Final Technical Report

DE-SC0003914 / DE-FG02-10ER16138, RECOVERY ACT—*Thylakoid Assembly and Folded Protein Transport by the Tat Pathway*, 04/15/2010-12/31/2015

Assembly of functional photosystems complete with necessary intrinsic and extrinsic proteins requires the function of at least three protein transport pathways in thylakoid membranes. Our research focuses on one of those pathways, a unique and essential protein transport pathway found in the chloroplasts of plants, bacteria, and some archaebacteria, the Twin arginine translocation (Tat) system. The chloroplast Tat (cpTat) system is thought to be responsible for the proper localization of ~50% of thylakoid lumen proteins, several of which are necessary for proper photosystem assembly, maintenance, and function. For example, of the handful of proteins experimentally shown to use the cpTat pathway, two components of the extrinsic oxygen evolving complex, PsbP and PsbQ, are transported to the thylakoid lumen via the cpTat system. PsbP and PsbQ have been shown to be important in photosystem II function and maintenance [2, 3].

The current project (April 2010 – December 2015) addresses the issues of what forms the translocation conduit, how the translocon complex is arranged and what role Tha4 may have in the process.

Identify the Tat component(s) that interact with the mature domain of precursor during transport. The Tat system is unique because it transports proteins in a folded conformation, without compromising the ion impermeability of the membrane and without the hydrolysis of NTP for energy, relying solely on the protonmotive force (PMF). Three membrane-bound protein components comprise the cpTat system, cpTatC, Hcf106, and Tha4. cpTatC is predicted to contain six transmembrane spans and serves as the initial precursor receptor [4, 5]. Hcf106 and Tha4 are structurally similar, yet functionally distinct having a predicted single transmembrane span at the N terminus followed by hinge region, a predicted amphipathic α-helix, and a loosely structured carboxy tail (C-tail). Hcf106 is found in the thylakoid in association with cpTatC while Tha4 is found as a separate complex and is found in roughly 8-fold molar excess over TatC and almost 2-fold molar excess over Hcf106. Unlike many other transport systems that maintain a gated pore, Tat systems are transient: assembling to allow transport of precursor, but dissembling after the translocation event. Biochemical data demonstrates that cpTatC/Hcf106 bind the precursor mostly through the signal sequence of the precursor in an energy-independent manner. Upon precursor binding and the presence of a PMF (i.e. upon illumination of the thylakoid), Tha4 then assembles with the precursor-bound cpTatC/Hcf106 complex and subsequently transport occurs. After transport of the precursor, Tha4 dissembles from the cpTatC/Hcf106 complex thus resetting the system for subsequent rounds of translocation.

What environment precursors actually encounter as they cross the membrane is unknown, although this project made some headway on that front. Several criteria must be met for the system however. First, the system must be able to accommodate folded mature domains of different sizes, varying from a diameter ~2.1 nm (relative hydrodynamic volume of ~3.5 nm^3) to ~5.4 nm (relative hydrodynamic volume of ~22 nm^3). Second the system must do this without compromising the ion impermeability of the membrane. Due to the molar excess and the regulated assembly of Tha4 with the precursor-bound receptor complex, Tha4 likely has a key role in transport. We hypothesized that at least a portion of the translocation channel comprises Tha4 and proposed to investigate this using a crosslinking strategy.

Using an affinity pull-down assay based on a His-tag on the precursor, we have surveyed Tha4 for interactions with the mature domain of pPsbQK99C and have incorporated additional Cys-substitutions in the precursor at different locations. We were very excited to see this interaction, as it is the first direct interaction between a cpTat precursor and Tha4. A summary of these data indicates that the precursor preferentially interacts with the APH region of Tha4 (**Fig. 1A**). To more fully understand the nature of the interaction, we analyzed the samples by blue native PAGE (BN-PAGE) and can demonstrate that Tha4 is cross-linking with the precursor in the translocon, the same 700 kD complex as cpTatC and Hcf106 (**Fig. 1B**). To determine if the interaction between Tha4 and the precursor is actually with the precursor or with the mature protein, i.e., before or after cleavage of the signal peptide, we performed reciprocal affinity precipitations using a FLAG-tagged Tha4 to pull-down the precursor (**Fig. 1C**). The reciprocal pull-downs demonstrate that the interaction between the two proteins occurs while the protein to be transported is still in the precursor form. This is an important point because it points to the interaction being 'on pathway' for transport of the precursor instead of an artifactual interaction after the transport event and cleavage of the signal peptide. Our evidence supports a method of transport that does not involve a topology inversion of Tha4 and may involve the formation of a cage-like structure around the precursor (**Fig. 1D**). **This work was published in *Plant Physiology* doi:10.1104/pp.112.207522.**

