Analysis of the dynamic property of Tat translocase and the fate of Tat signal peptides

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Herrn **Enguo Fan**

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Gutachter /in
1. Prof. Dr. Andreas Kuhn
2. Prof. Dr. Klaus Humbeck
3. Prof. Dr. Ralf Bernd Klösgen

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List of abbreviations

Alb3	Albino 3
amp	Ampicillin
APS	Ammonium peroxodisulphate
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
Bis-acrylamide	N′N′-methylene-bisacrylamide
BN-PAGE	Blue-native polyacrylamide gel electrophoresis
BSA	Bovine serum albumin
CAP	m7G(5′)ppp(5′)G
cDNA	copy (or complementary) DNA
CFoII	Chloroplast Fo ATP synthase subunit II
C-terminal	Carboxyl-terminal
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
DTT	1,4-Dithiothreitol
ECL	Enhanced chemiluminescence
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetra-acetic acid
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
Ffh	Fifty-four homologue
FtsY	Filamentous temperature sensitive mutant Y
g	Gram
g	Gravity
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HM	Hepes/magnesium buffer
Hsp	Heat shock protein
IgG	Immunoglobulin G
IPTG	Isopropyl-beta-D-thiogalactopyranoside

IVT	*in vitro* translation
kDa	Kilo-Dalton
l	Liter
Leu	Leucine
LHC	Light harvesting complex
LHCP	Light harvesting chlorophyII a/b binding protein
M	Molar
Met	Methionine
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimole per litre
mRNA	Messenger RNA
μg	Microgram
μl	Microlitre
nm	Nanometer
NMR	Nuclear magnetic resonance
N-terminal	Amino-terminal
NTP	Nucleoside triphosphate
OD	Optical density
OEC16	16 kDa oxygen evolving complex protein
OEC23	23 kDa oxygen evolving complex protein
OEC33	33 kDa oxygen evolving complex protein
Oxa-1	Cytochrome oxidase assembly 1
PAA	Polyacrylamide
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PC	Plastocyanin
PCR	Polymerase chain reaction
PE	Phycoerythrin
Pftf	plastid fusion/protein translocation factor
PMSF	Phenylmethylsulfonyl fluoride
PS I	Photosystem I
PS II	photosystem II
PsbW	Photosystem II subunit W
PsbX	Photosystem II subunit X
PsbY	Photosystem II subunit Y
REMPs	Redox enzyme maturation proteins
Rieske	Rieske iron-sulfur protein of the cytochrome complex

RIP	Regulated intramembrane proteolysis
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rounds per minute
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SDS	Sodium dodecyl sulphate
Sec	Secretory
SPP	Stromal processing peptidase
SRP	Signal recognition particle
STD	Stroma targeting domain
Tat	Twin arginine translocation
TEMED	N,N,N',N'-tetramethylethylenediamine
Tic	Translocon at the inner chloroplast envelope membrane
TMAO	Trimethylamine N-oxide
Toc	Translocon at the outer chloroplast envelope membrane
TPP	Thylakoidal processing peptidase
Tris	Tris(hydroxymethyl)methylamine
Tween20	Polyoxyethylenesorbitan monolaurate
v/v	Volume/volume
w/v	Weight/volume
°C	Degree Celsius
ΔpH	Proton gradient
$\Delta\psi$	membrane potential

Summary

Translocation of folded proteins across the thylakoid membrane of chloroplasts and the plasma membrane of bacteria distinguishes the Tat pathway from the other protein transport pathways. The work presented in this thesis characterizes the Tat pathway in the following aspects

(1) Evolutionary conservation of the targeting information of the Tat protein transport pathway.
In contrast to plant plastids derived from endosymbiosis of a cyanobacterium, cryptophytes acquire their plastids by engulfing and stably integrating a red algal cell, leading to a eukaryote-eukaryote chimera. The light-harvesting apparatus in cryptophytes is differentially arranged in comparison with that found in the thylakoids of cyanobacteria and red algae. In cryptophytes, the photosynthetic pigments like phycobilin and the relative phycobiliproteins are located on the lumenal rather than the stromal side of the thylakoid membrane. However, how and by which mechanism these phycobiliproteins like phycoerythrin (PE) are sorted is not known.

The transport properties as well as the organelle localization of one such PE protein, PEα, was analyzed in this work. The results show that the PEα subunit is transported into the thylakoid lumen and that the Tat translocase mediates this transport. This analysis, from the evolutionary point of view, strongly suggests that a protein transport pathway corresponding to the Tat pathway of higher plant chloroplasts exists also in cryptophyte plastids and that their targeting information is evolutionary conversed.

(2) Mechanism analysis of the Tat transport process.
Many models have speculated that the Tat translocase is a dynamic and transient translocon as it is formed only in the presence of a Tat transport substrate and the proton gradient across the membrane. To provide experimental evidence for the dynamic properties of the Tat translocon and thus to understand the Tat transport mechanism, a "train-like"protein (16/23-EGFP), in which EGFP (enhanced green fluorescent protein) was attached to the C-terminus of the 16/23 chimeric protein by use of a small peptide linker, has been constructed and analyzed in this work. The results show that the thylakoid transport of this chimeric protein was significantly retarded at indivi-

dual steps giving rise to three transport intermediates. Time course, competition as well as immunoprecipitation experiments were carried out to further characterize these transport intermediates. The results indicate that a single Tat-targeting signal peptide allows the transport of two different mature proteins. Furthermore, the 16/23-EGFP chimera is probably transported in a step-by-step manner. This supports the idea that the Tat translocase could dynamically adapt to different sizes and shapes of the cargo substrates in the course of the transport process.

(3) Analysis of the fate of Tat signal peptides after release by the signal peptidase.

Tat signal peptides play a key role in mediating the Tat transport. After translocation, the signal peptide is cleaved off from the precursor by the signal peptidase. However, what happens to these small signal peptides after signal peptidase cleavage is totally unknown so far.

To analyze the fate of Tat signal peptides, a "tandem-substrate‘" which is composed of two precursors fused in series as well as derivatives thereof have been constructed. The results show that Tat signal peptides are cleaved into subfragments after Tat-transport and processing by signal peptidase. Both events are necessary for the subsequent signal peptide cleavage. Different types of protease inhibitors have been tested for elucidation of the protease involved. It turned out that probably a metalloprotease catalyzes this cleavage. Additionally, the distance between the cleavage site and the C-terminal end of the signal peptide as well as the properties of the signal peptide, like the folding state, have an effect on the cleavage event. These data provide the first analysis of the fate of Tat signal peptides.

1 Introduction

Chloroplasts are organelles found in plant cells and eukaryotic algae that conduct photosynthesis. It has been estimated that about 3,500 proteins are required to build up a functional chloroplast (The Arabidopsis Genome Initiative, 2000; Emanuelsson et al., 2000). Among these 3,500 proteins, only about 100 proteins are encoded by the plastid genome while all the others are encoded by nuclear DNA and synthesized in the cytosol. Thus, to perform their function, all these nuclear-encoded proteins must be transported from outside into the chloroplast (Keegstra and Cline, 1999; Jarvis and Robinson, 2004).

However, transport of these proteins is complicated due to the existence of biological membranes which compartmentalize the chloroplast and maintain the characteristic differences between the contents of the chloroplast and the cytosol. Thus, for transporting of these nuclear-encoded proteins, elaborate protein transport systems have been developed in the membranes of chloroplast.

1.1 The structure of chloroplasts

The chloroplast of higher plants is made up of three types of membranes (Figure 1.1):

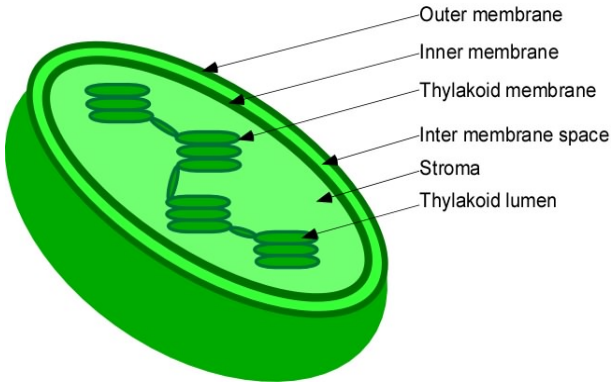

Fig 1.1: The structure of chloroplast.

(1) Outer membrane which is freely permeable to *small* molecules.
(2) Inner membrane which contains many transporters and is highly specialized with transport proteins.

(3) Thylakoid membranes which form a network of flattened discs called thylakoids. In the thylakoid membranes, the proteins responsible for photosynthesis and electron transport are embedded forming at least five multisubunit oligomeric complexes for photosynthesis, including the photosystems I and II and their light harvesting antenna (LHC, light harvesting complex), the cytochrome complex and the ATP synthase (Andersson and Barber, 1994; Herrmann, 1996). Some of these complexes work together to carry out the so-called "light-reactions" of photosynthesis.

Accordingly, separated by these three membranes, the chloroplast is divided into three distinct internal compartments:
(1) The intermembrane space between the two membranes of the chloroplast envelope;
(2) The stroma which lies inside the envelope but outside the thylakoid membrane. The stroma contains for example: (a) the enzymes, like RuBisCO, required to carry out the "dark-reactions" of photosynthesis; that is, the conversion of CO_2 into organic molecules like glucose; (b) a number of DNA molecules, each of which carries the complete chloroplast genome that encode around 100 proteins.
(3) The thylakoid lumen which contains many proteins that are important for photosynthesis processes like water splitting, electron transport etc.

1.2 Protein transport in chloroplasts

To allow protein passage through these three different membranes, chloroplast has developed different molecular machines in each membrane (Figure 1.2): for the outer and inner envelope membranes, the translocons referred to as Toc (Translocon at the outer envelope membrane of chloroplasts) and Tic (Translocon at the inner envelope membrane of chloroplasts), respectively. However, for transport into or across the thylakoid membrane, at least four transport mechanisms, called SRP (Signal Recognition Particle), Spontaneous, Sec (Secretory) and Tat (Twin arginine translocation)-dependent pathway, have been identified (Keegstra and Cline, 1999; Jarvis and Robinson, 2004; Gutensohn et al., 2006).

1.2.1 Passing through the envelope membrane (Toc and Tic)

The Toc translocon is composed of the receptor components, including Toc159 and Toc34, as well as Toc64 (Kessler et al., 2004; Qbadoua et al., 2007) for precursor recognition, and the translocation channel component (Toc75) (Schnell et al., 1994). Another component of the Toc complex is Toc12, which recruits the Hsp70 (Heat shock protein 70) of outer envelope membrane to the intermembrane space and facilitates the interaction of Hsp70 with the precursors (Becker et al., 2004). One recent model

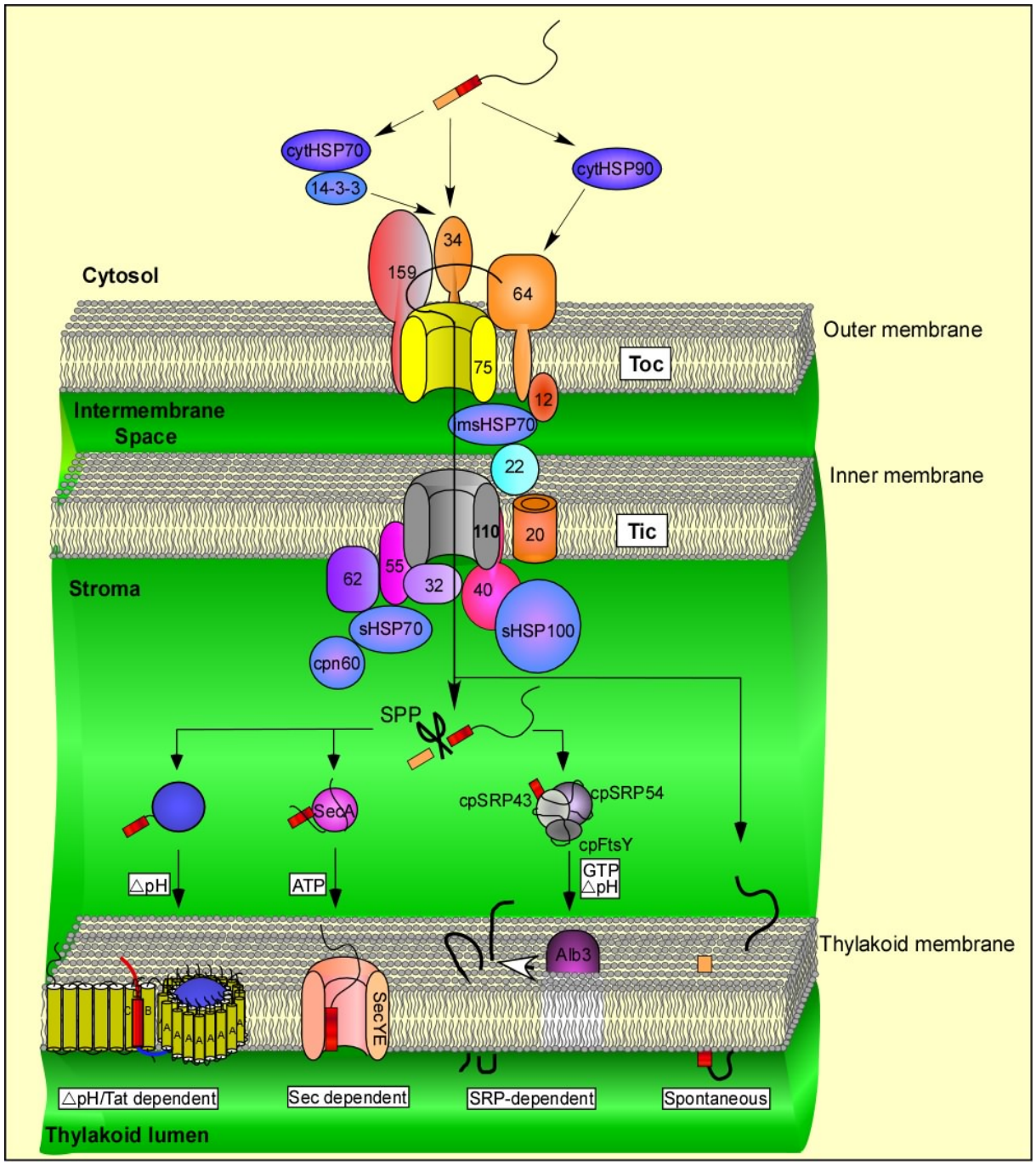

Fig 1.2: **Overview of the protein transport pathways in chloroplast.** The components of the Toc and Tic complexes are designated according to their molecular weight. For each of the four protein transport pathways operating at the thylakoid membrane, the stromal and thylakoidal factors involved are shown. The signal peptide of the stroma targeting domain (STD) is depicted as orange rectangle, thylakoid lumen targeting domain (LTD) as red rectangle. Likewise, the stromal processing peptidase (SPP) is shown as black scissors but thylakoid processing peptidase is not shown. For each pathway, the respective energy sources or driving forces are indicated. All further details are explained in the text. (14-3-3: 14-3-3 protein, cpFtsY: chloroplast SRP receptor FtsY, cpn60: chaperonin 60, cpSRP: chloroplast signal recognition particle, cyt-/ims-/s-HSP70: cytosolic-/intermembrane space-/stromal-heatshock protein 70 kDa, SPP: stromal processing peptidase.).

suggests that Toc translocon is formed by a single central Toc159 molecule that is surrounded by four copies of Toc75/Toc34 (Stengel et al., 2007). During the transport process, one set of precursors is directly recognized by Toc34 receptor, while another set of the precursors will be handled through either a cytosolic guidance complex with a 14-3-3 protein as the central component or cytosolic Hsp90 (May and Soll, 2000; Qbadou et al., 2006) and is then recognized by Toc34.

Tic translocon is probably composed of at least Tic110, Tic62, Tic55, Tic40, Tic32, Tic22, and Tic20 (Fig. 1.2; Soll and Schleiff, 2004; Gutensohn et al., 2006). Among them, the presumed basic components of Tic machinery include: (1) Tic22, a soluble protein that is peripherally associated with the inner envelope membrane from the inter membrane space and is assumed to be the first Tic component interacting with the incoming precursor protein (Kouranov and Schnell, 1997; Kouranov et al., 1998); (2) two integral membrane proteins, Tic20 and Tic110, both are assumed to be the translocation pore components (Kessler and Blobel, 1996; Kouranov et al., 1998; Heins et al., 2002); (3) several molecular chaperones including Hsp100 and Chaperonin-60 (Cpn60), which have been reported to interact from the stromal side with the Tic complex (Kessler and Blobel, 1996; Akita et al., 1997; Nielsen et al., 1997). At present, more works are required for understanding the Tic complex and its transport mechanism.

1.2.2 Passing through the thylakoid membrane

After passing through the Toc and Tic complexes, the proteins arrive in the stroma (Fig 1.1 and 1.2), where the N-terminal transit peptide, the so-called stroma-targeting-domain (STD), is proteolytically removed by a large monomeric enzyme called stromal processing peptidase (SPP) (Van der Vere et al., 1995). Depending on their final destination, further sorting of these proteins into or across the thylakoid membrane is handled by at least four protein transport pathways: the SRP-dependent and 'spontaneous' pathways mainly for insertion of proteins into the thylakoid membrane, and the Sec-dependent and ΔpH/Tat-dependent pathways mainly for transport of proteins into the thylakoid lumen (Fig 1.2 and Jarvis and Robinson, 2004; Gutensohn et al., 2006).

The SRP dependent pathway

The well known substrate of this pathway (Fig 1.2) is light-harvesting chlorophyII a/b-binding protein (LHCP). The analysis of this protein has shown that, after the signal peptide being removed by SPP inside the stroma, the substrate will be recognized and bound by a soluble 54 kDa GTPase (cpSRP54) and a plant specific stromal 43-kDa protein (cpSRP43). Together, they form a "transit complex"(Schünemann, 2004). The transit complex interacts further with cpFtsY – a protein that possibly functions as

a receptor (Kogata et al., 1999). Then the complex targets to a not yet identified translocase for membrane integration. Alb3, one of such an integral membrane protein, has been shown to be involved in this integration process (Sundberg et al., 1997; Moore et al., 2000). However, it is still not known, how many other membrane components are involved in LHCP integration and how the LHCP inserts into the thylakoid membrane. GTP hydrolysis provides the power for LHCP integration and ATP stimulates this process if it is present in combination with GTP (Hoffman and Franklin 1994; Yuan et al., 2002). Possibly, GTP is required to regulate the interaction of the GTPases cpSRP54 and cpFtsY during delivery of LHCP to the translocon (Schünemann, 2004). However, the role of ATP in LHCP insertion is completely unresolved since no ATP-binding proteins involved have yet been identified (Schünemann, 2004). Additionally, plastome encoded thylakoid membrane proteins, like D2, CP43, PSI-A and CFoIII, possibly also use SRP-dependent pathway to integrate into the thylakoid membrane but in a co-translational manner (Pasch et al., 2005).

The spontaneous pathway

Another set of thylakoid integral membrane proteins seem to require neither any known protein transport machinery or essential targeting factors nor energy for insertion into the thylakoid membrane. This feature leads to the designation of "spontaneous insertion mechanism" which constitutes a mainstream pathway for bitopic membrane proteins (Schleiff and Klösgen, 2001). Example substrates of this pathway are CFoII, the photosystem II subunits PsbW, PsbX, and PsbY as well as PsaK from Photosystem I and the SecE subunit (cpSecE) (Michl et al., 1994; Lorkovic et al., 1995; Kim et al., 1998; Thompson et al., 1998). The key feature requirements for the substrates of this pathway are: (i) they must have two hydrophobic domains provided one by the membrane anchor of the mature protein and the other by the signal peptide; (ii) the hydrophilic domain between the two hydrophobic domains is negatively charged while the extreme termini of the two hydrophobic domains, i.e. the N-terminus of the first hydrophobic domain and the C-terminus of the second hydrophobic domain, must be positively charged (Michl et al., 1994, 1999).

The Sec pathway

Beside the SRP dependent and the spontaneous pathway, which integrate most of the thylakoid membrane proteins, some other membrane proteins, like PSI-F, Cytochrome f, plastid fusion/protein translocation factor (Pftf), and Rieske protein, can also be integrated into the thylakoid membrane but by the other two transport pathways: Sec- (Cytochrome f, PSI-F) and Tat-dependent pathway (Pftf, Rieske) (Karnauchov et al., 1994; Nohara et al., 1996; Summer et al., 2000; Molik et al., 2001). However, these

latter two pathways are mainly responsible for the transport of proteins that function inside the thylakoid lumen. These two pathways have been characterized based on their transport requirements.

One subgroup of precursors is absolutely dependent on ATP as well as stromal extract and is stimulated by the thylakoidal ΔpH. This is the Sec pathway, which resembles the well-characterized Sec systems in bacterial inner membranes.

***The Sec signal peptides*:** The Sec signal peptides comprise three domains: a short, positively charged amino-terminal domain (N-domian); a central hydrophobic domain (H-domain); and a more polar carboxy-terminal domain (C-domain) containing the signal peptidase cleavage site (von Heijne, 1998). The length of N-domain is variable for different precursors while the H-domain is ∼15 residues long, on average. The end of the N-domain (to the H-domain terminus) is often occupied by charged residues. In the C-domain, basic residues are always lacking in contrast to the Tat signal peptides which frequently contain basic residues (Mori and Cline, 2001).

***The Sec translocon*:** In comparison with the Sec system in bacteria, thylakoid Sec components, SecA, SecY and SecE (Fig. 1.2) have been cloned (Berghöfer et al., 1995; Berghöfer and Klösgen, 1996) and shown to be involved in thylakoid protein transport process using *in vitro* assays. Presumably, SecY and SecE form the translocation pore in the thylakoid membrane (Mori and Cline, 2001; Jarvis and Robinson, 2004; Gutensohn et al., 2006). In bacteria, additional components like SecB, SecG, SecD, SecF or YajC, are also involved in the Sec transport. However, no chloroplastic homologous subunits have been identified in the *Arabidopsis* genome. Thus, it remains to be seen whether additional components are involved and how the thylakoidal Sec translocon is organized.

***The chaperones involved in Sec transport*:** Like its bacterial counterpart, proteins transported by thylakoid Sec pathway are also in an unfolded state which has been demonstrated experimentally using dihydrofolate reductase (DHFR) as a transport substrate (Endo et al., 1994; Hynds et al., 1998). In bacteria, this unfolded state is maintained by the action of chaperones like SecB (Manting and Driessen, 2000). Several chaperones like Hsp70, Hsp60, Rubisco activase were found in the stroma of chloroplasts (Jackson-Constan et al., 2001). However, no such chaperones affecting the Sec pathway have been identified.

SecA functions as an ATPase that powers the translocation of the polypeptides across the Sec translocase (Lill et al., 1989). Chloroplast SecA (cpSecA) has a dual localization in both the stromal and thylakoid fractions (Nakai et al., 1994; Yuan et al., 1994). The

presence of galactolipid and only a small fraction of anionic lipid optimally stimulate the SecA activity (Sun et al., 2007). Furthermore, SecA activity could only be stimulated by thylakoidal Sec-dependent signal peptides but not *E.coli* Sec signal peptides indicating that cpSecA probably has been evolved to be specifically well suited for the environment of the chloroplast thylakoid and to recognize thylakoidal Sec-dependent proteins thus ensures the pathway specificity (Sun et al., 2007). In this regard, chloroplast SecA could be considered as a chaperone.

***The energetics of Sec transport*:** When stroma extract was preincubated with anti-cpSecA antibodies prior to *in vitro* import assays, the transport of Sec precursors like OEC33 kDa protein and Plastocyanin (PC) into thylakoids was completely blocked (Nakai et al., 1994). Chloroplast SecA can be azide insensitive (spinach) or azide sensitive (pea) (Berghöfer et al, 1995). Furthermore, depletion of ATP by apyrase (Hulford et al., 1994) or by use of the ATP-analog AMP-PNP all result in a Sec-transport abolishment (Berghöfer, 1998) suggesting that ATP hydrolysis is absolutely required for thylakoid Sec-dependent translocation. A trans-membrane potential is not essential for Sec-dependent transport, however, translocation of some precursor proteins could be stimulated by the presence of ΔpH (Yuan and Cline, 1994; Mant et al., 1995).

Even though only limited number of experiments have fosused on this aspect, it seems that thylakoidal Sec transport and its bacterial counterpart are highly similar in mechanism. For example, spinach plastocyanin can be transported by the Sec pathway of bacteria (Haehnel et al., 1994). Briefly, when thylakoidal Sec precursors arrive in the stroma of chloroplast, cpSecA binds (Sun et al., 2007) and directs these precursors to the thylakoid membrane (Keegstra and Cline, 1999). Then, they form a stable complex within the membrane that also contains chloroplast SecY (Mori and Cline, 2001). Functioning as a translocation motor, cpSecA partially inserts into the lipid bilayer and pushes the precursors through the Sec translocon (Fig 1.2).

The ΔpH/Tat pathway

In sharp contrast to Sec pathway, Tat pathway is very unique in several features (Mori and Cline, 2001; Robinson and Bolhuis, 2004; Müller and Klösgen, 2005; Gutensohn et al., 2006). First, *in vitro* thylakoid experiments have shown that the transport solely and strictly depends on the transmembrane proton gradient while no requirements of soluble factors or nucleoside triphosphates were found to be involved (Mould and Robinson, 1991; Cline et al., 1992; Klösgen et al., 1992). Second, a twin pair of arginine residues is located at the boundary of N- and H-domain of the Tat signal peptides which give rise to the name of Tat (Twin-arginine translocation) (Chaddock et al., 1995). Third, probably the most remarkable feature is, however, that this pathway is

able to transport folded polypeptide chains across the membranes. This pathway was evolutionary conserved both in thylakoid membranes of chloroplast and in bacteria and thus the known features of this pathway will be summarized together here.

