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Targeting of a foreign protein into the thylakoid lumen of pea chloroplasts

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A chimaeric gene was constructed which encodes the pre-sequence of the 33 kDa protein of the oxygen-evolving complex, a thylakoid lumen protein, linked to dihydrofolate reductase, a cytoplasmic protein. The encoded fusion protein is transported into the thylakoid lumen of isolated pea chloroplasts, with concomitant removal of the pre-sequence.

Protein transport; Retargeting; Processing; Chloroplast; Thylakoid lumen

1. INTRODUCTION

Nuclear-encoded thylakoid lumen proteins are synthesized as larger precursors and imported into the chloroplast by a two-step process. Precursors are initially transported into the stroma and processed to intermediate forms by a stromal processing peptidase, after which the intermediates are transferred across the thylakoid membrane and processed to the mature size by a thylakoidal peptidase [1–4]. Analysis of the pre-sequences of lumenal proteins has shown that they consist of two domains, which are believed to direct 'envelope transfer' of the precursor and then 'thylakoid transfer' of the stromal intermediate [1,5].

The targeting properties of the two domains have been tested in studies using chimaeric proteins. In this work the pre-sequence of *Silene* plastocyanin was found to direct the transport of a foreign product, yeast superoxide dismutase, into the chloroplast stroma. However, neither this protein nor a stromal protein, ferredoxin, was targeted into the thylakoid lumen by the plastocyanin pre-sequence [1,6], suggesting that the mature sequences of lumenal proteins may contain some information essential for transport across the thylakoidal membrane.

In the present work, we fused the pre-sequence of another lumenal protein, the 33 kDa protein of the photosynthetic oxygen-evolving complex (33K) in front of mouse dihydrofolate reductase (DHFR), a cytoplasmic protein which has been used as a passenger protein in mitochondrial protein transport studies [7]. We show that DHFR is efficiently targeted to the thylakoid lumen in vitro.

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2. MATERIALS AND METHODS

2.1. *Materials*

Pea seedlings (*Pisum sativum*, var. Feltham First) were grown, and chloroplasts isolated as described [8]. Radioactive materials were obtained from Amersham International, England.

2.2. *Construction of p33K-DHFR*

A mouse DHFR cDNA clone, pDHFR2Z, was kindly provided by Dr J.V. Cullimore (Warwick). This vector contained the coding region from pDS5/2 [9] excised using *Bam*HI/*Hin*dIII and ligated into pGem2Z (Promega Biotech) which had been digested with *Bam*HI and *Sma*I. A cDNA clone encoding wheat pre-33K, p33K-2 [3] was cut with *Eco*RI and *Kpn*I to remove the pre-sequence coding region together with some of the mature sequence; this fragment was blunt-ended using S1 nuclease. pDHFR2Z was cut with *Bam*HI, the ends were filled in with Klenow fragment, and the 33K fragment was ligated to generate p33KDHFR. Clones in which the pre-sequence region

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was in-frame with the DHFR sequence were transcribed using T7 polymerase and transcripts were translated in a wheat germ system in the presence of [^{35}S]methionine [10,11]. Mature DHFR for use as a marker was synthesized in the same way from pDHFR2Z.

2.3. *Import and processing studies*

Chloroplast import assays were carried out essentially as described [8]. After incubation, non-imported proteins were digested using protease K (150 $\mu g \cdot ml^{-1}$, 45 min, 4°C), and the chloroplasts were washed once, lysed in 20 mM Tris-HCl, pH 8.0, and centrifuged at 10000 × *g* for 10 min to generate stromal and thylakoid fractions. Thylakoids were protease K-treated as above where appropriate. For processing studies, 2 μl translation product was incubated with 20 μl stromal or thylakoidal peptidase for 60 min at 27°C. Samples were analysed by polyacrylamide gel electrophoresis followed by fluorography.

3. RESULTS AND DISCUSSION

A chimaeric gene, p33KDHFR encoding the presequence of wheat 33K protein (with 22 residues of mature protein) linked to DHFR, was constructed as described in fig.1. The encoded protein (33KDHFR) was synthesized by in vitro transcription/translation and incubated with isolated pea chloroplasts. Fig.2 shows that the fusion protein is imported and converted to two forms, the larger of which is located in the stroma and the smaller associated with the thylakoids. The latter polypeptide is ~1–2 kDa larger than mature DHFR, possibly indicating that the entire 33K presequence has been removed, leaving a processed protein of 22 residues linked to DHFR. This protein is resistant to protease-digestion of the thylakoids. Control tests have confirmed that this protein is degraded if the vesicles are sonicated to allow access of the protease to the lumenal space contents (not shown). We conclude that this polypeptide is located inside the vesicles. The polypeptide is released from the vesicles by a very brief sonication, indicating that it is essentially soluble in the thylakoid lumen (fig.2B).

