divert the precursor onto the ΔpH-dependent pathway to any significant extent. This finding indicated that transfer peptides contain at least one further essential determinant. In the present study we show that the second characteristic element of transfer peptides is the presence of a highly hydrophobic residue either two or three residues after the twin-arginine. The observed consensus motif of (S/T)-R-R-x-F-L-K for predicted bacterial transfer peptides [16] suggests that this determinant is likely to be equally important in the bacterial signals.

2. Materials and methods

2.1. Import experiments

Precursor proteins were synthesised by transcription-translation of cDNA clones in the presence of [^{35}S]methionine, and imported into isolated pea chloroplasts essentially as described by Mould and Robinson [6]. Incubation times were 20 min in all cases, and incubations involving the use of nigericin were as detailed by Mould and Robinson [6]. Import into isolated thylakoids, and the use of apyrase, was as described by Hulford et al. [18]. Pre-23K was prepared and used in competition assays using the procedure of Cline et al. [19]. Import data were quantified using a phosphorimager.

2.2. Mutagenesis

Mutagenesis of the spinach PC/RR construct [17] or wheat pre-23K involved subcloning into M13mp19 and oligonucleotide-directed mutagenesis as described by Kunkel [20]. 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.

2.3. Sequence data

The targeting signals shown in Fig. 1 were essentially as listed in [17]. Expressed Sequence Tag sequences were taken from the data-

*Corresponding author. Fax: (44) (1203) 523701.
E-mail: cg@dna.bio.warwick.ac.uk

PII: S0014-5793(98)01028-X

base; accession numbers are: H36850 and T76472 (*Arabidopsis* pre-16Ks), and R64829 (*Arabidopsis* pre-PSII-T).

3. Results

3.1. Structures of thylakoid-targeting signals

The structures of representative thylakoid-targeting signals are given in Fig. 1. The list illustrates the point that all of the peptides are superficially very similar, containing the characteristic charged amino-terminal, hydrophobic core and more polar C-terminal domains (N-, H- and C-domains). Transfer peptides for the ΔpH-dependent pathway contain the conserved twin-Arg motif immediately prior to the H-domain but few other differences are apparent between the two groups of peptide. However, we noted that transfer peptides invariably contain a large, hydrophobic residue at the third position after the twin-Arg motif, and we considered this to be potentially significant. The functional importance of this characteristic was assessed by increasing the hydrophobicity of a Sec-type signal peptide in this region, using a mutant pre-PC (PC/RR; see Fig. 1) in which a twin-Arg has been inserted prior to the H-domain. This mutant has been shown to utilise only the Sec pathway to any significant extent [17], and our objective was to determine whether alterations in the H-domain would enable the precursor protein to engage the ΔpH-dependent pathway. One of these mutant signal peptides (PC/RRAAL) contained a five-residue sequence (the twin-Arg motif plus the following three residues) transposed from the presequence of the wheat 23-kDa photosystem II protein (23K), a typical substrate for the ΔpH-dependent pathway. Another mutant contained the same amino acid residues but with the sequence altered to RRALA. Other mutants contained only one or two 23K-type residues within the three residues after the twin-Arg. Finally, the wheat 23K transfer peptide was also mutated to test the importance of hydrophobicity in this region (the Wh23K/L55S mutant).

3.2. Mutations in the H-domain enable PC/RR to be targeted by either pathway

Preliminary studies (not shown) indicated that all of the mutant precursors can be imported into intact chloroplasts with similar efficiencies, and that nigericin had very little effect on the targeting of any of the proteins into the lumen. Since this proton ionophore totally blocks transport by the ΔpH-dependent pathway [6,7] it is clear that all of the PC mutants remain competent for targeting by the Sec pathway. However,

Transfer peptides

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Sp 23K                AQKQDDNEANVLNSGVSRRLALTVLIGAAAVGSKVSPADA
Wh 23K                 AQKNDEAASDAAVVTSRRAALSLLAGAAAIAVKVSPAAA

Sp 16K                   --AQQVSAEAETSRRAMLGFVAAGLASGSFVKAVLA
Ma 16K                  --ASAEGDAVAQAGRRAVIGLVATGIVGGALSQAARA
Ara 16Ka                --LVVRAQQSEETSRRSVIGLVAAGLAGGSFVQAVLA
Ara 16Kb                --RAQQNVSVPESSRRSVIGLVAAGLAGGSFVKAVFA