***Tat signal peptides*:** Beside the common principles of signal peptides like tripartite structure (Schatz and Dobberstein, 1996; von Heijne, 1998), Tat signal peptides have several specific features particularly when compared to the Sec signal peptides:
(i) In the N-domain, the most notable one is the presence of a characteristic -RR- motif just prior to (i.e. on the N-terminal side of) the H-domain. Even at this point two highly atypical Tat substrates (Pftf and the Rieske FeS protein) could be considered as exceptions (Summer et al., 2000; Molik et al., 2001). Mutagenesis studies have shown that replacement of both arginine residues, even by lysine, lead to a complete block in Tat dependent translocation while the conservative substitution of a single Arg by Lys usually affects the translocation rate only (Chaddock et al., 1995; Stanley et al., 2000; DeLisa et al., 2002; Ize et al., 2002). The RR-motif is, however, not diagnostic for Tat-specific export. In *Bacillus subtilis*, only two of a large number of RR containing signal peptides have so far been proven to direct their passenger proteins to a Tat translocase (Jongbloed et al., 2000; van Dijl et al., 2002). Thus an RR-consensus motif even if predicted by improved algorithms (Dilks et al., 2003) is not compelling for a Tat-dependent export.
(ii) The H-domain of Tat signal peptides is less hydrophobic and relatively long when compared to that of Sec signal peptides. These features have been considered to be one of the so-called "Sec-avoidance" determinants (Cristobal et al., 1999). Further studies have also shown that, beside the important -RR- motif, the presence of a highly hydrophobic residue at the + 2 or + 3 positions, relative to the second arginine residue, was almost equally important for Tat transport (Brink et al., 1998).
(iii) The C-domain of Tat signal peptides is characterised by a high proportion of basic amino acids and is often positively charged when compared to the Sec signal peptides. This feature has been suggested as another determinant of the "Sec-avoidence" (Bogsch et al., 1997; Ize et al., 2002; Blaudeck et al., 2003). However, since only a subset of Tat substrates possesses this "Sec-avoidance" signature, other potential determinants for escaping the Sec pathway have to be identified or characterized (Müller and Klösgen, 2005). Additionally, it has been proven that the polarity or the charge of this domain has an effect on the signal peptidase cleavage (Frielingsdorf and Klösgen, 2007).

The 3D structure of Tat signal peptides is not very well characterized to date, except for two examples from bacteria. By use of Nuclear Magnetic Resonance (NMR) and H/D exchange mass spectrometry, it has been shown that the N- and H-regions of the signal sequence, including the twin-arginine motif, form an unfolded conformation regardless

of a completely and correctly folded mature domain following it (Kipping et al., 2003). In another study, analysis of SufI signal peptide by Circular Dichroism (CD) which is the simplest indication of protein and peptide secondary structure, it was concluded that this Tat signal peptide has two distinct states depending on the surrounding environment: In aqueous solution, the signal is unstructured while in membrane-mimetic environments such as SDS micelles or water/trifluoroethanol, the signal peptide contains α-helical structure which located in the center of the peptide, starting either just before or at the twin-arginine motif (Miguel et al., 2003). Further works are required at this point.

***The Tat translocon*:** Owing to the remarkable feature of Tat pathway to transport folded proteins (Clark and Theg, 1997; Hynds et al., 1998; Marques et al., 2003, 2004) and the fact that transported Tat substrates are highly variable in size (from 10-100 kDa), shape and surface features (Berks et al., 2000; Müller and Klösgen, 2005), the Tat translocase must be able to either change its pore size or to form series of different size pores to accommodate its divergent transport substrates, meanwhile avoiding the leakage of ions across the membrane. This special character indicates that possibly the Tat translocase is a dynamic or an active translocation pore. Indeed, recently it has been suggested that a functional Tat translocase is assembled only on demand, i.e. in the presence of transport substrate and a trans-membrane proton gradient (Mori and Cline, 2002).

The current model identifies TatA as the pore-forming component. It has been reported that TatA monomers oligomerize to form a transient pore to translocate the Tat substrates (Mori and Cline, 2002; Alami et al., 2003; Dabney-Smith et al., 2006). After transport, the translocon will be disassembled (Mori and Cline, 2002) for the next round of transport. In line with this, the *E. coli* TatA protein assembles when overexpressed in the membrane as series of oligomers, at least homotrimers or homotetramers (de Leeuw et al., 2002). This oligomerization is a intrinsic property of the transmembrane helix as its removal results in the recovery of TatA monomers (Porcelli et al., 2002). Using single particle electron microscopy, the low-resolution 3D structures show that these TatA oligomers of different sizes are similar in shape, i.e. a ring-shaped structure. Each thick-walled ring has an asymmetric lid at one end. The ring is approximately 50Å deep (enough to span the bilayer) and ∼30Å wide (Gohlke et al., 2005). Most importantly, their internal cavities of these rings increased with the number increase of the TatA monomer. Even though these results possibly only stand for a resting stage of Tat system, they strongly suggest that it is possible to change the pore size by adding or removing the TatA monomers. However, how TatA oligomerize together to form a functional translocase, whether an appropriately sized TatA channel is selected or if

the channel is formed by active recruitment of TatA monomers remains to be an open question (Sargent et al., 2006).

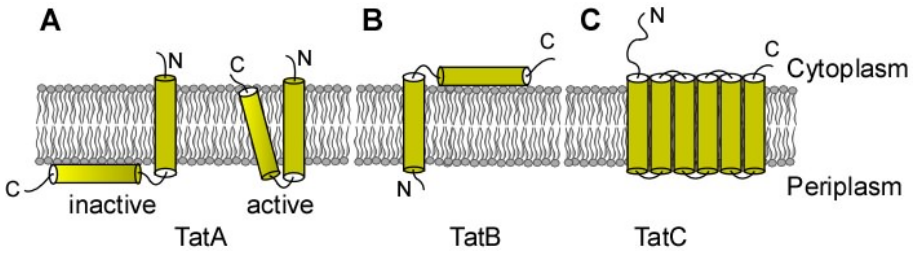

Fig 1.3: **The predicted topology of Tat proteins in *E.coli*. A**, TatA has a dual topology: (a) The inactive state with single transmembrane orientation in which N-terminus located on the cytoplasm side while C-terminus on the periplasm side; (b) The active state with double transmembrane orientation in which both N- and C-terminus located on the cytoplasm side. While it has to be pointed out that this topology is not yet fully settled. **B**, TatB is with its C-terminus on the cytoplasmic side and with its N-terminus on the periplasmic side while both N- and C-terminus of TatC are on the cytoplasmic side **(C)**. In thylakoids, cytoplasm corresponds to the stroma side and periplasm corresponds to the lumenal side. For TatA from plants, no such dual topology shift was predicted or shown so far. According to Berks et al. (2000) and Chan et al. (2007).

TatA is an integral membrane protein anchored in the membrane by one N-terminal transmembrane helix. Secondary structure predictions and circular dichroism spectroscopy suggest that TatA consists of two α-helices at its N-terminus, one hydrophobic and one amphipathic, followed by a larger, unstructured C-terminus (Porcelli et al., 2002). The transmembrane helix has been postulated to play an important role during the oligomerization process. Particularly, a conserved glutamate residue in the transmembrane helix is essential for the activity of TatA as conservative substitutions by aspartate and the structurally conserved glutamine impair Tat transport (Dabney-Smith et al., 2003). Another conserved residue, glycine, that is located in the amphipathic helix and seems to be part of a flexibility-conveying hinge region (Barrett et al., 2003; Hicks et al., 2003), probably plays a role for the interactions of TatA with its neighboring monomers as well (Chan et al., 2007). The N-terminus of TatA has been implied to be located in the periplasm based on predictions using the "positive-inside rule" and protease sensitivity experiments (Porcelli et al., 2002). However, recent experimental data have shown an opposite result, i.e. the N-terminus of TatA located in the cytoplasm in bacteria (Chan et al., 2007). Thus, it is not yet fully settled for the topology of TatA. In contrast, the C-terminus of TatA has a dual topology conformation depending on the presence of an intact membrane potential (Fig 1.3; Gouffi et al., 2004; Chan et al., 2007). This might also be true for plant TatA but needs experimental confirmation.

TatB and TatC are the other two components that have been identified to be required for a functional Tat transport. Like TatA, both proteins are also integral membrane

proteins (Fig 1.3; Settles et al., 1997; Walker et al., 1999; Motohashi et al., 2001). TatB, in several aspects, shares limited, but significant, sequence similarities with TatA (Sargent et al., 2006). For example, their amino acid residues are 25% identical in *E. coli*; both possess a transmembrane α-helix at their extreme N-terminus, followed by an amphipathic α-helix. In the case of TatB, however, the amphipathic helix is longer than that of the TatA protein and is probably not exposed to the trans-membrane side under any circumstances (Bolhuis et al., 2001). Finally, like TatA, the extreme C-terminal region of TatB is predicted to be unstructured and is not essential for a successful Tat translocation (Lee et al., 2002). Despite of these similarities, however, the two proteins have a different topology and fulfil different functions during the transport process (Müller and Klösgen, 2005; Lee et al., 2002).

TatC is the largest and most highly conserved component of the Tat machinery (Sargent et al., 2006). TatC has six transmembrane domains as predicted and experimentally confirmed by analysis of TatC reporter fusions (Gouffi et al., 2002; Behrendt et al., 2004; Ki et al., 2004). TatC protein also contains a number of conserved residues and some of them, when mutated, indeed interfere with the activity of TatC (Allen et al., 2002; Buchanan et al., 2002). However, our current knowledge on TatC structure and function is surprisingly rudimentary (Müller and Klösgen, 2005).

The stoichiometric ratio of TatA:TatB:TatC in the *E. coli* cytoplasmic membrane has been estimated to be approximately 20-30:1:0.4 (Berks et al., 2003). In plants, however, the amounts and ratios of Tat proteins varied depending on the species (like pea or Arabidopsis) and the developing stages of the plant (M. Jacob et al., submitted). On blue-native polyacrylamide gel electrophoresis (BN-PAGE), these components have been found to form series of high molecular weight complexes (Berghöfer and Klögen 1999; Bolhuis et al., 2001; Cline and Mori, 2001; Sargent et al., 2001; de Leeuw et al., 2002; Oates et al., 2003; Oates et al., 2005; Behrendt et al., 2007). TatA and TatB form complexes in a ladder-like pattern after solubilization of the cytoplasmic membrane of bacteria after overexpression. Depending on the number of monomers involved, the complexes ranged from about 100 kDa to over 880 kDa for TatB (Behrendt et al., 2007) and from 100 kDa to over 600 kDa with average differences of 34 kDa between the ladders of TatA complexes (Oates et al., 2005). Further, there are also cross-reactions between these three Tat components. In equimolar quantities, TatB and TatC form a complex with molecular weight of 560 and 620 kDa in thylakoids (Berghöfer and Klögen 1999; Cline and Mori, 2001) and of $\sim$600 kDa in bacteria (Bolhuis et al., 2001). It has been reported that TatC is highly unstable in the absence of TatB (Sargent et al., 1999), and that the TatBC complex is also unstable without TatA (Mangels et al., 2005), suggesting the important relevance of these three components.

***Chaperones involved in Tat transport*:** Tat system can transport folded proteins and some Tat substrates are cofactor containing proteins. However, it is still a mystery how the folding state is sensed and how the cofactor-containing proteins are held in a transport-waiting state before the cofactors become correctly incorporated (Palmer et al., 2005). It is likely that some chaperones or not yet identified accessory proteins contribute to these features (Müller and Klösgen, 2005).

Interestingly, specific proteins that bind to Tat signal sequences (Oresnik et al., 2001; Dubini and Sargent, 2003; Jack et al., 2004) and function as specific molecular chaperones in the targeted insertion of cofactors into Tat substrates have been described in bacteria (Driessen et al., 2001; Jack et al., 2004; Hatzixanthis et al., 2005; Graubner et al., 2007; Maillard et al., 2007; Perez-Rodriguez et al., 2007).

In the thylakoidal Tat system, such chaperones have not yet been identified. Generally, no stroma was added in the *in thylakoido* import assay which is widely used for Tat transport analysis. In other words, without any stroma factors, the Tat substrates could still be efficiently imported into the thylakoid lumen. This strongly suggests that probably chaperones are not involved in the thylakoid Tat transport system. However, the addition of stroma in such import assays sometimes increases the amount of imported substrates (S. Frielingsdorf, personal communication) indicating that probably for thylakoid Tat machinery, some yet-to-be-identified chaperones which probably increase the transport efficiency do exist. Interestingly, the analysis of one Tat substrate, Rieske protein, demonstrated that indeed stromal components, including cpn60 chaperonine, are involved in the targeting process (Molik et al., 2001).

***The driving force of Tat transport*:** In contrast to most of the protein transport powered by NTP hydrolysis (Alder and Theg, 2003b), Tat protein translocation is very unique as this transport solely depends on the transmembrane proton gradient (Cline et al., 1992; Klösgen et al., 1992; Santini et al., 1998; Yahr and Wickner, 2001; Alder and Theg, 2003a) which costs 3% of the total energy output of the chloroplast (Alder and Theg 2003a). This feature gave rise to the initial name of this pathway as ΔpH-dependent pathway. This character is further enhanced by the finding that overproduction of PspA, a protein involved in the maintenance of the H^+-motive force, favours Tat export in *E. coli* cells (DeLisa et al., 2004). It remains unknown, however, how the proton gradient was coupled to the Tat transport process.

However, the requirement of ΔpH in the Tat pathway has been challenged recently by *in vivo* analysis of *Chlamydomonas reinhardtii* (Finazzi et al., 2003) and transfected tobacco protoplasts (DiCola et al., 2005). The reason for the current discrepancy bet-

ween *in vitro* and *in vivo* data is not known, but two possibilities have been suggested (Theg et al., 2005): First, some factor(s) probably missing in the *in vitro* system, which the *in vivo* experiments might contain, alter the energetic requirements of the transport reaction; The second, the transmembrane $\Delta\psi$ and/or ΔpH contribute(s) to power the Tat pathway. It has been shown that the steady-state $\Delta\psi$ substantially decreased in isolated thylakoids, which have been used generally for *in vitro* assay for Tat transport analysis. If $\Delta\psi$ indeed was involved, this decrease could contribute to the discrepancy observed for the differences between *in vitro* and *in vivo* results. Interestingly, it has been reported in *E. coli* recently that ΔpH is not required, instead two kinds of $\Delta\psi$ with short and long duration are required, respectively. The short one is required for an early transport step while the long duration one is necessary to drive a later transport step (Bageshwar and Musser, 2007). In thylakoid system, it has also been reported recently that $\Delta\psi$ can replace ΔpH as a driving force for Tat transport (Braun et al., 2007).

***Current working model of the Tat transport process*:** Combining the results from cross-linking, immunoprecipitation and immunoblotting, as well as the analysis of chimeric proteins, our present understanding of the Tat protein transport process could be summarized as following: After most of the Tat precursors, if not all, first insert

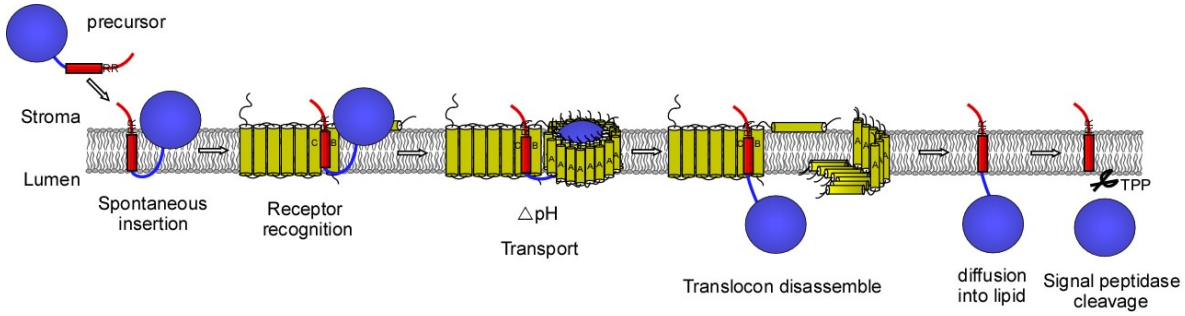

Fig 1.4: **Current working model of Tat protein transport process.** Based on the analysis of 16/23 chimera, Tat transport could be divided into the following steps: (1) Membrane insertion and receptor recognition (Transport intermediate Ti-1); (2) Translocation; (3) Translocon disassemble and precursor diffusion into the lipid (Transport intermediate Ti-2); (4) Maturation (TPP cleavage). For details see text.

into the thylakoid membrane or plasma membrane in an unassisted or spontaneous manner (Hou et al., 2006; Shanmugham et al., 2006) by use of their signal peptide forming a loop structure (Fincher et al., 1998), the precursors will be recognized by a receptor complex, which is 560-620 kDa in size (Berghöfer and Klösgen, 1999; Hou et al., 2006) and composed of TatB and TatC (Bolhuis et al., 2001; Cline and Mori, 2001; Alami et al., 2003). Then, in the presence of a trans-membrane proton gradient, the precursor is transported by the Tat translocase which is formed by recruitment of TatA

(Mori and Cline, 2002; Alami et al., 2003). After being successfully transported across the membrane, the precursors are probably released by lateral diffusion into the lipid membrane (Frielingsdorf and Klösgen, 2007). Then the signal peptidase will cleave off the Tat signal peptide and the mature proteins will be released into the lumenal side of the membrane. Meanwhile, as soon as the precursors being released into the lipid bilayer, the Tat translocon will be disassembled for next round transport (Müller and Klösgen, 2005). The oligomerization of TatA to form a transient translocon (assemble) (Dabney-Smith et al., 2006) and disassembled back into monomers could explain how the Tat system can accommodate folded proteins of varied size. It also explains in part how the system can exist in the membrane without compromising its ion and proton permeability barrier (Mori and Cline, 2002). For moving the substrate across the membrane, probably the dual topology of TatA has some functional indications: in the presence of proton motif force, the C-terminus of TatA will be oriented in the cytoplasm-side of the membrane probably in a flip-flopping manner, which probably could facilitate the formation of the translocon from a structure point of view (Chan et al., 2007).

1.2.3 The goal of the work

The goal of this work was to characterize the mechanism of thylakoid Tat protein transport. To this end, *in vitro* protein transport experiments were performed using isolated intact chloroplasts (*in organello*) or thylakoid vesicles (*in thylakoido*). Specifically, this thesis aimed to answer the following three questions: (I) In cryptophytes, the phycobiliproteins are located at the thylakoid lumenal side, but how these proteins are sorted is not known. To analyze the mechanism involved, one of the phycobiliproteins, notably phycoerythrin alpha, has been analyzed with the heterologous thylakoid system; (II) Tat transport machinery can transport folded proteins, but it is not known how the Tat system can accommodate the Tat substrates with different sizes. For this purpose, a "train-like" chimeric 16/23-EGFP protein has been constructed and analyzed. This allows for the analysis of the translocation steps and to get an idea about how Tat transport machinery can transport substrates with different sizes; (III) After Tat protein transport, Tat signal peptides are cleaved off by thylakoid processing peptidase, but it is not known what happens afterwards to these small peptides. To analyze the fate of Tat signal peptides, a "tandem-substrate", in which two precursors have been fused in a sequential order, has been constructed and used. This chimeric protein gives rise to an easier detection of the Tat signal peptide and one of its subfragment after a cleavage event.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals were purchased from the following companies:
Sigma-Aldrich Chemie (Deisenhofen), Roth GmbH & Co. (Karlsruhe), Serva Feinbiochemica (Heidelberg), Merck AG (Darmstadt), Fluka (Neu-Ulm). If not mentioned, all chemicals used were of analytical grade. Protein A-Sepharose was from Amersham Biosciences (Amersham-Pharmacia) (Freiburg). Nitrocellulose membranes were obtained from Schleicher & Schuell (Dassel) and Polyvinylidenfluorid (PVDF) transfer membranes were from Millipore Corporation (Bedford, MA). Secondary antibodies were obtained from Sigma. Radiochemicals were purchased from Amersham Biosciences and ICN Biomedicals GmbH (Mechenheim).

2.1.2 Marker

DNA standard	1kb Ladder	Gibco BRL (Eggenstein)
Protein standard	SDS-7L	Sigma-Aldrich
	SDS-prestained	Fermentas
	HMW (high molecular weight)	Amersham Biosciences

2.1.3 cDNA clones

For construction of the chimeric proteins, a collection of plasmids (23/23, 16/16, PC/PC, 16/23, 16/EGFP etc.) available from the previous works in our laboratory has been used. These plasmids contain cDNA sequences encoding either the authentic precursors of various polypeptides of chloroplast, or the cassettes encoding fusion proteins composed of one transit peptide and one mature portion from different precursor proteins. These plasmids have been described previously (Berghöfer, J., 1998; Hou, B., 2005; Molik, S., 2005) and, if desirable, are specified in detail in the text.

2.1.4 Bacterial strains and Vectors

Bacterial strains		
	E.coli DH5α	Hanahan, 1983
	E.coli strain BL21 (DE3)	Studier and Moffat, 1986
Vectors		
	pGEM-T easy	Promega
	pBluescript II KS-	Stratagene, San Diego
	pBAT	Annweiler et al., 1991

2.1.5 Enzymes

Molecular biology enzymes were purchased from: Roche Diagnostics GmbH (Mannheim), MBI Fermentas (Vilnius, Lithuania), New England Biolabs (Schwalgach), Stratagene (La Jolla, CA) and USB (Cleveland, OH). Proteases and protease inhibitors were from Sigma. RNase inhibitors were from MBI Fermentas.

2.1.6 Oligonucleotides

Oligonucleotides used for polymerase chain reaction (PCR) and mutagenesis were synthesized by Metabion GmbH (Planegg-Martinsried).

For construction of the "train-like" chimera, the linker plus EGFP part was directly taken from the pEGFP-N2 plasmid (GenBank Accession #: U57608) by use of the enzymes SmaI and NotI. The first parts (i.e. 16/23, 23/23, PC/PC) of the chimera were cloned by use of either T3 or T7 primer as forward primers and the reverse primers were as following:

16/23 reverse:	GCCGGCAACACTGAAAGAACTGGTAGC
PC reverse:	AATATTGACAGTTACTTTTCCCACCATAC

For construction of the "tandem-substrate" as well as its derivatives, the primers for the first part (i.e. 16/23, 23/23) were the same as used for the "train-like" chimera. For the second part, 16/EGFP is the template and T7 primer was used as reverse primer. The forward primers were used as following:
For construction of 23-16$_{LTD}$/EGFP, the 16/23-16$_{LTD}$/EGFP was used as template and the forward primer, 23mATG: ATGGCCTATGGAGAAGCTGCTAATG, and T7 primer was used as reverse primer.

PC_{LTD} forward:	CCCGGGGCTTCCTTGAAGAATGTCGG
16_{LTD} forward:	CCCGGGGCTCAGCAAGTGTCAGCTGAG
$16_{LTD-\Delta(1-5)}$ forward:	CCCGGGGCTGAGGCTGAGACTAGCC
$16_{LTD-\Delta(1-11)}$ forward:	CCCGGGCGCCGAGCTATGTTGGGCTTC
$16_{LTD-\Delta(1-13)}$ forward:	CCCGGGGCTATGTTGGGCTTCGTCGC
$16_{LTD-\Delta(1-20)}$ forward:	CCCGGGCTGGTTTGGCTTCTGGTTC

For construction of 16/23-16_{LTD} and its derivatives, the 16/23-16_{LTD}/EGFP was used as template, the T3 primer was used as forward primer and the reverse primer were as following:

16_{LTD} reverse:	TTAAGCAAGAACAGCCTTAAC
16_{LTD-L} reverse:	TTATAAAAGAACAGCCTTAAC
$16_{LTD-His}$ reverse:	AGCAAGAACAGCCTTAAC
PC_{LTD} reverse:	TTAGGCCATGGCGTTTCCGGCTAG

For the relative mutations, either 16/23 or 16/EGFP were used as template as indicated in the name of primer and the following pairs of primers were used:
16/23(A83L):
GGTTCGTTTGTTAAGGCTGTTCTTTTAGCCTATGGAGAAGCTGCTAATG
CATTAGCAGCTTCTCCATAGGCTAAAAGAACAGCCTTAACAAACGAACC(antisense)

16/EGFP(A83L) :
GTTCGTTTGTTAAGGCTGTTCTTTTAGGGATCCACCGGCCGGTCG
CGACCGGCCGGTGGATCCCTAAAAGAACAGCCTTAACAAACGAAC(antisense)

16/EGFP(AA69LL):
GAGCTATGTTGGGCTTCGTCCTGCTTGGTTTGGCTTCTGGTTCG
CGAACCAGAAGCCAAACCAAGCAGGACGAAGCCCAACATAGCTC(antisense)

16/EGFP(SG74LL):
CGTCGCAGCTGGTTTGGCTTTGCTTTCGTTTGTTAAGGCTGTTC
GAACAGCCTTAACAAACGAAAGCAAAGCCAAACCAGCTGCGACG(antisense)

2.1.7 Plant materials

Pea (*Pisum sativum*) seedlings were grown in green house at 25 °C with long day light (10 hours per day), and harvested on the 7th-10th day after sowing.

2.2 Methods

2.2.1 Standard methods

Basic molecular methods were performed according to Sambrook et al. (1989). Ligation, plasmid transformation in *E. coli*, DNA isolation from plasmid were performed according to Birnboim & Doly (1979). DNA-Restriction, Agarose gel electrophoresis, Plasmid preparation, DNA-extraction were made as detailed in the instructions given in the kits. DNA sequencing was performed either by using the dideoxynucleotide chain termination method (Sanger et al., 1977) or by use of ABI PRISM method (Applied Biosystems).

2.2.2 Construction scheme of the train-like protein

For construction of the "train-like" protein (16/23-EGFP), the DNA templates of 16/23 (Clausmeyer et al., 1993) and EGFP from pEGFP-N2 plasmid have been prepared. The fragment of 16/23 part was amplified by PCR by use of T7 and 23-resverse primers.

