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Evidence for a loop mechanism of protein transport by the thylakoid Delta pH pathway

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Abstract The thylakoid Delta pH pathway is a protein transport system with unprecedented characteristics. To investigate its mechanism, the topology of precursor insertion was determined. A fusion protein comprising a large polypeptide domain fused to the amino terminus of pOE17 (a Delta pH pathway precursor) was efficiently processed by thylakoid membranes. The amino terminus, including the targeting peptide, remained on the *cis* side of the membrane. Mature OE17 was transported to the lumen. These experiments demonstrate that Delta pH directed precursors enter the thylakoid membrane in a loop, implying that the Delta pH pathway has evolved from an export-type protein translocation system.

Key words: Chloroplast; Endosymbiotic; SecY; Signal peptide; Translocation; Twin arginine

1. Introduction

Many thylakoid proteins of plant chloroplasts are nuclear encoded, translated in the cytosol as precursors, and localized by a two-step process (see [1] for review). In the first step, precursor proteins are imported across the chloroplast envelope, a process directed by an amino terminal stroma-targeting domain (STD) of the presequence. The STD is removed from the precursor by a stromal processing protease. Proteins destined for the thylakoid lumen carry a second targeting sequence immediately following the STD called the lumen-targeting domain (LTD). All LTDs contain an essential motif that is closely related to endoplasmic reticulum (ER) and bacterial export signal sequences, having a charged N domain, a hydrophobic H domain, and a C domain governing proteolytic processing. Transport into the lumen of thylakoids results in cleavage of the LTD and release of the mature protein. Biochemical and genetic studies show that there are two pathways for protein entry into the lumen, the cpSec pathway and the Delta pH pathway. Precursor proteins transported by the cpSec pathway have similar transport requirements as exported bacterial proteins; ATP, the proton motive force, and a chloroplast homologue of the bacterial SecA protein (cpSecA) [2,3]. A plant thylakoid SecY homologue (cpSecY) is presumed to function in thylakoid transport in cooperation with cpSecA [4]. In contrast, protein substrates of the Delta pH pathway have only a single transport requirement, the trans-thylakoid pH gradient (see [1] for review). This characteristic sets the Delta pH pathway apart from other known translocation systems. The Delta pH mechanism does appear to utilize proteinaceous machinery as thylakoids lose transport competence following treatment with protease [5]. In addition, precursor competition studies demonstrate the existence of a saturable Delta pH component on the thylakoid membrane [6].

Because the Delta pH pathway exhibits such unusual characteristics, we have investigated the topology of its transport mechanism. Several different protein transport systems are recognized in eukaryotic cells, including export-type systems, organellar protein import systems, the peroxisomal protein transport system (see [7] for review), and endocytic-type processes. Export-type systems, i.e. the Sec system and the ER transport system, initiate transport via a transmembrane loop consisting of the signal peptide and carboxyl flanking region [8,9]. In contrast, current models of protein import into mitochondria and chloroplasts depict insertion and transport as proceeding amino terminus first (see [10,11] for review); peroxisomal and endocytic processes are capable of transporting folded proteins and even gold particles [12]. Here we present evidence that during transport on the Delta pH pathway, the amino terminus of the precursor protein remains on the *cis* side of the membrane. This is consistent only with the loop mechanism used by export-type systems. Thus, our data suggest that the Delta pH transport system shares a common evolutionary origin with the export system present in the bacterial cytoplasmic membrane.

2. Materials and methods

2.1. Materials

All reagents, enzymes, and standards were from commercial sources. In vitro transcription plasmids for precursors to pOE23 from pea, pPC from *Arabidopsis*, and pOE17 from maize have been described elsewhere [6]. *Escherichia coli* produced iOE23 has been previously described [6]. pOE23 was produced in *E. coli* [6] and then used for antibody preparation in rabbits. Antibody to pOE17, prepared against the fusion protein pOE17 maltose binding protein, was the generous gift of Dr. Alice Barkan (University of Oregon). Primers used in PCR reactions were manufactured by DNAgency (Malvern, PA).