Bar PSI-N              --AAAKRVQVAPAKDRRSALLGLAAVFAATAASAGSARA

Cot PSII-T              --VQMSGERKTEGNNGRREMMFAAAAAAICSVAGVATA
Ara PSII-T              --TPSLEVKEQSSTTMRRDLMFTAAAAAVCSLAKVAMA
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Mutant transfer peptide

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Wh 23K/L55S           AQKNDEAASDAAVVTSRRAASSLLAGAAAIAVKVSPAAA
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Signal peptides

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Wh 33K       AFGVDAGARITCSLQSDIREVASKCADAAKMAGFALATSALLVSGATA
Sp 33K  AFGVESASSSGGRLSLSLQSDLKELANKCVDATKLAGLALATSALIASGANA

Sp PSI-F        --QENDQQQPKKLELAKVGANAAAALALSSVLLSSWSVAPDAAMA
Bar PSI-F       --SGDNNNSTATPSLSASIKTFSAALALSSVLLSSAATSPPPAAA

Bar PC                                ASLGKKAASAAVAMAAGAMLLGGSAMA
Sp PC                                   ASLKNVGAAVVATAAAGLLAGNAMA
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Mutant signal peptides

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Sp PC/RR                              ASRRNVGAAVVATAAAGLLAGNAMA
Sp PC/RRAAL                           ASRRAALAAVVATAAAGLLAGNAMA
Sp PC/RRALA                           ASRRALAAVVATAAAGLLAGNAMA
Sp PC/RRNVA                           ASRRNVAAAVVATAAAGLLAGNAMA
Sp PC/RRNVL                           ASRRNVLAAVVATAAAGLLAGNAMA
Sp PC/RRNAA                           ASRRNAAAAVVATAAAGLLAGNAMA
Sp PC/RRNAG                           ASRRNAGAAVVATAAAGLLAGNAMA
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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 lumenal 33-kDa protein (33K) peptides have been delineated but the remainder have yet to be identified. Also shown are the mutant PC signal peptides described in this study together with a mutated wheat 23K transfer peptide. Charged residues are given in bold and the H-domains are underlined; residues introduced by mutagenesis are shown italicised.

this type of assay cannot be used to characterise the thylakoid-targeting pathway(s) in detail because no totally effective pathway-specific inhibitors are available. Assays for the import of proteins by isolated thylakoids are more effective, because the import of Sec substrates such as pre-PC is completely dependent on ATP [18], and the translocation of ΔpH-dependent substrates can be blocked by saturating concentrations of over-expressed pre-23K [19]. The import of Sec substrates is also stimulated to some extent by the presence of stromal extract, although we now find that import takes place with fairly high efficiency into washed thylakoids. One reason appears to be a much greater concentration of SecA in the wheat germ translation system used (our unpublished observation).