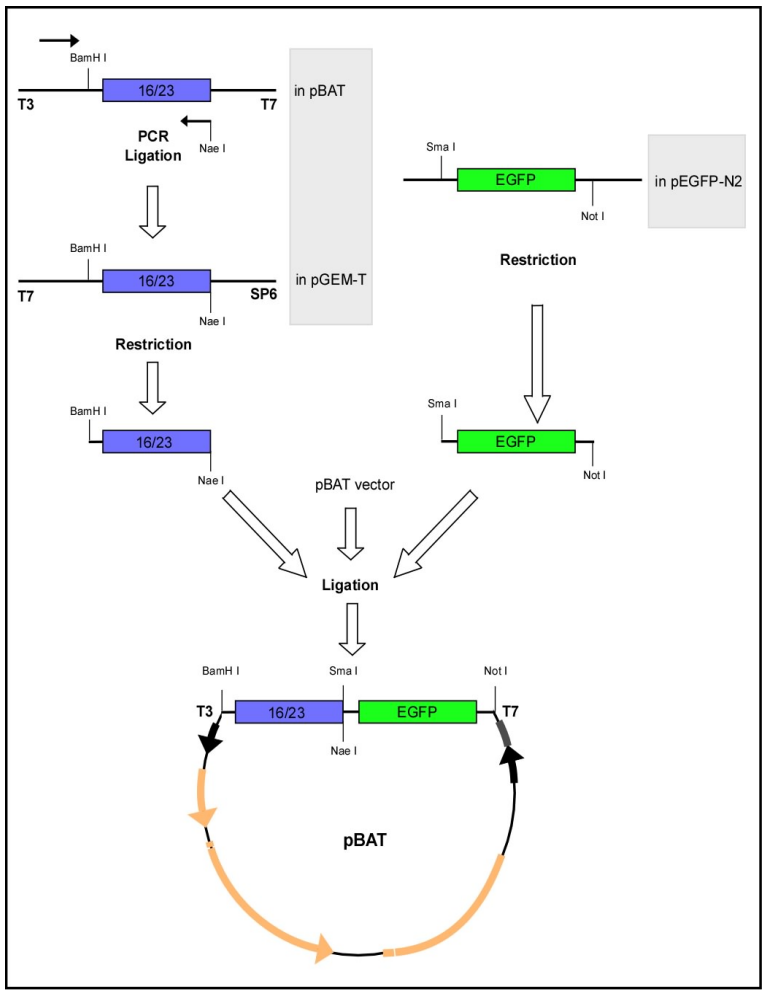

Fig 2.1: **Construction outline of the "train-like" chimera.**

Then the PCR products were ligated into the pGEM-T easy vector for amplification. The fragment was digested with restriction endonucleases BamHI and NaeI. The linker plus EGFP fragment was digested by use of SmaI and NotI directly from pEGFP-N2 plasmid. The isolated 16/23 and EGFP fragments were ligated into pBAT vector which contains the BamHI and NotI recognition site. The resulting recombinant fusions were verified by DNA sequence analysis. The construction procedure was summarized in Fig. 2. 1.

The same procedure was applied for construction of the "tandem substrate". The mutation has been performed according to the instruction manual from Stratagene (QuikChange Site-Directed Mutagenesis Kit, Catalog #200518). The respective "tandem substrates" then were used as templates for generation of derivatives used for the analysis of the fate of Tat signal peptide.

2.2.3 *In vitro* transcription and *in vitro* translation

In order to produce analytical amounts of radioactively labelled proteins, *in vitro* transcription and translation of respective cDNA clones encoding original, chimeric or mutant proteins were performed.

In vitro transcription

In vitro transcription of cDNAs was performed from gene cassettes cloned in either pBluescript KS or pBAT vectors. After linearization of the plasmid DNAs downstream of the gene sequence by proper restriction enzymes, the linearized plasmid DNAs were subjected for transcription reactions, using either T3 or T7 RNA polymerase according to manufactural recommendations (Stratagene and New England Biolabs, respectively).

Composition of the *in vitro* transcription reaction was as following:

H_2O (DEPC-treated)	6.5 μl
5 x reaction buffer	5.0 μl
2.5 mM rNTP mixture (GTP: 0.25 mM)	5.0 μl
100 mM DTT	2.5 μl
5 mM m^7GpppG (capping nucleotide)	2.5 μl
40 U/μl RNase inhibitor	0.5 μl
linerarized plasmid DNA (2 μg)	2.5 μl
RNA polymerase (40 U/l)	0.5 μl
Total volume	25 μl

The reaction mixture was pre-incubated at 37 °C for 30 min to allow the formation of

the cap structure. Incubation was continued for additional 1 h at 37 °C after adding 1 μl of 11.25 mM rGTP to the reaction, then the reaction was terminated by addition of 100 μl ice-cold DEPC-treated H_2O. To check the transcription products, 4 μl was taken out and subjected to 1% Agarose gel (1×MOPS buffer: 5 mM Na-Acetate pH 7,0, 20 mM MOPS, 1 mM EDTA). Finally, all the rest of the synthesized RNAs were precipitated by addition of 0.1 volume of 4 M NH_4OAc and 3 volumes of EtOH. RNA-ethanol suspension can be stored at −20 °C for years. RNAs were collected by centrifugation at 15,000 rpm for 30 min before subjected to *in vitro* translation reaction.

***In vitro* translation**

Synthesis of radioactively labelled proteins was performed by *in vitro* translation of mRNA obtained from *in vitro* transcription in the presence of ^{35}[S] -methionine (Amersham) using an reticulocyte lysate-based cell-free translation system.

DEPC-H_2O	4.65 μl
1 M KCl	0.6 μl
Amino acids mixture (-Met)	0.25 μl
100 mM DTT	2.5 μl
^{35}[S]-Met	0.5 μl
Reticulocyte lysate	0.5 μl
Total volume	12.5 μl

The reaction was carried out for 60-90 min at 30 °C. The resulting *in vitro* translation products were used for import experiments directly or stored at −80 °C for up to one week.

2.2.4 Isolation of chloroplasts from pea leaves

Green house-grown pea seedlings were harvested 7-10 days after germination; the leaves were homogenized in 400 ml of ice-cold SIM buffer by use of a Waring Blendor. Homogenate was filtered through two layers of Miracloth and centrifuged in a Serva SLC-250T rotor for 2 min at 4,000 rpm. The crude chloroplast pellet was resuspended with approx. 8 ml of SRM and loaded onto a 35% Percoll cushion. After centrifugation for 7 min at 4,000 rpm in a Serva SL-50T rotor, the pellet of intact chloroplasts was washed twice with 1 x SRM, and the chloroplasts were collected by centrifugation for 2 min at 3,000 rpm in a SL-50T rotor. The chloroplasts were finally resuspended in 2 ml 1x SRM.

The concentration of the chloroplast suspension was defined by its chlorophyll concentration. Chlorophyll was extracted from 10 μl of the chloroplast resuspension with 1 ml 80% acetone, and the solution was subjected to a Schimadzu spectrophotometer.

The total concentration of chlorophyll a and b was obtained according to the formula (Arnon, 1949): $C_{(Chlorophyll)}[\mu g/\mu l]=(A_{663}$ x 8.02 + A_{645} x 20.2)/10

1x SIM	Hepes/KOH, pH 7,6	25 mM
	EDTA	2 mM
	Sucrose	350 mM
5x SRM	Sorbitol	1,65 M
	Hepes/KOH pH 8,0	250 mM
35% Percoll solution	5 x SRM	2 ml
	Percoll	3.5 ml
	H_2O	4.5 ml
HM buffer	Hepes/KOH, pH 8.0	10 mM
	$MgCl_2$	5 mM

2.2.5 Import of proteins into intact chloroplasts

The standard *in organello* import reaction was performed as following:

	volume	final concentration
chloroplasts	equal to 60 μg chlorophyll	
250 mM methionine	3 μl	5 mM
100 mM Mg-ATP	12 μl	2 mM
1 M $MgCl_2$	1.5 μl	10 mM
in vitro translation product	12.5μ l	
1 x SRM	to 150 μl	

Import reaction with Nigericin

chloroplasts	equal to 60 μg chlorophyll	
250 mM methionine	3 μl	5 mM
100 mM Mg-ATP	12 μl	2 mM
1 M $MgCl_2$	1.5 μl	10 mM
250 mM KCl	6.0 μl	10 mM
0,3 mM Nigericin	3,0 μl	6 μM
in vitro translation product	12.5μ l	
1 x SRM	to 150 μl	

Complete assays without the *in vitro* translation products were briefly preincubated at 25 °C, and the import reactions were initiated by addition of *in vitro* translation product and carried out for 30 min at 25 °C in the light. After incubation, samples were

transferred onto ice and diluted with 350 μl of ice-cold 1x SRM buffer. Chloroplasts were collected from a 50 μl aliquot of the sample by centrifugation at 6,000 rpm for 3 minutes, and denatured with 2 x Laemmli buffer (C^- fraction). Chloroplasts collected from the rest of the sample were resuspended in 1x SRM containing 150 μg/ml thermolysin, and the reaction mixture was chased on ice for 20 min to remove the envelope-bound radioactive protein. Thermolysin treatment was terminated by addition of EDTA to 25 mM. Chloroplasts collected from one tenth aliquot of the assay were denatured with 2 x Laemmli buffer (C^+ fraction), and the rest of the chloroplasts were reisolated by centrifugation through a 35% Percoll cushion at 8,000rpm for 8 min. The intact chloroplasts were thoroughly washed with 1 ml of 1 x SRM buffer supplemented with 10 mM EDTA, and collected by centrifugation for 1 min at 6,000 rpm. Stroma and thylakoid fractions were separated by osmotically lysing chloroplasts in 100 μl of HM buffer containing 10 mM EDTA for 5 min, followed by centrifugation at 10,000 rpm for 5 min. Stromal proteins in the supernatant were supplemented with equal volume of 4 x Laemmli buffer (S fraction). Thylakoid membranes were washed with HM buffer and resuspended in 200 μl of the same buffer. One half of the thylakoids were mock-treated directly (T^- fraction), and the other half was treated with 200 μg/ml thermolysin for 30 min on ice to remove proteins exposed at the surface of the thylakoid membrane. The thermolysin treatment was terminated by addition of EDTA to 10 mM, and the resulted thylakoids were collected by centrifugation at 10,000 rpm for 4 min followed by denaturing with 2 x Laemmli (T^+ fraction). Protein samples were analyzed by SDS-PAGE and autoradiography.

2.2.6 Import experiments with isolated thylakoids

In thylakoido import experiments were generally carried out using thylakoids obtained from pea chloroplasts. To isolate thylakoids, chloroplasts were lyzed in HM buffer at a concentration of 0.75 mg/ml chlorophyll for 5 min on ice and then centrifuged for 5 min at 10,000 rpm at 4 °C. The supernatant containing stroma was separated from thylakoid pellets, collected and stored on ice. Thylakoids were washed twice with HM buffer by centrifugation (5 min, 10,000 rpm) and finally resuspended either in HM buffer or in stroma fraction at a chlorophyll concentration of 0.75 mg/ml. Routinely, thylakoid import reactions were conducted for 30 min at 25 °C in the light. The standard *in thylakoido* import assay includes the following compounds:

thylakoid suspension	40 μl
in vitro translation product	5 μl
HM buffer	5 μl
Total volume	50μl

The resulting thylakoid vesicles were re-isolated by centrifugation for 5 min at 10,000 rpm at 4 °C and washed twice with HM buffer. Then, half of the thylakoid vesicles were resuspended in 2x Laemmli sample buffer, while the second half were resuspended in HM buffer containing 200 μg/ml thermolysin. After incubation for 30 min on ice, protease treatment was terminated by addition of HME (10 mM Hepes/KOH, pH 8.0, 5 mM $MgCl_2$, 10 mM EDTA) buffer. Thylakoids were collected by centrifugation at 10,000 rpm for 5 min and resuspended in 2x Laemmli sample buffer. After denaturation by heating for 3 min at 100 °C, samples were analyzed by gel electrophoresis followed by autoradiography.

To examine the influence of inhibitors on the thylakoid translocation of proteins, assays were supplemented with nigericin (to 2 μM) or sodium azide (10 μM). To examine the role of NTPs, apyrase was added (1 U per 50 μl Assay). Competitor proteins were added to the concentration indicated in the respective assays. For characterization of the unknown protease cleaving cleaving within the signal peptides, respective inhibitors were incubated with thylakoids on ice for 10 min, then proceeded with standard import procedure.

2.2.7 Electrophoresis of proteins

SDS polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis of SDS-denatured proteins was performed according to Laemmli (Laemmli, 1970). As a rule, polyacrylamide gels with an acrylamide gradient from 10-15% were used except being mentioned (Hou, 2005; Molik, 2005).

Non-denaturing electrophoresis of membrane proteins (Blue Native electrophoresis (BN-PAGE))

To isolate the photosynthetic complexes from the thylakoid membrane, the blue native gel electrophoresis was used (Schäger and Jagow, 1991; Schäger et al., 1994; Karnauchov, 1998). By use of the mild detergent digitonin, the thylakoid membrane could be solubilized and the oligomeric complexes of the thylakoid membrane could be reproducibly separated with high-resolution.

- Preparation of samples

  To solubilize the membrane protein complexes, thylakoid membranes (equivalent to 30 μg of chlorophyll) were resuspended with 15 μl of lysis buffer and 7.5 μl of 5% digitonin (freshly prepared and 98 °C dissolved). After incubation for 30-60 min at 4 °C under agitation, nonsolubilized membrane materials were spun down at 40,000 g for 1 h at 4 °C. The supernatant was supplemented with 1.5 μl of

5% Coomassie Briliant Blue G-250 in lysis buffer, bound on ice for 10 min, and centrifuged at 40,000 g for 3 min. The supernatant of this centrifugation was used for loading onto the blue native gel.

Lysis buffer:

Stock solutions	Volume	Final concentration
0.5 M Bistris, pH7,0	500 μl	50 mM
2M aminocaproic acid	2500 μl	1 M
0.5 M EDTA, pH 8.0	50 μl	5 mM
0.1 M $MgCl_2$	25 μl	0.5 mM
0.1 M PMSF (in isopropanol)	50 μl	1 mM
0.1 M DTT	50 μl	1 mM
H_2O	add to 5 ml	

- Gel electrophoresis:

  10 x running buffer:

  0.5M tricine, 0.15 M Bistris, pH 7,0

  For preparation of the blue native gel:

Component	Stacking gel 4%	Separation gel 13,5%	Separation gel 10%
H_2O	5,1 ml	1,38 ml	3,08 ml
10 x Bistris (0,5M, pH 7,0)	1,0 ml	1,5 ml	1,5 ml
30% acrylamide/bisacrylamide	1,3 ml	6,75 ml	5 ml
2M ε-aminocaproic acid	2,5 ml	3,75 ml	3,75 ml
87,5% glycerol	–	1,5 ml	1,5 ml
5% digitonin	60 μl	90 μl	90 μl
10% APS	90 μl	46 μl	50 μl
TEMED	9 μl	4,6 μl	5 μl
Total volume	10 ml	15 ml	15 ml

  Protein samples were resolved at approx. 10 mA (with power restricted at 280 V) at 4 °C. After separation, the gel was fixed with 50% Methanol and 12% Acetic acid for about 15 min before drying. 1x running buffer contained 0,0075% of Coomassie G250. Detection of proteins in these gels was performed by either silver staining or autoradiography.

Immunodetection of proteins - Western blot

For detection of proteins using specific antisera, immediately after electrophoresis, the gels were incubated in transfer buffer (150 mM glycine, 20 mM Tris, 10% methanol)

for 30 min. Then the proteins were blotted onto a PVDF-Membrane (Immobilon-P, Millipore) with a semi-dry transfer apparatus (Gibco-BRL) following the manufacturer instructions (Immobilion-P Transfer Membrane User Guide). The transfer time is 1 h at 2 mA/cm^2 membrane. Prehybridization, hybridization with primary and secondary antisera were carried out in 1 x PBS containing 1% v/v Tween 20 and 5% dry skimmed milk. Specific antisera were used usually in a 1:1,000 dilution. The incubation time is 1-2 h. After 4 x 10 min washing with milk buffer, the secondary antisera conjugated to horseradish peroxidase (Anti-Rabbit-IgG Peroxidase-Conjugate, Sigma-Aldrich) were used in a 1:30,000 dilution for 1-2 h incubation. After this hybridization with the secondary antisera, the PVDF membranes were washed three times with 1 xPBS containing 0.1% v/v Tween 20. For visualization of protein bands, the secondary antibodies were developed with ECL (enhanced chemiluminescence) reaction (Voelker and Barkan, 1995). The developing reagent was set up before the reaction freshly from stock solution. After incubation for 1 min in the developing reagent, PVDF membranes were wrapped in plastic foil and exposed to an X-ray film for an appropriate time.

10 x PBS	NaCl	750 mM
	KCl	30 mM
	Na_2HPO_4	45 mM
	KH_2PO_4	5 mM

ECL reagent

stock solution	volume	concentration
1 M Tris-HCl, pH 8.5	500 μl	50 mM
250 mM luminol (in DMSO)	50 μl	1.25 mM
90 mM p-coumaric acid (in DMSO)	22 μl	200 μM
30% H_2O_2	3 μl	2.7 mM
H_2O bidist.	Add to 10 ml	

Immunoprecipitation

Washed thylakoid membranes (30μg chlorophyll) were solubilized in 100 μl resuspension buffer with Triton-X 100 (50 mM Hepes/KOH pH 8.0, 100 mM NaCl, 0.5 mM $MgCl_2$, 0.05% BSA, 1 mM PMSF, 25 mM EDTA and 1% Triton-X 100). After incubation for 45 min at 4 °C with agitation, the solubilized membrane material was gained from the supernatant of centrifugation at 13,000 rpm for 20 min, and was mixed with 1-5 μg IgG. 20 μl of 10% protein A-Sepharose CL4B (Pharmacia) was then added to the mixture, and the suspension was incubated for 1 h at 4 °C with agitation. The unbound proteins

were recovered by centrifugation for 5 min at 10,000 rpm, and the protein A-Sepharose beads were washed with resuspension buffer as described above, except containing only 0.1% Triton-X 100. Bound proteins were recovered by incubation in SDS-sample loading buffer at 100 °C for 5 min then followed by centrifugation. Protein samples were separated by SDS-PAGE followed by autoradiography.

Coomassie Staining of proteins

Staining buffer	45% (v/v)	Methanol
	9% (v/v)	Acetic acid
	0,25% (w/v)	Coomassie G-250
Destaining buffer I	20% (v/v)	Methanol
	7% (v/v)	Acetic acid
Destaining buffer II	50% (v/v)	Methanol
	10% (v/v)	Acetic acid

For detection of protein bands on gels, staining with Coomassie Brilliant Blue R-250 was routinely used. The gel was firstly stained in the staining buffer for 30 min at 50 °C and stopped by incubation in destaining buffer I at 50 °C until a better overview of the protein bands (approx. 1 h). After incubation with destaining buffer II for 10 min at room temperature, the gel was dried for 2 h at 80 °C in vacuum and exposed to Phosphorimaging plate (FUJIFILM). Phosphorimage analyzer Fujifilm FLA-3000 and the Programm AIDA (advanced image data analyzer, RAYTEST/FUJIFILM) were used for data analysis.

3 Results and Discussion

3.1 Evolutionary conservation of the Tat targeting information

Cryptophytes are an unusual group of flagellate algae common in marine and fresh water. In contrast to plant plastids derived from endosymbiosis of a cyanobacterium, cryptophytes acquire their plastid by engulfing and stably integrating a red algal cell, leading to an eukaryote-eukaryote chimera. Another peculiarity of cryptophytes is a differential arrangement of the light-harvesting apparatus in comparison with that found in the thylakoids of cyanobacteria and red algae. In cryptophytes, the photosynthetic pigments like phycobilin and the respective phycobiliproteins are located on the lumenal rather than the stromal side of the thylakoid membrane. However, it is not clear how these phycobiliproteins like phycoerythrin are sorted.

Sequence analysis of the phycobiliprotein–phycoerythrin alpha (PEα)–revealed that all genes encode preproteins containing a bi- or even tripartite topogenic signal, which is composed of an N-terminal signal peptide for co-translational import into the ER lumen via the Sec61 complex, followed by a transit peptide-like region mediating transport across the remaining three membranes into the plastid stroma (Gould et al., 2006). Additionally, more than half of them carry an additional, third topogenic signal comprising a twin arginine motif which is indicative of Tat-specific targeting signals. To analyze the transport property as well as the organelle localization of PEα, *in organello* as well as *in thylakoido* import assays have been performed. The results show that PEα is transported into the thylakoid lumen and that the Tat-dependent pathway catalyzes the transport process which indicates that the Tat targeting information is conserved between cryptophytes and higher plant.

3.1.1 Localization of PEα protein within chloroplast

The PEαC (the full-length PEα) as well as its derivatives, PEαTP (a deletion derivative lacking the ER-targeting signal), and PEαRR (a deletion derivative lacking both the N-terminal ER-targeting signal and the chloroplast transit peptide) were analyzed by incubation of their *in vitro* translation products with isolated pea chloroplasts

under standard import conditions (see Materials and Methods). As shown in Fig 3.1,

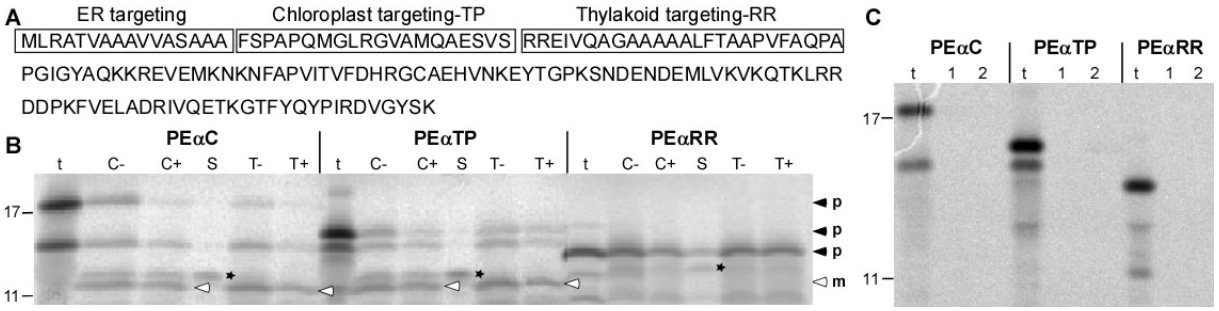

Fig 3.1: ***In organello* import of the phycoerythrin derivatives. A**, The full size protein sequence of PEαC. The predicted targeting signals are marked with rectangles and indicated on the top of the sequence. **B**, *In organello* import assay. The *in vitro* translation products of PEαC, PEαTP and PEαRR were incubated with isolated pea chloroplasts for 20 min at 25 °C in the light. After the import reaction, the chloroplasts were either treated with thermolysin (150 μg/ml) for 30 min on ice (lanes C^+) or mock-treated (lanes C^-), and then re-isolated by centrifugation through a Percoll cushion. Aliquots of the protease-treated chloroplasts were additionally fractionated into stroma (lanes S) and thylakoids. The thylakoid fractions were treated with either thermolysin (200 μg/ml, 30 min on ice, lanes T^+), or mock-treated (lanes T^-). Stoichiometric amounts of each chloroplast fraction, corresponding to 12.5 μg chlorophyll, were separated on 10-17.5% SDS-polyacrylamide gradient gels and visualized by phosphor-imaging. In lanes t, 1 μl of the respective *in vitro* translation products were loaded. Positions of the precursor (p) and mature proteins (m) are indicated by closed and open arrowheads, respectively. Putative degradation bands in the stroma fraction are marked with stars. **C**, Control of protease sensitivity of PEαC, PEαTP and PEαRR. The respective *in vitro* translation products were subjected to import buffer lacking chloroplasts and treated with thermolysin (150 μg/ml, 30 min on ice, lanes 1, or 200 μg/ml, 30 min on ice, lanes 2).

both PEαC and PEαTP, regardless of the presence of ER targeting signal, were successfully transported into the organelles and processed to their predicted mature form which migrates with an apparent molecular weight of 12 kDa upon SDS-PAGE. These mature proteins are resistent to externally added protease indicating their chloroplast internal localization (Fig 3.1, B, lanes C^+). In contrast, the absence of a chloroplast transit peptide (i.e. PEαRR) leads to the failure of the accumulation of mature PEα proteins, even though some of the precursors tightly associated with the isolated chloroplasts which to some degree were also resistent to the protease treatment (Fig 3.1, B). It is not clear at present what the reasons for this association as well as for protease treatment resistance are, since in the absence of chloroplasts, the *in vitro* translation products were completely degraded (Fig 3.1, C).

To examine the localization of PEα within the chloroplast, the chloroplasts were further fractionated, after import of PEαC and PEαTP, into stroma and thylakoids. The results (Fig 3.1, B) show that in both instances the presumed mature protein of approximately 12 kDa is found exclusively in the thylakoid fraction where it is resistant to protease added from the stromal side to the vesicles (Fig 3.1, B, lanes T^+). It should be pointed

out that inside the stroma fraction, a product around 13 kDa has been found (Fig 3.1, B, marked with stars). At first glance it might represent a stromal intermediate in which the chloroplast-targeting transit peptide has been removed by stromal processing peptidase (SPP), while the thylakoid targeting signal peptide is still present. However, the size difference of only ∼1 kDa compared to the mature protein argues against such a transport intermediate, because the thylakoid targeting Tat-signal comprises at least 24 residues (Fig. 3.1, A). Furthermore, a polypeptide of similar size is sometimes found also in the stromal fraction of the import assay when analyzing PEαRR (Fig. 3.1, B), which is not imported into chloroplasts. While the reason for the formation of this band is not clear, it likely represents a degradation product formed in the presence of organelles upon thermolysin treatment.

3.1.2 Transport of PEα protein across the thylakoid membrane is mediated by Tat-dependent pathway

The above result indicates that PEα protein can be transported into the thylakoid system of higher plant chloroplasts. For further examination, *in thylakoido* experiments were performed. As shown in Fig 3.2 A, all three derivatives were successfully imported into the thylakoid lumen including the PEαRR which is not imported in the *in or-*

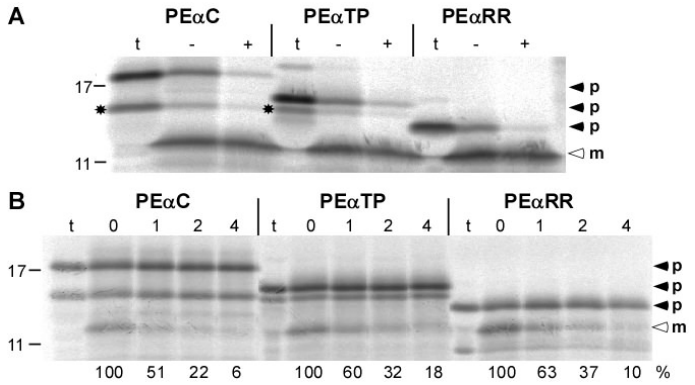

Fig 3.2: **Import of phycoerythrin derivatives into thylakoid vesicles isolated from pea chloroplasts. A**, Isolated thylakoids were incubated with radiolabelled precursor proteins for 15 min at 25 °C in the light. After the import reaction, thylakoids were treated with either thermolysin (200 μg/ml, 30 min on ice, lanes +), or mock-treated (lanes -). In lanes t, 1 μl of the respective *in vitro* translation assays were loaded. The asterisks indicate the putative N-terminally truncated translation product, which presumably derive from translation initiation at an internal start codon. **B**, Saturation of the Tat-dependent pathway inhibits transport of PEα across the thylakoid membrane. Thylakoid transport experiments were performed in the presence and absence of increasing amounts of precursor of the 23 kDa subunit of the oxygen-evolving system (OEC23 kDa) that were obtained by overexpression in *E. coli*. The concentration of competitor protein (in μM) present in each assay is indicated above the lanes. After the import reaction, the assays were loaded without further treatment to 10-17.5% SDS-polyacrylamide gradient gels. Mature PEα accumulating in the thylakoid lumen was quantified for each protein, and the relative amounts (in terms of percentage of mature PEα accumulating in the absence of competitor protein) are given below the lanes. For further details, see the legend to Fig. 3.1

ganello assay since the putative thylakoid targeting transport signal of PEαRR is not sufficient for organelle import. Additionally, this result further confirms the observation that the N-terminal extension of the Tat signal peptide has no effect on the ability of mediating transport by the signal peptide (Mould et al., 1991; Klösgen et al., 1992; Fincher et al., 1998).