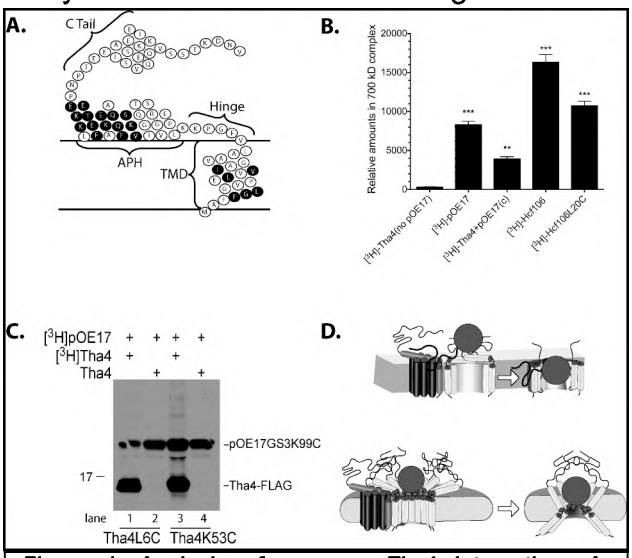

Figure 1. Analysis of precursor-Tha4 interaction. ***A,*** Schematic of Tha4 indicating the points of interaction (filled circles) with pOE17K99C-His_6. ***B,*** BN-PAGE analysis of the receptor 700 kD complex in the presence of precursor and Tha4. [^{3}H]-Tha4(no pOE17) indicates no precursor present in the sample. [^{3}H]-pOE17 indicates [^{3}H]-precursor in sample, whereas [^{3}H]-Tha4+pOE17(c) indicates no [^{3}H]-precursor. [^{3}H]-Hcf106 and [^{3}H]-Hcf106L20C were used as markers for the 700 kD complex. ***C,*** FLAG-tagged Tha4 interacts directly with the precursor. Cys-substituted Tha4 containing a FLAG-tag at the C terminus was used to affinity precipitate precursor. ***D,*** Possible models for a Tha4-mediated precursor translocation point of passage. *Upper,* channel model of Tha4 showing conformational change such that the APH lines the channel. *Lower,* Alternative model whereby overall topology of Tha4 does not change. This model is favored by our results. In the right part of each panel, the cpTatC-Hcf106 receptor was removed to focus on the contribution of Tha4. Panels C and D are from *Plant Phys* **161:**990-1001.

We are now using the reciprocal pull-downs to further study the progression of the transport process. By placing cysteines in different regions of Tha4 (e.g., the C-terminal half of the APH, the N-terminal half of the APH, the TMD) we are using Tha4 as a ruler to measure progress of the precursor during transport. Initial results indicate that precursor reaches and interacts with Cys-substitutions in the C-terminal portion of the APH before Cys-substitutions in the TMD. This is really exciting as we think we have identified the translocation pore.

In addition, we began to use a similar strategy to study the interaction of the mature domain of the precursor with Hcf106 (**Fig. 2**) and cpTatC. Hcf106 is found as a heterotrimer with cpTatC and Tha4 forming the receptor complex. By placing cysteines in different regions of Hcf106 (e.g., the C-terminal half of the APH, the N-terminal half of the APH, the TMD) we will map the preferred regions of interaction between Hcf106 and the mature domain of the precursor. In addition, previous work demonstrating interactions between TatC and precursor showed the primary interaction was between the signal sequence on the precursor and TatC [5-7]. Therefore, we also began to investigate the ability of cpTatC to interact with the mature domain of the precursor, thus contributing significantly to our understanding of the environment of the precursor if not during transport, at least immediately prior to transport.