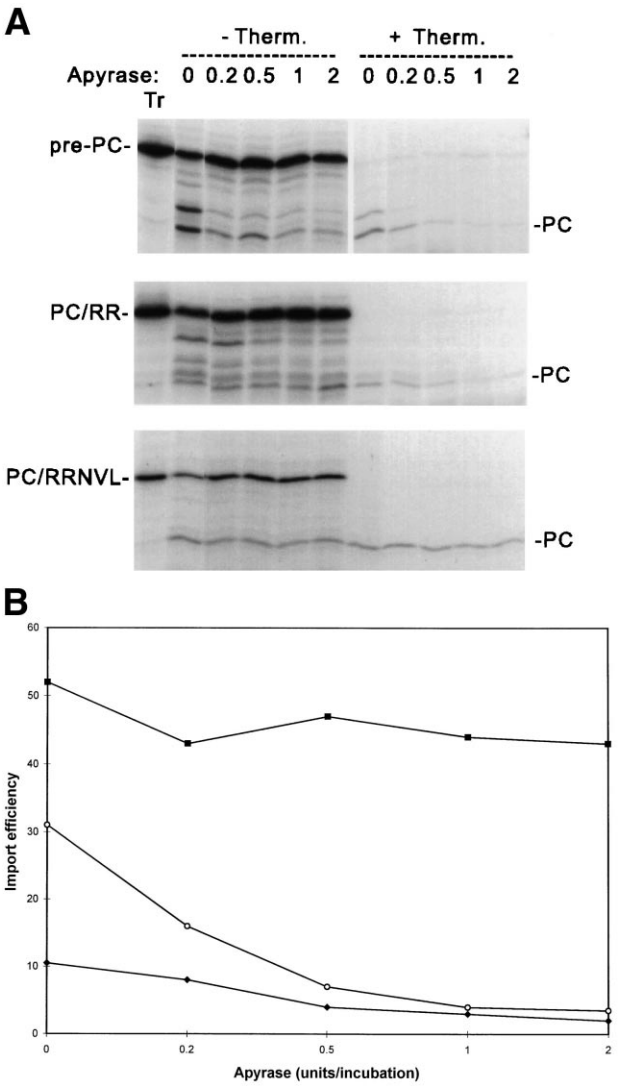

Fig. 2. Sec-independent import into isolated thylakoids of the PC/RRNVL mutant. Pre-PC, PC/RR and the PC/RRNVL mutant were incubated with isolated pea thylakoids after pre-incubation for 10 min on ice with increasing amounts of apyrase (units per import mixture are indicated). After the import incubation, equal samples were analysed directly or after thermolysin (Therm) treatment of the thylakoids (as indicated). PC: mature-size plastocyanin. Lower panel shows the import efficiencies (as percentage of available precursor) quantitated as a function of apyrase concentration. The graphs represent pre-PC (squares), PC/RR (open circles) and PC/RRNVL (diamonds).

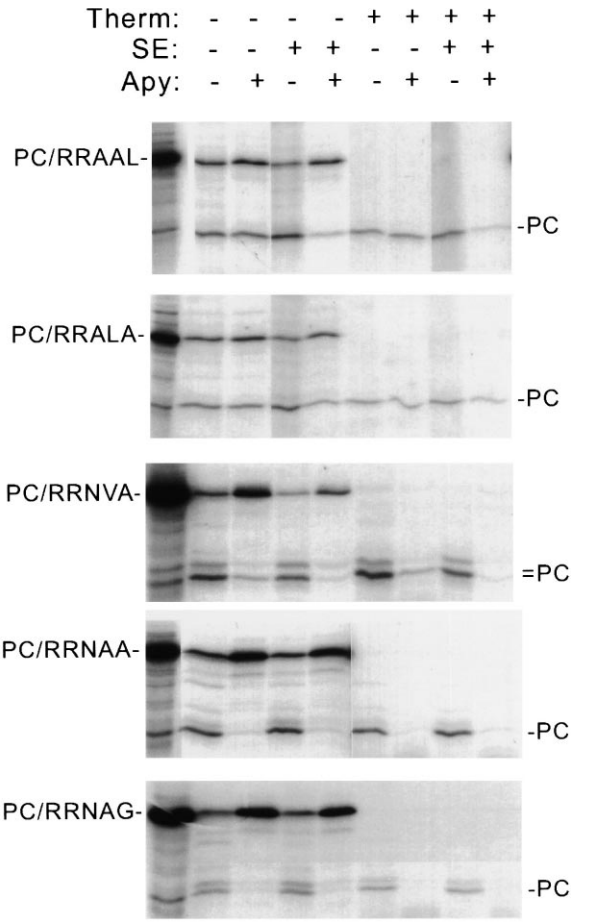

Fig. 3. Sec-independent import of other PC/RR derivatives. PC/RR and the mutant derivatives were incubated with isolated pea thylakoids in either the presence or absence of stromal extract (SE) as indicated. Before incubation, samples were preincubated for 10 min on ice with 2 units of apyrase (Apy) or with an equal amount of boiled apyrase. Samples were analysed directly or after thermolysin treatment (Therm) of the thylakoids.