2.2. Construction of chimeric precursors

Coding sequences for recombinant proteins were constructed by PCR-based methods using the above plasmids as templates. Amplifications were performed with *Pfu* polymerase (Stratagene, La Jolla, CA). Cloned constructs were verified by DNA sequencing. Sequencing was done with ABI Prism Dye Terminator cycle sequencing protocols developed by Applied Biosystems (Perkin-Elmer Corp., Foster City, CA) and an Applied Biosystems model 373 Stretch DNA Sequencer (Perkin-Elmer Corp.).

The chimeric precursor m23p17 is a fusion of coding sequences for amino acids constituting mOE23 (MAYGEAAN..TASSFSVA), a nine amino acid spacer (QKEKNLGAE), and the complete pOE17 sequence. In an effort to eliminate internal initiation in m23p17, the methionines in the presequence of pOE17 were replaced. The natural presequence beginning MAQAMASM.... was changed to LAQA-

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LASL.... Two stages of PCR reactions were used in the construction of the clone for m23p17. In the first stage, two PCR reactions produced fragments coding for mOE23 (containing an *Xba*I site near the 5′ end) and for the nine amino acid spacer plus pOE17 (containing a *Hin*dIII site near the 3′ end). The forward primer for the nine amino acid spacer plus pOE17 contained an overlap corresponding to the last 17 bases of mOE23. The two PCR products were purified and spliced by overlap extension (SOE) in a third PCR reaction [13]. The SOE product was restricted and ligated in the SP6 direction into pGEM 4Z cut with *Xba*I and *Hin*dIII.

The chimeric protein m23p is a fusion of coding sequences for the amino acids constituting mOE23, the same nine amino acid spacer, and the complete presequence of pOE17 (MAQAMASM...ALSQAARA). m23p was amplified in a single round of PCR using as template a version of m23p17 in which the original methionines were unmodified.

2.3. Preparation of radiolabeled precursors by in vitro translation

Capped RNA for the various precursors was produced in vitro with SP6 polymerase essentially as described by Cline, 1988 [14]. Translation in the presence of [^{3}H]leucine was in rabbit reticulocyte (Promega) or a wheat germ system [6], as indicated in the figure legends. Translations were terminated by transfer to ice, dilution three-fold, and adjustment to import buffer (50 mM HEPES (pH 8), 0.33 M sorbitol) and 30 mM unlabeled leucine.

2.4. Preparation of chloroplasts, thylakoids and lysate

Intact chloroplasts were isolated from 9- to 10-day old pea seedlings (Laxton's Progress 9) as described [6] and were resuspended in import buffer. Lysate and washed thylakoids were prepared from isolated chloroplasts [6]. Chlorophyll concentrations were determined according to Arnon [15].

2.5. Chloroplast import and thylakoid protein transport assays

Import of radiolabeled precursors into isolated chloroplasts or transport into washed thylakoids or chloroplast lysate was conducted in microcentrifuge tubes in a 25°C water bath illuminated with 70 μE m^{-2} s^{-1} white light [6] for 10 min or the time indicated in the figure legend. For assays conducted in the presence of inhibitors, chloroplast lysates were preincubated with azide (8 mM final concentration) or nigericin (0.5 μM final concentration) and valinomycin (1 μM final concentration) on ice for 10 min prior to the addition of Mg-ATP (5 mM final concentration) and radiolabeled precursor. For assays conducted in the absence of ATP, lysate (50 μg chlorophyll in 50 μl) and diluted translation product (25 μl) were each preincubated separately for 10 min on ice with 1 U apyrase. Competition assays for thylakoid transport were conducted as described previously [6]. Assays were generally terminated by transfer to 0°C. Where indicated, recovered chloroplasts or thylakoids were protease post-treated with thermolysin. Chloroplasts were repurified on Percoll cushions; thylakoids were recovered by centrifugation.

