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Targeting of ricin A chain into pea chloroplasts

Lynne M. Roberts, Julie W. Meadows and Colin Robinson

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

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A chimaeric gene was constructed encoding the pre-sequence of the 33 kDa oxygen-evolving complex protein from wheat (a thylakoid lumen protein) linked to ricin A chain. The fusion protein is efficiently imported by isolated pea chloroplasts and localised partly in the stroma, with the remainder bound to the stromal surface of the thylakoids. The imported protein is fully processed by both the stromal and thylakoidal processing peptidases, indicating that partial or complete translocation across the thylakoid membrane has taken place.

Protein transport; Retargeting; Ricin; Chloroplast

1. INTRODUCTION

Most chloroplast proteins are synthesised in the cytosol as larger precursors, after which they are transported into the organelle by an ATP-requiring process [1,2]. Imported stromal proteins are synthesised with amino-terminal pre-sequences which are typically 30–60 residues in length; after import, these pre-sequences are removed by a specific stromal processing peptidase [3]. The import of proteins into the thylakoid lumen is more complex, and can be divided into 2 phases. Initially, these proteins (e.g. plastocyanin and the 33 kDa protein (33K) of the oxygen-evolving complex) are synthesised as precursors and imported into the stroma, where they are cleaved to intermediate forms by the stromal processing peptidase. Thereafter, the intermediates are targeted across the thylakoid membrane and processed to the mature size by a thylakoidal peptidase [4,6].

The targeting properties of chloroplast protein pre-sequences have been analysed in studies using chimaeric proteins. The pre-sequences of stromal proteins, such as ribulose bisphosphate carboxylase-oxygenase, have been shown to direct the transport of a wide variety of foreign proteins into the stroma [7–9]. Much less is known of the potential for directing proteins into the thylakoid lumen. The plastocyanin pre-sequence was found to direct ferredoxin and yeast superoxide dismutase into the chloroplast stroma but not as far as the thylakoid lumen [10,11]. In contrast, the 33K pre-sequence has been used to mediate the transport of mouse dihydrofolate reductase and the small subunit of ribulose bisphosphate carboxylase into the lumen [6,12] although a third protein, glycollate oxidase, was targeted only as far as the stroma [6]. In this study we describe the import into pea chloroplasts of a fusion protein consisting of the wheat 33K pre-sequence followed by the toxic A chain polypeptide of ricin. The results indicate a third and unexpected variation amongst examples of retargeting into chloroplasts: the protein is fully processed by the thylakoidal processing peptidase but accumulates in the stroma.

Correspondence address: C. Robinson, Department of Biological Sciences, University of Warwick, Coventry, CV4 7AL, UK. Fax: (44) (203) 523 701

2. MATERIALS AND METHODS

2.1. *Construction and expression of p33-RTA*

The vector p33-RTA, encoding the 33-RA fusion protein, was constructed as follows. The coding sequence for ricin A chain was isolated by digestion of pUC8Neu [13] with *Xho*I and *Hind*III. This fragment was end-filled using T4 DNA polymerase and ligated into p33K-2 [5] which had been digested with *Kpn*I and end-filled to remove the coding sequence for mature 33K from the full precursor. Clones which contained the 33K pre-sequence and A chain sequence in-frame were expressed by in vitro transcription using SP6 RNA polymerase, and capped transcripts were translated in a wheat-germ lysate in the presence of [^{35}S]methionine as described [5].

2.2. *Import and processing studies*

In vitro synthesised 33-RA was imported into isolated intact chloroplasts by the procedure of Hageman et al. [4]. Thylakoidal processing peptidase was isolated as described by Kirwin et al. [14].

3. RESULTS AND DISCUSSION

The fusion protein used in this study consisted of the pre-sequence of the wheat 33 kDa oxygen-evolving complex protein (33K; a thylakoid lumen protein) linked to ricin A chain. The characteristics of this fusion protein, 33-RA, are illustrated in Fig. 1; briefly, 33-RA consists of the 33K pre-sequence plus 22 residues of mature 33K protein preceding 4 residues of a linker peptide followed by full length mature A chain. Complete removal of the 33K pre-sequence during import would therefore leave ricin A chain preceded by 26 resi-