Fig. 2 shows the effects of increasing concentrations of apyrase on the import of three proteins: pre-PC, PC/RR and the PC/RRNVL mutant. Apyrase hydrolyses NTPs and sufficiently high concentrations are able to completely inhibit translocation by the Sec pathway [18,21]. The data were also quantitated using a phosphorimager, taking into account the loss of 50% of the labeled methionine residues when the presequence is removed (lower section). In the control using pre-PC, the precursor is imported by thylakoids and processed to mature PC; a minor intermediate-size band is also apparent which appears to represent partially processed stromal intermediate. As found in [21], import of pre-PC is drastically reduced at higher concentrations of apyrase (from 31% to 3.5% in this experiment). PC/RR is imported with lower efficiency (10.5% in the absence of apyrase) but, significantly, apyrase inhibits import to a similar extent: down to 2% with 2 units of apyrase. Clearly, blocking the Sec pathway does not result in diversion of PC/RR onto the ΔpH-dependent pathway to any significant extent, and this result confirms our earlier proposal that a twin-Arg motif alone is not sufficient to support targeting by this pathway. However, the tar-

geting properties of the PC/RR presequence can be dramatically transformed by a single amino acid substitution at the third position after the twin-Arg, from RRNVG (in PC/RR) to RRNVL. The lower panel of Fig. 2 shows that this mutant is imported with much higher efficiency than either pre-PC or PC/RR (52% import in the control import), and that import remains high even in the presence of the highest concentrations of apyrase, down only to 43%. This reflects very efficient targeting by a Sec-independent mechanism, which we presume to be the ΔpH-dependent pathway.

Fig. 3 shows the results of similar assays using the remaining mutants, and in this experiment we also tested the effects of including stromal extract. Control assays (not shown) confirmed that pre-23K is imported by the ΔpH-dependent pathway in either the absence or presence of stromal extract, and apyrase has no effect on import, as found previously [6,7]. The data shown in Fig. 3 using the mutant constructs are of interest from three standpoints. First, the effects of apyrase treatment vary considerably among the different PC/RR derivatives. Like PC/RRNVL (Fig. 2), PC/RRAAL and PC/RRALA are both efficiently imported in the presence of apyrase, especially in the absence of stromal extract. PC/RRNVA is affected to a greater extent by this treatment but there is again a significant level of apyrase-insensitive import. The import of PC/RRNAA and PC/RRNAG, on the other hand, is drastically inhibited by apyrase treatment, and essentially no import is apparent. The latter two mutants are thus similar to wild-type pre-PC and PC/RR in terms of requirement for NTPs. These data indicate that several of the mutants can be imported into thylakoids with high efficiency by a Sec-independent mechanism. This Sec-independent targeting

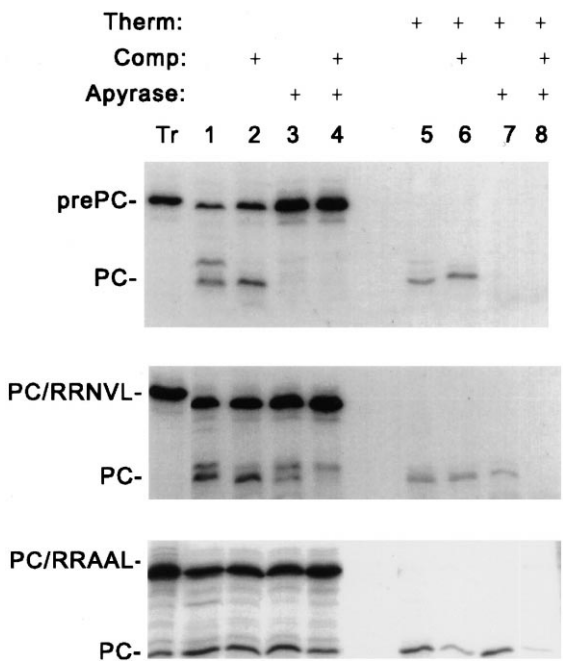

Fig. 4. PC/RR derivatives are imported by the ΔpH-dependent pathway. Pre-PC, PC/RRAAL and PC/RRNVL were incubated with thylakoids under control conditions, after preincubation with 2 units apyrase (as indicated), or in the presence of 4 μM over-expressed competitor pea pre-23K (Comp), or both. Lane Tr: translation product. Samples were analysed directly or after thermolysin treatment of the thylakoids (Therm).