2.6. Immunoprecipitation

Samples for immunoprecipitation were boiled 1 min in SDS (final concentration 1%) and then diluted 1:11 with 10 mM Tris/HCl (pH 7.5), 140 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 1 mM PMSF. Rabbit antiserum (5 μl) was added, the samples incubated over-night at 4°C, and then rotated slowly for 2 h at room temperature. Protein A-Sepharose slurry (30 μl) in 10 mM HEPES/KOH (pH 8.0), 10 mM $MgCl_2$ was added and rotation continued at room temperature for 1 h. The pellet was recovered and washed four times with 10 mM Tris/HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA and 0.2% Triton X-100 followed by washing twice with 10 mM Tris/HCl (pH 7.5). The pellet was boiled 2 min with SDS sample buffer and the recovered supernatant analyzed by SDS-PAGE/fluorography.

2.7. Analysis of samples

Samples were subjected to SDS-PAGE and visualized by fluorography [16]. Quantification of the radiolabeled protein in a gel band was accomplished by scintillation counting of the excised and extracted gel band [16]. In the time course experiment, the relative molar quantity of m23p17 was calculated as the cpm (minus background) in the m23p17 band divided by the number of leucines in m23p17. The relative molar amount of each product was calculated as the number of cpms (minus background) in each product band divided by the number of leucines expected in the protein at that location. The relative molar percent of each protein is the relative molar amount multiplied by 100 and divided by the relative molar amount of m23p17 at time zero.

3. Results

3.1. A chimeric precursor protein for examination of transport topology was constructed

To investigate the topology of transport on the Delta pH pathway, we constructed a protein, m23p17, consisting of the mature domain of OE23 (mOE23) fused to pOE17 (Fig. 1). If the thylakoid Delta pH translocation machinery transports proteins via a loop structure, the internally located LTD must engage the translocon while both mOE23 and mOE17 are on the *cis* side of the membrane. Subsequently the amino acids carboxy-proximal to the LTD would enter the lumen. Cleavage by the lumenal processing protease would release mOE17 on the *trans* side of the membrane, leaving the LTD and amino acids amino-proximal to it, m23p, on the *cis* side (Fig. 1).

Incubation of m23p17 with isolated thylakoid membranes under transport-permissive conditions produced two polypeptide products (Fig. 2A). At time zero only the translation product, m23p17, and a faint band apparently resulting from internal initiation were visible. By 1 min, polypeptides with the expected molecular weights of m23p and mOE17 began to accumulate. Quantification of radioactivity in the bands demonstrated that the two products accumulated progressively and stoichiometrically throughout the 15-min experiment (Fig. 2B). Furthermore, the relative molar decrease in m23p17 was equal to the relative molar increase in the two products. This processing was very efficient; approximately 70% of the substrate was cleaved to the two products in the 15-min assay.

The identity of the products was confirmed by co-migration with authentic polypeptides and by immunoprecipitation (Fig. 2C). The presumptive m23p migrated identically to an authentic m23p translation product prepared by recombinant methods (lanes 3 and 4) and was immunoprecipitated by antibodies to OE23 (lane 8), but not by preimmune serum (lane 7) or by

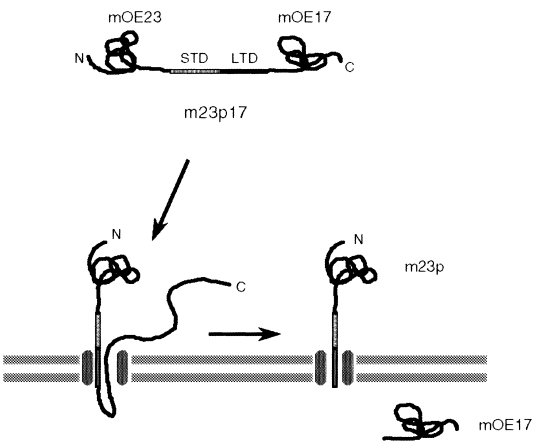

Fig. 1. Model for translocation of a fusion protein, m23p17, by a loop initiation mechanism. The mature form of OE23 was attached amino terminal to the full sequence of pOE17. The model depicts the substrate and products as well as their relative locations if the Delta pH pathway initiates transport with a loop.