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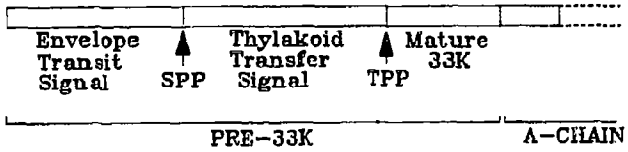

Fig. 1. Structure of the 33-RA fusion protein. 33-RA consists of the 33K pre-sequence (79 residues) together with 22 residues of mature 33K protein, followed by linker peptide (boxed) Glu-Asp-Asn-Asn and mature A chain (open dashed line). The 33K pre-sequence contains 2 targeting signals in tandem: an envelope transit signal, which is removed by a stromal processing peptidase (SPP), followed by a thylakoid transfer signal which is removed by a thylakoidal processing peptidase (TPP).

dues of the mature 33K protein and the linker polypeptide. The wheat 33K pre-sequence has previously been used to target mouse dihydrofolate reductase into the thylakoid lumen [12]. 33-RA was synthesised by in vitro transcription–translation and incubated with isolated intact pea chloroplasts. Fig. 2A shows that the 33-RA translation product (40 kDa) is converted to two polypeptides of mol. wt. 34 kDa and 32 kDa (lane 1) of which the 32 kDa is most abundant. Both polypeptides are resistant to trypsin treatment of the chloroplasts (lane 2) indicating that they represent imported forms. In contrast, residual 33-RA which is bound to the chloroplast surface is completely digested (compare the position of 33-RA in lanes 1 and 2). In order to determine the precise locations of the imported polypeptides, import incubations were carried out and the organelles were then lysed and fractionated into soluble (stromal) and membrane components (lanes 3 and 4). The membrane fraction consists primarily of thylakoids, since the envelope membranes contribute only 1% of total chloroplast membrane. The 32 kDa and 34 kDa polypeptides are both found in each fraction and both polypeptides are digested if the stromal and thylakoid fractions are incubated with proteinase K (lanes 5 and 6). These data indicate that neither of the imported forms are located in the thylakoid lumen. Instead, a proportion of each of the 34 kDa and 32 kDa forms are soluble in the stroma, with the remainder bound to the outer surface of the thylakoids and hence accessible to proteinase K.

As controls for the fractionation procedure and protease treatments, we carried out parallel import incubations using pre-33K. Fig. 2B shows that this precursor is imported, processed to the mature size, and localised almost exclusively in the thylakoid fraction. Mature-size 33K is resistant to proteinase K digestion of the thylakoids, confirming that this protein is located in the lumen and demonstrating that proteinase K, used under the conditions described, does not penetrate the thylakoid membrane.

Authentic, mature ricin A chain migrates in this gel system as a 30 kDa polypeptide [13] which would suggest that the major, 32 kDa imported polypeptide in Fig. 2A has been fully processed by both the stromal

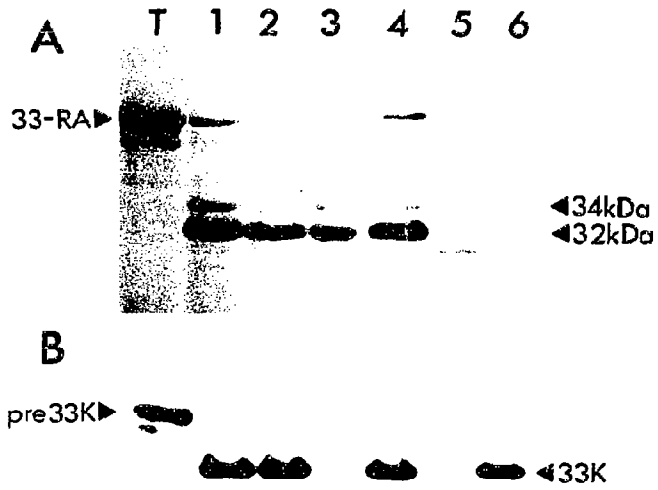

Fig. 2. Import of 33-RA and pre-33K into isolated chloroplasts. (A) The 33-RA translation product (lane T) was incubated with intact isolated chloroplasts for 30 min, after which samples were analysed directly (lane 1) or after trypsin treatment of the chloroplasts (lane 2). Other import mixtures were lysed (without prior trypsin treatment) and centrifuged. Samples of stromal and membrane fractions were then analysed directly (lanes 3 and 4, respectively) and after incubation of each fraction with 50 $\mu g \cdot ml^{-1}$ proteinase K for 20 min on ice (lanes 5 and 6). (B) Import reactions and fractionations were carried out exactly as above but with the pre-33K translation product (lane T). Arrowheads indicate polypeptides of mol. wts. 34 and 32 kDa, imported A chain forms of given M_r 33K and mature-size 33K.

