Identification of novel chloroplast membranes involved in thylakoid biogenesis in the green alga *Chlamydomonas reinhardtii*

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ABSTRACT
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Oussama Rifaï

In chloroplasts, thylakoid membranes contain the complexes responsible for the light-dependent reactions of photosynthesis. While much is known about their functioning, the biogenesis of these membranes and their different components is less well understood. Previous work using *in situ* approaches to confocal microscopy found evidence for the existence of a specific zone where the synthesis of thylakoid proteins takes place in the chloroplast of the green alga *Chlamydomonas reinhardtii*. Building on these findings, I used a modified approach of cellular subfractionation by sucrose-density gradient centrifugation to identify sites of thylakoid biogenesis in the chloroplast of this organism. I found evidence for the existence of distinct membrane fractions that might be involved in thylakoid biogenesis. Named high density membranes (HDM), they are higher in density than thylakoids and fractionate below them on a sucrose gradient. HDM’s were found to be qualitatively different from thylakoids and contained markers of protein synthesis. Pulse-labelling experiments did not confirm that these membranes are involved in protein and lipid synthesis; however, these results also showed a departure from current models. Finally, these results suggest a potential role for these membranes in chlorophyll synthesis. We propose that these membranes are at the very least involved in
the early steps of synthesis and assembly of thylakoid proteins. They are high in density in conditions of active thylakoid membrane biogenesis, whereas in the absence of thylakoid biogenesis they are not laden with ribosomes, RNA-binding proteins and newly-synthesized proteins and thus are light in density. The significance of my work is that it lays the groundwork for further characterization of HDM’s in Chlamydomonas and higher plant species.
Acknowledgments

First and foremost, I would like to thank my supervisor Dr. William Zerges for his mentoring and continual support throughout the project. I also thank my committee members; Drs. Michael Sacher, Muriel Herrington and Paul Joyce for their sound guidance during long committee meetings. I thank all my friends and colleagues in the Zerges lab past and present: Ying Zhang, James Uniacke, Nadia Ait Ali, Madhav Soowamber, Yu Zhan, Matthew Peters, Marco Schotkowski, Jamieson Dhaliwal and Kassim Moujaber. This work was initiated by Ying Zhang, who identified membranes enriched in RB38 and chloroplast r-proteins, but which are distinct from thylakoid membranes.

Finally, I cannot thank enough my friends, family and loved ones for all their help and support through good times and bad times.
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<td>Alb3.1</td>
<td>Albino 3.1</td>
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<tr>
<td>CAP</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>CHX</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>DGDG</td>
<td>Digalactosyldiacylglycerol</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>HDM</td>
<td>High density membrane</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HSM</td>
<td>High-salt minimal medium</td>
</tr>
<tr>
<td>LDM</td>
<td>Low density membrane</td>
</tr>
<tr>
<td>LHCI</td>
<td>Light-harvesting complex I</td>
</tr>
<tr>
<td>LHCII</td>
<td>Light-harvesting complex II</td>
</tr>
<tr>
<td>MGDG</td>
<td>Monogalactosyldiacylglycerol</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PLB</td>
<td>Prolamellar body</td>
</tr>
<tr>
<td>PSI</td>
<td>Photosystem I</td>
</tr>
<tr>
<td>PSII</td>
<td>Photosystem II</td>
</tr>
<tr>
<td>RBP</td>
<td>RNA-binding protein</td>
</tr>
<tr>
<td>T zone</td>
<td>Translation zone</td>
</tr>
<tr>
<td>TAP</td>
<td>Tris-acetate-phosphate</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin-layer chromatography</td>
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Introduction

Life on Earth as we know it today owes much to the evolution of photosynthesis. Chloroplasts are the organelles of plant and algal cells that are specialized for oxygenic photosynthesis, whereby light energy from the sun is used to produce chemical energy (in the form of ATP and NADPH) to drive the fixation of inorganic carbon (from CO₂) into organic molecules that the organism can use for its metabolism. Molecular oxygen (O₂) is released as a by-product. These organelles are thought to have originated from an ancient cyanobacterial lineage that went through an endosymbiotic event with an aerobic eukaryote several billion years ago (Bhattacharya et al; 2007). As a consequence; and not unlike mitochondria, the chloroplast retains a small bacterial-like genome that encodes between 80-200 genes translated on 70S ribosomes within the organelle (Joyard et al; 2009, Schwarz et al; 2007, Bhattacharya et al; 2007, Goodenough; 1971, Bourque et al; 1971).

Since chloroplasts are thought to contain about 3000 proteins (Joyard et al; 2009) most are encoded by the nuclear genome, translated in the cytosol by the eukaryotic cytoplasmic translation machinery and imported into the chloroplast. As highly specialized organelles, chloroplasts show a great degree of compartmentalization. They are bound by a double membrane system consisting of the outer and inner envelope and contain a fluid medium called the stroma which is analogous to the cytoplasm of eukaryotic cells. Finally a network of flattened connected vesicles called thylakoid membranes form the third major compartment. Meaning “sac-like” in Greek, the thylakoids enclose a continuous space inside of them called the lumen. They can be found either stacked upon one another forming structures called grana or can consist of single

1
unappressed vesicles extended out into the stroma. Most thylakoids are usually stacked in grana (Ohad et al; 1967). All these different compartments play critical roles in the functioning of the chloroplast and the cell as a whole. The envelope for instance is thought to be involved in lipid and pigment synthesis for use inside and outside the chloroplast while the stroma contains enzymes for the Calvin cycle responsible for carbon fixation (Suss et al; 1993) and also is the site of synthesis of free fatty acids. Thylakoids are the *bona fide* photosynthesis membranes. They are made of a lipid bilayer in which several protein complexes (containing pigments and other accessory factors) are embedded. These complexes function in the photosynthetic electron transport chain which creates an electrochemical gradient across the thylakoids between the stroma on one side and the lumen on the other to generate ATP and NADPH. The major complexes are photosystem II (PSII); which in the green alga *Chlamydomonas reinhardtii* consists of 15 chloroplast-encoded protein subunits and 6 nuclear-encoded subunits (Minai et al; 2006) and photosystem I (PSI) which has most of its subunits encoded by the nucleus. The two other main complexes of the thylakoid membranes are the ATP-synthase and the cytochrome b6/f complex. Antennal complexes called the light-harvesting complexes I and II (LHCI and LHCII) are associated with PSI and PSII, respectively. They contain the pigment chlorophyll and transfer energy from light as excitation energy to the reaction centers of the photosystems where a pair of proteins (PsaA and PsaB for PSI, D1 and D2 for PSII) in complex with chlorophyll can harness this energy and transduce it to the rest of the electron carriers of the electron transport chain.

The three main elements of thylakoid membranes; proteins, lipids and chlorophyll all depend on one-another for their synthesis, transport and functioning and complex
mechanisms are at play both inside and outside the chloroplast to regulate these processes. It is known, for instance, that light plays a key role in modulating chlorophyll and lipid synthesis and the expression of chloroplast and nuclear genes encoding for subunits of the two photosystems and their antenna (Ohad et al; 1967, Barthélemy et al; 2000, Janero and Barneitt; 1981, Joyard et al; 1998, Adir and Ohad; 1990, Zerges et al; 2002). Furthermore, chlorophyll association was found to be necessary for the import, stability and assembly of the nuclear-encoded light-harvesting complexes (Reinbothe et al; 2006, Hoober et al; 1991, Hoober and Eggink; 2001) and for completion of translation of chloroplast-encoded chlorophyll-binding proteins and their insertion into thylakoid membranes (von Wettstein; 2001). Interactions between the LHC’s and thylakoid lipids are essential for the formation of the lipid bilayer since the main constituent lipid of the thylakoid membranes (monogalactosyldiacylglycerol, MGDG) does not readily form lipid bilayers (Vothknecht and Westhoff; 2001). Conversely MGDG synthesis is required for the synthesis of proteins of the photosynthetic apparatus (Kobayashi et al; 2007). Last but not least, the different subunits of the two photosystems regulate their own expression as well as that of other subunits and stabilize the association of the different components of a functional photosystem (Minai et al; 2006, Ozawa et al; 2010).

While much is known about the organization of the thylakoid membrane and the different mechanisms governing its different components, thylakoid biogenesis per se is still not well understood; that is the “molecular processes connected to the origin, synthesis, maintenance and adaptation” of thylakoid membranes and the spatial organization of these processes (Vothknecht and Westhoff; 2001, von Wettstein; 2001, Benning; 2009, Andersson et al; 2001, Räntfors et al; 2000, Uniacke and Zerges; 2007).
My Master's thesis project looked at thylakoid biogenesis in the green alga *Chlamydomonas reinhardtii*, focusing on the localization of sites of synthesis of chloroplast-encoded proteins and also touching upon the sites of chlorophyll and lipid synthesis. In this introductory chapter, I present this model organism in the next section, followed by brief reviews of protein, lipid and chlorophyll synthesis in chloroplasts. Finally, I shall be presenting my research question.