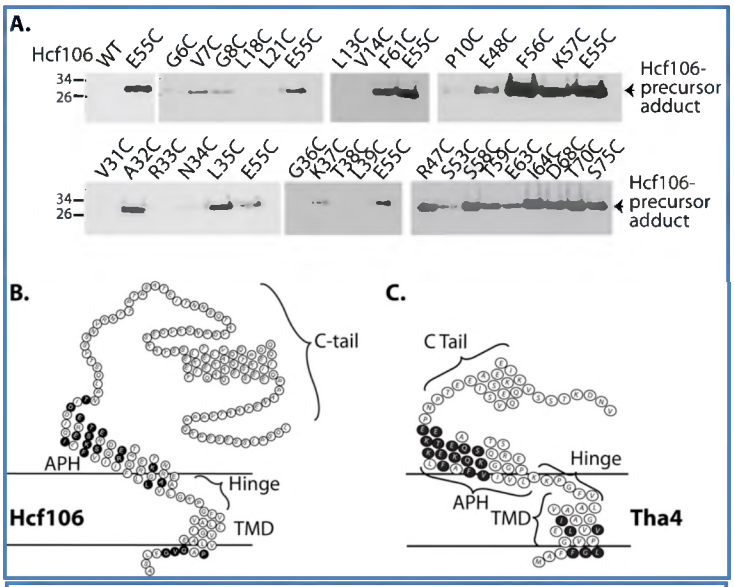

Figure 2. Preliminary analysis of precursor-Hcf106 interaction. ***A,*** Hcf106 interacts directly with His-tagged precursor, pOE17K99C. The His-tagged precursor was used to affinity precipitate Hcf106 with the indicated cysteine substitution. ***B,*** A schematic of Hcf106 summarizing cysteine substitutions able to form disulfide bonds with the precursor (black filled circles). ***C,*** For comparison, a schematic of Tha4 summarizing cysteine substitutions that interact with precursor (black filled circles) adapted from [1].

Determine the organization of the cpTat translocon. Hcf106 is similar in structure to Tha4, both have single transmembrane domains at the N terminus followed by a hinge region, a predicted amphipathic helix, and a loosely structured carboxy terminus (**Fig. 2B-C**). However, despite their structural similarities, they have distinct and different functions. In order to investigate the interactions between all of the components in the membrane we need to have ways to manipulate at least two of the three components. Thus we have made single cysteine substitutions throughout Hcf106 to enable us to investigate the interactions with itself or between Tha4 and Hcf106 or Hcf106 and cpTatC, the third transport component.

Attempts to identify areas of interaction between Hcf106 and cpTatC using crosslinking and mass spectrometry as originally proposed were not successful. However in the process we discovered that we could get information about Hcf106 concerning its oligomerization status in the thylakoid at rest or during active transport. We are pursuing this to provide us more information about Hcf106 and through structure/function analysis will try to characterize its role in transport. We took a two-pronged approach to investigate (1)Hcf106 interaction with a synthetic lipid bilayer and (2)Hcf106-specific interactions in the thylakoid. First, the synthetic lipid bilayer is mimetic of chloroplast membranes in that it contains <15% (mole ratio) monogalactosyl diacylglycerol (MGDG). MGDG is an interesting lipid because it is not a phospholipid and has a tendency towards forming inverted micelles because its headgroup occupies less space than the tail groups. We synthesized peptides corresponding to the transmembrane domain or amphipathic helix of Hcf106 and used SS-NMR (solid-state NMR) to measure the membrane activity of the peptides. Hcf106 and Tha4 are thought to insert into thylakoid membrane in a spontaneous manner, meaning no protein components or energy are

required. We tried to characterize that ability of Hcf106 to understand the process so that we can then apply what we learn to the process of spontaneous insertion of photosystem proteins such as PsbX, which are though to insert spontaneously. In addition, these studies will also allow us to probe membrane structure using both SS-NMR and EPR. Being able to generate multi-lamellar vesicles with Hcf106 incorporated is the first step towards generating MLVs with both Hcf106 and cpTatC incorporated in order to perform more structural studies using pulsed EPR, such as DEER (double electron-electron resonance) or ESEEM (electron spin echo envelope modulation). **This work was published in *Chemistry and Physics of Lipids* doi: 10.1016/j.chemphyslip.2013.09.002 and *BBA: Biomembranes* doi: 10.1016/j.bbamem.2013.10.007.**