Are the stromal and thylakoidal DHFR forms products of the stromal and thylakoidal processing peptidases? This question was addressed by incubating the fusion protein with the individual peptidases, both of which have been extensively purified [12,13]. Fig.3 shows that the stromal peptidase converts 33KDHFR to a form which is slightly larger (as judged by SDS-polyacrylamide gels) than the stromal polypeptide generated

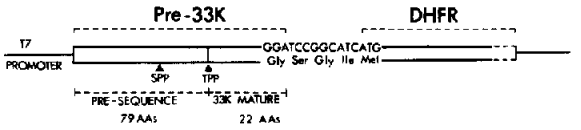

Fig.1. Structure of the 33KDHFR fusion protein. p33KDHFR was constructed as detailed in section 2. The cloning procedure introduced a 3-residue linker between the 33K and DHFR polypeptide sections. Sites of cleavage by the stromal and thylakoidal processing peptidases (SPP, TPP) are denoted by arrowheads. The precise site of cleavage by SPP is not known.

during import. The difference in mobilities is slight but reproducible. The thylakoidal peptidase processes the fusion protein to a polypeptide of identical mobility to imported, thylakoidal DHFR. These findings strongly suggest that the imported, thylakoidal protein has been correctly processed by the thylakoidal peptidase, but raise the possibility that the stromal form has been generated by proteolytic degradation or aberrant processing. This form may not, therefore, be a true import intermediate en route to the thylakoids.

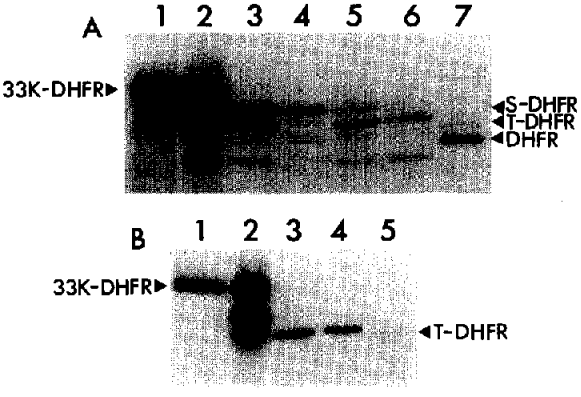

Fig.2. Transport of 33KDHFR into isolated pea chloroplasts. (A) 33KDHFR (lane 1) was synthesized in vitro, imported into peak chloroplasts, and samples were analysed without (lane 2) or with (lane 3) subsequent protease K treatment of the organelles. Lanes 4 and 5, chloroplasts were protease-treated after import and fractionated into stromal and thylakoid samples, respectively. Lane 6, as in lane 5 except that the thylakoids were protease K-treated. Lane 7, mature DHFR. (B) 33KDHFR (lane 1) was imported and samples analysed without protease treatment (lane 2) and after protease treatment of the thylakoids (lane 3). Lanes 4,5: thylakoid samples were sonicated for 5 s and then centrifuged to generate soluble and membrane fractions, respectively. S-DHFR, T-DHFR, stromal and thylakoidal DHFR forms, respectively.

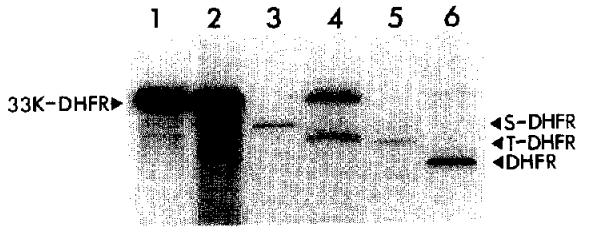

Fig.3. Processing of 33KDHFR by the stromal and thylakoidal peptidases. 33KDHFR (lane 1) was incubated with partially purified stromal (lane 2) or thylakoidal (lane 4) processing peptidase. Lanes 3,5, imported stromal and thylakoidal DHFR forms as in fig.2A, lanes 4 and 5. Lane 6, mature DHFR. Symbols as in fig.2.

The combined results indicate that the wheat 33K pre-sequence is capable of mediating the transport of a foreign protein into the thylakoid lumen of pea chloroplasts. Other work has ruled out the possibility that DHFR is itself capable of traversing the thylakoid membrane: the pre-sequence of *Silene pratensis* ferredoxin (a stromal protein) directs the transport of DHFR into the stroma, but no transport of the processed, mature DHFR across the thylakoid membrane is observed (Hageman, J. and Weisbeek, P., unpublished data).

Our findings contrast with those from previous studies [1,6] in which a plastocyanin pre-sequence failed to direct two soluble proteins into the thylakoid lumen. One possible explanation may be that the wheat 33K pre-sequence contains a more efficient thylakoid transfer signal (at least in pea chloroplasts) than the corresponding *Silene* plastocyanin sequence. Alternatively, DHFR may be more easily transported across the thylakoid membrane than ferredoxin or yeast superoxide dismutase. Finally, the residues of mature 33K protein may have enhanced the thylakoid lumen-targeting properties of the pre-sequence used in this study.

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