As the signal peptide of PEα contains a RR-motif, which is indicative of Tat-mediated transport, competition assays were performed using the authentic Tat-specific transport substrate OEC23 kDa protein (Fig 3.2, B). The accumulation of the mature PEα protein was impaired substantially in a dose-dependent manner when the concentration of the competitor was increased which unequivocally proves that they are translocated by the Tat pathway across the thylakoid membrane.

3.1.3 Discussion of the transport of PEα protein

The unusual localization of the photosynthetic pigments in cryptophytes makes its light-harvesting system different from others like red algae, glaucophytes and cyanobacteria (Gould et al., 2007). In the present work, the import analysis into the pea chloroplast provides additional experimental proof for the thylakoid lumen localization of the PEα protein, one subunit of the light-harvesting system of cryptophytes.

In contrast to red alga, glaucophytes and cyanobacteria, whose phycobiliproteins are associated to the thylakoid membrane from the stromal or cytosolic side, the thylakoid lumen localization of PEα in cryptophytes suggest that a new targeting information mediating their transport across the intraorganellar membranes had been developed upon evolution. As indicated by the presence of the RR-motif in its signal peptide, the PEα could be a Tat-pathway substrate. By analyzing this prediction using isolated thylakoid vesicles from pea chloroplast, it could be concluded that indeed the Tat translocase mediates the transport of PEα. From the evolutionary point of view, this result strongly suggests that the targeting information of PEα protein was evolutionary conserved between cryptophytes and higher plant and probably a protein transport pathway corresponding to the Tat pathway of higher plant chloroplasts exists also in cryptophyte plastids.

3.2 Analysis of the Tat transport mechanism across the thylakoid membrane.

Transporting folded proteins across the membranes makes Tat pathway so unique (Mori and Cline, 2001; Robinson and Bolhuis, 2004; Müller and Klösgen, 2005; Gutensohn et

al., 2006). However, the big challenge for Tat transport machinery is how to transport substrates with divergent size (from 10-100 kDa), shape and surface features (Berks et al., 2000; Müller and Klösgen, 2005), meanwhile avoiding the leakage of components like ions. Diverse transport mechanisms have been suggested (Musser and Theg, 2000; Brüser and Sanders, 2003; Müller and Klösgen, 2005), and now it is generally accepted that Tat translocase is formed only transiently, probably by oligomerization of TatA monomers (Müller and Klösgen, 2005; Dabney-Smith et al., 2006). Furthermore, it was suggested that these oligomerized TatA form a transient pore to perform the transport of Tat substrates (Mori and Cline, 2002; Alami et al., 2003). It is, however, still not clear how TatA oligomerizes to form a functional translocase and if the same translocation pore is used for different substrates or if an appropriate pore is selected based on the substrate?

Substrates which are blocked at specific transport stages giving rise to the so-called transport intermediates (Ti) are valuable tools for analyzing transport mechanism, since their characterization is always helpful for obtaining a greater understanding of the protein transport process. For this purpose, construction of chimeric proteins which contain protein domains from different transport substrates has been used successfully to obtain such transport intermediates (Fincher et al., 1998; Berghöfer and Klösgen, 1999; Marques et al., 2003, 2004; Müller and Klösgen, 2005; Hou et al., 2006). For example, attaching a tightly folded protein behind the precursor which is transported in an unfolded manner allows often to block the transport and to get such Ti for subsequent analyzes (e.g. Vestweber and Schatz, 1988; Pfanner et al., 1992; Schülke et

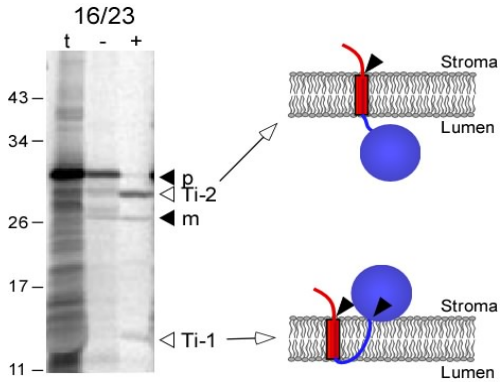

Fig 3.3: ***In thylakoido* transport of the 16/23 chimera.** Protease treatment of the import reaction mixture of *in vitro* translated 16/23 chimera with thylakoid vesicles generates two transport intermediates Ti-1 and Ti-2 (lane +). The formation of the two transport intemediates was cartooned beside the SDS-gel as indicated with arrows: The signal peptide of OEC16 kDa protein is in red and the mature OEC23 kDa is in blue; the predicted thermolysin cleavage sites for the formation of Ti-1 and Ti-2 were given with closed arrowheads in the cartoon. For further figure legend details, see the legends to Fig. 3.1 and Fig. 3.2. For further details about Ti-1 and Ti-2 see Berghöfer and Klösgen (1999) and Hou et al., (2006).

al., 1997). In the case of Tat protein transport research, however, it is more difficult to block the transport and to find translocation intermediates because Tat translocase can transport folded proteins (Müller and Klösgen, 2005). Interestingly, a chimeric protein, 16/23, which is composed of the transit peptide from OEC16 kDa protein and the mature OEC23 kDa protein, both from oxygen-evolving complex (OEC) that are targeted by Tat pathway, turned out to be a particular suitable candidate for Tat research, since this protein is not blocked but rather retarded during membrane transport (Fig 3.3; Berghöfer and Klösgen, 1999; Hou et al., 2006; Frielingsdorf and Klösgen, 2007). However, because of the capability of Tat translocase to translocate folded protein domains, it is still not possible using 16/23 chimera to get an idea how the Tat transport machinery arranges different substrates. To this end, we wondered if it is possible to construct a "train-like" protein as substrate in which two different proteins with different size and shape are combined in a sequential order. If there is a successful translocation event, it should be possible by analyzing how this substrate is transported to get an idea about the dynamic properties of the Tat translocase. For this purpose, based on the unique transport performance property of the 16/23 chimera, a new "train-like" 16/23-EGFP chimera, in which EGFP (enhanced green fluorescent protein) was attached to the C-terminus of the 16/23 protein by use of a small peptide linker, was generated (Fig 3.4). The construction of this new chimera was based on the following considerations: (i) both mature OEC23 kDa and EGFP have been shown to be transported successfully into the thylakoid lumen when fused to Tat specific signal

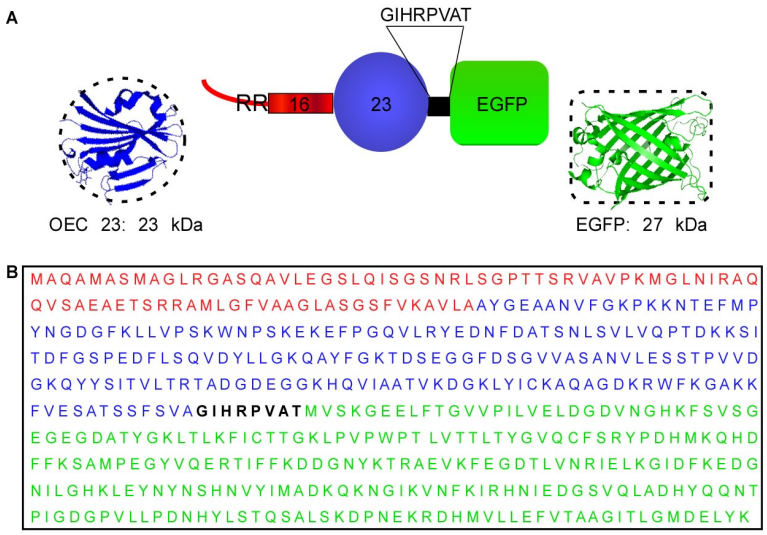

Fig 3.4: **Schematic representation of the "train-like" chimera 16/23-EGFP. A**, Based on the 16/23 chimera, the "train-like" chimera was constructed by attaching the mature EGFP behind the 16/23 chimera using a linker which was shown on the top of the cartoon. The 3D structure as well as the molecular weight of OEC23 kDa and EGFP were also shown. Accordingly, amino acid sequence of 16/23-EGFP is given in **B**. The signal peptide of OEC 16 kDa protein is given in red, the mature OEC23 kDa in blue, the linker in bold and black, and the mature EGFP in green. Similarly, EGFP was fused to the C-terminus of 23/23 and PC/PC to form 23/23-EGFP and PC/PC-EGFP chimera.

peptides (Berghöfer and Klösgen, 1999; Marques et al., 2003, 2004; Hou et al., 2006); (ii) most importantly, the 3D crystal structures of OEC23 kDa and EGFP are known (Fig 3.4) (Yang et al., 1996; Ifuku et al., 2004). OEC23 kDa is a globular protein with a diameter of ∼25 Å in average (minimum 25 Å and maximum 45 Å) (Martin Caffrey, personal communication), which is composed of two distinct domains, in which six antiparallel β-sheets form the central part and one α-helix covers the β-sheets from both sides (Ifuku et al., 2004). In contrast, EGFP shows a cylindrical property with a diameter of 30 Å and 40 Å in length. The crystal structure of EGFP shows that it is comprised of two quite regular β-barrels with 11 strands on the outside of the cylinder (Yang et al., 1996). Both 23 kDa and EGFP have a tightly folded core fragment which is largely protease-resistant (Miyao et al., 1988; Creighton et al., 1995). (iii) EGFP is not of plant origin and displays no significant homology to any known plastid protein. Thus, it can be considered as a "neutral" passenger protein that is unaffected by any internal plant regulatory circuits (Marques et al., 2003, 2004).

3.2.1 Two mature proteins can be transported by a single Tat signal peptide

Incubation of *in vitro* translated 16/23-EGFP protein with isolated thylakoid vesicles

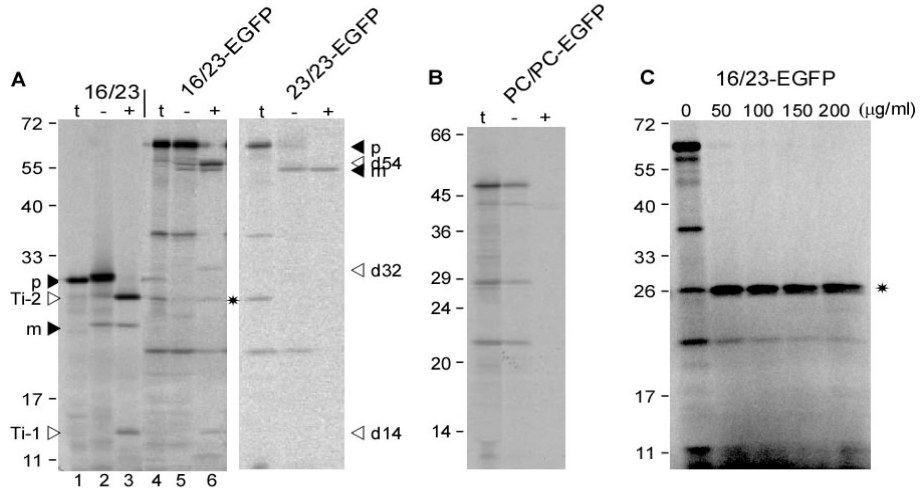

Fig 3.5: ***In thylakoido* transport of "train-like" chimeric proteins. A**, *in vitro* translated precursors of 16/23-EGFP and 23/23-EGFP were incubated with isolated thylakoid vesicles under import conditions. After protease treatment of the import reaction of 16/23-EGFP, three protease-resistant degradation products indicative of putative transport intermediates (Ti) are indicated by open arrowheads (d14, d32 and d54). As a control, the import result of 23/23-EGFP as well as 16/23 chimera are shown. The two transport intermediates, Ti-1 and Ti-2, from 16/23 chimera are indicated on the left. **B**, Import assay of PC/PC-EGFP shows no transport of this "train-like" chimera. **C**, Control of protease sensitivity of 16/23-EGFP. The respective *in vitro* translation products were subjected to import buffer lacking thylakoids and treated with thermolysin at the concentration indicated above the figure. The bands generated by thermolysin treatment in A and C are indicated by a star. For further details, see the legends to Fig. 3.1 and Fig. 3.2.

from pea under standard import conditions (Hou et al., 2006) showed that the "train-like" precursor protein is efficiently imported into the thylakoid lumen and processed by thylakoid processing peptidase (TPP) to a protein of approximately 51 kDa (Fig 3.5, A, lanes -), which corresponds well to the size expected for the "mature" 23-EGFP chimera after removal of the transport signal. This putative mature 23-EGFP protein is resistant against externally added protease which is an indication of its internal localization (Fig 3.5, A, lanes +). The successful transport of this "train-like" precursor protein is not limited to the signal peptide of 16 kDa protein as the same result was found also for 23/23-EGFP which contains the transit peptide from 23 kDa protein (Fig 3.5, A). In contrast, if instead of a Tat signal peptide, a Sec-signal peptide, e.g. from PC (plastocyanin), is used in the train-like construct forming PC/PC-EGFP, no such transport can be observed (Fig 3.5, B). The reason for the transport incompentence of PC/PC-EGFP is not known, but might be due to the folding of EGFP in the import assays which is not suitable for Sec transport, as only unfolded proteins can be transported by Sec pathway. Taken together, these results demonstrate that a single Tat specific signal peptide is sufficient to transport two passenger proteins.

3.2.2 Three transport intermediates can be distinguished during the transport of the "train-like" protein

During the transport of the 16/23 chimera, protease treatment generates two consecutive transport intermediates (Fig 3.3, 3.5). Among them, Ti-1 represents the stage of membrane insertion with both N- and C-termini located outside of the thylakoid membrane, while Ti-2 represents the stage of successful transport of the passenger but before TPP cleavage, i.e. with the N-terminus located outside of thylakoid membrane while the C-terminus is located inside the thylakoid lumen (Fig 3.3; for details see Berghöfer and Klösgen, 1999; Hou et al., 2006). Interestingly, protease treatment of transport reaction of the "train-like" precursors gave rise to three potential transport intermediates, named d14, d32 and d54, respectively (Fig 3.5 A). Similarly, like with

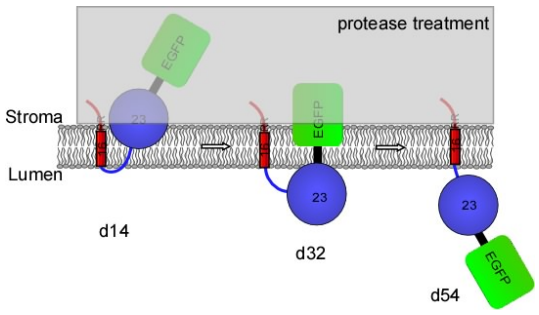

Fig 3.6: **A simplified schematic representation of d14, d32 and d54.** The shadow represents the stroma exposed part is degraded after protease treatment, while the membrane protected part forms d14, d32 and d54 respectively.

the 16/23 chimera, d14 of the "train-like" precursor stands for the loop insertion stage with both N- and C-termini protruding into the stromal side of thylakoid membrane, because its size is identical to that of Ti-1 of 16/23 (Fig 3.5, compare lanes 3 and 6; Fig 3.6). Potentially, d54 of the train-like protein corresponds to the stage when the whole 23-EGFP was transported into the thylakoid lumen, while TPP has not yet performed its function to release the mature 23-EGFP (Fig 3.6). This situation is identical to the Ti-2 stage of 16/23 chimera. Concerning d32, it possibly represents a stage between d14 and d54 during Tat transport (Fig 3.6), as its size is smaller than the mature protein but larger than d14 as well as Ti-2 of 16/23 chimera (Fig 3.5 A). This needs further characterization (see next). Beside the three potential transport intermediates, there is another degradation fragment formed (Fig 3.5, marked with star). However, this band is not a transport intermediate. Instead, it is a degradation band directly resulting from the protease treatment of the precursor (Fig 3.5, C).

To confirm that the observed respective degradation fragments are real transport intermediates from the 16/23-EGFP transport, three independent approaches have been used. Fig 3.7 shows the time course experiments which clearly demonstrate that with

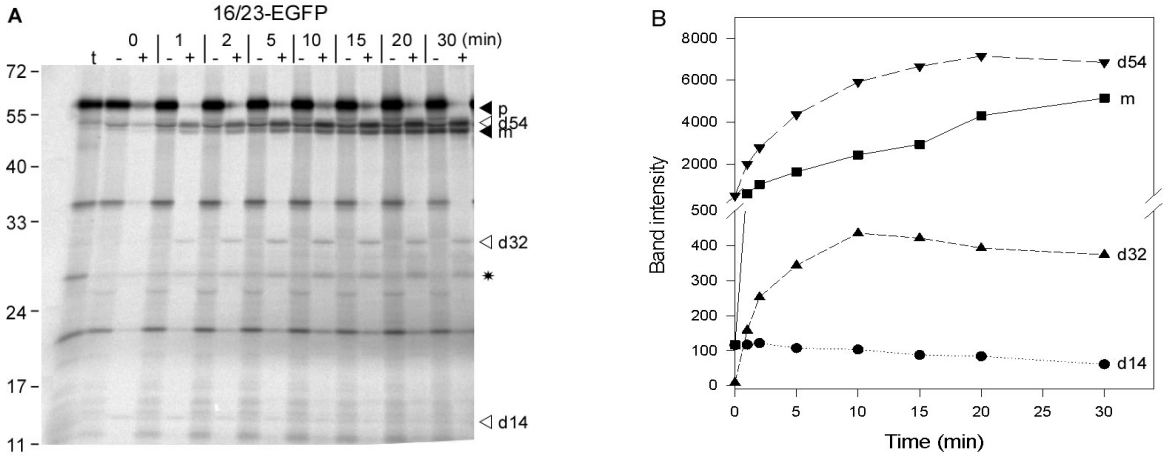

Fig 3.7: **Time course experiment of the 16/23-EGFP shows the kinetics of the three potential transport intermediates. A**, Import reactions were conducted for the incubation time as indicated above the figure, then treated as described in Fig 3.2 and analyzed by SDS-PAGE. **B**, The band intensities of the three potential transport intermediates as well as the mature protein shown in A were quantified to show the kinetics of the process. For further details, see the legends for Fig 3.1 and 3.2

increasing incubation time, the amount of d14 decreases gradually, while both d54 and mature 23-EGFP accumulate with time. In contrast, the amount of d32 first increases but after 10 minutes decreases substantially. These kinetic patterns indicate that d14 appears first even at 0 min, which is only shortly incubated on ice, then comes d32 and following d54. All of these transport intermediates finally decrease leading to the accumulation of mature 23-EGFP.

As the second strategy, the TPP cleavage site was mutated at position -1 from Ala to Leu, since it has been shown that TPP cleavage occurs at the site of Ala-X-Ala, and cleavage is abolished if -1 Ala was mutated to Leu (Shackleton and Robinson, 1991). This mutation should thus block the transport at the stage of d54, and as a result, the transport might be "frozen" at the stage of d32 as well. This effect could even saturate the Tat translocase. In contrast, because the formation of d14 is independent of Tat translocase, thus it should still be inserted substantially which should form even more d14. As shown in Fig 3.8, this Ala to Leu mutation indeed abolished the appearance of the mature 23-EGFP proving that the TPP cleavage was not anymore occurring. In contrast, the transport intermediates d32 and d54 are slightly increased in the TPP mutation (Fig 3.8, B for d32 and d54). This strongly suggests that a "traffic-jam" effect exists, because otherwise one can speculate that there should be no differences after

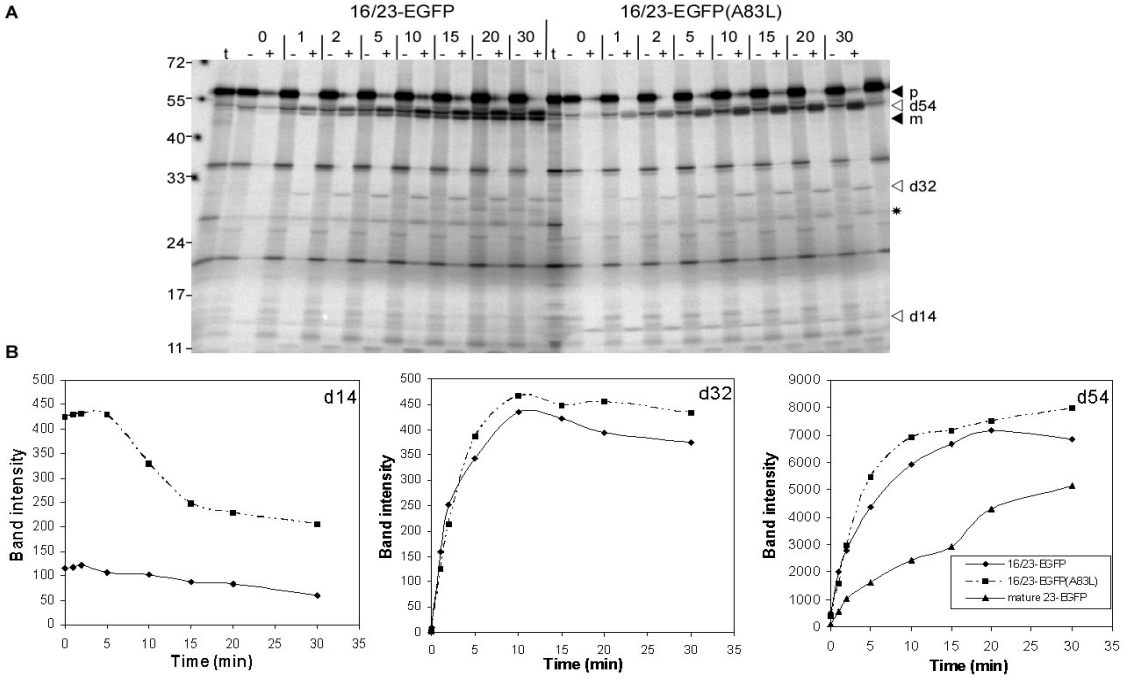

Fig 3.8: **Abolishment of TPP cleavage enhances the formation of transport intermediates. A**, *In vitro* translated 16/23-EGFP and 16/23-EGFP(A83L) were incubated at import conditions with isolated thylakoid vesicles for the time periods indicated above the lanes. **B**, The band intensity of the three potential transport intermediates as well as of the mature protein shown in A were quantified. For comparison between 16/23-EGFP and 16/23-EGFP(A83L), each transport intermediate was put in the same figure (d14, d32, d54 in Fig. B, respectively). The dash lines represent the 16/23-EGFP(A83L) and the solid lines represent the 16/23-EGFP. For details see text and legends of Fig 3.1 and 3.2.

TPP mutation if each transport step is independent (i.e. the three transport intermediates form independently). Interestingly, in the mutant d14 has a higher intensity when compared with the original "train-like" precursor under the same import reaction time (Fig 3.8, compare lanes "+" for d14 at each time point in A and in B for d14) which is in line with the proposed saturation effect. This observation further indicates that these three transport intermediates are formed in a consecutive manner, since the

final TPP cleavage was abolished and because of the "traffic jam" effect, thus leading to the accumulation of d14 at earlier steps. Another conclusion of this TPP cleavage site mutation result is obviously that TPP functions only after the Tat transport has successfully been finished. This correlates with the results of Frielingsdorf and Klösgen (2007). Furthermore, these authors provide also the evidence that the bitopic translocation intermediate Ti-2 of 16/23 is not necessarily associated with the Tat translocation pore, i.e. laterally released into the lipid bilayer, before TPP cleavage (Frielingsdorf and Klösgen, 2007). In line with this result, probably the biotopic translocation intermediate d54 of 16/23-EGFP is also laterally released into the lipid bilayer. As shown in Fig 3.8 B (quantification of d54 and d32), the amount of d54 of the 16/23-EGFP(A83L) is not significantly accumulated compared with 16/23-EGFP. If d54 is still associated with the active translocase until cleavage occurs, one would expect that the accumulation of d54 of 16/23(A83L) should be similar as of 16/23-EGFP. In contrast, if d54 is completely laterally released from translocase, significant amount accumulation of d54 of 16/23(A83L) will be expected. Slight accumulation of d54 of 16/23(A83L) indicates maybe only a small amount of the bitopic translocation intermediate d54 is laterally released into the lipid bilayer before TPP cleavage.

The third approach was a competition experiment. In such assays, *in vitro* translated "train-like" substrates were mixed with thylakoids in the presence of excess amounts of the over-expressed, non-radiolabled authentic OEC23 kDa precursor which was frequently used in such competition experiments (Berghöfer and Klösgen, 1999; Molik et al., 2001; Hou et al., 2006). The Tat translocase could be saturated by this authentic precursor, which should lead to a competition effect between the radiolabeled "train-

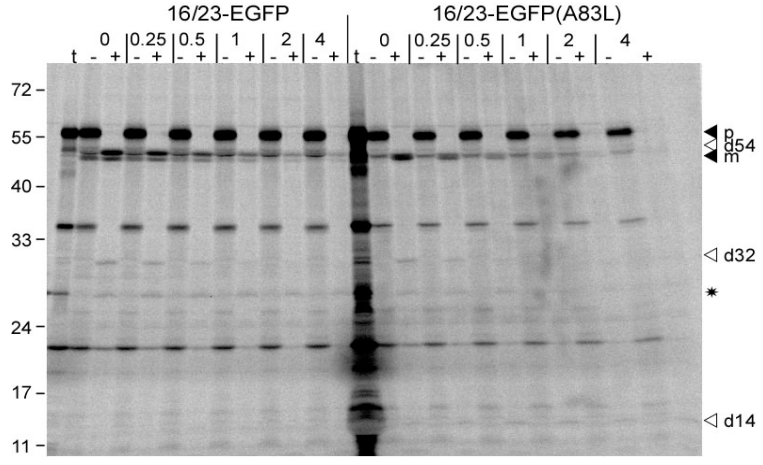

Fig 3.9: **Saturation of the Tat-dependent pathway affects the formation of the potential transport intermediates Ti-2 and Ti-3 as well as the accumulation of mature 23-EGFP.** Import experiments were performed in the presence of increasing amounts of *E. coli* overexpressed OEC23kDa at the concentration (μM) indicated above the lanes in each assay.

like" precursors and the overexpressed OEC23 kDa precursors. As a consequence, it can be expected that the amount of imported "train-like" substrate will be substantially decreased. Indeed it turned out that under such competition conditions, appearance of the transport intermediates as well as the mature 23-EGFP were impaired with increasing amount of overexpressed authentic Tat pathway protein (Fig 3.9). This further confirms that the observed bands are indeed transport intermediates resulting from the transport of the "train-like" precursor by Tat translocase.