Before investigating interactions of Tha4 with either Hcf106 or cpTatC, we developed assays to introduce *in vitro* expressed forms of Hcf106 and cpTatC into thylakoid. We chose to focus on Hcf106 first because of its similarity to Tha4. In order to better understand Hcf106 and its oligomerization status in the thylakoid at rest or during active transport, we first developed a battery of tests to confirm that integrated, *in vitro* expressed Hcf106 behaved in the membrane similar to what is known about endogenous Hcf106. For example, we used biochemical methods to characterize integrated, *in vitro* expressed Hcf106 in thylakoid membranes with regard to resistance to alkaline extraction and migration in a complex containing cpTatC. Endogenous Hcf106 is resistant to alkaline extraction of isolated thylakoid and when analyzed by blue-native PAGE (BN-PAGE) from detergent-solubilized membrane, migrates in a complex with cpTatC to ~700 kD [8]. We can show that integrated Hcf106 (e.g., wild type and Cys-substituted forms) are largely resistant to alkaline extraction similar to the endogenous Hcf106 protein and migrate as a band ~700 kD when analyzed by BN-PAGE (**Fig. 3**).

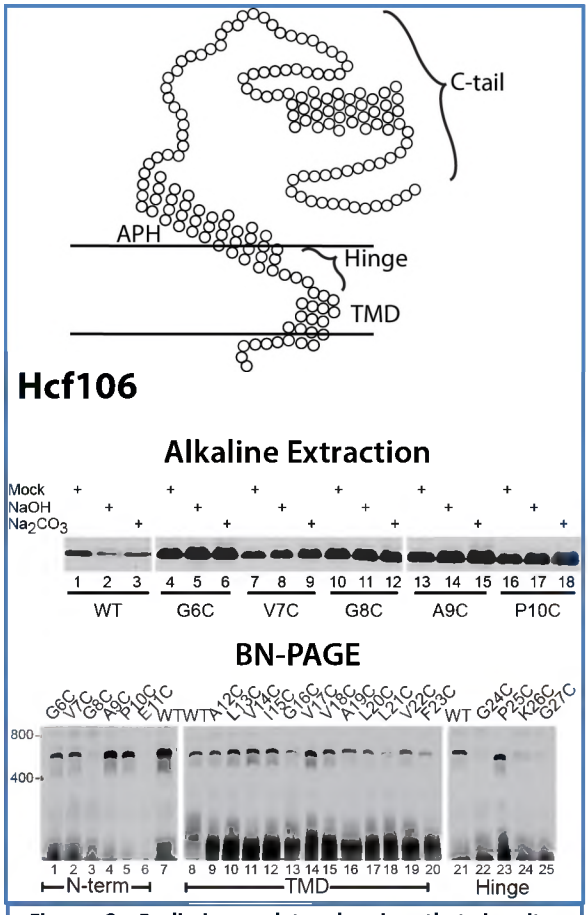

Figure 3. Preliminary data showing that *in vitro* expressed Hcf106 integrates into thylakoid. *A,* Schematic of Hcf106 showing the different regions of the protein. The transmembrane region is at the N terminus, followed by a hinge region, an amphipathic helix and a loosely structured C tail. *B,* Resistance to alkaline extraction for wild type and single Cys-substituted Hcf106. *C,* BN-PAGE analysis of digitonin solubilized thylakoid showing integrated Hcf106 migrates in a ~700 kD comlex.

Second, we used biochemical methods to characterize the oligomer state of Hcf106 in thylakoid membranes (**Fig. 4A**). This approach is very similar to the approach taken with Tha4 in that we can integrate single Cys-substituted Hcf106 into thylakoid and using an oxidant such as CuP (copper(II) phenanthroline) determine the propensity to form disulfide bonds. Sulfhydryls need to be within ~5Å in order to covalently bond to each other, and this can be used as a probe to orientation of the proteins in the membrane. If the Cys on one Hcf106 protomer is close enough to the same Cys on a neighboring protomer, a disulfide bond will form. If however, the Cys on one Hcf106 protomer

is not within ~5Å, then no disulfide bond will result even in the presence of an oxidant. **Fig. 4B** demonstrates the propensity to form disulfide bonds when Cys are substituted throughout the N terminus and transmembrane domain of Hcf106. Helical wheel projections of the transmembrane domain indicate a preferred face for interacting with neighboring Hcf106 molecules with the same single Cys substitution (**Fig. 4C, *left***). When similar assays were done with Hcf106 protomers containing single Cys substitutions in the amphipathic helix, two different faces arise. The first half of the helix has on preferred face as indicated by the stars (**Fig. 4C, *middle***), whereas the second half of the helix has two preferred faces (**Fig. 4C, *right***). How this correlates to the presence of Hcf106XnC with cpTatC in the receptor complex is an area of active investigation. Demonstrating the presence of the Cys-substituted Hcf106 in the receptor complex, would then allow us to activate the system for transport to further study the cpTatC-Hcf106 interaction.