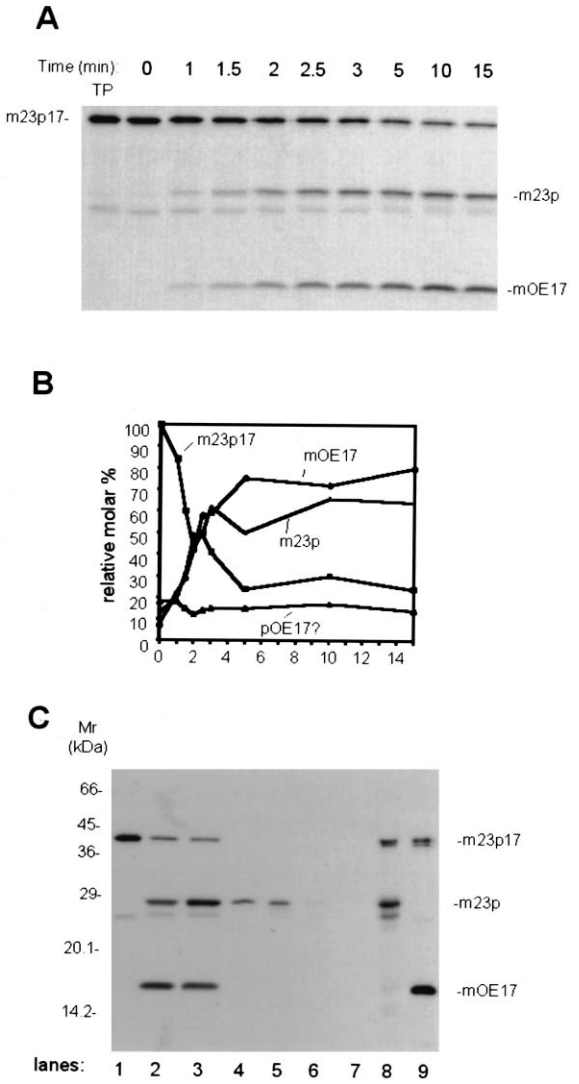

Fig. 2. Temporal appearance and identities of peptides produced during a transport reaction with m23p17. A: Rabbit reticulocyte produced m23p17 was incubated with washed thylakoid membranes in an illuminated bath at 25°C. Samples were taken at the times indicated, boiled in SDS buffer, and analyzed by SDS-PAGE and fluorography. The radiolabeled precursor (TP) represents an amount equivalent to that in each assay sample. B: Quantification of bands shown in A. C: m23p17 and m23p were generated by translation with rabbit reticulocyte and are seen in lanes 1 and 4, respectively. A 10-min transport assay with m23p17 was divided into 5 aliquots. Samples for lanes 2 and 3 were boiled in SDS. The remaining material was processed for immunoprecipitation (see Section 2). Lane 3 contains a mixture of transport products (90% of the amount in lane 2) and translation-generated m23p (equal to the amount in lane 4). Lanes 5 and 6 contain m23p subjected to immunoprecipitation with antibodies to OE23 and OE17, respectively. Lanes 7, 8 and 9 contain transport assay samples subjected to immunoprecipitation with preimmune, anti-OE23, and anti-OE17, respectively.

antibodies to OE17 (lane 9). The mOE17 band migrated identically to authentic mOE17 (see below) and was immunoprecipitated only by antibodies to OE17 (lane 9). The m23p17 present in transport mixtures was immunoprecipitated by both OE23 and OE17 antibodies as expected (lanes 8, 9).

In previous studies with ER membranes, the signal peptide was further cleaved in the hydrophobic core prior to release of the amino terminal portion into the supernatant [17]. No processing of the LTD or degradation of m23p was apparent in our experiments. We estimate that our gel analysis would have detected the loss of as few as six amino acids.