3.2.3 d32 represents the "train-like" protein spanning the thylakoid membrane with mature EGFP located outside but mature 23 kDa located inside the thylakoid lumen

As discussed above, d14 and d54 of the "train-like" chimera represent the early and late steps of transport process, respectively. In case of d32, it is assumed that this transport intermediate represents an intermediate step between d14 and d54. Since its size (32 kDa) is even larger than the 16/23 precursor (31 kDa),it must contain some part from the mature EGFP. This consideration strongly indicates that part of mature EGFP

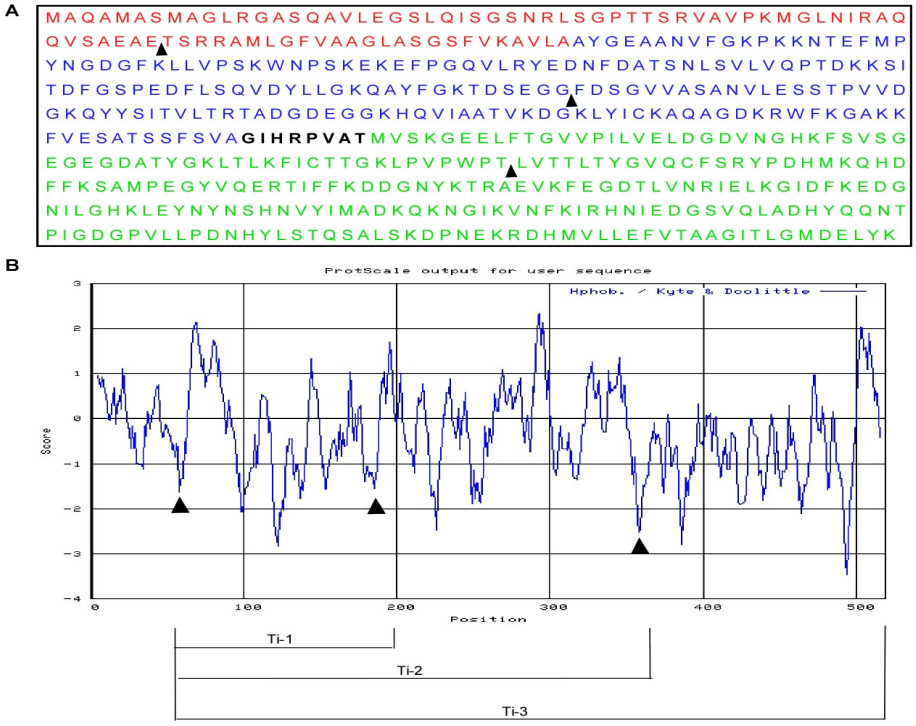

Fig 3.10: **Predicted thermolysin cleavage sites of 16/23-EGFP. A**, Amino acid sequence of 16/23-EGFP. The signal peptide of OEC 16 kDa protein is given in red, the mature OEC23 kDa in blue, the linker is in bold and black, and the mature EGFP in green. The predicted thermolysin cleavage sites important for the formation of three potential transport intermediates are shown with closed arrowheads. **B**, Hydropathicity of 16/23-EGFP was calculated by use of the program ProtScale (http://www.expasy.org/cgibin/ protscale.pl). The predicted thermolysin cleavage site for the formation of three potential transport intermediates are indicated by closed arrowheads and indicated at the bottom of the figure.

is protected by the thylakoid membrane. In other words, EGFP is very likely present in a membrane-spanning conformation. To get an idea about d32, first a detailed calculation of its size, based on the molecular marker as standard, using the computer program (Compute pI/Mw tool, http:/ca.expasy.orgtoolspi_tool.html) was carried out. The molecular weights of d14, d32, d54 were approximately 14 kDa, 32 kDa, 54 kDa and the predicted thermolysin cleavage sites based on these calculations are shown in Fig 3.10. Obviously the thermolysin cleavage site for the formation of d32 (from the C-terminus) is located inside the mature EGFP domain (Fig 3.10 A). This part is located in a hydrophilic valley of EGFP according to the hydrophobicity examination (Fig 3.10 B).

To examine the above results experimentally, immunoprecipitation experiments using anti-OEC23 kDa and EGFP specific antibodies was conducted (Fig 3.11). When anti-OEC23 kDa antibody was used, all three transport intermediates could be immunoprecipitated indicating that all of them contain the whole or at least part of the mature OEC23 kDa protein (Fig 3.11). In contrast, when anti-EGFP antibody was used,

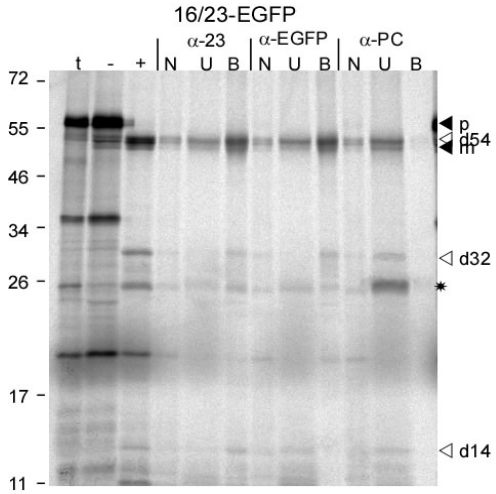

Fig 3.11: **Immunoprecipitation of three transport intermediates from 16/23-EGFP.** Radiolabelled 16/23-EGFP was incubated with pea thylakoids at 25 °C for 5 min. After import, thylakoids were reisolated and washed twice to remove unbound 16/23-EGFP proteins, and finally solubilized in buffer containing 1% Triton-X 100 and a protease inhibitor cocktail. After sedimentation of the nonsolubilized material (N), the solubilized thylakoids were incubated with 20 μg of purified IgGs. After incubation over night under 4 °C with agitation, protein A-Sepharose was added to a final concentration of 1% (w/v) for additional 1 hour agitation. After centrifugation, the supernatants (U, unbound material) and pellets (B, bound material) were recovered. The pellets were once washed with binding buffer and subsequently boiled in the presence of SDS-loading buffer. After centrifugation, the supernatant was loaded as bound material (B). Finally the samples were analyzed by SDS-PAGE and autoradiography.

as expected, d14 could not be immunoprecipitated, while both d54 and mature 23-EGFP could be immunoprecipitated. Most importantly, d32 was also precipitated by the EGFP antibody indicating that this transport intermediate contains at least part

from EGFP, which correlates with the prediction results discussed above. As a control, the immunoprecipitation experiments were performed using a PC-specific antibody. The results showed that neither of the transport intermediates nor the mature 23-EGFP could be immunoprecipitated which further confirms the specificity of immunoprecipitation results using OEC23 kDa and EGFP antibodies (Fig 3.11 and data not shown). Taken together, both calculation and experimental results suggest that d32 represents a membrane-spanning transport stage of the "train-like" chimera, in which the mature OEC23 kDa protein as well as the N-terminal part of EGFP are protected by the thylakoid membrane, while the C-terminal part of mature EGFP is still located outside of the thylakoid membrane that is still accessible to the externally added thermolysin.

3.2.4 Two high molecular weight Tat complexes can be identified by BN-PAGE.

Previous work has shown that two putative complexes of approximately 560 and 620 kDa are found by blue-native gel electrophoresis (BN-PAGE) containing the 16/23 chimera during thylakoid transport (Fig 3.12; Berghöfer and Klösgen, 1999; Frielingsdorf and Klösgen, 2007). This result is an indication of interaction between the transport substrates and the Tat transport machinery, presumably thylakoidal TatB/C comple-

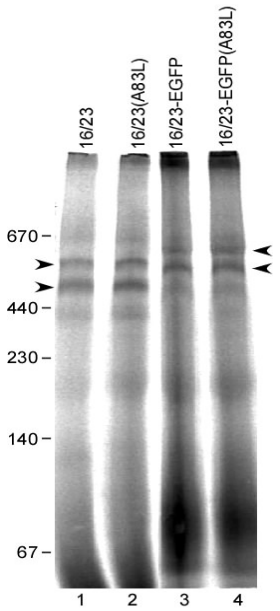

Fig 3.12: **Two high molecular weight complexes could be identified on BN-PAGE.** *In vitro* synthesised 16/23 and 16/23-EGFP chimera, as well as the corresponding TPP cleavage site mutatant 16/23-EGFP(A83L) proteins, were incubated for 15 min with isolated thylakoids under standard import conditions. After the import reaction, the membranes were washed twice and separated, after mild solubilisation with 1% digitonin, on a 5-13.5% blue native polyacrylamide gel as described in materials and methods. The arrows point to two putative Tat complexes to which the radiolabelled translocation intermediates of the 16/23 and 16/23-EGFP chimeras have bound.

xes (Cline and Mori, 2001). Since in 16/23-EGFP, an additional protein was attached to the C-terminus of the 16/23 chimera, it can be speculated that there must be a band shift on BN-PAGE when comparing the two complexes from 16/23-EGFP and 16/23, respectively. This, indeed, holds true, as shown in Fig 3.12 (compare lanes 1 and 3). Even though it is difficult to get a precise view of the exact molecular weight for these complexes from BN-PAGE, the molecular weights of the two complexes from the import reaction of 16/23-EGFP were deduced from the molecular masses of the molecular markers. The calculated molecular weights of the two complexes from the 16/23-EGFP chimera were roughly 610 and 644 kDa. Comparison of the complexes recovered from 16/23 (560 and 620 kDa) and 16/23-EGFP (610 and 644 kDa), the band shift for the upper complex corresponds approximately to the size of mature EGFP (27 kDa), while the lower band shift (∼50 kDa) corresponds approximately to two copies of EGFP (27 kDa). This suggests 1-2 copies of precursor per complex.

Furthermore, as shown in Fig 3.8, probably part of the biotopic translocation intermediate d54 of 16/23-EGFP and Ti-2 of 16/23 is laterally released into the lipid bilayer. The released d54 transport intermediates will migrate in the BN-PAGE faster, while only those intermediates which are still bound to the translocation pore will contribute to the intensity of the detectable signal on BN-PAGE. As a consequence, no differences for the formation of the two high molecular weight complexes have been observed between the authentic chimera and the mutated derivatives (3.12, compare lanes 1 and 2, 3 and 4).

3.2.5 Discussion of the Tat transport mechanism across the thylakoid membrane.

It is a general approach to attach a folded protein domain behind the transport substrate to generate a chimera which could block the transport process in order to identify transport intermediates for the characterization of the translocase (Vestweber and Schatz, 1988; Joly and Wickner, 1993; Schülke et al., 1997; Müller and Klösgen, 2005). Because of the ability of the Tat pathway to transport folded proteins across membranes, however, this approach was not successful in the case of Tat protein transport analysis (Clark and Theg, 1997; Hynds et al., 1998; Musser and Theg, 2000). Fortunately, due to the unique hydrophobicity and/or polarity pattern of the 16/23 chimera within the TPP cleavage site region (Frielingsdorf and Klösgen, 2007), the transport velocity of 16/23 was significantly reduced, thus allowing to identify transport intermediates (Berghöfer and Klösgen, 1999; Hou et al., 2006). On the basis of this unique chimera, a new “train-like” chimera has been constructed in the present work. With this new chimera, a new transport intermediate (d32) which spans the membrane could

be identified for the first time. It is tempting to speculate that this special membrane-spanning transport intermediate can provide a useful tool for the isolation of the Tat translocase as well as for further characterization of this unique transport machinery.

The time course experiment indicated that the three transport intermediates are sequentially formed. The competition experiment further confirmed that they are really formed during the Tat transport process. Furthermore, sequential formation of the respective transport intermediates is further evidenced from the analysis of the TPP cleavage site mutatant 16/23-EGFP(A83L). The situation of stopped transport at the stage of the mature protein located on the lumenal side of the thylakoid membrane but before the TPP cleavage is not surprising since some of the membrane anchored proteins like the Rieske protein is positioned in the membrane in such a configuration (Karnauchov et al., 1997; Molik et al., 2001).

Because some spontaneously inserting thylakoid proteins, like CFo-II or PsbW, also carry cleavable signal peptides but do not depend on either Sec or Tat translocase for membrane transport, it seems that the thylakoidal processing peptidase (TPP) is not associated with protein transport machinery but instead floating freely within the membrane bilayer, i.e. probably TPP is not a component of the Tat transport machinery (Frielingsdorf and Klösgen, 2007). In other words, it indicates that the bitopic protein (i.e. d54) is not necessarily associated with the Tat translocase for cleavage by the thylakoidal processing peptidase. Instead, they might be released from the membrane complex prior to terminal processing. This data are in line with the finding of Frielingsdorf and Klösgen (2007) who also demonstrates that TPP cleavage can take place independently of Tat transport. Together, it is very likely that most, if not all, Tat substrates, after being successfully transported into the thylakoid lumen, are released laterally into the lipid bilayer. If the protein functions in the membrane, it will remain anchored in the thylakoid membrane without further TPP cleavage, while for those proteins that have to function in the thylakoid lumen, a TPP cleavage event is required to release the mature proteins into the thylakoid lumen (Molik, 2005).

The phenomenon of transporting two mature proteins by use of a single transit peptide as described in the present work seems not be restricted to the thylakoidal Tat translocase, because a similar finding has been reported recently in bacteria (Fisher et al., 2006). Even though the aim of these authors was different from the work presented here, the strategy used by Fisher et al. was very similar. In their study, series of tripartite fusion proteins were constructed in which one ssTorA Tat signal peptide was used for directing the transport of different target proteins followed by the TEM1-βlactamase protein (Fig 3.13). In each case, the chimeric protein was successfully exported across

the bacterial plasma membrane and obtained its native structure as proven by the functional activity of TEM1-βlactamase. Considering the evolutionary conservation of the Tat system in bacteria and thylakoids, it is reasonable to assume that all of these

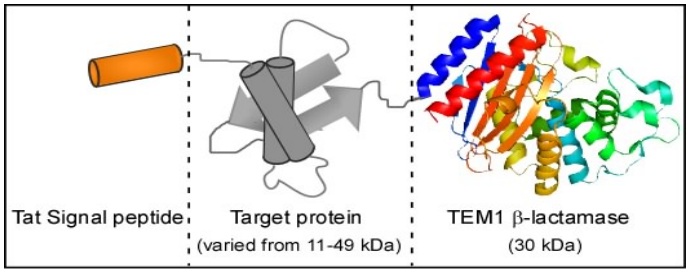

Fig 3.13: **The schematic representation of the tripartite proteins constructed by Fisher et al. (2006).** According to Fisher et al., 2006.

constructs are transported by bacterial Tat translocase also in a step-by-step manner as described in the present work (Fig 3.14). Taken together, these results suggest that it should be possible to fuse a biomedically interesting protein behind the Tat transport substrate and take advantage of translocating folded proteins by Tat system to get fusion proteins for biotechnology purposes.

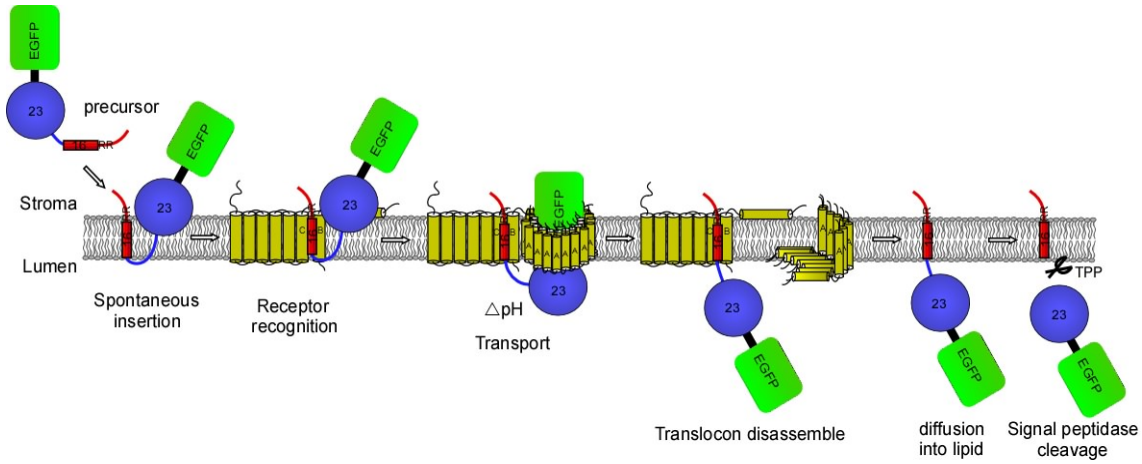

Fig 3.14: **Working model for the transport of the "train-like" chimeric protein.** The Tat-dependent translocation process of 16/23-EGFP can be dissected into the following steps: 1, Unassisted or spontaneous loop insertion of precursor protein into the thylakoid membrane (d14). 2, Recognition by the TatBC receptor complex (d14). 3, Oligomerization of TatA to form the translocon and translocation of mature 23-EGFP in a step-by-step manner (d32). 4, Translocon disassembly and diffusion of the precursor into the lipid phase (d54). 5, TPP cleavage and release of the mature protein. For further details see text.

Results from many labs have suggested that TatB and TatC form the receptor complex, while TatA forms a transient translocase only on requirement (Müller and Klösgen, 2005). All the previous work to characterize the Tat translocase led to the speculation that Tat translocase is a dynamic transport machinery. This speculation is further supported in the present work by the identification of the membrane-spanning transport intermediate (d32), which suggests that the two mature 23 kDa and EGFP proteins are

transported in a "step-by-step" manner (Fig 3.14). Even though the size of these two mature proteins is not significantly different, it is obvious that their 3D structure and shape are divergent. Remarkably, the size differences are more obvious in the tripartite chimeras constructed by Fisher et al. (2006) (Fig 3.13): the sizes of the target proteins (in total 12 different proteins) that followed the Tat signal peptide varied from 11 kDa to 49 kDa, while the C-terminal protein TEM1-βlactamase had always the molecular weight of 30 kDa. Considering that the two differently shaped mature proteins of the "train-like" precursor must pass through the same Tat translocase (Fig 3.14), it appears reasonable to assume that the Tat translocase could dynamically change its pore size to accommodate transport substrates with variable sizes.

Many working models regarding the membrane transport of the Tat pathway have postulated that the Tat translocation pores are composed predominantly or even exclusively of TatA (Mori and Cline, 2002; Gohlke et al., 2005; Dabney-Smith et al., 2006; Sargent et al., 2006). This suggests that the number of TatA should be present in the membranes in excess amounts. However, this is not always the case when compared in *Arabidopsis thaliana*, *Pisum sativum*, as well as in *E. coli* and significant discrepancies have been observed (M. Jacob et al., submitted). This observation argues against the TatA function as main constituent of Tat translocation pores. Just recently, another suggestion that TatA facilitates transport by "weakening"the membrane in a yet unknown manner has been postulated (Natale et al., 2007). Such weakening might enable the substrates to pass the lipid phase either directly or assisted by a conformational change of the TatB/C receptor complex. At present it is hard to say which hypothesis is more suitable to explain how the "train-like" protein is transported. Furthermore, the band shift of the upper complex on BN-PAGE of 16/23-EGFP in comparison to the complex of 16/23 indicates that this shift could be attributed to another component except the mature EGFP. However what is this unknown component and what is its function are not clear at this moment.

3.3 Analysis of the fate of the Tat signal peptides

Transit peptides play a key role in mediating membrane insertion, receptor recognition and interaction of the precursors with translocation apparatus during protein transport. However, after transport and release from the precursors, these small amino acid sequences have been shown can be severely harmful for the structural and functional integrity of the membranes as tested with both natural and artificial lipid bilayers (Zardeneta and Horowitz, 1992; Maduke and Roise, 1993; Wieprecht et al., 2000). Several groups have reported that these peptides can result in membrane lysis, uncoupling of respiration, and dissipation of the mitochondrial membrane potential, possibly by

forming pores or inducing channels (Glaser and Cumsky, 1990; Nicolay et al., 1994; Matsuzaki et al., 1996; Lu and Beavis, 1997). Thus, to avoid potentially harmful effects of these free peptides, either fast degradation processes or export mechanism are urgently required for removing these signal peptides after release from precursor proteins.

In human ER membranes and *E. coli* inner membranes, the released signal peptides are cleaved rapidly into subfragments for further degradation (Novak and Dev, 1988; Lyko et al., 1995; Weihofen et al., 2002) to avoid their harmful effects. The fate of the Tat signal peptides, after release by TPP, however, is totally unknown to date. To analyze the fate of Tat signal peptides, a series of chimeric proteins were constructed. The results show for the first time, that these small peptides are indeed further cleaved into subfragments after transport. TPP cleavage is even a prerequisite for the subsequent cleavage events. Furthermore, by testing different types of protease inhibitors, it could be concluded that a metalloprotease is involved. Several preliminary analyzes about the determinants affecting the cleavage event were additionally performed.

3.3.1 Construction of a "tandem-substrate" for analyzing the fate of the Tat signal peptide

Generally, the analysis of the fate of the released signal peptides and of the putative cleavage products are complicated by the following facts: (i) there are few methionine or cysteine residues located within the signal peptides which complicates the efficient radio-labelling for their detection; (ii) the signal peptides are too small to be distinguished from those small peptides that are produced upon *in vitro* translation as a result of premature chain termination; (iii) small peptides can be only separated in specific modified gel systems; (iv) probably the most important reason is that the signal peptides are only transiently stable and possibly undergo a fast turnover process that makes it not easy to detect the fragments derived from cleavage reactions.

To overcome the above mentioned difficulties for the analysis of what happens to the Tat signal peptide after TPP cleavage, a "tandem-substrate", 16/23-16_{LTD}/EGFP (Fig 3.15 A), was constructed. In this chimera, the two chimeric proteins 16/23 and 16_{LTD}/EGFP (both chimera have been thoroughly used in the lab for Tat analysis) were ligated in a sequential order. Because ① the transit peptide of OEC16 kDa protein contains two parts: the stroma targeting domain (STD) which is responsible for the translocation across the Toc and Tic complexes, and the thylakoid lumenal targeting domain (LTD) which is responsible for the translocation across the thylakoid membrane; ② during the transport process, the STD will be removed in the stroma and only LTD remains for mediating further Tat transport (see Fig 1.2), thus in the "tandem-substrate", the

first transport signal is the full length transit peptide of OEC16 kDa protein, while the second signal contains solely the LTD, (hereafter named 16_{LTD}). This mimics the real situation in Tat protein transport.

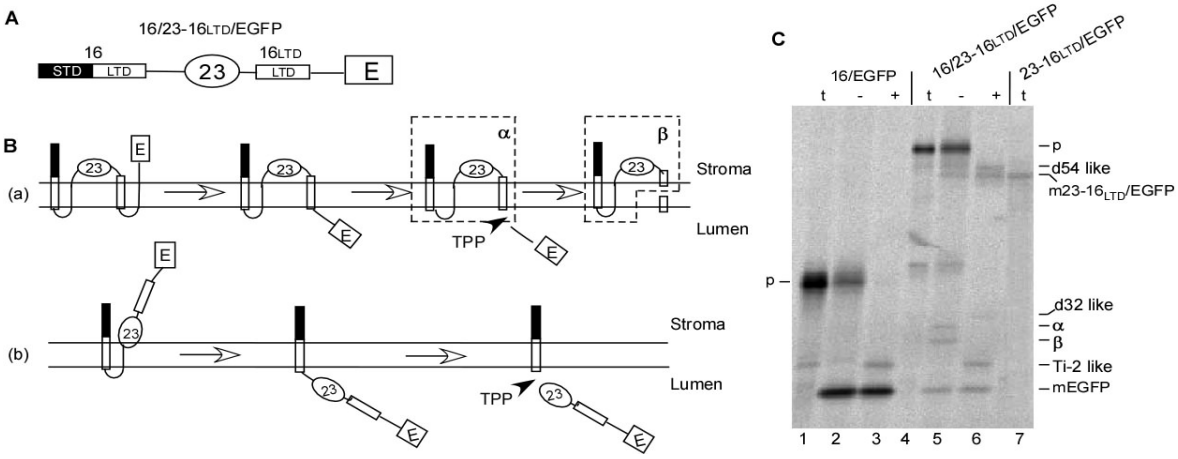

Fig 3.15: ***In thylakoido* transport of the "tandem-substrate".** **A**, Schematic representation of the "tandem-substrate". The STD of the transit peptide of OEC16 kDa protein is given in black and LTD in open square, respectively. 23 means the mature part of OEC23 kDa protein and E means mature EGFP. **B**, Due to the presence of two transport signals within the "tandem-substrate", two potential transport possibilities exist: (a), the internal signal peptide (16_{LTD}) mediates the transport of mature EGFP. After TPP cleavage, EGFP is released while the 16/23-16_{LTD} part is probably hooked into the membrane (α); β represents a hypothesized event related to the 16_{LTD}. (b) first signal peptide (16) mediates the transport of the whole protein, 23-16_{LTD}/EGFP, as illustrated like the train-like chimera. **C**, Incubation of 16/23-16_{LTD}/EGFP with isolated thylakoid vesicles generates two mature proteins: ①mature EGFP, which is identical to the result from the import reaction of 16/EGFP (compare lanes 2, 3 and 5, 6 for mEGFP). "Ti-2 like" represents the transport intermediate of 16/EGFP. This Ti corresponds to the Ti-2 of 16/23 (for 16/EGFP, see Marques et al., 2003, 2004); ② mature 23-16_{LTD}/EGFP, which is identical to the *in vitro* translated 23-16_{LTD}/*EGFP*. "d54 like" and "d32 like" represent two transport intermediates corresponding to d54 and d32 of 16/23-EGFP (see Fig 3.5). α and β represent two hypothesized products as illustrate in B, (a) panel marked with dash line. For further details see text the legends to Fig 3.5.