Compare Tha4 topology in thylakoids during active transport and at rest. Previous reports from studies in bacteria have presented some conflicting data regarding the topology of TatA, the bacterial homolog of Tha4 before, during, and after transport [9, 10]. The topology of Tha4 has not been investigated. Our initial approach was to utilize cysteine accessibility to membrane permeant and impermeant reagents, and we had some success with this strategy, albeit in a different way than originally proposed. We have a Tha4, lacking native cysteine, with cysteines substituted throughout the amino acid sequence. These Cys-substituted Tha4s are fully functional and can replace endogenous Tha4 to support transport of precursor [11]. The advantage of this system is that we can query Tha4 topology before, during and after transport reactions. Tha4 is predicted to have an N-terminal transmembrane domain (TMD) followed by a hinge region an amphipathic alpha helix (APH) and a loosely structured carboxy tail region. In the predicted model, the hinge, APH, and C-tail are exposed to the stroma, but could also adopt alternative topologies. Structural studies of $TatA_D$ from the gram-positive bacterium, *Bacillus subtilis,* demonstrate a topology supporting the prediction, but those studies used purified protein in a lipid vesicle system that in no way could support transport [12, 13]. If Tha4 in thylakoid is similar in topology to *B. subtilis* $TatA_D$, the N terminus should be insensitive to labeling with a membrane impermeant molecule, such as streptavidin. We have adopted a topology labeling strategy where we label single cysteine Tha4 protomers with biocytin (a biotin-containing molecule) that freely diffuses across the membrane and contains a maleimide moiety to bind the free sulfhydryl on cysteine. The rationale is illustrated in **Fig. 5**.

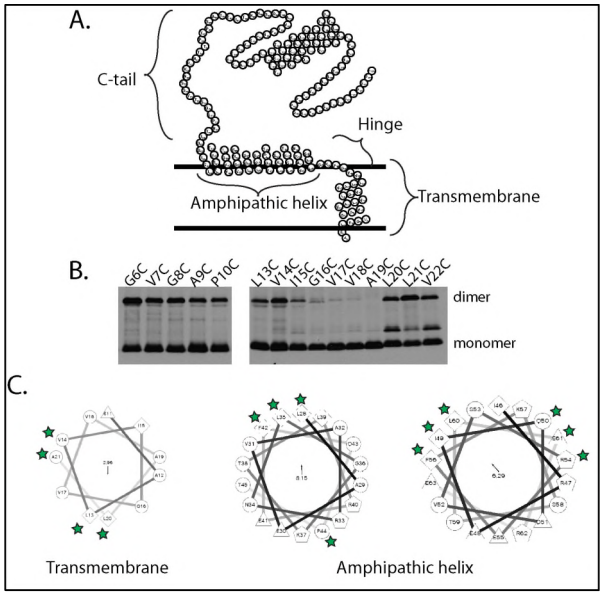

Figure 4. Characterization of Hcf106 interactions. *A,* Schematic of Hcf106 showing the different regions of the protein. The transmembrane region is at the N terminus, followed by a hinge region, an amphipathic helix and a loosely structured C tail. *B,* Dimerization studies of single Cys-substituted Hcf106 using Cu-Phen (copper(II) phenanthroline) as an oxidant to stimulate disulfide bond formation. *C,* Helical wheels summarizing the results of the disulfide bond crosslinking studies in the transmembrane or amphipathic regions. Stars indicate areas of dimer formation.

Through collaboration with Ken Cline (University of Florida) we obtained control proteins (cpTatC-X_nC) that contained Cys-substitutions on the stromal face, the transmembrane

domain, or the lumenal face. We can show that biocytin can freely cross the thylakoid membrane and label cysteines found on the lumen side (**Fig. 6A**).