and thylakoidal processing peptidases (leaving A chain plus the 26-residue N-terminal extension). We further investigated this possibility by specifically removing the pre-sequence from 33-RA using partially purified thylakoidal processing peptidase [14] and comparing the gel mobilities of the cleaved product with that of the imported 32 kDa form. Fig. 3A shows that these polypeptides do indeed have identical mobilities, which strongly suggests that the 32 kDa form has been processed during import by the thylakoidal peptidase. These observations, in turn, suggest that the A chain must be translocated, at least partially, across the thylakoid membrane, because the active site of the thylakoidal peptidase is known to be located on the lumenal face of the thylakoid membrane [15]. We tested the effects of inhibiting the thylakoidal protein transport system using the uncoupler nigericin. The transport of 33K across the thylakoid membrane (but not the envelope membranes) requires a trans-thylakoid proton gradient, and thus presence of nigericin leads to an accumulation of the stromal intermediate form during import [16]. Fig. 3B shows that, in the presence of nigericin, 33-RA is efficiently imported into chloroplasts but the formation of the 32 kDa protein is drastically inhibited. There is a concomitant accumulation of the 34 kDa protein such that this form is now by far the more abundant of the two imported forms. These data rule out the possibility

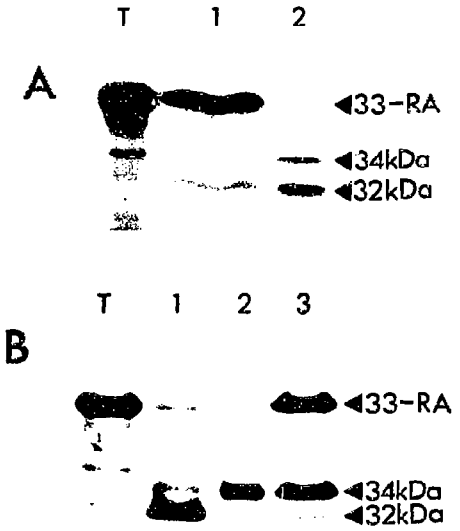

Fig. 3. Imported 33-RA is cleaved by the thylakoidal processing peptidase. (A) 2 μl of 33-RA (lane T) was mixed with 20 μl partially purified thylakoidal processing peptidase and incubated for 60 min at 27°C (lane 1); (lane 2) sample of imported 33-RA prepared as in Fig. 2A, lane 2. (B) 33-RA was incubated with isolated chloroplasts in the absence of nigericin (lane 1) or in the presence of 1 μM nigericin, 15 mM KCl, after which the organelles were lysed and centrifuged to generate stromal (lane 2) and membrane (lane 3) fractions. Arrowheads are as in Fig. 2.

that the 32 kDa polypeptide is generated by a stromal protease, and indicate instead that the A chain must be targeted, at least partially, into the thylakoid lumen, such that the terminal cleavage site becomes accessible to the thylakoidal processing peptidase. The data do not indicate the extent to which the A chain is transported across the thylakoid membrane.

The A chain may be fully translocated into the lumen and subsequently returned to the stromal phase by a mechanism which is as yet obscure. Ricin A chain is known to have membrane interactive properties [17]. During normal cellular intoxications with whole ricin, the A chain is believed to accomplish a reverse membrane translocation step from an oxidising endocytic compartment into the cell cytoplasm [18]. It remains a possibility that the micro-environment within the thylakoid lumen in some way resembles that normally encountered by the A chain during its endocytosis, thereby allowing it to translocate the thylakoid membrane after its complete import and processing. Alternatively, it is possible that 33-RA is transported only partially across the thylakoid membrane, just sufficient for processing the N-terminus and then released back into the stroma after maturation has taken place. The consistent observation that a significant proportion of 33-RA is always associated with the thylakoid membranes in a protease-sensitive fashion, suggests that this alternative remains a strong possibility.

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