1.1 *Chlamydomonas reinhardtii*

The unicellular green alga *Chlamydomonas reinhardtii* is a good model organism for chloroplast research (Appendix 1). Belonging to the phylogenetic clade Viridiplantae, it is closely related to higher plants. It has a single large chloroplast which contains a small genome consisting of ≈100 genes, most of which encode proteins of thylakoid membrane complexes and rRNA. Both nuclear and chloroplast genomes are fully-sequenced and annotated and they are easily amenable to genetic manipulation. As a model organism in use since the mid 1940's, a number of mutant strains are available (see Togasaki and Surzycki; 1998 in “The Molecular Biology of Chloroplasts and Mitochondria” for a good historical review). Because this organism can grow heterotrophically on a medium supplemented with acetate it is ideal for studies using photosynthesis mutants. A large proteinaceous structure found in the chloroplast called the pyrenoid plays a role in carbon-concentrating mechanisms for this alga which in nature inhabits wet soils or freshwater ponds where concentrations of available CO₂ are much lower than in air.

The following sections deal with chloroplasts in general and are not limited to *Chlamydomonas*. 
1.2 Protein synthesis

Early work in the 1960's and 1970's showed that chloroplasts contain bacterial-like 70S ribosomes that are active in translation. Inhibition experiments using bacterial antibiotics showed that they do indeed synthesize proteins in vivo (Bourque et al; 1971, Goodenough; 1971, Eytan and Ohad; 1970, Chua et al; 1973). To investigate the localization of protein synthesis within the chloroplast, researchers turned to electron microscopy. Initial results showed an even distribution of ribosomes throughout the chloroplast (Goodenough; 1971, Bourque et al; 1971). Further refinements such as treatment with the antibiotic chloramphenicol (CAP) which inhibits the elongation step of translation effectively “freezing” the ribosomes in a translationally-active form, led to the finding of ribosome clusters (polysomes) bound to thylakoid membranes preferentially at the unappressed stromal thylakoids (Chua et al; 1976, Chua et al; 1973, Margulies and Michaels; 1974). Another line of evidence for the localization of protein synthesis came from radioactive pulse-labelling experiments followed by cellular subfractionation of the different chloroplast compartments on sucrose-density gradients. It was concluded from these experiments that thylakoids were indeed the sites of translation in the chloroplast, with more translation observed on stromal thylakoids (Herrin et al; 1981, Margulies; 1983, Herrin and Michaels; 1985, Edelman and Mattoo; 1986).

Therefore, the commonly accepted model states that translation in chloroplasts occurs through polysomes bound to stromal thylakoid membranes, with the de novo synthesized proteins directly integrating into the membrane. Since the distribution of photosynthetic complexes on thylakoids is heterogeneous; for instance granal membranes are enriched in PSII whereas stromal membranes are enriched in PSI (Adir et al; 1990), it
was proposed that lateral migration within the membranes allows the newly-synthesized proteins to reach their final destinations (Ojakian and Satir; 1974, van Wijk et al; 1995, Adir et al; 1990). While this model is supported by several lines of evidence, many doubts remain about whether it is the only mode of translation operating in the chloroplast. Indeed, several inconsistencies exist within the early electron microscopy reports. In some cases, structures that look highly similar are categorized as both stromal and granal thylakoids without much reasoning by the authors (Chua et al; 1976, Margulies and Michaels; 1974). Furthermore, many of the procedures used for isolating and separating chloroplast membranes are highly crude and induce a significant amount of cross-contamination (Edelman and Mattoo; 1986, Adir et al; 1990, Ossenbühl et al; 2002). One report showed that ribosomes were, in fact, mostly found on a membrane that had very different properties from thylakoids in terms of protein and chlorophyll content and density (Margulies and Weistrop; 1980). In addition, many studies examined the synthesis of the D1 subunit of PSII alone, which is known to have a very short half life in thylakoids (approximately 8 hours) and thus is constantly turned over and shows high levels of repair synthesis compared to most other subunits of the photosystems (Adir et al; 1990).

1.3 Lipid synthesis

The lipids of thylakoid membranes are very different from those found outside the chloroplast (Joyard et al; 1998). They are mostly composed of galactolipids and phospholipids which account for 80-90% and 10-20% of total lipids, respectively (Awai et al; 2001, Jouhet et al; 2007, Vothknecht and Westhoff; 2001). The major galactolipids,
mono- and digalactosyldiacylglycerol (MGDG and DGDG), account respectively for roughly 50-60% and 20-30% of total thylakoid galactolipids (Kobayashi et al; 2007, Jouhet et al; 2007).

Initial work on characterizing the sites of lipid synthesis in the chloroplast used radioactive pulse-labelling coupled with electron microscopy to look for newly-synthesized lipids in situ. The labelled lipids were found equally distributed in the envelope and thylakoid membranes (Goldberg and Ohad; 1970). Later research also used lipid pulses but followed these by cellular subfractionation on sucrose-density gradients. These methods reported lipid synthesis activity to be highest on envelope membranes (Douce; 1974, Douce and Joyard; 1980, Joyard and Stumpf; 1980, Janero and Barrnett; 1981); particularly at the inner envelope (Andrews and Mudd; 1985, Miquel and Dubacq; 1992). More recently, experiments were conducted to localize the enzyme responsible for the last step in MGDG synthesis (MGDG synthase) by performing Western blots on membrane extracts and the results seemed to point toward a localization on the inner envelope membrane (Awai et al; 2001, Miège et al; 1999, Räntfors et al; 2000, Kobayashi et al; 2007, Kelly and Dormann; 2004). One major concern with most of these studies, however, is the fact that they tend to use equal loading amounts of protein or chlorophyll. Since the envelope has about 1000 times less chlorophyll and proteins compared to thylakoids (Barthélemy et al; 2000), these studies tend to grossly over-estimate the contribution of the envelope in lipid synthesis. Studies done on a per fraction basis would be more biologically relevant, yet they are few and far between in the literature (Joyard and Stumpf; 1980).

Nevertheless, since the inner envelope and thylakoid membranes have a very
similar lipid composition (Jouhet et al; 2007), the major polemic currently surrounding lipid research is not whether lipids are made on the envelope but how they get from the envelope to the thylakoids. Some evidence exists for zones of contact between the inner envelope membrane and thylakoids where lipids could be transferred from the former to the latter through direct contact or invagination of the envelope. However, this evidence is mostly in the form of early microscopy studies with very few recent supportive studies (referred to in Douce; 1974, Hoober et al; 1991, Kobayashi et al; 2007). A recent model proposed that vesicular transport might bring lipids from the inner envelope membrane to the thylakoids. Originally based on the observation of structures which look like vesicles budding off the envelope (Hoober et al; 1991, Morré et al; 1991, Kaneko and Keegstra; 1996), this hypothesis gained more ground with the description of a protein found in Arabidopsis, Chlamydomonas, and the cyanobacterial relative of chloroplasts Synechocystis that was found to be necessary for thylakoid biogenesis and whose absence was correlated to the disappearance of vesicle structures inside the chloroplast. This protein was named Vipp1; vesicle-inducing protein in plastids 1 (Kroll et al; 2001, Fuhrmann et al; 2009, Aseeva et al; 2007, Benning et al; 2006).

So far, there has been little direct evidence supporting the involvement of this protein in vesicle formation. Furthermore, no mechanism for its action has been demonstrated yet. Except for reports of it interacting with the chloroplast heat-shock protein HSP70B and a protein of the chloroplast import machinery (Benning et al; 2006, Jouhet and Gray; 2009) there has been no evidence of interacting partners that would be necessary for vesicular formation. Recently, it has been shown that deletion of Vipp1 in cyanobacteria causes first the loss of photosynthetic activity and then a loss of thylakoid
formation per se (Gao and Xu; 2009). Since this protein is a homologue of a bacterial protein involved in stress response and membrane integrity and bacteria are thought to entirely lack the capacity for a vesicular transport system, there is considerable doubt as to whether this mechanism really exists inside chloroplasts (Gao and Xu; 2009, Aseeva et al; 2007, Benning; 2009).

1.4 Chlorophyll synthesis

In some cyanobacteria, green algae and higher plants most of the chlorophyll is found in two forms: chlorophyll a and chlorophyll b which have identical structures except that chlorophyll b has an aldehyde side-chain instead of a methyl side chain as in chlorophyll a. Chlorophyll a is bound with the reaction center proteins of the two photosystems and the LHC’s whereas chlorophyll b is almost exclusively found in the LHC’s (Hoober and Eggink; 2001, Hoober et al; 2007, Oster et al; 2000). As a highly reactive molecule, chlorophyll absorbs light resulting in the production of single-state excited chlorophyll molecules. This excitation energy can be dissipated by a decay process, one outcome of which can lead to the production of singlet oxygen which are extremely reactive oxygen species that can cause significant damage within the chloroplast (Müller et al; 2001). Therefore its synthesis has to be tightly coordinated with that of the proteins, lipids and carotenoids it binds with to prevent an accumulation of free, unbound chlorophyll (Joyard et al; 2009).