Using this assay, all cysteine locations are able to bind biocytin, except those located in the hydrophobic core of the membrane as evidenced by an inability of SA to bind the cpTatC-L92C control protein even after detergent solubilization (**Fig. 6B, *upper panel***). By contrast, cpTatC-K72C was labeled with SA in intact membranes and digitonin-solubilized membranes, indicating that it is on the stromal face. However, SA labeling of cpTatC-L126C only occurred after digitonin solubilization and not in intact membranes. These data indicate that biocytin can cross the membrane and label thiols, but cannot label thiols in the hydrophobic core of the membrane. We surveyed the entire Tha4 protein by selecting Cys-variants containing a cysteine in each of the domains or regions; e.g., F3C and F4C (N-terminal), V13C (transmembrane), V21C (hinge), F48C (APH) and T78C (C-tail); for accessibility of streptavidin to bind the biocytin label during non-transport conditions (**Fig. 6B, *lower panel***). Biocytin-activated cysteine placed in the C-terminal half of Tha4 were accessible to SA (see Tha4F48C, Tha4T78C) in intact membrane or after detergent solubilization. However, cysteine placed in the hinge and transmembrane domains were unable to become biocytin labeled, suggesting they are in the hydrophobic core of the membrane (Tha4V21C, Tha4V13C). Lastly, biocytin-activated cysteine at the N terminus are not accessible to SA in intact thylakoid, but are accessible to SA after detergent solubilization, thus strongly suggesting that they are on the lumenal side of the membrane.

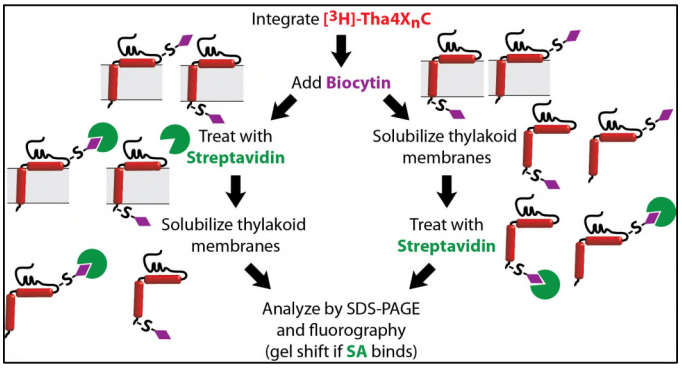

Figure 5. Topology labeling scheme. Cys-substituted Tha4 is integrated into thylakoids and activated by biocytin. Samples are divided and one half receives streptavidin (SA) treatment before detergent solubilization. Biocytin that is stromal can bind SA, whereas biocytin that is lumenal cannot. The second half is solubilized in detergent, before SA is added. All biocytin bound to Tha4 can bind SA. See text for more details.

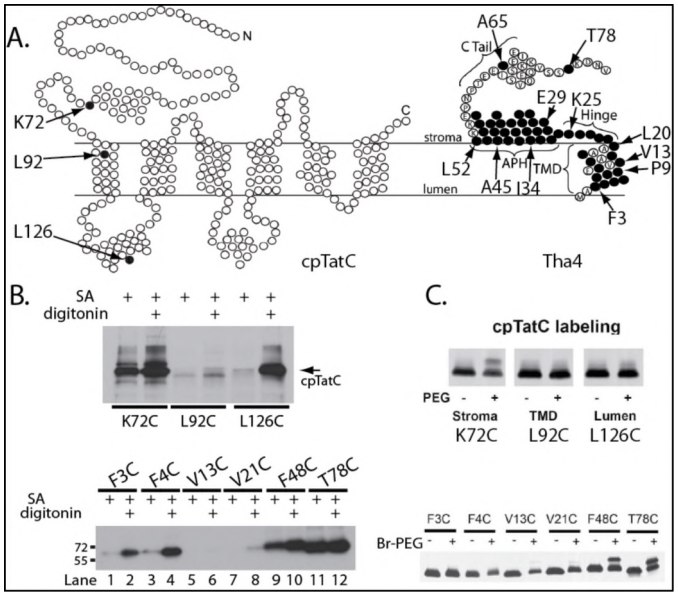

Figure 6. Streptavidin accessibility to biocytin activated Tha4. ***A,*** Schematic representation of the control protein, cpTatC, and Tha4. The filled circles indicate the location of Cys substitutions. ***B,*** Accessibility of Streptavidin to biocytin-activated cysteine. *Upper panel*, cpTatC. *Lower panel*, Tha4. ***C,*** PEG'ylation of the same cysteine probed in ***B*** using a branched PEG maleimide. *Upper panel*, cpTatC. *Lower panel*, Tha4. See text for details.