3.2. *m23p is localized to the cis side of the membrane, whereas mOE17 is in the lumen*

To determine the location of the above products, soluble and membrane fractions were obtained from a transport assay. In time course reactions such as the one shown in Fig. 2, all of the mOE17 and most of the m23p were recovered with thylakoids (not shown). Accordingly, thylakoids from a 10-min transport assay were treated with thermolysin to distinguish surface-exposed from lumenal species (Fig. 3). The m23p17 and m23p bands were degraded by protease treatment, indicating that they were exposed to the stromal surface of the membranes, whereas the mOE17 band was protease resistant, indicating a lumenal location (lane 5). When the thylakoid lumen was opened by sonication or 1% Triton X-100, the mOE17 was degraded by protease (data not shown). For comparison, in vitro translated pOE17 (lane 6) was similarly assayed with thylakoids (lanes 9 and 10). Membrane-associated pOE17 and mOE17 displayed the expected protease sensitivity and insensitivity, respectively.

Also shown in Fig. 3, m23p17 was not imported into isolated chloroplasts and even failed to bind significantly to the chloroplast surface (lanes 2 and 3). In chloroplast import experiments with rabbit reticulocyte translated m23p17, a very faint band at the location of mOE17 was produced (data not shown). This apparently resulted from import of a small amount of internally initiated pOE17 that occurs in the reticulocyte system (see Fig. 2). These results can be compared to the robust import achieved with the authentic pOE17 precursor (lanes 7 and 8). The lack of import or binding of m23p17 by chloroplasts is apparently due to their inability to recognize the internally located transit peptide. This agrees with current models of organellar import in which translocation is initiated at the N terminus of the targeting peptide, followed by movement of the precursor linearly through the pore [11].

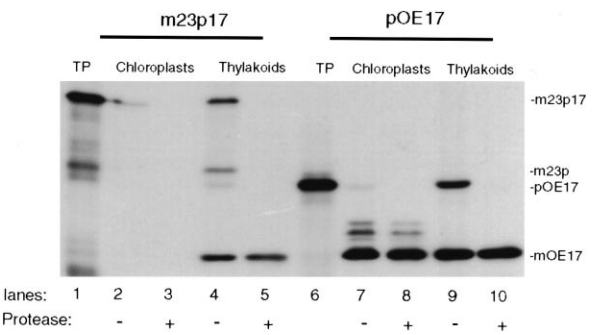

Fig. 3. Transport of m23p17 delivered mOE17 to the lumen, leaving m23p on the stromal face of the thylakoids. Wheat germ-translated m23p17 and pOE17 were assayed for import into isolated chloroplasts and transport into isolated thylakoids at 25°C for 10 min (see Section 2). Recovered chloroplasts or thylakoids were treated with or without thermolysin as shown below the panel. Lanes 1 and 6 represent 1% of the m23p17 and pOE17, respectively, added to each assay. Lanes 2 through 5 and 7 through 10 contain chloroplasts or membranes equivalent to 6% of that present in each assay.