The biochemical pathways of chlorophyll synthesis are well described; however, the spatial organization of these pathways is still a matter of debate. It is well agreed upon that early steps that synthesize soluble precursors occur in the chloroplast stroma. Later steps have been shown to occur on membranes, with some groups reporting activities on
either envelope, thylakoids or both membranes for the same enzymes (Tottey et al; 2003, Eckhardt et al; 2004, Barthélemy et al; 2000, Hoober et al; 2007, Reinbothe et al; 2006, Oster et al; 2000, Joyard et al; 2009). Some controversy surrounds the final steps of chlorophyll synthesis, where the precursor protochlorophyllide is turned to chlorophyllide then to chlorophyll a or b. The enzyme catalyzing this last reaction (chlorophyll synthase) has been found exclusively in the thylakoids (Joyard et al; 2009, Beale; 1999) which would make sense if one considers that synthesis of chloroplast-encoded thylakoid proteins is co-localized with the synthesis of the chlorophyll that binds to these proteins. The problem is how the final form of chlorophyll binds to LHC’s which are imported from the cytosol in the absence of chlorophyll synthase in the envelope, considering that this binding is required for import and stability of LHC proteins as discussed before. It has been proposed that a precursor to chlorophyll binds to these proteins upon import, and somehow gets catalyzed into chlorophyll once the LHC reaches the thylakoids. This has yet to be definitively demonstrated (Eckhardt et al; 2004, Hoober and Eggink; 2001, Beale; 1999, Reinbothe et al; 2006).

1.5 Research question

As described in the previous reviews, thylakoid biogenesis as a whole is still poorly understood. Many reports are purely biochemical in nature and have not been corroborated in situ. In a cell, the structure and the location of a process can say much about its role and mode of functioning in vivo; and the relationships between the different elements of a process depend on the biologically available concentrations of each element. Therefore, in situ approaches that take these factors into account can tell us much about what happens inside a cell. Previous work in our lab using in situ
hybridization and confocal microscopy showed the existence of distinct regions in the chloroplast of *Chlamydomonas* located around the pyrenoid that are the sites of *de novo* synthesis of proteins of PSII (Uniacke and Zerges; 2007). This is in contradiction with the aforementioned model of protein synthesis on stromal thylakoids which are evenly distributed within the chloroplast. Furthermore, other work showed the existence of a distinct membrane compartment that is associated with thylakoids that had markers of protein synthesis (Zerges and Rochaix; 1998).

Building on these reports, my project used cellular subfractionation to try to identify where translation markers are found in the chloroplast of *Chlamydomonas*. Results from these experiments showed that these translation markers were enriched in a distinct membrane fraction that was denser than thylakoids isolated by sucrose-density gradient centrifugation. Pulse-labelling approaches, however, were less conclusive, with most of the newly-labelled proteins found in thylakoids. These results could either be interpreted as refuting the previous results or simply indicating very fast transport of these newly-synthesized proteins to their final destination in the thylakoids. I investigated whether this membrane compartment is involved in lipid and chlorophyll synthesis. The results were also inconclusive.

I will be presenting these results in the next sections and discussing their implications.
2 Materials and methods

I- Chlamydomonas reinhardtii culture conditions

Wild-type, cell wall and photosynthesis mutant strains were cultured heterotrophically on Tris-acetate-phosphate (TAP) medium, (Gorman and Levine; 1965) until mid-log phase (1-5 x 10^6 cells/mL) at 24°C with continuous shaking at a light intensity of 100-150 µE·m^{-2}·s^{-1}.

II- Cellular subfractionation

Cells cultured in 500 mL of culture medium were incubated with the antibiotic chloramphenicol (CAP) to a final concentration of 100 µg/mL at 24°C for 10 minutes prior to harvesting. All subsequent steps were carried out at 4°C. Cells were harvested by centrifugation at 4000 g for 5 minutes and then resuspended in 7 mL of TKMD buffer (Chua et al; 1973) containing 25 mM KCl, 25 mM MgCl2, 25 mM Tris-HCl pH 7.5 and 100 µg/mL CAP. The cells were broken by passing twice through a chilled French pressure cell at 1000 psi (breakage was monitored by light-microscopy and this method was found to be highly efficient). The crude cell extract was transferred to a 12 mL Beckman ultracentrifuge tube and the volume was adjusted using the same buffer. Crude membranes and cell debris were pelleted during a 2 hour centrifugation at 100,000 g using the SW-41 Ti rotor in a Beckman ultracentrifuge. This pellet was then resuspended in 2 mL of a 2.5 M sucrose solution containing 25 mM MgCl2, 20 mM KCl, 10 mM tricine and 2 mM of the protease inhibitor PMSF. This was loaded at the bottom of a 12 mL Beckman tube and a continuous gradient of 2.0-0.5 M sucrose concentrations (in the same buffer as above) was layered on top of it using a gradient maker connected to a Bio-
Rad peristaltic pump. The gradients were centrifuged for 16 hours in an SW-41 Ti rotor at 100,000 g. The fractions were collected from the top using micropipettors and great care was taken to avoid fraction cross-contamination. See Figure 1 for the location of these fractions on the gradient. Thylakoids are clearly visible as a dense green band in the middle of the gradient. These fractions were then each transferred to new 12 mL tubes, diluted with TKMD buffer and pelleted during a 2 hour centrifugation at 100,000 g. Membrane pellets were resuspended in an appropriate volume of D buffer (20mM HEPES-KOH pH 7.8, 60 mM KCl, 0.2 mM EDTA and 20% glycerol) and stored at -80°C for further use.

III- Immunoblot analyses

Samples of the various extracts were incubated for 1 hour at 42°C in protein loading buffer containing 10% glycerol, 50 mM Tris-HCl pH 6.8, 2% SDS, 0.28 M beta-mercaptoethanol (added to 2% of sample volume from the 14 M stock) and 0.01% bromophenol blue. They were then loaded on a 12% SDS-polyacrylamide gel for the resolving step (Laemmli; 1970). Proteins were electroblotted onto nitrocellulose membranes (0.45 µm pore diameter) and protein transfer was verified by staining with Ponceau S. The filters were blocked in a solution containing 5% non-fat dry milk in 1X phosphate buffer-saline (PBS) for 1 hour at room temperature. Primary antibody incubation was done in the same milk solution for 2 hours at 4°C. The filters were washed three times for 10 minutes each in 1X PBS then incubated with the secondary antibody (in milk) for 1 hour at room temperature. After three 10 minute washes with 1X PBS ECL solution was added to the filters according to the manufacturer's specifications.
and the membranes were exposed to X-ray film to reveal the signals. Signal intensity was determined with the ImageJ software.

**IV- In vivo protein pulse-labelling and cell lysis conditions**

Cultures were grown on TAP medium as described previously. Cells were pelleted by centrifuging for 5 minutes at 4000 g at room temperature and then resuspended in TAP medium lacking sulfur (TAP-S) in 15 mL Corex tubes. The antibiotic cycloheximide (CHX) was added to a final concentration of 10 µg/mL to inhibit translation by cytoplasmic ribosomes and the tubes were incubated for 5 minutes at room temperature then 5 minutes at 4°C under white fluorescent lighting and with continuous shaking. [³⁵S] H₂SO₄ (250 µCi) were added per 4 X 10⁷ cells and the reaction was allowed to proceed for 15 minutes at 4°C. Tubes were then put on ice and CAP added to 100 µg/mL to stop protein synthesis in the chloroplast and freeze 70S ribosomes on membranes. Chases, when needed, were performed for 1 hour at room temperature using an excess of unlabelled MgSO₄ to a final concentration of 2 mM MgSO₄. The cells were pelleted and resuspended in 9 mL of isotonic breaking buffer (0.3 M sorbitol, 50 mM HEPES, 5 mM MgCl₂, 100 µg/mL CAP) then broken using a Yeda press nitrogen bomb apparatus (Goldschmidt-Clermont; et al 1989). The crude cell extract was centrifuged at 4000 g for 3 minutes and the chloroplasts in the pellet were lysed by resuspending in hypotonic buffer containing 2 mM EDTA and 100 µg/mL CAP by repeated vortexing and pipetting. Proper lysis was verified by light microscopy. Successful lysis is revealed by observing an undifferentiated mass of green globular material (Appendix 2). The presence of chloroplast fragments or unbroken cells indicates incomplete lysis. Many
lysis conditions were assayed and were found to have different efficiencies depending on the cell strain. For instance, using a hypotonic buffer containing 10 mM Tricine, 5 mM MgCl₂ and 5 mM beta-mercaptoethanol (Zerges and Rochaix; 1998) gave a lysis efficiency of approximately 50% for the CC-503 cell-wall mutant strain and an efficiency approaching 100% for the #6 cell-wall mutant strain. Lysing with 2 mM EDTA was almost 100% efficient for both strains. Thus, strain #6 and 2 mM EDTA were chosen in order to ensure optimal lysis conditions. Fragments of lysed chloroplasts and membranes were pelleted by centrifuging for 2 hours at 100,000 g, the pellet was then resuspended in 1 mL of 2.5 M sucrose, transferred to a 5 mL Beckman ultracentrifuge tube and a 2.0-0.5 M continuous sucrose gradient (in the same buffer as described above) was layered above it as previously described. Gradients were centrifuged for 16 hours at 100,000 g at 4°C in an SW-55 Ti rotor and membrane fractions in the gradients were collected and pelleted as described. Samples were prepared for electrophoresis then loaded on a 12-18% gradient SDS-polyacrylamide denaturing gel containing 8M urea. After electrophoresis, gels were stained with Coomassie blue and dried with a gel drier. They were then exposed to a phosphoimager screen and signal was revealed by scanning the screen in a Typhoon trio imager (GE Healthcare).