As illustrated in Fig 3.15, a panel, the internal signal peptide (16_{LTD}) within the "tandem-substrate" should be able to mediate the transport of EGFP because it has been shown that extension at the N-terminus of Tat signal peptide has no effect on its function (Fincher et al., 1998; Berghöfer et al., 1999; Hou et al., 2006; Gould et al., 2007). After TPP cleavage, 16_{LTD} should still bind to the C-terminus of 16/23 thus forming 16/23-16_{LTD} (α). As a result of the loop insertion mechanism of Tat signal peptides (Fincher et al., 1998), 16/23-16_{LTD} must be located outside of the membrane. Furthermore, because Tat signal peptides can spontaneously insert into the thylakoid membrane (Hou et al., 2006), probably 16/23-16_{LTD} is still bound to the the thylakoid membrane by use of the first signal peptide. In this way, the events occurring to the 16_{LTD} part might be easily detectable using 16/23 as a reporter protein (Fig 3.15, B, a panel).

As shown in Fig 3.15 C, incubation of the *in vitro* translated chimeric "tandem-substrate" of 16/23-16$_{LTD}$/EGFP with isolated thylakoids under standard import conditions generates a band corresponding to mature EGFP as its size is identical to the mature EGFP resulting from the import assay of 16/EGFP (Fig 3.15 C, lanes 2, 3 and 5, 6). This indicates that in this case the internal signal peptide is functional and mediates the transport of EGFP, as is the case for 16/EGFP. In addition, a band (Fig 3.15 C, Ti-2-like, lanes 3 and 6) which corresponds to the stage of Ti-2 of the 16/23 chimera is observed with both, 16/EGFP and 16/23-16$_{LTD}$/EGFP (Marques et al., 2003, 2004). The appearance of this transport intermediate further indicates the identity for the transport of both chimera regardless of the N-terminal extension located in front of the internal 16$_{LTD}$ signal peptide within the "tandem-substrate". More interestingly, two additional bands (Fig 3.15 C, lane 5) appear, named band α and β. Among them, the band α corresponds well in size (∼33 kDa) with the expected band 16/23-16$_{LTD}$ hypothesized in Fig 3.15 B, a panel. This indicates that band α probably represents the situation that after TPP cleavage, the 16$_{LTD}$ part remains bound to the C-terminus of the 16/23 chimera as suggested in Fig 3.15 B a panel (see also next). Band β probably correlates with an event as illustrated in Fig 3.15 B (a) panel and needs further characterization. Furthermore, externally added proteases remove both bands suggesting that band α and β must be accessible from the outside of the thylakoid membranes as suggested above.

The above results are based on the internal signal peptide mediated protein transport. However, because of the existence of the first signal peptide within the "tandem-substrate", it is also reasonable that the N-terminal signal peptide mediates the transport process thus leading to the whole substrate, 23-16$_{LTD}$/EGFP, being transported. This is indeed the case as shown in Fig 3.15, C, as the mature 23-16$_{LTD}$/EGFP shows the expected electrophoretic mobility on SDS-PAGE as was confirmed by comparison of the *in vitro* translated 23-16$_{LTD}$/EGFP (Fig 3.15 C, lanes 5, 6 and 7). This indicates that also the first transit peptide is functional and can mediate the transport of 23-16$_{LTD}$/EGFP as an entity. In this situation the internal signal peptide has no targeting function but operates only as a linker peptide, corresponding to the linker in the "train-like" chimera 16/23-EGFP. After protease treatment, the presence of the d54- and d32-like transport intermediates further confirm this scenario of transport like the "train-like" chimera (Fig 3.15 B, b panel and C).

In both cases, the mature proteins are resistant to the externally added protease which is a characteristic of thylakoid lumen localization. The existence of the transport intermediates further confirms successful transport taking place by both transport signals (Fig 3.15 B). However, it is not clear how Tat translocase deals with the "tandem-

substrate" in two ways and further investigations are required.

3.3.2 Band α and β contain the mature 23 part.

If the formation of band α and β indeed works as hypothesized in Fig 3.15 B, a panel, they must both contain the mature OEC23 kDa protein. As a consequence, these two bands should be detectable by OEC23 kDa specific antibodies. To test this idea, immunoprecipitation experiments were performed using OEC23 kDa protein specific antibodies. As shown in Fig 3.16 C, both bands can be recognized and precipitated by

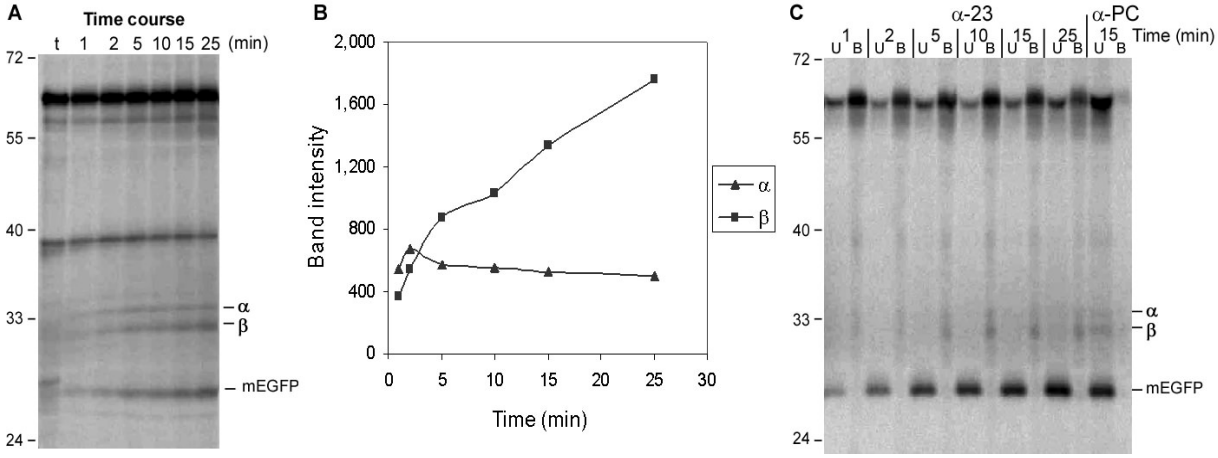

Fig 3.16: **Time course and immunoprecipitation experiments.** *In vitro* synthesized "tandem-substrate" 16/23-16$_{LTD}$/EGFP was incubated with thylakoid vesicles under standard import conditions for the time period indicated above the lanes. After washing twice with HM buffer, aliqots of the sample were subjected directly to SDS-PAGE without further treatment (**A**). The band intensities of α and β in A were quantified to show the kinetics of the two band formation (**B**). The other aliquot was subjected to the immunoprecipitation experiment using anti-23 kDa antibodies (**C**). U, unbound material; B, bound material. For further details see the legend to Fig 3.11

OEC23 kDa protein specific antibodies, while mature EGFP cannot be precipitated as expected. Additionally, in the control assay, both bands can not be recognized by PC specific antibody which further confirms the specificity of the precipitation experiment. Furthermore, this result strongly indicates that these two bands are formed as supposed in Fig 3.15 B, a panel.

The time course experiment shown in Fig 3.16 A demonstrates that the formation of bands α and β occurs in a time-dependent manner. With increasing time, band α first slightly increases but finally substantially decreases (Fig 3.16 B). In contrast, band β constantly accumulates with time. Together, these results suggest that band α forms at earlier time points and is then processed to band β.

3.3.3 Formation of band α and β depends on the internal signal peptide mediated transport

The results shown above are in line with the transport model depicted in Fig 3.15 B, a panel. However, because of the two transport possibilities, further analysis for the formation of band α and β are required. Since Tat transport depends strictly on the presence of the RR-motif in its signal peptide (Chaddock et al., 1995; Stanley et al., 2000; DeLisa et al., 2002; Ize et al., 2002), the RR-motifs of either the first signal peptide or the internal signal peptide (16_{LTD}) were mutated to KK (Fig 3.17 A). Analyzes

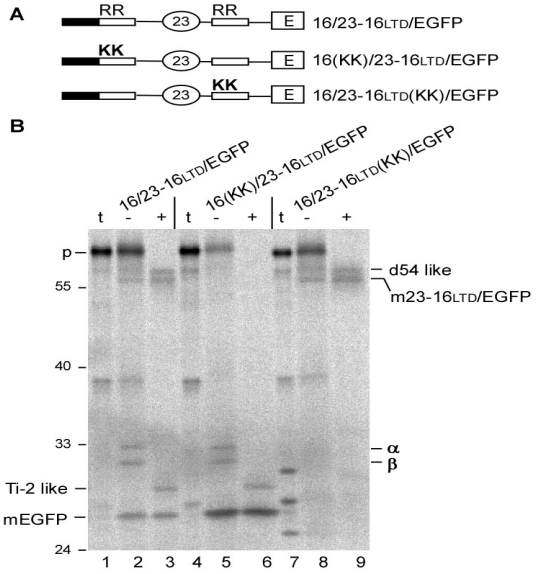

Fig 3.17: **Formation of band α and β depends on transport mediated by the internal signal peptide. A**, Schematic representation of 16/23-16_{LTD}/EGFP and its derivatives. The mutated amino acids of RR to KK are in bold. **B**, *In vitro* synthesized 16/23-16_{LTD}/EGFP as well as the derivatives were incubated with thylakoid vesicles under standard import conditions for 15 min, then were treated as described in Fig 3.1 and analyzed by SDS-PAGE. For further details, see text.

of these mutants by *in thylakoido* assays showed that if RR was mutated in the first signal peptide, the appearance of mature 23-16_{LTD}/EGFP as well as of the d54 like transport intermediate were abolished (Fig 3.17 B, lane 6). However, this mutation has no effect on the transport mediated by the internal signal peptide because mature EGFP still accumulates inside the thylakoid lumen and the corresponding Ti-2 like transport intermediate is still present too (Fig 3.17 B, lane 6). Most interestingly, the RR to KK mutation in the first signal peptide has no effect on the appearance of band α and β suggesting that transport by the first signal peptide is not responsible for their formation.

In contrast, RR to KK mutation of the internal signal peptide (Fig 3.17 A) results in a block of EGFP transport as well as in the formation of its corresponding Ti-2 like transport intermediate, while the appearance of mature 23-16_{LTD}/EGFP and its

corresponding d54 like transport intermediate remains unchanged (Fig 3.17 B, lane 9). Furthermore, the appearance of bands α and β is completely abolished (Fig 3.17 B, lane 8), demonstrating that formation of bands α and β is correlated with Tat transport mediated by the internal signal peptide.

3.3.4 Formation of band α and β depends on the TPP cleavage of the internal signal peptide

In case of band β, there are at least two possibilities (Fig 3.18 A): (i), after transport, the 16/23-16$_{LTD}$ is further processed by TPP which could have access to the cleavage site of the first signal peptide (Michl et al., 1994) thus generating a fragment "23-16$_{LTD}$" which might represent band β (possibility I); (ii), after transport of EGFP and TPP cleavage at the internal signal peptide (16$_{LTD}$), this internal signal peptide is further cleaved into subfragments. This cleavage generates band β (possibility II).

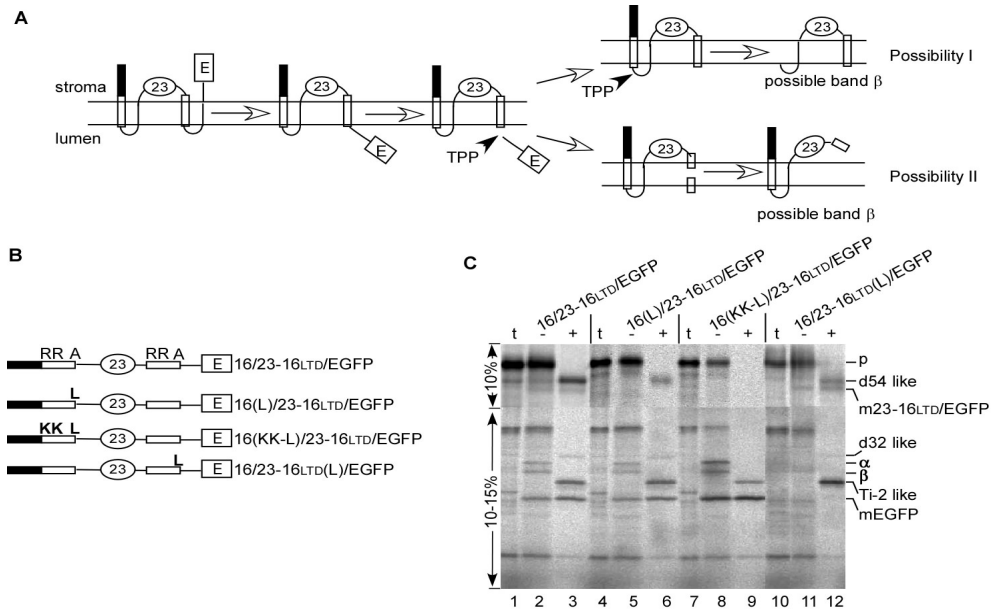

Fig 3.18: **Formation of band α and β depends on TPP cleavage of the internal signal peptide. A**, After TPP cleavage of the internal signal peptide, there are two possibilities for the formation of band β: either from TPP cleavage of the first signal peptide (possibility I) or from a further cleavage of the internal signal peptide (possibility II). **B**, The "tandem-substrate" derivatives of the TPP cleavage site mutation. The mutated cleavage sites are shown in bold. **C**, *In vitro* synthesized 16/23-16$_{LTD}$/EGFP as well as the TPP cleavage site mutated derivatives were incubated with thylakoid vesicles under standard import conditions at 15 min and analyzed by SDS-PAGE (marked on the left). For further details see text.

To distinguish between these two possibilities, a series of TPP cleavage site mutations within the parent "tandem-substrate" was performed. These mutations were either in the first or in the internal signal peptide or in both (Fig 3.18 B) subsequently analyzed by import experiments. It turned out that when the TPP cleavage site of the first signal peptide was mutated, no mature 23-16$_{LTD}$/EGFP could accumulate in the thylakoid

lumen. In contrast, the d54-like transport intermediate is still visible (Fig 3.18 C, lane 6). This indicates that transport mediated by the first signal peptide still works but TPP cleavage is abolished. Interestingly, in this case the formation of band α as well as band β is not affected (Fig 3.18 C, lane 5) which demonstrates that the formation of these two bands is independent from the TPP cleavage of the first signal peptide which excludes possibility I in Fig 3.18 A. This conclusion is further confirmed by the result of double mutation experiments, in which RR-motif was mutated to KK-version in order to abolish the first signal peptide mediated thylakoid import and TPP cleavage. In this case, no transport takes place by the first signal peptide but still bands α and β are present (Fig 3.18 C, lanes 8 and 9).

In contrast, TPP cleavage site mutation within the internal signal peptide from Ala to Leu at -1 position abolishes as expected the appearance of mature EGFP (Fig 3.18 C, lanes 11 and 12). The existence of Ti-2 like transport intermediate (Fig 3.18 C, lane 12) after protease treatment demonstrates, however, that the transport still occurs. While this mutation has no effect on the first signal peptide mediated Tat transport (Fig 3.18 C, lanes 11 and 12), the formation of band α and β are abolished as a consequence of this mutation (Fig 3.18 C, lane 11). This proves that the formation of band α and β depends on the TPP cleavage of the internal signal peptide. This conclusion was further confirmed by using an *in vitro* translated 23-16$_{LTD}$ as a reference protein. It turned out that band β and 23-16$_{LTD}$ have different electrophoretic mobilities upon SDS-PAGE (see Fig 3.24 A, compare lanes 2 and 4). Furthermore, this result suggested that TPP cleavage of the internal signal peptide is a prerequisite for the formation of band α and β. Taken together, the hypothesis shown in Fig 3.18 A, possibility II is responsible for the formation of band β. All these data point to the conclusion that band β is very likely a subfragment from the cleavage of the 16$_{LTD}$ signal peptide as hypothesized in Fig 3.15 B, a panel.

3.3.5 Tat signal peptides are cleaved into subfragments

As shown above, band α probably represents the internal signal peptide bound to the C-terminal end of 16/23 chimera after TPP cleavage. To provide direct evidence, a reference protein, 16/23-16$_{LTD}$ (Fig 3.19 A) was produced. Consequent experiments on SDS-PAGE showed that band α and the reference protein 16/23-16$_{LTD}$ are identical in size (Fig 3.19 B, compare band α in lane 2 with lane 3) therefore confirming the hypothesis shown in Fig 3.15 B, a panel.

The formation of band α results from the TPP cleavage at the internal signal peptide, which is a prerequisite for the formation of band β. The finding that a single site

mutation in the internal 16_{LTD}-signal peptide prevents the formation of both band α and β and the fact that only a single band of the mEGFP is detected (Fig 3.15 C) excludes that the later part, 16_{LTD}/EGFP, of the tandem-substrate contains a second TPP-cleavage site that becomes recognized by the protease. Since the size of band α corresponds to the expected 16/23-16_{LTD} intermediate, it can be concluded that band α is the original TPP cleavage product and that band β results from further cleavage of band α. Thus, formation of band β from α should be a separate process that occurs independently from the TPP-catalyzed release of band α. In order to examine this possibility, the reference protein 16/23-16_{LTD} was directly used as a substrate for cleavage (Fig 3.19 B).

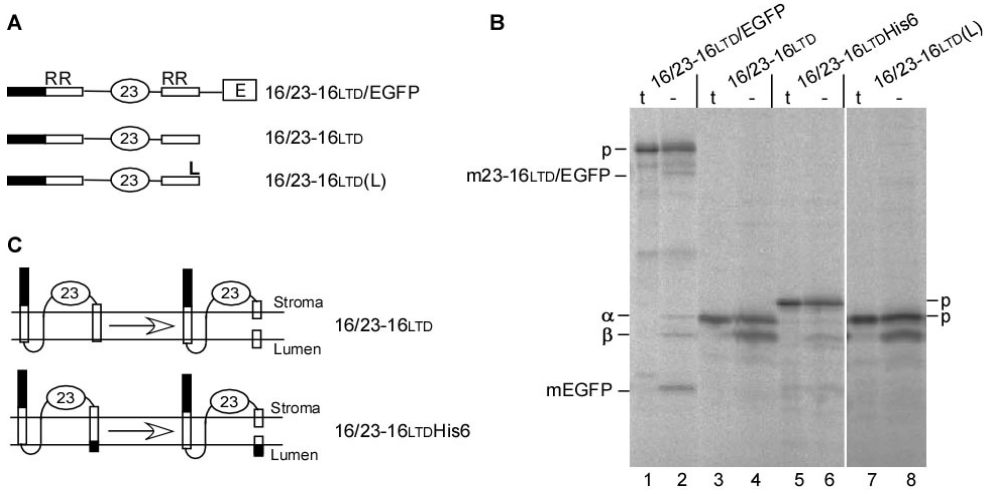

Fig 3.19: **Band α and β result from the cleavage events related to the 16_{LTD} signal peptide. A**, Schematic representation of 16/23-16_{LTD} and its derivative in which the the last amino acid Ala was mutated to Leu inside the second signal peptide. **B**, *In vitro* synthesized 16/23-16_{LTD}/EGFP and 16/23-16_{LTD} are incubated with thylakoid vesicles under standard import conditions for 15 min, then analyzed by SDS-PAGE without further treatment. And this process is explained in **C**.

After incubation of *in vitro* translated 16/23-16_{LTD} with isolated thylakoids, a band appears which corresponds in size to band β on SDS-PAGE (Fig 3.19 B, compare lanes 2 and 4). This identical cleavage pattern is expected because if the 16/23-16_{LTD} also spontaneously inserts into the membrane and the 16_{LTD}-part adopts a transmembrane orientation similar (or identical) to that formed in the course of EGFP-transport and TPP catalyzed cleavage of the "tandem-substrate". Furthermore, the signal intensity of band β generated by 16/23-16_{LTD} cleavage is even higher than that produced with the "tandem-substrate". This is reasonable if a sequential mode of processing, catalyzed by TPP-cleavage as a first step and a second cleavage step by another protease, is assumed. Using the "tandem-substrate", formation of band β depends on successful transport of EGFP, which might be the rate-limiting step, and TPP cleavage of the internal signal peptide. Afterwards, the free internal signal peptide is available for further cleavage. In contrast, reference protein 16/23-16_{LTD} directly can be used as a substrate for the

cleavage event after spontaneous insertion of 16_{LTD} into the membrane (see next).

In order to further confirm that band β results from cleavage of the 16_{LTD} signal peptide, a derivative of the 16/23-16_{LTD} was generated carrying a carboxy-terminal hexahistidine tag. As expected, the mobility of the *in vitro* translated precursor of 16/23-16_{LTD}-His6 on SDS-PAGE is higher than that from 16/23-16_{LTD} (Fig 3.19 B). The difference corresponds well to the size of the His6-tag. If cleavage occurs at the C-terminal tagged site of the precursor, i.e. inside the 16_{LTD} signal peptide, the cleavage product should be identical to band β (Fig 3.19 C), if the cleavage occurs at the first signal peptide, a size shift of the cleavage product, similar to that of the substrates, will be expected. After incubation of 16/23-16_{LTD} and 16/23-16_{LTD}-His6 with isolated thylakoids, cleavage bands appeared which are identical in size (Fig 3.19 B, compare lanes 4 and 6). This demonstrates that band β indeed represents a cleavage fragment resulting from removal of the C-terminal part of the 16_{LTD} signal peptide.

Additionally, the His-tag experiment also shows that possibly the length (and/or the amino acid composition) of the attached sequence influences the efficiency of Tat signal peptide cleavage (see also Fig 3.27). Only 10% of the His-tagged precursor was cleaved after 10 min incubation with isolated thylakoids, compared to about 48% cleavage of the 16/23-16_{LTD} substrate (Fig 3.19 B, compare lanes 4 and 6).

Cleavage of the internal signal peptide normally depends on the preceding transport of the passenger protein and its cleavage by TPP. Mutation of the TPP-cleavage site still allows transport to take place but prevents EGFP release and thus also the following signal peptide cleavage. The latter was assumed to be an indirect consequence of the prevented TPP-cleavage, because a single site mutation several amino acid residues apart from the mutagenesis site should not influence a proteolytic step taking place. In order to examine this hypothesis, the same single site mutation was introduced into the 16/23-16_{LTD}. In this new derivative, the terminal Ala residue was mutated to Leu forming 16/23-16_{LTD}(L) (Fig 3.19 A). After incubation with isolated thylakoids the same cleavage products as described for 16/23-16_{LTD} and the "tandem-substrate" (band β) were found (Fig 3.19 B, compare lanes 2, 4 and 8) although the efficiency decreased slightly. This observation demonstrates that the amino acid residue occupying the TPP cleavage site is not important for further cleavage of Tat signal peptide.

3.3.6 The first signal peptide is important for the analysis

Because of its uniquely retarded transport behavior (Berghöfer and Klösgen, 1999; Hou et al., 2006; Frielingstof and Klösgen, 2007), the 16/23 chimera contributes significantly

to the convenience for the analysis of the fate of Tat signal peptide by use of the "tandem-substrate" as shown above. If the 16/23 part was replaced by the authentic OEC 23 kDa protein, most of the precursor (87%) will be transported as an entity (i.e. 23-16$_{LTD}$/EGFP), as confirmed by *in vitro* translated 23-16$_{LTD}$/EGFP as a reference protein (Fig 3.20, compare lanes 7 and 8), while only 13% percent are transported by the internal signal peptide (Fig 3.20). This makes it difficult to detect the subfragments described for the 16/23-16$_{LTD}$/EGFP, although a faint band corresponding to band β does exist (Fig 3.20, lane 7).

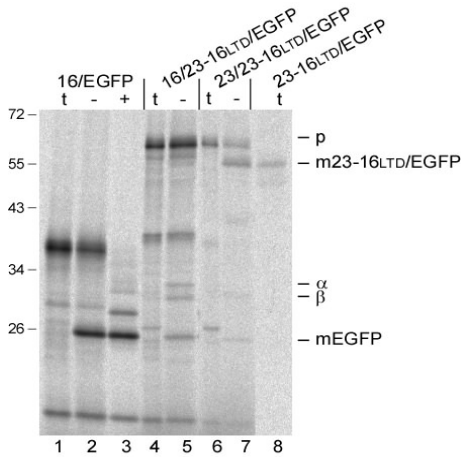

Fig 3.20: ***In thylakoido* transport of the 23/23-16$_{LTD}$/EGFP chimera.** *In vitro* translated 23/23-16$_{LTD}$/EGFP was incubated with isolated thylakoid vesicles for 10 min and treated as described in Fig 3.1. The *in vitro* translated 23-16$_{LTD}$/EGFP was used as a control. For details see text.

On the other hand, without the first signal peptide (i.e. like in the reference protein 23-16$_{LTD}$/EGFP used in Fig 3.15 C and Fig 3.21 A), the results showed that expected

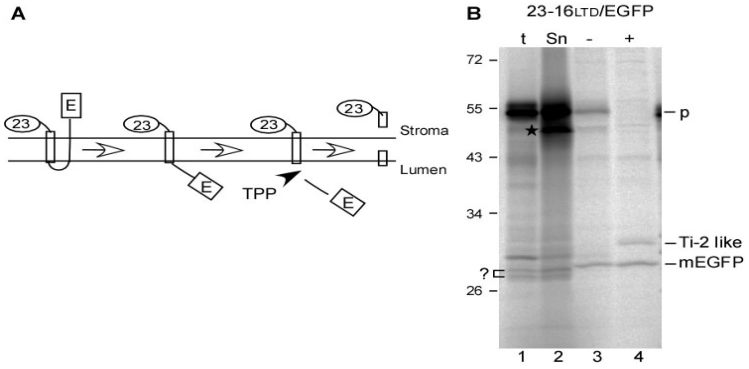

Fig 3.21: ***In thylakoido* transport of the 23-16$_{LTD}$/*EGFP* chimera. A**, Schematic representation of the events during the transport of 23-16$_{LTD}$/EGFP. **B**, After incubation of *in vitro* translated 23-16$_{LTD}$/EGFP with isolated thylakoid vesicles for 15 min under standard import conditions, the reaction mixture was centrifuged for 4 min at 10,000 rpm, then the supernatant (Sn) was loaded on SDS-gel. After washing with import buffer two times, the sample was treated as described in Fig 3.1 and analyzed by SDS-PAGE as well as phosphor-imaging visualization. The unknown band in Sn (star) and the two putative bands correlating with band α and β are indicated with a question mark. For further details, see legend to Fig 3.2

bands do appear in the supernatant (Fig 3.21 B, lane 2, marked with **?**). After transport, the 16_{LTD} remains attached to the 23 part and undergoes the cleavage process (Fig 3.21 A). In this situation the cleaved subfragments will probably be released into the supernatant and do not keep bound to the thylakoid membrane. However, in the *in vitro* translated products two bands (Fig 3.21 B, lane 1, marked with **?**) also appear because of the internal initiation of the translation, which makes it difficult to analyze the results. Furthermore, in the supernatant resulting from the import assay of this chimera, one unexpected band which is running at the range of 50 kDa (Fig 3.21 B, lane 2, marked with a star) appears in the experiments performed. At present there is no reasonable explanation about the formation of this band which further indicates that the 23-16_{LTD}/EGFP is not suitable for the analysis of the fate of Tat signal peptides.