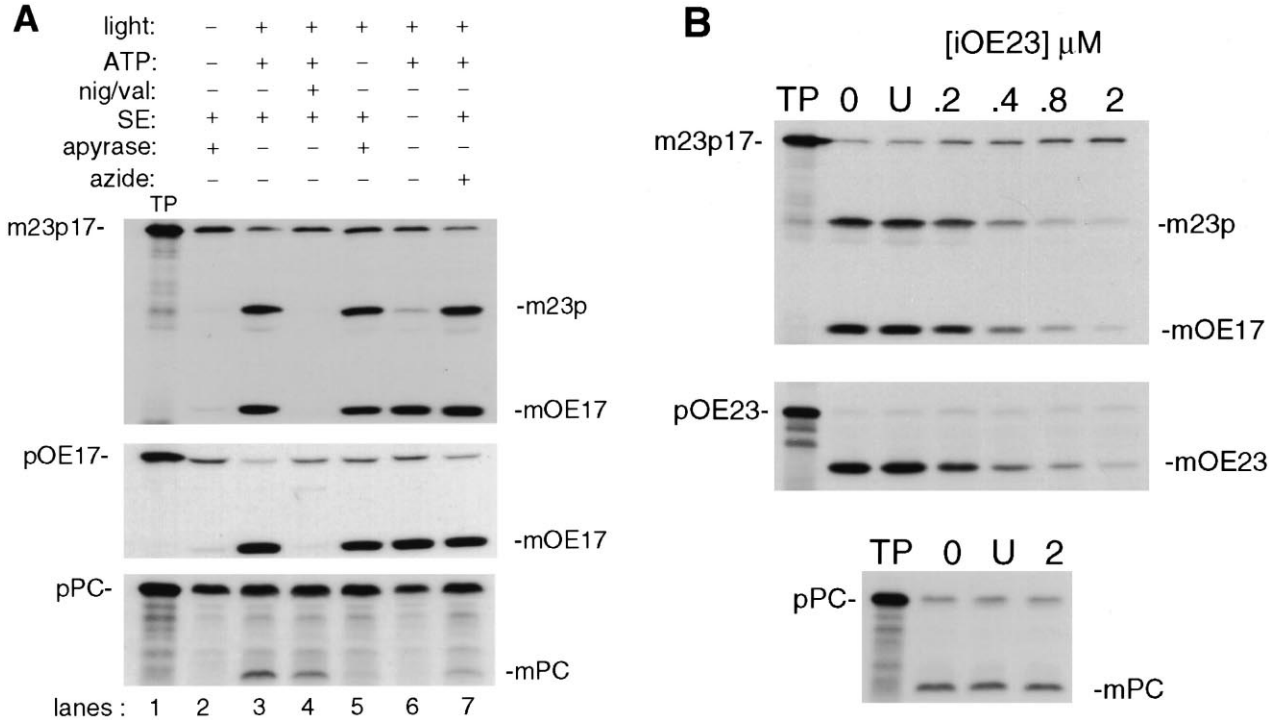

Fig. 4. m23p17 was transported exclusively on the Delta pH pathway. A: Transport of wheat germ produced precursors across thylakoid membranes was conducted for 10 min at 25°C in light or darkness as shown, with lysate to provide stromal extract (SE), or with twice washed thylakoids (see Section 2). Assay conditions were designed to examine the requirement for SE, ATP, a ΔpH, or sensitivity to azide. Apyrase was used to eliminate residual ATP in lysate and translation products. These conditions are designated above the fluorogram panels. Recovered thylakoids were washed and analyzed by SDS-PAGE and fluorography. The radiolabeled precursor (TP) represents 1% of the amount in each assay. Lanes were loaded with recovered thylakoids equivalent to 6.7% of each assay. B: Transport competition assays were conducted with chloroplast lysates and 5 mM ATP in the presence of increasing concentrations of unlabeled iOE23 for 10 min at 25°C in the light. Thylakoid membranes were washed and analyzed by SDS-PAGE and fluorography. Lanes were loaded with TP and sample amounts as in A above. The final concentration of iOE23 competitor is indicated above the fluorograms. The lane designated U represents a control assay containing no iOE23 competitor, but containing urea (U) at 167 mM (equal to the concentration of urea in the 2 μM competition assay).

3.3. *Production of m23p and mOE17 from m23p17 results from Delta pH pathway transport*

Because of the alterations necessary to make m23p17, it was important to determine if m23p and mOE17 were produced as a result of transport via the Delta pH pathway. Assays were conducted under conditions diagnostic for Delta pH-mediated transport (Fig. 4). In Fig. 4A the energy and stromal requirements for production of m23p and mOE17 are compared to those for transport of Delta pH pathway directed pOE17 and cpSec pathway directed pPC. As with pOE17 transport, production of m23p and mOE17 was abolished by ionophores that dissipate the trans-thylakoid ΔpH (lane 4) and was unaffected by removal of ATP (lane 5), by the absence of stromal extract (lane 6), or by sodium azide (lane 7), a SecA inhibitor [18]. In contrast, cpSec-mediated pPC transport was only slightly diminished by ionophores, but abolished by removal of ATP or stromal extract (the source of approximately 90% of cpSecA) and diminished by addition of sodium azide.