V- In vivo lipid pulse-labelling

This was done essentially as above, with the following notable exceptions. Cells were initially resuspended in HSM minimal medium (Harris; 1989) instead of TAP-S. Cells were allowed to recover for 10 minutes at room temperature and then 10 minutes at the experimental temperature (4°C) before adding [¹⁴C]-acetate dissolved in HSM
using 50 µCi for 4 X 10^7 cells. Chases were performed by adding 1 mL of a concentrated 40X TAP solution containing cold acetate. After collecting the membrane fractions from the sucrose gradients membrane lipids were extracted with chloroform/methanol (Wersel and Flügge; 1984). They were then dried under a stream of nitrogen and resuspended in an appropriate volume of chloroform. The samples were then loaded on a pre-coated Silica gel 60 thin-layer chromatography plate (EMD Chemicals) and separation was done in the following solvent system: chloroform: methanol: acetic acid: water in the ratios 85:15:10:3 (Wood et al; 1965). After the end of the run the plate was left to dry under nitrogen and was then exposed to a phosphor imaging screen as before. Lipid species were identified based on their relative retention factor (RF) values (Wood et al; 1965, Zerges and Rochaix; 1998).

VI- In organello protein pulse-labelling

Cultures were grown in 250 mL of TAP medium to cell densities of 1-5 X 10^6 cells/mL. Cells were pelleted as before and resuspended in the same breaking buffer. They were then broken in a Yeda press (Goldschmidt-Clermont et al; 1989), pelleted and washed again in the same buffer. All operations following breakage were performed at 4°C. This crude cell extract was diluted to a final chlorophyll concentration of 200 µg/mL (see below) and 700 µL of this dilute extract was added to a 15 mL Corex tube containing the following reaction mixture: 250 µL of 4X master mix (1.65 M sorbitol, 175 mM Hepes pH 7.6, 40 mM MgCl₂, 4 mM 3-PGA, 1.6 mM spermidine free base, 4 mM Mg-ATP, 40 mM DTT, 160 µM of each amino acid minus cysteine and methionine), [³⁵S] methionine-cysteine (10 µL), 40 µL of 100 mM Mg-ATP and 1 µL of 10 µg/mL CHX.
Labelling reactions were carried out for 15 minutes at 4°C under fluorescent lighting and with continuous shaking. If needed, chases were performed for 1 hour at room temperature using an excess of unlabelled methionine (final concentration 10 mM). The labellings were stopped by placing the tubes on ice and adding CAP to a final concentration of 100 µg/mL. Chloroplasts were pelleted (4000 g, 3 minutes) then resuspended in hypotonic buffer (2 mM EDTA, 100 µg/mL CAP) and lysed by vortexing and pipetting vigorously. Membranes were then pelleted and fractionated on a sucrose gradient as previously described. The fractions were then collected, membranes pelleted and electrophoresed on an SDS-PAGE using the same methods described for in vivo labelling.

**VII- Chlorophyll and protein concentration measurements**

Chlorophyll content was determined after extracting the pigments in cold methanol using a spectrophotometric assay (Porra; 2002). Protein concentrations were determined by using a BCA-BSA assay kit from Sigma-Aldrich according to the manufacturer's instructions. BSA was used to determine the standard curve.

**VIII- HPLC analysis of pigments**

Pigments were extracted with 80% acetone and dried under a stream of N₂. Dried extracts were resuspended in 100 µL of ethyl acetate and injected into a C18 reverse-phase column (Phenomenex, 5 µm pore size, 25 cm length). Separation was performed essentially according to Norris et al (1995) on an HP 1090 HPLC system. In brief, the solvents used were acetonitrile:H₂O:triethylamine in 9:1:0.01 ratios (solvent A) and ethyl acetate as solvent B. Starting conditions were 80% solvent A and 20% solvent
B. A 45-minute gradient saw the proportion of solvents going to 100% B and then back to the starting conditions at the end of the run; a flow rate of 1 mL/minute was used throughout. Detection was performed with a photodiode array detector scanning wavelengths between 350 and 500 nm at 2 nm intervals. Pigments were identified by comparison to published retention times (Norris et al; 1995) and their identities confirmed by mass spectrometry on a Quattro LC system connected to the HPLC output. The following settings were used for mass spectrometry: ionization mode ES\(^+\), mass range was 500 to 1000 Da and cone voltage was 40 V. All results were analyzed using the Masslynx software package and pigments were quantified by integrating the area present under their peaks of absorption.
3 Results

3.1 Membranes denser than thylakoids are enriched in translation markers

A previous student found membranes that are denser than thylakoid membranes and are enriched in translation markers (Ying Zhang, personal communication). Previous in situ work also revealed that certain translation markers co-localize to distinct regions around the pyrenoid during active de novo synthesis of thylakoid proteins (Uniacke and Zerges; 2007). The RNA-binding protein RBP40 which is thought to preferentially bind to the mRNA encoding for the D2 subunit of photosystem II (Schwarz et al; 2007) showed strong co-localization with the L12 protein of the chloroplast large ribosomal subunit. These areas were named T zones for translation zones (Uniacke and Zerges; 2007) (Appendix 3-A). I wanted to determine if this co-localization could be observed by molecular means and to find by sucrose-density gradient fractionation which sub-chloroplast fraction(s) show(s) enrichment for these markers. Treating the cells with the antibiotic CAP has been shown to prevent termination of translation allowing ribosomes attached to membranes by nascent polypeptide chains to remain there, preventing them from being released during subsequent steps of cell harvesting, breakage and fractionation. Membranes were fractionated by sucrose-density gradient ultracentrifugation. This way, membranes are resolved according to their buoyant density and cell debris and other non-membrane components of the cell will pellet at the bottom of the centrifuge tube.

Following an overnight centrifugation (Figure 1-A) membranes separated into a major thylakoid band (Fraction 2) identified as such based on the area of the gradient it migrates to, its dark green colour and on the presence of bona fide thylakoid markers (see Figure 10). The area above the thylakoids corresponds to the envelope membrane of the
chloroplast. Its yellowish colour indicates an enrichment in carotenoid pigments and chlorophyll is undetectable in this fraction. Previous work has established that envelopes are lighter in density than thylakoids and fractionate above them on a sucrose-density gradient (Zerges and Rochaix; 1998, Douce et al; 1973, Joyard et al; 1980). Denser membranes fractionated below the thylakoids (Fractions 3-5). Figure 1 shows an immunoblot probed for the T zone markers mentioned above along with the Ycf4 protein which is a photosystem I assembly factor thought to be present at thylakoids (Boudreau et al; 1997, Ozawa et al; 2010). The samples were loaded on a per fraction basis, not on a total protein basis so that the presence, absence or enrichment of a protein in one fraction compared to another is an accurate representation of the distribution of this protein within the cell. Thus, we see that Fraction 1 has no signal for any of the 3 marker proteins. Fraction 2 shows signal for all 3 proteins, as expected based on its green colour. The fractions below the thylakoid, however, show significant enrichment in the ribosomal protein L12 as well as enrichment in RBP40 in Fractions 4 and 5 compared to thylakoids. Ycf4 was not detected below the thylakoid, consistent with the prediction that it is a thylakoid protein. Furthermore, it was found that the fractions below the thylakoid have much less total chlorophyll and show a higher protein/chlorophyll ratio than thylakoids (Figure 2-A and 2-B respectively).