These results were confirmed by PEG-ylation using a branched PEG (polyethylene glycol) to bind thiols. The br-PEG cannot cross the membrane, but when it binds thiols it generates an adduct that can be detected by SDS-PAGE (**Fig. 6C**). The cpTatC control protein (*upper panel*) and Tha4 (*lower panel*) both demonstrate similar results as the biocytin-SA assay. Our collaborators at the University of Florida generated the PEG'ylation data. We have systematically tested Tha4 through the transmembrane domain and the APH and have seen that the N-terminal proximal portion of the APH is less accessible to SA in intact membranes than the C-terminal proximal portion is. This suggests a topology where Tha4 may be

tilted in the membrane much like the solution structure of $TatA_D$ indicates [12, 13].

How these data fit into the transport process is an active area of investigation. **This manuscript was published in *The Journal of Biological Chemistry,* 2012 287:34752-34763.**

In order to detect specific Hcf106-cpTatC interactions we developed an assay whereby we first import a cysteine labeled cpTatC into isolated chloroplasts, isolating the thylakoids from those chloroplasts, integrating Cys-substituted Hcf106 into those thylakoids and looking to see the formation of disulfide bonds between the imported cpTatCXnC and integrated Hcf106XnC (see **Fig. 7** for a general scheme). If the respective sulfhydryls were within 5Å of each other we would expect to see a higher molecular weight adduct when analyzed by SDS-PAGE.

Using this methodology, we have been able to map some interactions between cpTatC and Hcf106. cpTatC has six transmembrane domains (TM1-M6) connected by three stromal loops (S1-S3) and three lumen loops (L1-L3). Preliminary data shows that cysteine placed in the TMD of Hcf106 preferentially interacts with cysteine placed in TM5 of cpTatC. Likewise cysteine placed in the APH of Hcf106 preferentially interacts with the stromal loops (S1~S2>S3) of cpTatC (**Fig. 8**). We have therefore established that we can use *in vitro* expressed exogenous forms of cpTat proteins in native thylakoid to query the organization of the cpTat receptor and translocase. The current proposal seeks to expand upon this and through the use of doubly Cys-substituted cpTat components crosslink Tha4, Hcf106 and cpTatC into the same complex, which has not been demonstrated to date.

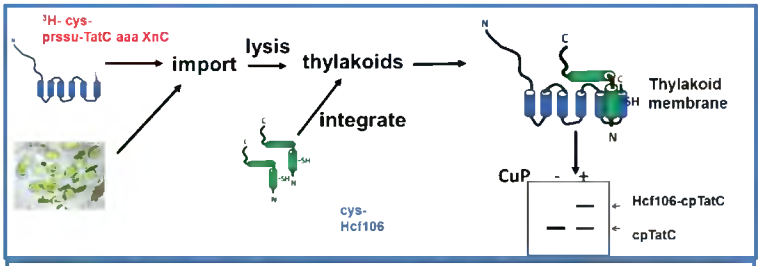

Figure 7. Import of cpTatC and integration of Hcf106 into the same thylakoids. *In vitro* expressed [³H]-prSSU-cpTatC is incubated with isolated chloroplasts to allow import of the precursor form of cpTatC. This precursor has been modified to include the transit peptide of the precursor to the small subunit of Rubisco (prSSU) at the N terminus, which greatly enhanced the import of pre-cpTatC (generous gift from Ken Cline, University of Florida). In addition, native cysteines were changed to alanine (aaa) to allow use of Cys-substitution crosslinking. After import, isolated, intact chloroplasts were lysed and the thylakoids harvested. *In vitro* expressed Hcf106 was incubated with the isolated thylakoids for integration. Ater integration, washed thylakoids were subjected to oxidation by Cu (II) phenanthroline (CuP) to promote formation of disulfide bonds, which could be detected as a higher molecular weight adduct on SDS-PAGE (Hcf106-cpTatC).