In the experiment shown in Fig. 4A, only the recovered membranes were analyzed. An unexpected result was that removal of stromal components and thorough washing of thylakoids used for the assay, while without effect on the amount of mOE17 produced from m23p17 (top panel, lane 6), resulted in much less membrane-associated m23p. In other experiments (not shown), we verified that m23p was indeed produced under these conditions.

As an additional test of pathway utilization by m23p17, precursor competition studies were conducted. Precursor proteins utilizing the Delta pH pathway are able to compete with one another for transport, but not with cpSecA utilizing precursors. As shown in Fig. 4B, the Delta pH pathway intermediate iOE23 competed for transport of pOE23 and m23p17, but not pPC transport. Taken together, these results demonstrate that transport of the mOE17 moiety of m23p17 occurs via the Delta pH pathway. Moreover, they substantiate that production of the *cis*-localized m23p is a transport-coupled phenomenon.

4. Discussion

A hallmark of a loop mechanism of insertion is that the amino-proximal peptide flanking the signal sequence remains on the *cis* side of the membrane, while the carboxyl flanking polypeptide is transported across the membrane. The loop mechanism was demonstrated for the ER by expression in HeLa cells of a vesicular stomatitis virus glycoprotein (VSV G) with a non-cleavable amino terminal extension [8]. The VSV G domain was translocated to the ER lumen, whereas the amino terminal extension remained exposed to the cytoplasmic side of the membrane. The existence of an inserted loop in the ER was further demonstrated by Mothes et al. [19] who used photo-reactive crosslinkers to show that the polypeptide chain spanned the ER membrane twice prior to, but

only once following signal sequence cleavage. Kuhn et al. [9] demonstrated a loop mechanism for protein translocation by the Sec pathway of *E. coli* with a similar strategy to that employed in our studies; i.e. a chimeric precursor protein having a large amino terminal extension derived from ribulokinase was fused to proOmpA.

Similar to the results obtained with the ER system and the *E. coli* system, the amino terminal domain of m23p17 did not interfere with signal peptide recognition or with translocation by the Delta pH mechanism of thylakoids. In addition, the m23p domain remained outside of the thylakoids, whereas the OE17 domain was translocated into the lumen (see Fig. 1 for model). In studies with ER membranes, the amino terminus remained transiently associated with the membranes prior to release into the supernatant [17]. We also observed binding of transport-generated m23p to membranes that varied with the specific conditions of the assay (Fig. 4A). Membrane-bound m23p was tightly associated, as much of it remained bound even after washing with high salt or urea. However the physiological relevance of such binding is unclear because m23p produced directly by in vitro translation also bound tightly to the membrane and was resistant to the same salt and urea extractions (Fincher and Cline, unpublished results). Thus, although it is possible that transport produced m23p remains transiently associated with the translocon as depicted in Fig. 1, we cannot currently distinguish this from non-specific association with the membrane surface.

The known export systems employ homologous components for translocation, i.e. Sec61α is closely related to bacterial SecY and Sec61γ to SecE. In addition, the thylakoidal cpSecA-dependent pathway appears to employ a homologue of the bacterial SecY protein. Thus, given the facts that the Delta pH system translocates proteins via a loop and that the presequence of Delta pH precursor proteins so closely resembles signal peptides of bacterial and ER export systems, it is likely that the Delta pH translocon is related to SecY/E and Sec61α/γ. One possibility is that both the thylakoid cpSec and the Delta pH pathways employ a SecY-based translocon, but rely on different accessory proteins. This appears to be the case for the co-translational and post-translational systems of the yeast ER protein translocation apparatus [7]. One candidate for such an accessory protein is the recently described Hcf106 protein, which is essential for transport on the Delta pH pathway [20]. Future studies will address this possibility.

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