These results show that, in addition to bona fide thylakoids, membranes denser than thylakoids show enrichment in T zone markers but not Ycf4 and that by virtue of their difference in protein and chlorophyll concentrations they represent a distinct membrane within the chloroplast. These membranes will be henceforth referred to as high density membranes (HDM).
Figure 1: Western blot showing presence of marker proteins in chloroplast fractions separated by sucrose-density gradient fractionation. Cells were disrupted and membranes floated on a 0.5-2.5 M linear sucrose gradient, with Fraction 2 corresponding to thylakoid membranes, Fraction 1 containing the envelope and Fractions 3-5 were named high density membranes (A). These were loaded on a per fraction basis on an SDS-PAGE gel (B) and probed with antibodies against RBP40, an RNA-binding protein specific for translation of the D2 subunit of PSII (Schwarz et al; 2007), Ycf4 an assembly factor for PSI (Boudreau et al; 1997) and the L12 protein of the chloroplast large ribosomal subunit.
Figure 2: Chlorophyll content and protein/chlorophyll ratio in membrane fractions separated by sucrose-density gradient centrifugation. Fractions were taken from the gradient presented in Figure 1 and the numbers represent the percentage of total chlorophyll present in each fraction (A). Protein/chlorophyll ratios for these same fractions are presented in (B); note that the Y axis is logarithmic.
3.2 *Alb 3.1* mutants accumulate unassembled D1 subunits in high density membranes

HDM's of a mutant cell strain were found to accumulate unassembled D1 subunits, a result similar to that observed by confocal microscopy. The *alb3.1* mutant of *Chlamydomonas reinhardtii* is defective in PSII assembly. The Alb3.1 protein interacts with both the nuclear-encoded LHCII proteins and the chloroplast-encoded D1 subunit of PSII and is required for integration of these proteins into functional PSII complexes in thylakoid membranes. In *Chlamydomonas* the *ac29* mutant has a disruption in the gene encoding for Alb3.1 and was found to accumulate unassembled D1 subunits in thylakoids (Bellaﬁore et al; 2002, Ossenbühl et al; 2004), more specifically in a ring-like fashion around the pyrenoid, i.e. in a region that encompasses the T zones (Uniacke and Zerges; 2007). I investigated whether I could detect subcellular fractions in which D1 accumulation occurs specifically in this mutant by comparing membrane fractions to those from a wild-type control. An enrichment in D1 in a mutant fraction compared to the same fraction in the wild-type indicates the regions where unassembled D1 is accumulating. In these two gradients (Figure 3), Fraction 1 represents the envelope, Fraction 2 the thylakoids and 3 and 4 are HDM fractions. In the wild-type, D1 was mostly found in thylakoids and the first high density fraction (3). In *ac29* on the other hand, D1 was also found in the second high density fraction (4) in addition to Fractions 2 and 3. Comparing the ratio of signal from D1 between thylakoids and HDM’s between the two strains we find that there is more D1 on a per fraction basis in HDM’s in the *ac29* mutant compared to the wild-type (ratios are 0.647 and 0.519 respectively). Probing for the L12 ribosomal protein showed that the mutant has fewer overall chloroplast ribosomes relative to the wild-type however the ratios of ribosomes below the thylakoid
to those in the thylakoid in both strains do not show much difference. Relating these results to the previous *in situ* work indicates that HDM's are similar in their distribution of these markers to the region around the pyrenoid that accumulates unassembled D1 subunits (Appendix 3-B).
Figure 3: Western blot showing enrichment in D1 protein in high density membranes from the alb3.1 mutants. Membranes from wild-type and alb3.1 mutants were separated by sucrose-density centrifugation (A) then probed for the D1 subunit of PSII and the L12 protein of the chloroplast large ribosomal subunit (B). Samples were loaded on a per-fraction basis.
3.3 High density membranes have a different chlorophyll a/b ratio than thylakoids

In order to further characterize HDM’s and examine any qualitative differences between them and thylakoids, I determined the ratio of chlorophyll a to chlorophyll b in the different membrane fractions using two approaches. The first consisted of separating the membrane pigments by thin-layer chromatography (TLC) in the appropriate solvent system. Pigments were then identified by their retention factor and positions relative to one-another (Quach et al; 2004). Figure 4-A shows the relative levels of these pigments. The position of both chlorophylls and beta-carotene are indicated. Figure 4-B shows the chlorophyll a/b ratio in each fraction. While thylakoids have a ratio of 0.6, these values are much higher in the HDM’s with the first fraction immediately below the thylakoids having a ratio of 1.4 and the second a value of 0.9. The second approach for determining this ratio was to separate the pigments by HPLC and analyze the area under the absorbance spectrum of each pigment. Chlorophyll a and chlorophyll b were identified by both their elution times and order of appearance and confirmed by mass spectrometry. Figure 5 shows two such absorbance spectra for thylakoids and the uppermost high density fractions (Fig. 5-A and 5-B respectively). Table 1 lists the ratio of chlorophyll a/b in the different fractions. Thylakoids had a value of 0.53 and the three HDM fractions had ratios of 1.03, 1.56 and 1.18, respectively. These values compare favourably to those obtained by TLC, the differences being due to the use of different sets of samples for the two experiments. What is clear however is that the pigment compositions of thylakoids and HDM’s differ substantially. This qualitative difference in itself can be used as an argument in favour of the HDM’s being at the very least a different sub-category of thylakoids. Since chlorophylls a and b are differentially associated with different
membrane protein complexes, these differences in ratio have profound implications on the protein composition of the HDM’s (see Discussion).
Figure 4: Thin-layer chromatography (TLC) separation of pigments contained in membranes separated by sucrose-density gradient (A). The chlorophyll a/b ratios were calculated for those fractions where the amount of chlorophyll was measurable (B).
Figure 5: Absorbance spectra of pigment extracts from thylakoids (A) and high density membranes (B). These extracts were separated by HPLC and the peaks representing 3 different pigments were eluted at characteristic times and confirmed by mass spectrometry.
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Chlorophyll a/b ratio</th>
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<tbody>
<tr>
<td>Thylakoid (2)</td>
<td>0.53</td>
</tr>
<tr>
<td>High density membrane (3)</td>
<td>1.03</td>
</tr>
<tr>
<td>High density membrane (4)</td>
<td>1.56</td>
</tr>
<tr>
<td>High density membrane (5)</td>
<td>1.18</td>
</tr>
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Table 1: Chlorophyll a/b ratio from membrane fractions determined by HPLC.
3.4 Protein synthesis determined by radio-isotope pulse-labellings

If HDM’s are the sites of thylakoid protein synthesis, we expect radioactive pulse-labellings to reveal newly-synthesized proteins there. Results were contrary to our expectations. *Chlamydomonas* cells were subjected to radioactive pulse and pulse-chase labellings using elemental $^{35}$S (in SO$_4$) for whole-cell labelling and [$^{35}$S]-methionine-cysteine for *in organello* chloroplast labelling. Compared to previous gradients, many fewer cells were used in these experiments in order to increase the specific activity of the label. Therefore, Fraction 2 in Figure 6, corresponding to thylakoids, is the most apparent to the naked eye while HDM’s are either invisible or appear as a mass of pale green specks dispersed in the sucrose (Fraction 3). The fraction above the thylakoids (envelope) is either invisible or appears as a faint yellow-green band (Fraction 1). Finally, the pellet (Fraction P) contains unbroken cells, unlysed chloroplasts, starch, cell debris and any non-membrane cellular component that does not float in sucrose. When labelling whole cells, the silver-staining patterns reveal a high enrichment in total proteins in the thylakoid fraction compared to the other fractions as expected. Note that the smearing observed here is due to the lower-than-usual amount of DTT used to denature the proteins. This was done to reduce background caused by reducing agents when silver staining according to the manufacturer's specification. The autoradiogram shows that most of the labelled newly-synthesized proteins are also found in thylakoids. The results following a one-hour chase show a similar pattern, except that in this case there seems to be comparatively more total and newly-synthesized proteins in the thylakoids.

Figure 7 shows the results for an *in organello* pulse-labelling experiment using [$^{35}$S]-methionine-cysteine. Two pulses were performed, one at the regular experimental temperature of 4°C and the other at room temperature (22-24°C). Results from the latter
can be likened to those coming from a chase in the sense that at room temperature translation happens at a faster rate than at 4°C. Furthermore, it has been estimated that polysomes bound to membranes spend 10 minutes or less to complete translation (Chua et al; 1976). Possible mechanisms for transport of proteins from their sites of synthesis to their final destination on thylakoid membranes by means of lateral diffusion or vesicular transport are slowed at 4°C (Morré et al; 1991, Kroll et al; 2001, Garcia et al; 2010). Thus, 4°C should be well-suited for observing newly-synthesized proteins right at their sites of synthesis while a pulse performed at room temperature would introduce the variables of transport as well as a high rate of turnover of translation and, therefore, its results can be favourably compared to those of a pulse-chase.