Obviously, the 16/23-16_{LTD} is more convenient for the analysis of the fate of Tat signal peptide, as transport and TPP cleavage are not required. However, two kinds of considerations could be argued: First, does the first signal peptide really insert into the thylakoid membrane (i.e. Fig 3.22 A, upper panel)? In other words, if the first

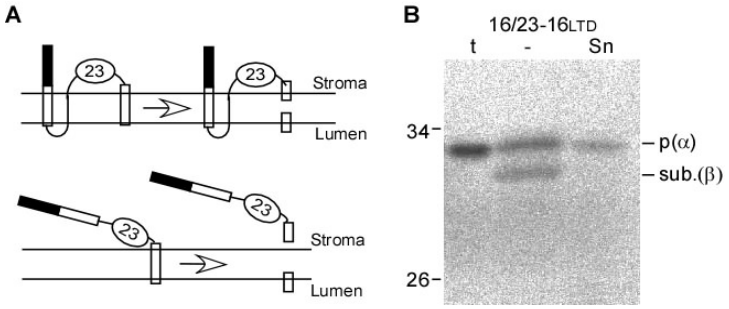

Fig 3.22: **The first signal peptide of the 16/23-16_{LTD} inserts into the thylakoid membrane. A**, Two alternative localization of the "16/23 containing part" after cleavage of the signal peptide. **B**, *in vitro* synthesized 16/23-16_{LTD} was incubated with isolated thylakoid vesicles for 10 min under standard import conditions, the reaction mixture was centrifuged for 4 min at 10,000 rpm. Then the supernatant (Sn) was loaded on SDS-gel and the pellet was treated as described in Fig 3.1 (marked as "-"). Both samples were analyzed by SDS-gel and phosphor-imaging visualization. For further details, see legend to Fig 3.2

signal peptide does not insert into the thylakoid membrane, it could be speculated that the cleaved subfragments will be released into the supernatant (Fig 3.22 A, lower panel). However, such bands could not be identified in the supernatant (Fig 3.22 B). Furthermore, one can speculate that without the first signal peptide, i.e. 23-16_{LTD}, the cleaved subfragments will be released into the supernatant (Fig 3.23 A). After checking the *in thylakoido* assay of 23-16_{LTD}, this is indeed the case as expected subfragments accumulate in the supernatant (Fig 3.23 B). Taken together, these data strongly suggest that without the first signal peptide, the cleaved subfragments are released into the supernatant. It can therefore be concluded that most, if not all, of the first signal peptide inserts into the thylakoid membrane.

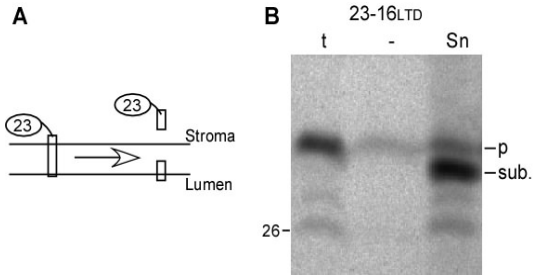

Fig 3.23: **Without the first signal peptide (i.e. 23-16$_{LTD}$), the subfragments are not hooked into the thylakoid membrane. A**, Without the first signal peptide, i.e. 23-16$_{LTD}$, it is speculated that the cleaved subfragments will be released into the supernatant. **B**, *in vitro* synthesized 23-16$_{LTD}$ was incubated with isolated thylakoid vesicles for 10 min under standard import conditions, the reaction mixture was centrifuged for 4 min at 10,000 rpm. Then the supernatant (Sn) was loaded on SDS-gel and the pellet was treated as described in Fig 3.1 (marked as "-"). Both samples were analyzed by SDS-gel as well as phosphor-imaging visualization. For further details, see legend to Fig 3.2

The second consideration could be: since the first signal peptide is still functional, why could not the 23-16$_{LTD}$ be transported as the mature protein? As shown in Fig 3.24 A, import experiments showed that one mature protein, which is protease resistant, does exist (Fig 3.24 A, lanes 2 and 3). However, its size is smaller than the expected mature protein 23-16$_{LTD}$ but larger than mature 23 kDa (Fig 3.24 A, compare lanes 3, 4 and 7 for mature proteins). Interestingly, its size (∼25 kDa) corresponds well to the size of

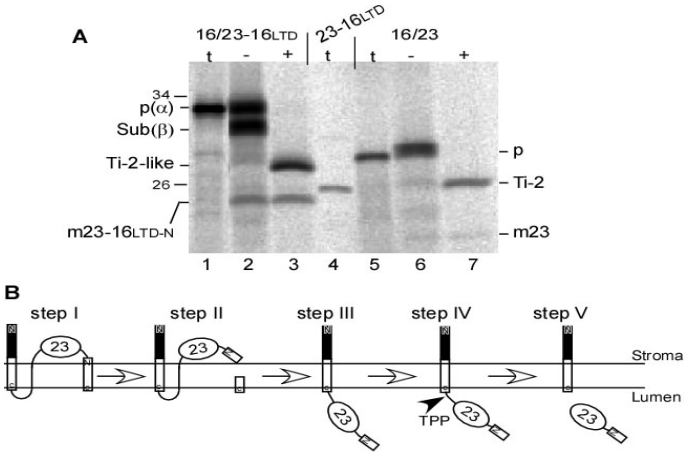

Fig 3.24: **The N-terminal subfragment is cotransported with 23 kDa by the first signal peptide after cleavage of 16$_{LTD}$. A**, *in vitro* synthesized 16/23-16$_{LTD}$ was incubated with isolated thylakoid vesicles for 15 min under standard import conditions, then treated as described in Fig 3.2. **B**, Schematic representation of the transport process of 16/23-16$_{LTD}$.

23-16$_{LTD-N}$, i.e. the N-terminal subfragment from the cleavage of 16$_{LTD}$ together with mature 23 kDa. Furthermore, one transport intermediate, Ti-2-like (Fig 3.24 A, lane 3), corresponds well in size to the Ti-2 of 16/23 plus the N-terminal subfragment after cleavage of 16$_{LTD}$ (Fig 3.24 A, compare lanes 3 and 7 for transport intermediates). Taken together, this demonstrates that most, if not all, of 16/23-16$_{LTD}$ first inserts into the thylakoid membrane with both signal peptides (Fig 3.24 B, step I), then the

N-terminal subfragment together with 23 kDa is further transported as one entity by the first signal peptide (step II-V).

3.3.7 The cleavage site is localized in the hydrophobic domain of Tat signal peptide

During the transport process, Tat transit peptide first inserts into the thylakoid membrane in an unassisted manner by forming a loop structure, i.e. both N- and C-terminus located on the outside of thylakoid membrane. After the mature protein was successfully transported across the thylakoid membrane, the transit peptides obtain a trans-

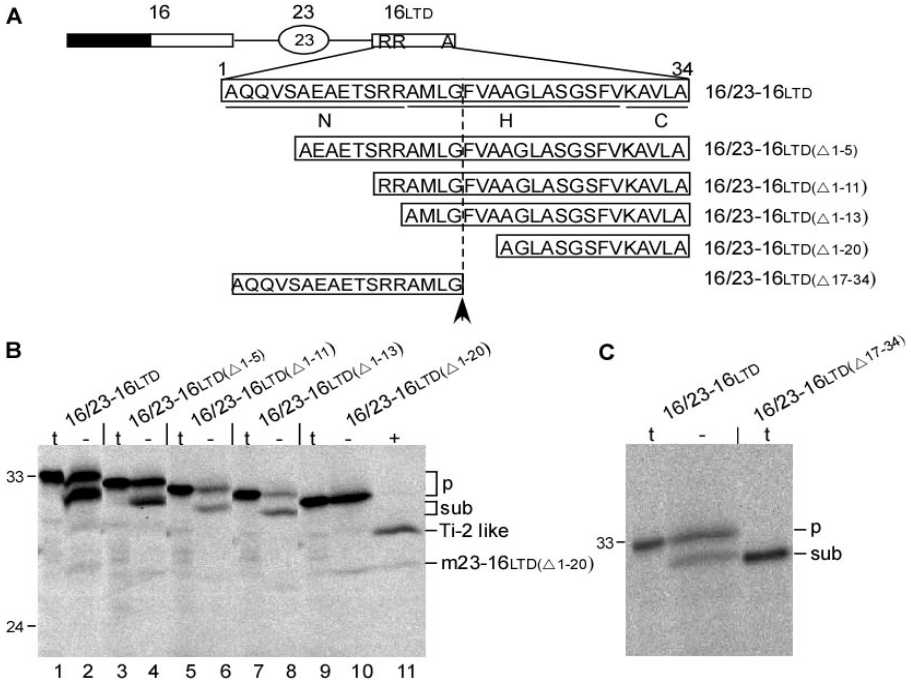

Fig 3.25: **The cleavage site is located in the hydrophobic domain of the Tat signal peptide. A**, Schematic representation of 16/23-16$_{LTD}$ and its deletion derivatives. The deletion started from the N-terminus of the second signal peptide (16$_{LTD}$). The sequence of 16$_{LTD}$ is numbered from the N-terminus and the N-, H-, C-domains are underlined. The determined cleavage site is indicated by an arrow. **B**, *In vitro* synthesized 16/23-16$_{LTD}$ and its deletion derivatives were incubated with isolated thylakoid vesicles under standard import conditions for 10 minutes, washed two times, and loaded onto SDS-PAA-gels for analysis without further treatment, except for the 16/23-16$_{LTD\Delta(1-20)}$ which was treated following the protocol described in Fig 3.2. The transport of 16/23-16$_{LTD\Delta(1-20)}$ following the process as 16/23 chimera and its transport intermediate related to Ti-2 stage for 16/23 as well as the mature protein are indicated. For further details see the legends to Fig 3.2, 3.3 and 1.4. **C**, Based on the prediction results, the 16/23-16$_{LTD\Delta(17-34)}$ derivative was constructed and after *in vitro* synthesis, loaded onto the SDS-gel for comparison with band β generated from the assay of 16/23-16$_{LTD}$. The subfragment (band β) from the cleavage event is marked as sub in this figure.

membrane topology, i.e. the N-terminus is located on the stroma-side of thylakoid membrane, while the C-terminus is located on the lumenal side of thylakoid membrane. To obtain an idea about the position of the cleavage site within the signal peptide,

a series of deletion constructs from the N-terminus of the 16_{LTD} signal peptide within the 16/23-$_{LTD}$ chimera was generated (Fig 3.25 A).

As shown in Fig 3.25 B, the deletions within the N-domain (16/23-$16_{LTD\Delta(1-5)}$, 16/23-$16_{LTD\Delta(1-11)}$ and 16/23-$16_{LTD\Delta(1-13)}$), have no effect on the cleavage of 16_{LTD} transit peptide (Fig 3.25 B, lanes 1-8), which indicates that the cleavage site is not located within the N-domain. However, when the deletion covers part of the H-domain (16/23-$16_{LTD\Delta(1-20)}$), the cleavage event was abolished. Instead, most of the substrate is then transported and accumulates as 23-$16_{LTD\Delta(1-20)}$ protein within the thylakoid lumen (Fig 3.25 B, lanes 10 and 11). This result indicates that the truncated second signal peptide cannot anymore insert into the thylakoid membrane and/or the cleavage site is lost thus leading to the abolishment of the cleavage event. In contrast, most of the precursor is transported by the first signal peptide. Together, the data strongly suggest that the cleavage site must be located in the hydrophobic domain of the signal peptide.

To further address this question, theoretical calculations have been performed based on these derivatives using the standard protein marker as molecular weight standard combined with the computer program "Compute pI/Mw Tool" from ExPASy (Gasteiger et al., 2003). The results of the molecular weight of each derivative on SDS-PAGE as well as their relative theoretical molecular weight from Compute pI/Mw Tool are shown below.

The derivatives	Size of precursors		Size of relative subfragments	
	SDS-PAGE	Computer	SDS-PAGE	calculated size
16/23-16_{LTD}	32.8	33.5	31.2	31.9
16/23-$16_{LTD\Delta(1-5)}$	32.3	33.0	30.7	31.4
16/23-$16_{LTD\Delta(1-11)}$	31.7	32.4	30.2	30.9
16/23-$16_{LTD\Delta(1-13)}$	31.2	32.1	29.7	30.4
16/23-$16_{LTD\Delta(1-20)}$	30.7	31.2	—	—

From the above table, it is obvious that there is a variability between the theoretical size obtained from computer program and running behavior upon SDS-PAGE of each derivative. Thus the relationship between the size on SDS-PAGE and computer program were expressed as Y=1.02328X (Y=computer program calculated molecular weight, X=calculated molecular weight from SDS-PAGE) based on the above table. After calculating the size of each derivative and its corresponding subfragment from SDS-PAGE, the size differences were calculated between each derivative and its corresponding subfragment. Finally the average size difference from all the derivatives on SDS-PAGE was 1572.5 Da, which corresponds to the theoretical size of 1609.11 Da

according to Y=1.02328X. This theoretical size (1609.11 Da) finally was used with "Compute pI/Mw Tool program" for prediction of the cleavage site. After these precise calculations, it could finally be concluded that the cleavage site is located between Gly-17 and Phe-18 of the 16_{LTD}, which is indeed located inside the H-domain, in line with the deletion result shown above (Fig 3.25). As a direct proof for this conclusion, a reference derivative (16/23-$16_{LTD\Delta(17-34)}$) was constructed (Fig 3.25 A) in which the amino acids from 18 Phe to the C-terminal end of the signal peptide were deleted. The electrophoretic mobility of the subfragment (i.e. band β) from the cleavage of 16_{LTD} with reference peptide were compared on SDS-PAGE. As shown in Fig 3.25 C, the size of the *in vitro* translated 16/23-$16_{LTD\Delta(17-34)}$ is identical to the size of the subfragement (band β) which further confirms that the cleavage site located inside the H-region and very likely between Gly-17 and Phe-18.

3.3.8 The RR-motif is not required for the cleavage of Tat signal peptide

A conserved RR-motif located at the boundary of N-domain and H-domain is a specific character of Tat signal peptides (Müller and Klösgen, 2005). However, deletion experiments shown above indicate that this RR-motif is not required, or at least its deletion has no strong effect on the cleavage of Tat signal peptides. To further examine this result, the conserved RR-motif within the 16_{LTD} was mutated to twin lysines (Fig 3.26 A), as it has been shown that this mutation completely abolishes Tat transport (e.g.

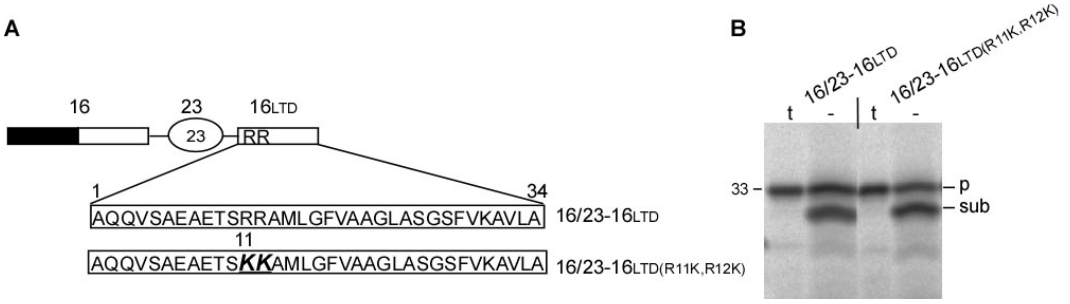

Fig 3.26: **The RR-motif is not required for the cleavage event. A**, Schematic representation of 16/23-16_{LTD} derivative in which the RR motif was mutated to KK inside the 16_{LTD} signal peptide to form 16/23-$16_{LTD(R11K,R12K)}$. The sequence of 16_{LTD} is numbered from the N-terminus and the mutated amino acids are in bold and numbered. **B**, *In vitro* synthesized 16/23-16_{LTD} and 16/23-$16_{LTD(R11K,R12K)}$ were incubated with isolated thylakoid vesicles for 10 min under standard import conditions and washed twice then loaded onto the SDS-gel without further treatment. The subfragment from cleavage event is marked as sub (corresponds to band β) in this figure. For further details see the legend to Fig 3.2.

Chaddock et al., 1995; Stanley et al., 2000; DeLisa et al., 2002). The results shown in Fig 3.26 B indicate that this mutation has no effect on the cleavage event of the 16_{LTD} signal peptide. This result strongly suggests that the cleavage event is probably is a more general phenomenon occurring at the thylakoid membranes for signal peptides

cleaved off by signal peptidase (TPP), rather than restricted to the Tat signal peptides only (see next).

3.3.9 The distance from the cleavage site to the C-terminal end of the Tat signal peptide is a determinant of cleavage efficiency

Previous results (Fig 3.18) have shown that TPP cleavage is a prerequisite for the cleavage of the 16_{LTD} signal peptide. In other words, only those signal peptides without a C-terminal extension can be substrates for the cleavage event. This indicates that probably the length (or size) from the cleavage site to the C-terminus of the cleavage substrate is important for occurring of this cleavage event. Furthermore, depending on the functional place and the presence of TPP cleavage site, precursors handled by Tat pathway have different final localizations, i.e. located inside the thylakoid lumen or within thylakoid membrane. For example, the signal peptide of OEC 16 kDa protein is cleaved off by TPP, whereas the transport signal of the Rieske protein functions as a membrane anchor after transport rather than being cleaved off (Molik et al., 2001). Obviously in the latter case, this signal anchor domain must not be destroyed or cleaved into subfragments, in order to perform its function. These considerations make it

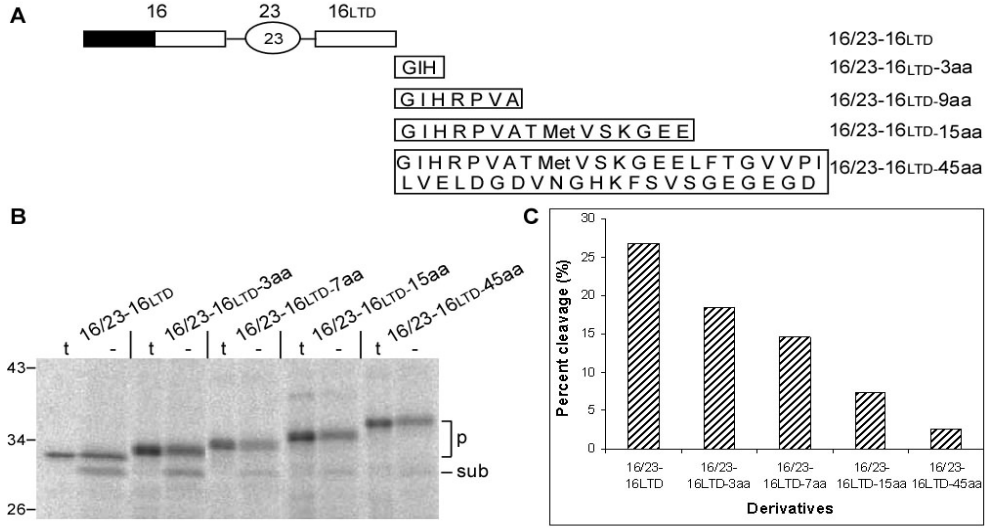

Fig 3.27: **The distance from the cleavage site to the C-terminal end of the signal peptide has an effect on the cleavage efficiency. A**, Schematic representation of 16/23-16_{LTD} extension derivatives in which different numbers of amino acids were added behind the 16_{LTD} signal peptide as indicated in their name. **B**, *In vitro* synthesized 16/23-16_{LTD} and the extension derivatives were incubated with isolated thylakoid vesicles for 10 min under standard import conditions, washed twice and then loaded onto the SDS-gel without further treatment. The subfragment from cleavage event is marked as sub (corresponds to band β) in this figure. For further details see the legend to Fig 3.2. **C**, Quantification result based on the B and the cleavage percentage were calculated as the band intensity of subfragment divided by sum of band intensity of subfragment and the remaining precursors.

reasonable to assume that the distance from the cleavage site inside the H-domain to the C-terminus of the signal peptide probably has an impact on the cleavage event of the signal peptide as indicated by the His-tag result from Fig 3.19.

To test this hypothesis, the distance from the cleavage site to the C-terminus of the signal peptide was gradually increased by adding between 3∼45 amino acids to the 16/23-16$_{LTD}$ C-terminus (Fig 3.27 A). The molecular weight of these derivatives increased correspondingly as seen by their electrophoretic mobilities upon SDS-PAGE (Fig 3.27 B). In contrast the subfragments appearing for each of these derivatives all at the same position on SDS-PAGE which indicates that cleavage occurs at the same site within the signal peptide regardless of the difference on its C-terminus (Fig 3.27 B). This result is in line with the observation of His6-tag result (Fig 3.19 B). However, the efficiency for this cleavage decreased significantly with increasing length of the extension on the C-terminus of the signal peptide, from 27% down to 2.5% (Fig 3.27 B and C). This result suggests that the distance from the cleavage site to the C-terminus of the transit peptide is an important determinant for the cleavage of Tat signal peptide into its subfragments.

3.3.10 The position of helix forming residues within the Tat signal peptide has an effect on the cleavage event

As the above results (Fig 3.25) have shown, the cleavage site within the Tat signal peptide is located inside the hydrophobic domain, i.e. in the plane of thylakoid membrane. This is an unusual phenomenon, because the proteolysis occurs in the membrane where is a hydrophobic environment. Recently, a so-called regulated intramembrane proteolysis (RIP) has been described, in which the proteolysis occurs inside the membrane (Wolfe and Kopan, 2004). One specific requirement for successful cleavage of RIP is the presence of helix-breaking or bending residues located inside the transmembrane domain of the substrates (Wolfe and Kopan, 2004). This specific requirement has been explained in such a way that the helix-breaking residues must unwind the helical structure of the transmembrane segment, thus making it more easier to be attacked by RIP protease (Ye et al., 2000; Lemberg and Martoglio, 2002; Akiyama and Maegawa, 2007).

Structural studies of signal peptides by use of nuclear magnetic resonance (NMR) in membrane-mimicking environments with anionic detergents have shown that a functional signal peptide adopts a dynamical “helix-break-helix” conformation (Rizo et al., 1993; Chupin et al., 1995). The helix propensities of amino acids are: Ile>Leu>Val>Met> Phe>Ala>Gln>Tyr>Thr>Ser>Asn>Gly>Pro. It has been suggested that these struc-

tural helix-breaking motifs probably play an important role for the efficiency of translocation initiation, the membrane integration of the signal sequence, and the substrate cleavability by RIP proteases (van Klompenburg and de Kruijff, 1998; Lemberg and Martoglio, 2004).

For successful cleavage into subfragments, Tat signal peptides might also have to adopt a helix-breaking structure. In other words, if helix-forming amino acids are introduced, an effect on the cleavage event will be expected. To test this idea, double Leucine motifs were introduced into the 16_{LTD} signal peptide within the 16/23-16_{LTD} chimera at three different positions starting from the predicted cleavage site to the C-terminus of 16_{LTD} (Fig 3.28 A). Although leucine and alanine residues are often predicted to have similar

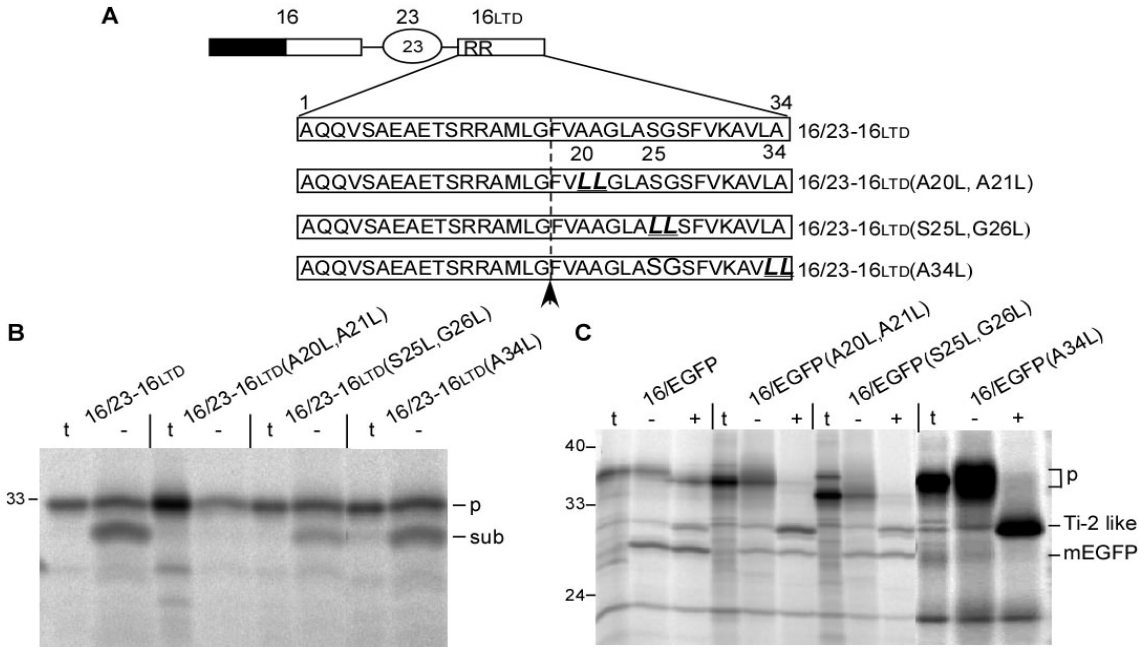

Fig 3.28: **The position of helix forming residues within the Tat signal peptide has an effect on the cleavage event. A**, Localization of double LL-motifs inside the 16/23-16_{LTD}. The sequence of 16_{LTD} is numbered from the N-terminus and the mutated amino acids are in bold and numbered. The predicted cleavage site is also indicated by an arrow and a dashed line. **B**, *In vitro* synthesized 16/23-16_{LTD} and mutation derivatives 16/23-16_{LTD}(A20L,A21L), 16/23-16_{LTD}(S25L,G26L) and 16/23-16_{LTD}(A34L) were incubated with isolated thylakoid vesicles for 10 min under standard import conditions, washed twice, and then loaded onto the SDS-gel without further treatment. The subfragment from cleavage event is marked as sub (corresponds to band β) in this figure. For further details see the legend to Fig 3.2. **C**, The introduction of LL-motif into 16_{LTD} has no strong effect on the Tat-dependent transport except a 16/EGFP(A34L). For further details, see the legend to Fig 3.2. and 3.3

propensities for forming an α-helix, a considerably higher α-helical content is observed in signal peptides which contain predominantly polyleucine core regions (Izard et al., 1995).