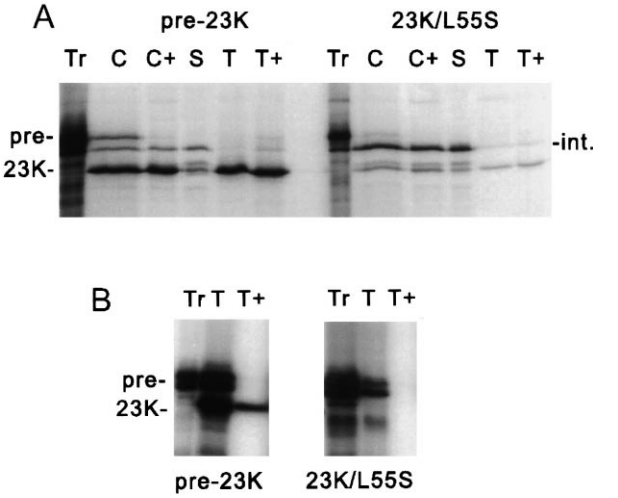

Fig. 5. The 23K/L55S mutant is severely compromised in thylakoid translocation. A: Pre-23K and the 23K/L55S mutant were imported into isolated chloroplasts and the organelles fractionated as shown in Fig. 1. Lane T+: protease-treated thylakoids. B: Pre-23K and the 23K/L55S mutant were incubated with isolated thylakoids after which samples were analysed directly (lanes T) or after protease-treatment of the thylakoids (lanes T+). Tr: translation product. 'Pre' denotes precursor form, int. denotes stromal intermediate.

is furthermore most efficient in mutants containing a more hydrophobic region following the twin-Arg motif.

Fig. 3 also shows that the presence of stromal extract enhances import in several cases, including PC/RRAAL and PC/RRALA. This strongly suggests that these proteins are imported by both pathways under these conditions, since stromal extract does not enhance targeting by the ΔpH-dependent pathway. Interestingly, apyrase has a more significant effect on the import of these mutants in the presence of stromal extract, and one possible explanation is that some precursor molecules may become dedicated to the Sec pathway, rendering them incapable of engaging the ΔpH-dependent pathway.

PC/RRNVL, PC/RRAAL and PC/RRALA are clearly good substrates for both the Sec-dependent and -independent pathways because import efficiency remains high in the presence of apyrase. Repeat experiments (data not shown) have revealed PC/RRAAL to be consistently imported with higher efficiency than PC/RRALA, but the difference is not great and the data shown in Fig. 3 are typical in this respect. PC/RRNVA is certainly able to use the Sec-independent pathway (presumably the ΔpH-dependent pathway) but with lower efficiency, and PC/RRNAA and PC/RRNAG are hardly transported at all. The final test on the PC mutants involved confirming that the observed Sec-independent targeting is indeed taking place by the ΔpH-dependent pathway. In this experiment we again suppressed the Sec pathway using apyrase and then tested whether the residual translocation into thylakoids was competed by saturating concentrations of over-expressed pea pre-23K. The basic procedure was as described by Cline et al. [19] and in control tests (not shown) the competitor pre-23K was able to completely inhibit import of radiolabeled, in vitro-synthesized pre-23K, indicating that the ΔpH-dependent pathway is saturated. Apyrase did not inhibit import as expected from previous studies. The effects of competitor and apyrase on translocation by the Sec pathway are shown in the control tests using pre-PC (Fig. 4, top panel). As expected,

competitor pre-23K has essentially no effect on import whereas apyrase again causes a complete block. In the case of PC/RRAAL, however, the presence of competitor pre-23K leads to a marked inhibition of import (down to 17% of the control value) whereas apyrase treatment has relatively little effect on import efficiency (91% of the control value). A combination of apyrase and competitor almost completely blocks import of the radiolabeled PC/RRAAL. The marked effect of the competitor pre-23K suggests that the ΔpH-dependent pathway is the preferred route for PC/RRAAL in the absence of stromal extract. The data for the PC/RRNVL mutant are slightly different: both apyrase and competitor inhibit import to a similar, limited extent but a combination of the two again leads to a total block (lane 8). These data confirm that the Sec-independent import of this protein is again taking place by the ΔpH-dependent mechanism, but we believe that the more limited effect of competitor in this case may reflect a more efficient targeting by the Sec pathway when compared with the PC/RRAAL mutant.