Figure 7-A shows the Coomassie blue staining pattern of the different fractions collected from the sucrose-density gradient, with Fraction 2 corresponding to thylakoids, 1 and 3 the above and below thylakoids (envelope and HDM’s) respectively and P the pellet. Even though great care was taken during collection, there was some cross-contamination of the fractions (especially Fraction 1) with thylakoids as revealed by the patterns of staining in Lanes 1 and 3. The autoradiogram in Figure 7-B shows that most of the signal appears in Lane 2 for both experimental conditions, with the amount of signal directly proportional to the amount of total protein loaded on the gel. We expect from a site of synthesis to have a higher ratio of labelled proteins to total proteins compared to a site where translation does not take place; therefore, it is not possible to say whether such a site was identified by the two previous experiments.
Figure 6: *In vivo* pulse-labelling of proteins from chloroplast membranes isolated by sucrose-density gradient centrifugation. Cells were subjected to a pulse-labelling with [\(^{35}\)S] or a pulse-labelling followed by a chase then fractionated on a sucrose density gradient (top). Membranes were collected and proteins separated on an SDS-PAGE which was then silver-stained (A). The gel was dried and exposed to a phosphor screen and radiolabelled proteins were revealed by autoradiography (B).
Figure 7: *In organello* pulse-labelling of proteins from chloroplast membranes isolated by sucrose-density gradient centrifugation. Chloroplasts were subjected to pulse-labelling experiments with [$^{35}$S]-methionine-cysteine at either 4°C or room temperature then fractionated on a sucrose density gradient (left). Membranes were collected and proteins separated on an SDS-PAGE which was then Coomassie stained (A). The gel was dried and exposed to a phosphor screen and radiolabelled proteins were revealed by autoradiography (B).
3.5 Lipid synthesis determined by radio-isotope pulse-labellings

As with protein synthesis, involvement of HDM's in thylakoid biogenesis might imply that these membranes are also involved in lipid synthesis. This was not confirmed by the following experiments. Pulse and pulse-chase experiments were performed using $[^{14}\text{C}]-\text{CH}_3\text{COOH}$ in order to label newly-synthesized lipids and follow them from sites of synthesis to sites of accumulation. Membrane fractions separated by sucrose-density gradient centrifugation were analyzed on TLC and signal was revealed by autoradiography. As Figure 8-A shows, almost all of the labelled lipids are seen in thylakoids (Fraction 2) after a 15 minute pulse at $4^\circ$C. If this is followed by a one-hour chase, we now see additional lipid bands appear in the other fractions. Thus it seems as if lipids are originally synthesized on thylakoid membranes then transported to the envelope and HDM’s; which is in contradiction with the accepted models for lipid synthesis and transport (see Discussion for further details). Neutral lipids, which include the chlorophyll pigments and the carotenoids, migrate near the solvent running front on the TLC plate. Thus, we can see most of the newly-synthesized pigments in the lane corresponding to thylakoids while some carotenoids seem to be chased into the envelope and HDM’s after the one-hour chase.
Figure 8: In vivo pulse-labelling of lipids from chloroplast membranes isolated by sucrose-density gradient centrifugation. Cells were subjected to a pulse-labelling with \[^{14}\text{C}]\) or a pulse-labelling followed by a chase then fractionated on a sucrose density gradient (A). Membranes were collected and the lipids were extracted then separated on TLC. The TLC plate was dried and exposed to a phosphor screen and radiolabelled lipids were revealed by autoradiography (B).
3.6 Interrupting chlorophyll synthesis does not affect the protein composition of chloroplast membranes

Arresting thylakoid biogenesis is expected to have qualitative and quantitative effects on the thylakoid biogenesis compartment; which we propose to be the HDM’s according to our model (see Discussion). Indeed, we observed some differences in HDM’s at the level of sucrose gradients but not at the molecular level in cells where chlorophyll synthesis was interrupted. To further characterize the chloroplast membranes in *Chlamydomonas*, I looked at the effect that shutting down thylakoid biogenesis has on the protein composition of these membranes. To do so, an indirect approach was used that takes advantage of the y-7 mutant cell strain which does not synthesize chlorophyll in the dark. Since chlorophyll synthesis is intimately linked to thylakoid protein and lipid synthesis (see Introduction), shifting a culture from light to dark for an appropriate amount of time should lead to a stoppage of chlorophyll synthesis which would then trigger the arrest of thylakoid protein synthesis (and thylakoid biogenesis as a whole). Thus, cell cultures were divided in two; one half was placed in the dark for 2 hours while the other was kept in the light prior to cellular subfractionation. This experiment was repeated 9 times, Figure 9 shows typical sucrose gradients obtained after ultracentrifugation. Figure 9-A shows two gradients, one derived from cell cultures kept in the light (control) versus another that was switched to the dark for 2 hours. These types of gradient profiles were obtained 4 times out of 9. One can see thylakoids (Fraction 3) shifting upwards in the dark compared to the control which indicates that they are comparatively lighter in density. Furthermore, we observe more lighter membranes above the thylakoid (Fraction 2) and fewer HDM’s below thylakoids in the dark-treated samples (Fractions 4 and 5). Figure 9-B shows gradients for which opposite results were seen.
These last results were obtained on two different occasions. Finally, 3 experiments showed no differences at the gradient level between dark and light treatments (not shown).

In order to determine whether these visual differences implied any differences at the molecular level, Western blots were performed using antibodies against a variety of chloroplast proteins. Figure 10-A and 10-B shows the results obtained using proportionally-loaded samples from the gradients shown in Figure 9-A and 9-B respectively. In addition to previously-described proteins, I probed for RBP63, an RNA-binding protein which is involved in repair synthesis of photo-damaged D1 subunits (Ossenbühl et al; 2002), cytochrome f which is a protein of the cytochrome b6/f complex, PsA which is a subunit of PSI, LHCII represents a protein present in the LHCII antennal complex of PSII, D2 is a protein subunit of PSII and finally I probed for the S20 ribosomal protein present in the chloroplast small ribosomal subunit.

The results in Fig. 10-A and 10-B show no significant differences between light and dark treatments for both types of gradients obtained. They do, however, show some differences from previously observed results (Fig. 1). For instance, RBP40 was undetectable in Fig. 10-A whereas the antibody also reacted with a protein of higher molecular mass than expected in Fig. 10-B (∼70 kDa versus ∼40 kDa respectively). The antibody used in this set of experiments was different from the one used for Fig. 1 and this second antibody might be weaker than the first. This could explain why no signal was detected. Furthermore, RBP40 has been shown to interact with the Nac2 protein to form a complex involved in D2 synthesis (Schwarz et al; 2007). This complex has a similar molecular mass as the upper bands reacting with the β-RBP40 antibody in Fig. 10-B. The fact that free RBP40 is found only in thylakoids in these samples while the
form that might correspond to a translation initiation complex is enriched (on a per fraction basis) in the two HDM fractions is a further argument for the involvement of these membranes in the synthesis of thylakoid proteins. Furthermore, the antibody against D1 used in this set of experiments only gave signal in the thylakoid fraction (Fig. 10-B). This is most likely due to a problem with the antibody itself (see Discussion).

Another interesting observation was that Psaa and RBP63 do not seem to be enriched in HDM's. Since Psaa is known to be preferentially located on stromal thylakoids (see Introduction) and RBP63 was found to localize as well to stromal thylakoids (Ossenbühl et al; 2002), these results indicated that HDM's are not stromal thylakoids. The implications of these findings will be further expanded upon in the discussion.
Figure 9: Sucrose gradients obtained after ultracentrifugation of samples exposed for 2 hours to the dark or kept in the light. This experiment was repeated 9 times and the results followed these two general patterns: 4 times out of 9, the sample exposed to the dark showed an upward shifting of thylakoids (Fraction 3), more light-density membranes in Fraction 2 and less high-density membranes in Fractions 4 and 5 (A). Opposite results were obtained 2 times out of 9 (B).
Figure 10: Western blots probed for different chloroplast proteins show no significant differences between cell cultures kept in the light and cultures transferred to the dark for 2 hours. Samples from the gradients shown in Fig. 9-A and 9-B were loaded on a per-fraction basis and probed for several proteins. (A) and (B) correspond to samples from the gradients in Fig. 9-A and 9-B respectively.
4 Discussion

4.1 Novel chloroplast membranes are resolved by increasing sucrose concentrations in gradients

Using a modified approach based on established protocols of cellular subfractionation, I was able to show the existence of a novel class of membranes in the chloroplast of *Chlamydomonas*, which previous studies would not have detected. Most studies in the literature use variations on two types of cellular subfractionation preparations by sucrose-density gradient centrifugation to separate envelope membranes from thylakoids. These approaches either recover a green so-called thylakoid pellet by sedimentation in 1.5-1.8 M sucrose or by floating thylakoids and collecting them at the interface of a sucrose solution of 1.2-1.5 M density with another solution of lower density (Keegstra and Yousif; 1986, Andersson et al; 2001, Ojakian and Satir; 1974, Douce; 1973, Margulies and Michaels; 1974).