(1) As shown in Fig 3.29, most of the Tat signal peptides have a surprisingly high amount of alanine residues in their H-domains. Thus, the first LL-motif was introduced

by mutagenesis of the double AA-motif inside 16_{LTD}, which is near the predicted clea-

		N-region	H-region	C-region
Sp	16 K	AQQVSAEAETSRR	**A**MLGFV**AA**GL**A**SGSFV	KAVLA
Ma	16 K	ASAEGDAVAQAGRR	**A**VIGLV**A**TGIVGG**A**LS	QAARA
Sp	23 K	AQKQDDNEANVLNSGVSRR	L**A**LTVLIG**AAA**VGS	KVSPADA
Wh	23 K	AQKNDEAASDAAVVTSRR	**AA**LSLL**A**G**AAA**I**A**V	KVSPAAA
Cot	PSII-T	VQMSGERKTEGNNGRR	EMMF**AAAAAA**ICSV**A**	GVATA
Ara	PSII-T	TPSLEVKEQSSTTMRR	DLMFT**AAAAA**VCSL**A**	KVAMA
Sp	PsbP	NVLNSGVSRR	L**A**LTVLIG**AAA**VGS	KVSPADA
Wh	PsbP	SDAAVVTSRR	**AA**LSLL**A**G**AAA**I**A**V	KVSPAAA
Ma	PsbQ	GDAVAQAGRR	**A**VIGLV**A**TGIVGG**A**LS	QAARA
Ara	PsbQ1	AQQSEETSRR	SVIGLV**AA**GL**A**GGSFV	QAVLA
Ara	PsbQ2	NVSVPESSRR	SVIGLV**AA**GL**A**GGSFV	KAVFA
Bar	PsaN	VQVAPAKDRR	SALLGL**AA**VF**AA**T**AA**S**A**	GSARA
Bar	PSI-N	AAAKRVQVAPAKDRR	S**A**LLGL**AA**VF**AA**T**AA**S**A**	GSARA
Cot	Psb T	EGNNGRR	EMMF**AAAAAA**ICSV**A**	GVATA
Ara	PsbT	KEQQSSTTMRR	DLMFT**AAAAA**VCSL**A**	KVAMA

Fig 3.29: **The sequences of thylakoid lumenal targeting domains of some Tat substrates.** The N-, H-, C-region are marked on the top of the sequences. The Ala amino acid inside the H-region is in bold. Sp, spinach; Ma, maize; Wh, wheat; Cot, cotton; Ara, *Arabidopsis*; Bar, barley.

vage site (Fig 3.28 A, 16/23-16_{LTD}(A20L, A21L)). Interestingly, the mutation of A20L, A21L completely abolished the cleavage of the Tat signal peptide. This indicates that either the AA-motif contributes to the recognition by the protease or the two leucines tend to form an α-helix which is not suitable for substrate recognition by the protease. This point needs, however, further confirmation. (2) The second mutation is located near the end of the H-domain of 16_{LTD}, since both amino acids, Ser25 and Gly26, are helix-breaking residues. The results shown in Fig 3.28 B (16/23-16_{LTD}(S25L,G26L) indicate that the 16_{LTD} signal peptide can still be cleaved into its subfragments. However, the efficiency decreases from 48% to 20% which suggests that the helix-breaking structure might play a role in the cleavage event. (3) The third LL-motif is located at the very end of 16_{LTD} (Fig 3.28 A) which is inside the C-domain and out of the H-domain of the signal peptide. For the formation of this LL-motif, the last amino acid Ala was mutated to Leu, together with the Leu amino acid in front to form a LL-motif. The results shown in Fig 3.28 B (16/23-16_{LTD}(A34L)) suggest that the LL-motif located at the very end of the signal peptide has no significant effect since the efficiency only slightly decreases from 48% to 37%. Taken together, the effect of LL-motif introduction is position-dependent which indicates that probably the structure of the signal peptide is more critical for the cleavage event. However, at present it still can not be excluded that the observed effect is more dependent on the amino acid composition rather than on the structure.

Finally, to rule out that the observed effects are only due to the fact that the mutated

signal peptides cannot anymore insert into the thylakoid membrane thus leading to the inhibition of the cleavage event, the 16/EGFP mutants bearing respective mutations in the signal peptide were tested for import and the results were shown in Fig 3.28 C. Even though the transport is not as efficient as that of the original 16/EGFP, transport still takes place, since mature EGFP still accumulates in the thylakoid lumen, except for the 16/EGFP(A34L) precursor. Because TPP cleavage in this later derivative is abolished, no mature EGFP protein accumulates. But the transport intermediate corresponding to the Ti-2 of 16/23 still exists in 16/EGFP(A34L) and its size is identical to the other mutated substrates which indicates that in this case transport still takes place. In summary, the mutations apparently have no or only minor effects on the membrane insertion of the signal peptide, since otherwise transport could not have taken place. Furthermore, the LL-motif theoretically should enhance the membrane insertion of the signal peptide according to the high hydrophobicity of leucine (Hessa et al., 2005) which provides further evidence that the introduction of LL-motif should not affect the membrane insertion of the Tat signal peptide.

3.3.11 Cleavage of signal peptides in the thylakoid membrane is not restricted to Tat signal peptides

The fact that RR-motif is not required for the cleavage event (Fig 3.25, 3.26) indicates that the cleavage process is probably not only restricted to Tat signal peptides but is instead a more general event for thylakoid targeting signal peptides. Furthermore,

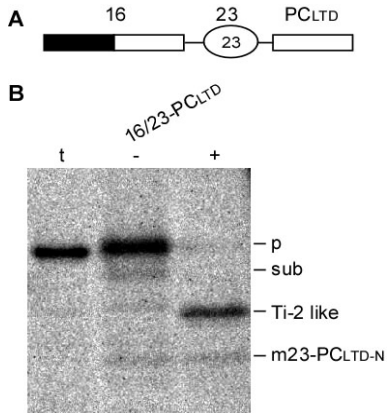

Fig 3.30: **The signal peptide of PC is also cleaved into subfragments. A**, Schematic representation of 16/23-PC$_{LTD}$. **B**, *In vitro* synthesized 16/23-PC$_{LTD}$ was incubated with isolated thylakoid vesicles in the presence of stroma for 10 min under standard import conditions, washed twice, and then loaded onto the SDS-gel without further treatment. The subfragment from cleavage event is marked as sub. After cleavage, the N-terminal part of the PC signal peptide still binds to the C-terminus of OEC 23 kDa protein and as a consequence of the first signal peptide (i.e. 16 kDa protein signal peptide), the mature 23 kDa protein plus the N-terminal part of PC signal peptide is transported and released by TPP cleavage (marked as m23-PC$_{LTD-N}$). Correspondingly, the transport intermediate relative to Ti-2 of 16/23 was also indicated as Ti-2-like. For further details, see the legend to Fig 3.2 and Fig 3.19

because Sec-signal peptides insert into the membrane also in a loop-mechanism which should adopt a similar topology as Tat signal peptide (Kuhn et al., 1994), it is reasonable to assume that probably similar cleavage event also occurs to Sec-signal peptides. To test this possibility, the signal peptide of plastocyanin (PC), a transport substrate of the Sec pathway, was attached to the 16/23 chimera to form 16/23-PC$_{LTD}$ (Fig 3.30 A). After incubation of *in vitro* translated 16/23-PC$_{LTD}$ with isolated thylakoid vesicles, the signal peptide of PC was successfully cleaved into subfragments as for the signal peptide of OEC16 kDa protein (Fig 3.30 B, marked as sub). However, at this point the efficiency is extremely low. The reason is not further analyzed yet. Still, this result strongly suggests that the cleavage of signal peptide is probably a more general event within thylakoid membranes to remove signal peptides after transport.

3.3.12 A metalloprotease is involved in the cleavage of Tat signal peptides

To determine which class of enzymes is involved in the proteolysis of the signal peptides, experiments in the presence of different protease inhibitors were performed with 16/23-16$_{LTD}$. The results show that serine-, aspartyl- and cysteine-protease inhibitors PMSF,

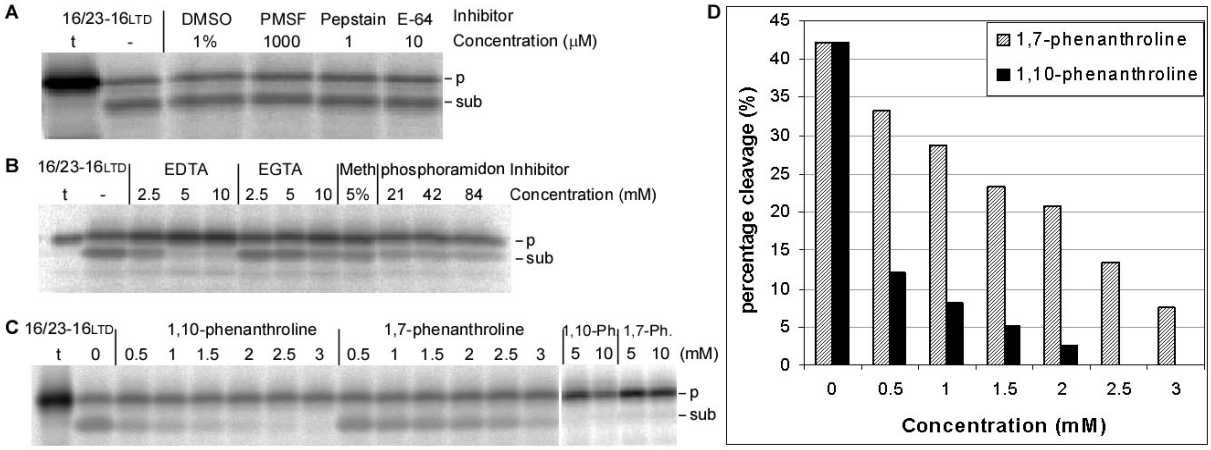

Fig 3.31: **A metalloprotease is responsible for the cleavage of Tat signal peptides.** **A**, Inhibitors from different protease families (serine-, aspartyl- and cysteine-proteases) were pre-incubated with isolated thylakoid vesicles on ice for 10 minutes. The *in vitro* translated 16/23-16$_{LTD}$ was added, transferred to the standard import conditions for another 10 minutes and further treated as described in Fig 3.26. The concentrations of the respective protease inhibitors are indicated above the lanes and 1% DMSO is the solvent for PMSF. **B**, Treatment of the reaction for the metalloprotease inhibitors EDTA, EGTA and phosphoramidon was as described in A. 5% methanol (Meth) was the solvent for phosphoramidon experiment. **C**, Treatment of the reaction for the metalloprotease inhibitors 1,10-phenanthroline and its control analogue 1,7-phenanthroline was as described in A. The band intensity in each lane was quantified to show the cleavage efficiency (in terms of the band intensity of the subfragments divided by the sum of subfragments and the remaining precursors). The quantification result is shown in **D**.

pepstain and E-64, respectively, had no detectable effect on 16/23-16$_{LTD}$ cleavage (Fig 3.31 A). Instead, the activity was inhibited by several metalloprotease inhibitors (Fig 3.31 B and C).

Among the metalloprotease inhibitors, EDTA has a significant effect indicating that the activity of the protease is dependent upon divalent cations. However, since this compound is an excellent chelator of calcium, which is required for many enzymes in biological systems, an indirect effect could not be excluded. In order to investigate whether the observed inhibition resides on calcium binding or not, EDTA was replaced by the calcium-specific chelator EGTA. The result shows that EGTA has no severe effect (Fig 3.31 B) demonstrating that calcium is probably not involved in this signal peptide cleavage process.

Another specific metalloprotease inhibitor, 1,10-phenanthroline, also severely inhibits the cleavage of 16$_{LTD}$. Since its analog, 1,7-phenanthroline, does not bind bivalent cations, it was used as a control (Fig 3.31 C). In the concentration range up to 3 mM, specific inhibition of protease by 1,10-phenanthroline was observed. When the concentration was increased to 5 mM, however, unspecific inhibition occurs, since in the assay containing 1,7-phenanthroline, an inhibition has been observed too (Fig 3.31 C).

Phosphoramidon, an inhibitor specific for thermolysin-like metalloproteases, only has a weak effect on the 16$_{LTD}$ cleavage (Fig 3.31 B), indicating that the protease responsible for the signal peptide cleavage does not belong to this sub-group of metalloproteases. Taken together, it can be concluded that a divalent cation dependent metalloprotease is responsible for the cleavage of thylakoid targeting signal peptides after transport.

3.3.13 Discussion of the fate of the Tat signal peptides

In this chapter, an analysis of the fate of a Tat-pathway signal peptide after release by TPP has been performed. The experiments demonstrate that Tat signal peptides are cleaved by a metalloprotease.

The “tandem-substrate” allows the detection of subfragment cleaved from the Tat signal peptide

Normally, it is difficult to monitor a fate of the signal peptide after its release by signal peptidase due to its small size. Previously, Lyko et al. (1995) successfully analyzed signal peptide processing in rough microsomes after introducing methionine into the signal peptide for detection. However, this method maybe is not ideal for analyzing the fate of Tat signal peptides, because features like hydrophobicity or polarity of the signal

peptides probably will be affected by amino acid substitution, and this may eventually affect membrane transport. For example, membrane insertion of the transported protein depends not only on the total hydrophobicity but also on amino acid composition (Hessa et al., 2005). It has also been shown that the Tat passenger protein can be completely rerouted to the Sec pathway by as few as two amino acid substitutions within the Tat signal peptide (Cristobal et al., 1999; Blaudeck et al., 2003). Additionally, amino acid substitution might affect also the signal peptide cleavage process.

To analyze Tat signal peptide cleavage, an alternative strategy has therefore been used in the present work. Two chimeric proteins, 16/23 and 16_{LTD}/EGFP, which both can be successfully transported by Tat pathway were ligated in tandem. Due to the property of Tat signal peptides to spontaneously insert into thylakoid membranes (Hou et al., 2006), the internal signal peptide should remain at the first precursor, which should allow to monitor the cleavage events. Previously, Fincher et al. (1998) used a similar stragety to show that the Tat signal peptide inserts into the thylakoid membrane in a loop mechanism. The key difference between their chimeric protein and the "tamdem-substrate" used in this work is the composition of the internal signal peptide. Their substrates contain the full length transit peptide composed of both STD and LTD from the OEC 16 kDa protein. However, under *in vivo* conditions, STD is already cleaved off by stroma processing peptidase (SPP) before targeting to the thylakoid membrane. These authors claimed that no further cleavage or processing of the Tat signal peptide was found (Fincher et al., 1998). The reasons that make the difference are not clear at present, but probably it is due to the existence of the STD. It has been observed that the presence of STD at least has an effect on the membrane interaction of the LTD with artificial membranes (P. Hanner, M. Jacob, R. B. Klösgen, personal communication). Furthermore, it has also been reported that sequential signal peptides could compete with each other for membrane insertion (Monne et al., 2005). However it is not clear at present how the presence of STD affects the subfragments cleavage event of Tat signal peptide.

Although the "tandem-substrate" constructed in this work is complicated, it provides the possibility to get a rather easy detection of the processed signal peptide. Even though one part of the "tandem-substrate" is translocated across the thylakoid membrane using the first transit peptide and all the rest is considered as a mature protein, it is not known how the Tat translocase deals with the "tandem-substrate" in two ways. Whatsoever, this strategy could overcome the problems that the small signal peptide cannot be distinguished from small peptides produced in *in vitro* translation systems as well as the problem of low radiolabeling efficiency. The first transit peptide within the "tandem-substrate" is also critically important for this analysis. First, by taking

advantage of its spontaneous membrane insertion property, the fragments can stick to the membrane after cleavage of the Tat signal peptide thus avoiding the difficulty to distinguish them from the *in vitro* translation bands within the supernatant of the import reaction mixture. Second, if the first signal peptide within the "tandem-substrate" is not 16_{LTD} but 23_{LTD}, i.e. the reporter protein as authentic OEC 23 kDa protein, the results show that most of the "tandem-substrate" is transported by using the first signal peptide and 23-16_{LTD}/EGFP as a complete passenger protein (Fig 3.20). Obviously, this transport behavior is not helpful for analyzing the fate of Tat signal peptide. This observation is not surprising if the different transport behavior of authentic OEC 23 kDa protein and the chimeric 16/23 are considered: the former one is transported too fast to give transport intermediates, while the latter one is significantly retarded during Tat transport thus giving rise of the transport intermediates (Berghöfer and Klösgen, 1999; Hou et al., 2006; Frielingsdorf and Klösgen, 2007).

Requirements for the subfragments cleavage of the Tat signal peptides

The cleavage process of Tat signal peptide occurs in a time-dependent manner, as the cleaved subfragments gradually accumulate over time as soon as TPP cleavage releases the free signal peptide (Fig 3.16). TPP activity is a prerequisite for signal peptide cleavage (Fig 3.18). This is critically important for the thylakoid system to avoid the erroneous cleavage of transport signals that function as membrane anchors. For example, Rieske protein inserts into the thylakoid membrane using Tat pathway (Molik et al., 2001). After Tat transport, the signal peptide is not cleaved off by TPP. Instead, it is used as a membrane anchor. Of course, this kind of Tat signal should not be cleaved. On the other hand, Tat signal peptides released by TPP cleavage into the thylakoid membrane now become potentially harmful for thylakoid membrane, and thus, should undergo a clearance process. In this regard, the cleavage of free Tat signal peptides is not only important to maintain the integrity of the thylakoid membrane, but also for the subsequent polypeptide translocation. Without clearance, they will possibly cause a "traffic jam", because it has been shown that inhibition of *E. coli* signal peptidase results in inhibition of translocation (Chen and Tai, 1989). Thus, the cleavage of Tat signal peptides possibly contributes to maintain a high efficiency of protein translocation across thylakoid membranes. As soon as the signal peptides are released by TPP cleavage from Tat precursors, possibly the subfragments cleavage of Tat signal peptides will start automatically as the reference 16/23-16_{LTD} which contains a free Tat signal peptide could be used as a substrate for cleavage directly. Additionally, the length of the cleavage site to the C-terminal end of the signal peptide has also an effect on the subfragments cleavage process, since the extension of the C-terminus of the Tat signal peptide significantly reduced the efficiency of the cleavage process (Fig 3.27).

As determined in Fig 3.25, the cleavage site is located in the hydrophobic domain of the signal peptide and probably embedded in the membrane which is an indication of regulated intramembrane proteolysis (Wolfe and Kopan, 2004). For successful cleavage, the signal peptide must be to some degree unstructured as to be accessible for the proteolysis. Interestingly, most signal peptides have a "helix-breaking-helix" structure (van Klompenburg and de Kruijff, 1998). In other words, α-helix formation can be expected to potentially affect cleavage. By mutation of AA to LL near the cleavage site, the α-helix formation was enforced and interestingly, the cleavage of Tat signal peptide was significantly affected (Fig 3.28). In contrast, replacement of helix-breaking residues far away from the cleavage site, e.g. SG mutated to LL, only decrease the cleavage efficiency. Furthermore, if the helix-forming motif LL locates outside of the H-domain, its effect is not anymore predominant (Fig 3.28). These preliminary data suggest that unwinding of the signal peptide can probably be another determinant as supposed for RIP proteolysis (Wolfe and Kopan, 2004). But at this point more efforts are required to elucidate the amino acid effect on the cleavage event.

Even though the analysis was based mostly on Tat specific signal peptides, it may well represent a common phenomenon for clearance of thylakoid membrane targeting signal peptides, since the RR-motif is not required (Fig 3.26). This hypothesis could be experimentally confirmed by use of PC-signal peptide which is a Sec-pathway specific transport substrate (Fig 3.30).

The protease involved in the cleavage of the Tat signal peptides

After being transported across the envelope membrane of chloroplast, the STD is cleaved off as a result of the function of stroma processing peptidase (SPP). After processing, SPP remains bound to STDs and cleaves them into subfragments for further turnover by transit peptide subfragment-degrading enzyme (TP-DE) (Richter and Lamppa, 1999; Richter and Lamppa, 2002). Obviously, this is not the case for Tat signal peptide cleavage, since it has been shown that the function of TPP is even stimulated by chelating agents such as EDTA and EGTA (Kirwin et al., 1987), while the protease responsible for cleavage of Tat signal peptide is inhibited by EDTA. This suggests that the tasks of signal peptide cleavage from its precursor and cleavage of free Tat signal peptide are fulfilled by different proteases.

The inhibitor experiments (Fig 3.31) showed that a calcium-independent metalloprotease is responsible for cleavage of the Tat signal peptides. It is too early at present to draw a conclusion what kind of metalloprotease is involved in this process, since we

know nothing about its characteristics and diverse proteases exist in chloroplast (Adam and Clarke, 2002; Rawlings et al., 2006). However, there are some potential candidates: (i) First, considering TPP cleavage is a critical prerequisite for occurring of Tat signal peptide cleavage, this correlates with the identification that FtsH, a thylakoid membrane bound, ATP-dependent metalloprotease, which in some cases requires also an initial cleavage for recognition and complete degradation of its substrate (Lindahl et al., 2000; Preiss et al., 2001). This indicates that possibly FtsH is involved in the cleavage of Tat signal peptide, too. FtsH belongs to a larger family of AAA proteins (ATPases associated with diverse cellular activities) (Langer, 2000). FtsH is an integral thylakoid membrane protein and its catalytic as well as ATP-binding domains are located at the stroma-exposed regions of thylakoid (Lindahl et al., 1996). This protease is involved in both the degradation of unassembled subunits of membrane complexes, such as the Rieske Fe-S protein of the cytochrome complex, and the degradation of oxidatively damaged protein such as the D1 protein of the photosystem II (PS II) reaction centre (Adam and Ostersetzer, 2001). In plant chloroplasts, six potential FtsH proteases have been identified (Adam and Ostersetzer, 2001). Recently, involvement of FtsH in Tat protein transport has been hypothesized (Brüser and Sanders, 2003). However, no findings of FtsH digestion of signal peptides have been reported yet and even the hypothesis of FtsH involvement in Tat pathway needs experimental support. (ii) The second possible candidate is a group of putative plant metalloproteases belong to the recently identified "regulated intramembrane-cleaving proteases" (iCLiP) (Kinch et al., 2006). Implications for this speculation are based on the observation that the cleavage site for subfragments cleavage of Tat signal peptide is located within the hydrophobic domain. This indicates that possibly the event of Tat signal peptide cleavage falls into the phenomenon of RIP story (Wolfe and Kopan, 2004). In addition, a preliminary cleavage is critically required for sequential occurring of intramembrane proteolysis which gives another clue that the proteolysis described here can be grouped into the RIP, as TPP is critically required. Furthermore, α-helix formation affects the cleavage event which gives further hints that it is probably a RIP-like phenomenon. Whatsoever, the questions about the protease involved and its characteristics as well as its physiological role in chloroplast biogenesis now are open for answering.

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List of publications

1, **Enguo Fan**, Mario Jakob, Ralf Bernd Klösgen, A train-like chimeric protein can be transported by the ΔpH/TAT pathway across the thylakoid membrane (in preparation).

2, **Enguo Fan**, Mario Jakob, Ralf Bernd Klösgen, The fate of Tat signal peptides. (in preparation)

3, **Enguo Fan**, Mario Jakob, Ralf Bernd Klösgen, Requirements for the cleavage of the Tat signal peptides. (in preparation).

4, Sven B. Gould, **Enguo Fan**, Franziska Hempel, Uwe-G. Maier, Ralf Bernd Klösgen, Translocation of a phycoerythrin α subunit across five biological membranes, *Journal of Biological Chemistry*, 2007, 282(41): 30295-30302.

5, Michael Gutensohn, **Enguo Fan**, Stefan Frielingsdorf, Peter Hanner, Bo Hou, Bianca Hust, Ralf Bernd Klösgen, Toc, Tic, Tat et al.: structure and function of protein transport machineries in chloroplasts, *Journal of Plant Physiology*, 2006, 163(3): 333-347

Erklärung

Ich erkläre an Eides statt, dass ich mich mit der vorliegenden Arbeit erstmals um die Erlangung des Doktorgrades bewerbe, die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Halle (Saale),

Enguo Fan

Curriculum vitae

Name: Enguo Fan
Birthday: 07, 11, 1975
Place of Birth: Shandong, China
[**Educations**]
Jul. 2003 – Mar. 2008 Martin-Luther University, Halle/Saale, Germany, PhD student.

Sep. 1999 – Jun. 2002 Nankai University, Tianjin, China, Master degree in Biochemistry.

Sep. 1997 – Jun. 1999 Ludong University, Shandong, China, Bachelor degree in Biology.

Sep. 1994 – Jun. 1997 Linyi Normal University, Shandong, China, Biology study.

[**Working Experiences**]
Apr. 2008 – present Wissenschaftlicher Mitarbeiter, University of Freiburg in the group of Prof. Dr. Matthias Müller.

Nov. 2002 – Jun. 2003 Wissenschaftlicher Mitarbeiter, Martin-Luther University Halle-Wittenberg, in the group of Prof. Dr. Ralf Bernd Klösgen.

Apr. 2002 – Oct. 2002, project leader, Beijing Purinatural Pharmaceuticals Institute Co., Ltd., Beijing, China.