To summarize, we have made great headway on all of our specific aims. Firstly, we have identified points of contact between precursor and Tha4. Secondly, we have demonstrated topology of Tha4 in non-transport active thylakoid and in transport active thylakoid. Currently, we are actively determining the points of contact between Tha4 and the precursor as it transports. This is the first demonstration of a direct and specific interaction between Tha4 and the precursor. Even more importantly it is the first demonstration of direct, specific interaction between any TatA homolog and the mature domain of the precursor. These data strongly implicate Tha4 in the translocation pore. We are also actively expanding our investigation of Tha4 during transport to get a complete picture of the changes the molecule undergoes during transport of precursor. We can also address Hcf106 organization in the thylakoid and indirectly interaction with cpTatC. Further we are gaining insight into the general mechanism of spontaneous insertion of a protein into the membrane through biophysical studies of the Hcf106 protein and peptides. We would then apply this knowledge to the insertion of several

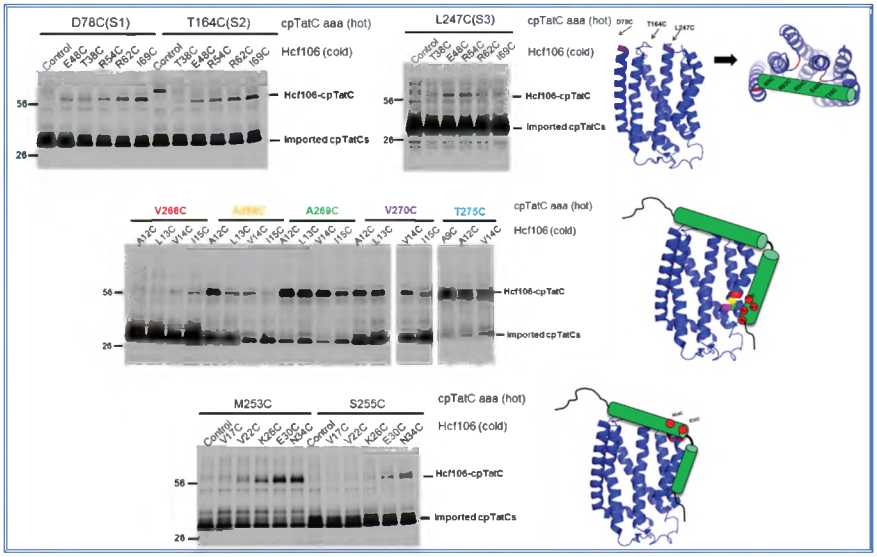

Figure 6. Preliminary mapping Hcf106 interactions with cpTatC. [^{3}H]-pre-cpTatC (hot) with the indicated cysteine substitution (X_nC) was imported into isolated chloroplasts Re-isolated chloroplasts were subject to osmotic lysis and the thylakoids harvested. Unlabeled Hcf106 (cold) was integrated into the isolated thylakoids and the oxidant CuP was added to promote disulfide bond formation. *Upper panel,* The APH of Hcf106 can interact with the stromal loop regions of cpTatC. *Middle panel,* the N-proximal portion of the transmembrane domain of Hcf106 preferentially interacts with the lumen proximal portion of TM5 on cpTatC. *Lower panel,* the N-proximal portion of the APH of Hcf106 preferentially interacts with the stromal-proximal portion of TM5 on cpTatC. Taken together, Hcf106 TMD sits along TM5 on cpTatC and the APH stretches across the stromal loops. Cartoon representations of the interactions between Hcf106 (green) and cpTatC (blue) are depicted to the right of each panel.

thylakoid proteins, which also seem to follow a spontaneous insertion pathway. We are currently updating our model of transport to account for these exciting new data.

Manuscripts published:

Debjani Pal, Kristen Fite, and Carole Dabney-Smith. Direct interaction between precursor mature domain and transport component Tha4 during Twin Arginine Transport (Tat) of chloroplasts. *Plant Physiology* **161:**990-1001. doi:10.1104/pp.112.207522.

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Manuscripts submitted:

Qianqian Ma, Kristen Fite, and Carole Dabney-Smith. Cysteine scanning and cross-linking study reveals the oligomeric property of Hcf106 in the chloroplast Tat system. PLoSONE *in review*.

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