There are many issues of concern with these two approaches; the main one being that they assume *a priori* that there are only two classes of membranes in the chloroplast (envelope and thylakoids) and therefore do not take into account the possibility of having different sub-populations of membranes (or different classes altogether) that would band differently on a sucrose gradient. What this means in practice is that any membranes that are denser than thylakoids would either be pelleted along with the thylakoids if using the former approach, whereas any membranes banding (or pelleting) below the thylakoids using the latter approach would tend to be discarded and only the desired thylakoid band would be kept for analysis. The second major problem applies mostly to the first approach. By pelleting thylakoids, any other non-membrane components such as starch, free ribosomes, cell debris, unbroken cells and unlysed chloroplasts will also pellet. This
can lead to many erroneous results. To avoid these two major issues with the classical techniques, I refined the membrane flotation approach by increasing the range of the sucrose gradient to 2.5-0.5 M concentrations and by loading the samples at the bottom of the centrifuge tube and then layering the gradient on top of the samples. Increasing the range of concentrations in the gradient should allow for an increased resolution of membranes in sucrose, especially any potentially dense membranes that would have pelleted if using lower sucrose concentrations. Loading samples at the bottom of the tubes should allow membranes only to float up to the region corresponding to their buoyant density in the gradient while non-membrane components should not float upwards and will instead pellet or remain in the 2.5 M sucrose cushion.

In my experience, breaking cells and lysing chloroplasts were found to be very critical steps in the procedure and specific conditions are required for different cell strains. I used a methodological approach for finding the right buffer conditions and physical breakage procedures to ensure optimal chloroplast lysis before fractionation. This was monitored by light-microscopy throughout the experiments to verify the state of the samples. Therefore, I was able to optimize most aspects of cellular subfractionation including those steps leading to the actual centrifugation (see Materials and methods).

This new approach allowed me to resolve a novel class of membranes that banded below the thylakoids (denser than thylakoids) that would have been previously missed by the classical approaches. These membranes were named high density membranes (HDM). They were found to be qualitatively different from thylakoids: they have much less chlorophyll (an order of magnitude less) yet have a high protein/chlorophyll ratio. Furthermore, they have a higher chlorophyll a/b ratio and are differently enriched in chloroplast proteins. Interestingly, I found that they are enriched in markers of T zones,
which are regions of the chloroplast involved in translation. Thus, they were found to be enriched in the RNA-binding protein RBP40 and the L12 protein of the chloroplast ribosome.

Furthermore, I originally observed an enrichment in the D1 subunit of PSII in HDM's of the alb3.1 mutant compared to the wild-type, an effect also seen in situ in T zones. This effect was proposed to be a potential quality-control feature of T zones "that ensures that only assembled PSII complexes are transported outward", in a manner analogous to how the rough endoplasmic reticulum (ER) retains nonnative and incompletely assembled protein complexes (Uniacke and Zerges; 2007). This enrichment was observed in the first three experiments while no such effect was seen in two subsequent experiments. These differences might be caused by the use of a different D1 antibody in the last two experiments. This second antibody was found to cross-react with samples coming from the D1-deficient strain Fud7 that do not show any reactivity with the antibody used previously (data not shown). This second antibody must therefore be reacting with another protein of similar molecular weight which is equally present in wild-type and mutant strains. A potential candidate might be a protein of the cytochrome b6/f complex that was found to co-migrate with D1 on an SDS-PAGE (Metz and Miles; 1982). Thus, it can be reasonably assumed that the results obtained from the first three experiments are more likely to be representative of the actual enrichment levels in D1 between the two strains because they were obtained with an antiserum specific to D1.

4.2 High density membranes are involved in thylakoid biogenesis

In light of the aforementioned results, we propose that HDM's are located in T zones and are specialized in the synthesis and assembly of thylakoid membrane proteins. As T zones were found in punctate regions around the pyrenoid and seemed to be located
at the basal regions of the chloroplast in *Chlamydomonas*, we propose that HDM's are membranes continuous with mature thylakoids and are where chloroplast-encoded thylakoid proteins are made and early steps of protein complex assembly takes place. Consistent with their role as a thylakoid biogenesis compartment, HDM's feature quality-control checkpoints that retain improperly-assembled protein complexes to prevent them from being integrated into the mature thylakoids. Since protein synthesis is intricately linked with lipid and chlorophyll synthesis (see Introduction), we also propose that all these processes be linked together either by synthesis or assembly at the level of the HDM's. Previous work on thylakoid membrane biogenesis had identified membranes that are normally associated with thylakoids but when released by a low Mg\(^{2+}\) treatment turn out to be lighter in density and fractionate above the thylakoids on a sucrose-density gradient. These so-called low-density membranes (LDM) were found to be enriched in RNA-binding proteins and were proposed to be involved in RNA metabolism and translation. However, ribosomes were not detected at these membranes (Zerges and Rochaix; 1998).

We propose that LDM's are in fact HDM's that are not involved in translation and as such are not laden with ribosomes and nascent protein chains that, if present, would make them much heavier in density and consequently lead to them fractionating below the thylakoids on a sucrose gradient. One way to test this hypothesis is by inhibiting thylakoid membrane biogenesis and then fractionating the cell culture. We should expect to see more of the LDM's and fewer HDM's on the gradient following such a treatment compared to a control. I used the \(\gamma\)-7 mutant strain which does not synthesize chlorophyll in the dark and therefore transferring a cell culture of this mutant to the dark should lead to shutting-down the synthesis of chlorophyll, thylakoid proteins and lipids. This
experiment was performed 9 times, observations with the naked eye revealed 4 results that fit the predictions, while 3 results were either difficult to interpret or showed no differences between both treatments. Finally, 2 results were opposite to what is expected.

In addition to visual observation alone, I performed Western blots on 6 different experimental replicates representing the 3 aforementioned results to see if there are any differences at the molecular level between treatments. No significant differences were observed between dark-treated cells and cells kept in the light. My treatment time was relatively short at 2 hours and this was chosen as a good compromise between the need to have a sufficient minimal time to shutdown chlorophyll synthesis causing a downstream effect that stops the other components of thylakoid biogenesis; and the desire to avoid leaving the cultures for too long in the dark which would dramatically alter the pattern of gene expression in the cell and change its phenotype to that of a dark-adapted cell. These latter cells show profound differences in chloroplast ultrastructure and protein content compared to a cell growing in the light. Since such large-scale changes require much longer periods in the dark, on the order of several hours to several days (Janero and Barrnett; 1981, Janero and Barrnett; 1982, Ohad et al; 1967), it can be reasoned that my experimental design allowed the shutdown of thylakoid membrane biogenesis without changing the proteome of the chloroplast membranes in a way that can be detected by Western blots which cannot distinguish between newly-synthesized proteins and those that were already present. My experiments however showed with a certain degree of reproducibility a shift from HDM's to LDM's when thylakoid biogenesis is shutdown that is consistent with the model presented earlier. Similar experiments were previously conducted and gave corroborating results (Zerges, unpublished results).
4.3 **Protein pulse-labelling experiments showed inconclusive results**

Pulse and pulse-chase experiments were performed in order to determine whether HDM's are the major sites of *de novo* protein and lipid synthesis in the chloroplast of *Chlamydomonas*, thereby answering one of the questions brought up by the aforementioned experiments. Results were inconclusive. Since I was working with radioactive materials, a great deal of time and effort was spent on modifying existing fractionation protocols to better suit the cell strains I was using here. It was found that proper chloroplast lysis prior to fractionation is a critical step to ensure a good resolution of membranes on a sucrose-density gradient. While most of the labellings were performed in the midst of optimization trials, I was able to carry out three experiments under what I found to be ideal conditions. If HDM's are indeed the sites of thylakoid biogenesis, we should expect to find newly-synthesized proteins, lipids and chlorophyll there and we should be able to chase them out into the mature thylakoids.

My results, however, did not agree with these expectations as radio-labelled proteins and lipids were not found to be particularly enriched in the HDM's and most of the signal was instead found in the thylakoids. Furthermore, a few cases showed lipids and proteins actually chasing out of the thylakoids and into the other membranes of the chloroplast. While these results seem to contradict the proposed model for HDM's being the site of thylakoid biogenesis, one should keep in mind the difficulty of trapping newly-synthesized proteins and lipids at their sites of synthesis in other membranous compartments. Indeed, elucidating the secretory pathway in eukaryotic cells required the specific use of cell strains mutant for every step along the pathway or treatment with chemicals targeting these individual steps. This work showed residency times in the ER or Golgi apparatus for certain newly-synthesized proteins in the order of seconds or a few
minutes which would make locating these proteins moving through this pathway almost impossible to detect without the use of blocks (Green et al; 1987, Fries et al; 1984, Yeo et al; 1985, Hirschberg and Lippincott-Schwartz; 1999).