3.3. Importance of the third residue of the H-domain in the wheat 23K transfer peptide

In order to confirm the importance of the first part of the H-domain in an authentic transfer peptide, we replaced the third residue after the twin-Arg in the wild-type wheat 23K transfer peptide (Leu) with Ser, a more hydrophilic amino acid, converting the sequence from RRAAL to RRAAS. Chloroplast import assays using this 23K/L55S mutant (Fig. 5A) reveal that this single mutation drastically affects targeting within the chloroplast. In the control import, the vast majority of imported protein is found as the mature size form in the thylakoid lumen, and the stromal intermediate is only a minor form. The imported 23K/L55S, on the other hand, is found predominantly as the stromal intermediate, demonstrating that translocation across the thylakoid is severely affected. The extent of inhibition is revealed more clearly by thylakoid import assays (panel B), which demonstrate very efficient import of wild-type pre-23K whereas that of the mutant is totally blocked. Clearly, this substitution has a massive detrimental effect on targeting by the ΔpH-dependent pathway.

4. Discussion

In this report we have shown that a typical Sec-type signal peptide can be converted to a highly efficient transfer peptide by minor substitutions. The introduction of a twin-arginine is of course a prerequisite, given the essential nature of this motif [17], but this is not on its own sufficient and the observed level of ΔpH-dependent transport is very low indeed in the case of the PC/RR mutant. However, the additional presence of a highly hydrophobic residue at the +2 or +3 position (relative to the twin-arginine) enables the precursor to effectively engage the ΔpH-dependent transport machinery, to the extent that targeting into thylakoids is as efficient as that observed using a 23-PC chimera containing an authentic transfer peptide. This suggests that these two determinants, within the overall context of a signal peptide, represent the definitive features of thylakoid transfer peptides. It is, however, worth pointing out that our assays are based on re-routing a Sec signal onto the ΔpH-dependent pathway and it remains possible that other critical determinants remain to be characterised, simply because they are coincidentally present within the PC signal peptide.

Another study on this topic emerged recently [22] but in this work the preferred approach was to exchange entire domains between Sec- and ΔpH-dependent precursors. These authors reached rather different conclusions but these may well reflect the structures of the particular chimeras used. A fusion (termed DT-PC) of the N-domain of the *Arabidopsis* 23K transfer peptide (containing the twin-Arg motif) with the H- and C-domains of the pea PC signal peptide was shown to target mature PC by both the Sec- and ΔpH-dependent pathways, and it was concluded that the twin-Arg was the only important factor required for the latter pathway. However, the import efficiency of the DT-PC construct was low: 7% of precursor imported by thylakoids using the ΔpH-dependent pathway. Because the PC/RRNVL and PC/RRAAL constructs can be imported with efficiencies approaching 50%, and the 23K/L55S mutant is so drastically affected in targeting, we conclude that the H-domain does in fact contribute a major targeting determinant. This determinant is indeed almost as important as the twin-Arg motif because the PC/RRNAA mutant, lacking a hydrophobic residue at either the second or third positions after the twin-Arg, is completely unable to utilise the ΔpH-dependent pathway in thylakoid import assays. It is, however, interesting that the DT-PC construct was imported at all by the ΔpH-dependent pathway since it is similar in overall structure to the PC/RR mutant used in the present study. This may reflect the fact that the H-domain of pea PC in the DT-PC construct is more hydrophobic than that of the spinach sequence in PC/RR, especially at the third position (RRFGV compared with RRNVG).

Our mutagenesis data are entirely consistent with the structures of known thylakoid transfer peptides, all of which contain a hydrophobic residue (Leu, Ile, Met or Phe) at the +2 or +3 positions. The results are also likely to be of direct relevance to studies on the Sec-independent export of proteins in bacteria. These proteins are likewise synthesised with twin-arginine-containing targeting signals and the available data suggest that the bacterial/chloroplast systems are structurally related [11–16]. Analysis of the proposed substrates for the bacterial systems reveals that highly hydrophobic residues are invariably present at the +2 or +3 positions relative to the twin-arginine, and we predict that this feature will be as important for bacterial Sec-independent export processes as for thylakoid protein targeting. It has already been shown that several of these bacterial transfer peptides can direct efficient targeting into isolated thylakoids [23,24], indicating that at least some facets of this targeting process have been very tightly conserved, and a twin-arginine alone was insufficient to promote Sec-independent export of one protein analysed [25].

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