While my experimental approach was based on the assumption that incubating the cells and carrying out the labelling reactions at $4^0\mathrm{C}$ should slow down the transport of newly-made thylakoid components from their site(s) of synthesis to their final destinations on mature thylakoids, it is difficult to gauge how effective these conditions were. The only differences observed between labellings done at $4^0\mathrm{C}$ versus room-temperature were at the level of total protein synthesis rather than distribution of labelled products between the different membrane compartments (room-temperature showed more overall protein synthesis than $4^0\mathrm{C}$). Finally, lysis conditions were such that no Mg$^{2+}$ was present in the lysis buffer. It is known that low Mg$^{2+}$ can cause ribosome disassembly leading to a release of ribosomes from membranes and a possible loss of HDM's from the sucrose gradient fractions due to them being no longer high in density. Taking all these arguments into consideration and the fact that good fractionation was very hard to obtain, results from these experiments are more ambiguous to interpret than what it originally seems; and while they do contradict the model I propose these results are not enough to completely dismiss it. A different approach might be useful to look for newly-synthesized proteins. GFP-tagging of proteins has been successfully used to track proteins in the secretory pathway moving from the ER to the Golgi apparatus. This approach used a temperature sensitive mutant that retains the tagged protein in the ER and only allows it to move to the Golgi at a specific permissive temperature. This method could be adapted for in vivo monitoring of translation in chloroplasts (Hirschberg and Lippincott-Schwartz; 1999).
4.4 High density membranes are enriched in chloroplast-encoded proteins

In agreement with our model for HDM's being the sites of thylakoid biogenesis, these membranes were found to be enriched in chloroplast-encoded proteins. Indeed, Western blots revealed an enrichment of proteins present in the three complexes of thylakoid membranes, namely D1 (Fig. 3) and cytochrome f (Fig. 10) of PSII and the cytochrome b6/f complex, respectively. Probing for non-chloroplast encoded proteins such as LHCII which is part of the antennal complex of PSII showed the absence of this protein from HDM's. Since LHCII is imported from the cytosol and assembled with the PSII core complex in the final steps of PSII assembly (van Wijck et al; 1995) these results indicate that HDM's are at the very least early thylakoid membranes. Furthermore, the lack of LHC associated with PSI and PSII (LHCI and LHCII, respectively) is further supported by the difference in chlorophyll a/b ratios between HDM's and thylakoids, the former having a ratio 2-3 times higher than the latter. Since chlorophyll b is almost exclusively found in both LHCI and LHCII, these results represent a second line of evidence for the enrichment of HDM's in chloroplast-encoded proteins. The PsaA protein of PSI was detected in HDM’s but was not found to be significantly enriched. Therefore, this is evidence that HDM’s are not simply stromal thylakoids that are dense because they are laden with newly-synthesized proteins, ribosomes and RNA-binding proteins.

The significance of these results lies in the fact that they are similar to those obtained from the very different in situ approaches used to characterize T zones. Indeed, the cellular subfractionation experiments presented here showed similar enrichment patterns in markers of protein synthesis to the in situ study on T zones (Uniacke and Zerges; 2007). Therefore, it is possible that HDM's are indeed involved in the synthesis of chloroplast-encoded proteins even if the evidence from pulse-labellings does not seem
supportive.

4.5 Chlorophyll and lipid synthesis results show differences from current models

In contrast with our proposed model and the current models proposed in the literature, thylakoids were found to contain most of the newly-synthesized chlorophyll and lipids. While these results may not be definitive (as discussed earlier), one interesting observation was that lipids such as MGDG and DGDG and chlorophyll seemed to chase out of thylakoids into other membranes such as the envelope (Figure 8). Most current models for lipid synthesis assume that MGDG is made at both envelope and thylakoids while DGDG is exclusively made at the envelope. The contradiction between my results and those of others is likely due to the fact that almost all other studies made the mistake of over-representing the envelope several hundred fold by loading samples on an equal protein (or chlorophyll) basis rather than analyzing samples on a per fraction basis, taking into account the natural distribution of membranes in the chloroplast. My study therefore demonstrates the need for biological relevance when examining sub-cellular processes and shows how dramatically different results can be obtained if one were to disregard this need.

While there is less of a consensus regarding the sites of chlorophyll synthesis in the literature, my results agree with the studies demonstrating synthesis on the thylakoids. However, other results indirectly raise the possibility that HDM's might be involved in chlorophyll synthesis. There is disagreement in the field as to whether chlorophyll b is made from the oxidation of chlorophyll a or whether it is made directly from an earlier intermediate (Beale; 1999, Eckhardt et al; 2004, Oster et al; 2000). If the former
hypothesis is true, then the higher chlorophyll a/b ratio could be an indicator that HDM's are also sites of chlorophyll synthesis by virtue of their enrichment in the earlier-made chlorophyll a.

4.6 Summary and future directions

This study reports a novel membrane compartment in the chloroplast of *Chlamydomonas* that could be the site of thylakoid membrane biogenesis. Qualitatively different from thylakoids, this compartment is enriched in markers of protein synthesis such as RNA-binding proteins and ribosomes. Our working hypothesis is that the LDM's with the RNA-binding proteins are an early compartment in a spatial-temporal gradient of thylakoid biogenesis where mRNA's and translation components are recruited. We also hypothesize that HDM’s are intermediate in this pathway, having polysomes and assembling thylakoid membrane complexes, possibly accounting for their high density. Since they are enriched in the same markers as T zones, the HDM's could be membranes isolated by cellular subfractionation that correspond to the T zones observed by confocal microscopy. While pulse-labelling experiments did not show the enriched presence of newly-synthesized proteins, lipids and chlorophyll in HDM's, these results were not enough to dismiss a potential participation of HDM's in thylakoid membrane biogenesis especially since other lines of evidence offered support for this hypothesis.

Interestingly, early studies reported sub-membrane fractions denser than thylakoids where most ribosomes were found that were also qualitatively different (Margulies and Weistrop; 1980). However, these membranes were not further investigated and this paper is only cited once in the literature (Uniacke and Zerges; 2007).

The model proposed in this study attempts to explain the process of thylakoid
biogenesis as a whole instead of looking separately at the synthesis of the individual components of thylakoid membranes, as the current working models in the literature do. Its main advantage is its intuitive simplicity; since it has long been known that synthesis of thylakoid membrane proteins, lipids and pigments is coordinated in time, one can also wonder if these processes are not also coordinated in space. By having all these components made at the same place, this offers a solution that satisfies these constraints. Furthermore, this model is better suited to explain how ultra-reactive molecules such as chlorophyll reach the proteins they bind to or how lipids, for which no known transport mechanisms inside organelles exist, get to the sites they are needed at for forming thylakoid membranes. Finally, one can picture HDM's as primordial immature thylakoids which after synthesis and assembly of the different components of mature thylakoids has taken place are pushed laterally into the chloroplast in order to reach their final destination as fully-differentiated thylakoids. A complete elucidation and demonstration of this general model is beyond the scope of this study, however I was able to show preliminary evidence for involvement of HDM's in protein synthesis and propose a role for these membranes in the other aspects of thylakoid biogenesis.

Many questions arise from all the above such as the relevance of HDM's to thylakoid biogenesis in higher plants and whether or not they are only found in *Chlamydomonas*. In higher plants, it is known that a paracrystalline structure called the prolamellar body (PLB) is present in chloroplasts of dark-grown plants (the chloroplasts are referred to as etioplasts in this case) where mature thylakoids are absent. The PLB is composed mostly of lipids with very few proteins and contains enzymes involved in chlorophyll synthesis. Upon illumination, the PLB is converted into primordial thylakoids (prothylakoids) and then into mature thylakoids in a process that takes 24 to 48 hours
(Staehelin; 2003, Lütz et al; 1981, Barthélemy et al; 2000, Vothknecht and Westhoff; 2001). Could HDM's be analogous structures to PLB's in *Chlamydomonas* or are they only found in fully-developed chloroplasts where they play a role in the maintenance of existing thylakoid membranes? Conversely, are HDM's also found in mature chloroplasts of higher plants? Current work is being undertaken in our lab in order to determine if HDM's are present or not in higher plants and to further characterize their role and function. Preliminary results are promising and support the presence of dense membranes involved in protein synthesis in the chloroplast of spinach.

As with any scientific endeavour, this study leads to more questions than answers. Further work is still needed to test the validity of our model first, then to purify the HDM's for their biochemical characterization and determination of their proteome by mass spectrometry.
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References


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Appendix 1: Electron micrograph of a *Chlamydomonas reinhardtii* cell. Structures labelled are: envelope membrane of the chloroplast (E), pyrenoid (P), cell nucleus (N), thylakoids (T). Ohad et al. 1967. JCB 35: 521-552.
Appendix 2: Light micrographs showing unlysed (left) versus lysed (right) chloroplasts.