Low Density Membranes Are Associated with RNA-binding Proteins and Thylakoids in the Chloroplast of *Chlamydomonas reinhardtii*

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Abstract. Chloroplast subfractions were tested with a UV cross-linking assay for proteins that bind to the 5′ untranslated region of the chloroplast *psb*C mRNA of the green alga *Chlamydomonas reinhardtii*. These analyses revealed that RNA-binding proteins of 30–32, 46, 47, 60, and 80 kD are associated with chloroplast membranes. The buoyant density and the acyl lipid composition of these membranes are compatible with their origin being the inner chloroplast envelope membrane. However, unlike previously characterized inner envelope membranes, these membranes are associated with thylakoids. One of the membrane-associated RNA-binding proteins appears to be RB47, which has been reported to be a specific activator of *psb*A mRNA translation. These results suggest that translation of chloroplast mRNAs encoding thylakoid proteins occurs at either a subfraction of the chloroplast inner envelope membrane or a previously uncharacterized intra-chloroplast compartment, which is physically associated with thylakoids.

Plastids perform many diverse functions for plants and eukaryotic algae, e.g., photosynthesis, the assimilation of nitrogen and sulfur, and the synthesis of carbohydrates, amino acids, fatty acids, and glycerolipids. Plastids of plants and green algae are surrounded by two concentric envelope membranes, which regulate the trafficking of diverse molecules (e.g., amino acids, proteins, and metabolites) between the stroma and cytosol. In chloroplasts, one of the developmental fates of the plastids, the light-driven reactions of photosynthesis occur in the membranes of the disklike thylakoids. Multiple thylakoids are associated in stacks called grana.

Considerable progress has been made recently towards understanding the processes underlying the biogenesis of the thylakoid compartment. Thylakoid proteins encoded by chloroplast genomes are thought to be synthesized directly into thylakoids on membrane-bound ribosomes (for review see Harris et al., 1994). However, the glycerolipids, quinones, and pigments (i.e., carotenoids and chlorophyll) found in thylakoids are probably synthesized at the chloroplast envelope, and then transported to thylakoids by as yet unknown routes (for review see Joyard et al., 1991). Moreover, many thylakoid proteins are encoded by nuclear genes, synthesized on cytosolic ribosomes, and then imported through the chloroplast envelope and stroma to the thylakoid membrane or lumen (for review see Cline and Henry, 1996).

Translation in the chloroplast of the green alga *Chlamydomonas reinhardtii*, and in the mitochondria of *Saccharomyces cerevisiae*, requires genetic interactions between gene-specific, nucleus-encoded functions and sequences within the 5′ untranslated region (UTR)[1] of the organellar mRNA (for review see Hinnebusch and Liebman, 1991; Gillham et al., 1994; Fox, 1996; Rochaix, 1996). Potential regulatory proteins of chloroplast mRNA translation have been identified by their ability to bind to the UTRs of chloroplast mRNAs in vitro (Hsu-Ching and Stern, 1991; Nickelsen et al., 1994; for review see Gillham et al., 1994; Zerges and Rochaix, 1994; Chen et al., 1995; Hauser et al., 1996). The most extensively studied protein in this class is a 47-kD protein in *C. reinhardtii* called RB47. A multiprotein complex containing RB47 has been postulated to specifically regulate translation of the *psb*A mRNA in response to light intensity (Danon and Mayfield, 1991) via stromal ADP levels (Danon and Mayfield, 1994*a*) and redox potential (Danon and Mayfield, 1994*b*). There is yet no direct evidence that RB47 is localized to the chloroplast. Other studies have suggested translational roles for RNA-binding proteins with molecular masses in the 46–47

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1. *Abbreviations used in this paper*: DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; PSI, and PSII, photosystem I and II; UTR, untranslated region.

kD molecular mass range (Zerges and Rochaix, 1994; Hauser et al., 1996). In this study we have used biochemical approaches to address the intra-chloroplast locations of chloroplast RNA-binding proteins.

Materials and Methods

Media and Growth Conditions

Cultures were grown in Tris-acetate-phosphate medium (Gorman and Levine, 1965), exposed to a light intensity of 100 microeinsteins (μE) m^{-2} s^{-1}, to 2–5 $\times$ 10^6 cells/ml, and with aeration. Chloroplasts were isolated from a strain carrying the *CW15* mutation, which affects the cell wall (Davies and Plaskitt, 1971), to allow breakage of cells but not chloroplasts.

Isolation and Fractionation of Chloroplasts

Cells were concentrated by centrifugation for 10 min at 4,000 *g*, resuspended in isotonic solution (0.3 M sorbitol, 10 mM tricine-HCl, pH 7.8, 5 mM $MgCl_2$), at $\sim$5 $\times$ 10^7 cell/ml, and then broken by passage through a Nebulizer (Gascol Apparatus Co., Terre Haute, IN) at 22 bar. Samples were maintained at 0–4°C thereafter. Chloroplasts, other organelles, and unbroken cells were pelleted by centrifugation at 3,000 *g* for 1.0 min, gently resuspended in the same isotonic solution, loaded on a discontinuous (45/75%) Percoll gradient (with 0.3 M sorbitol, 10 mM tricine-HCl, pH 7.8, 5 mM $MgCl_2$), and then centrifuged at 6,000 *g* for 20 min. Chloroplasts were collected from the interphase of the 45% and 75% Percoll solutions. Two volumes of the isotonic buffered solution were added to dilute the Percoll so that the chloroplasts could be pelleted by centrifugation for 5 min at 4,000 *g*. The chloroplast extract (see Fig. 1, lane *1*) was prepared by lysis in 0.1% Triton X-100, 10 mM tricine, pH 7.8, 60 mM KCl, 5 mM β-mercaptoethanol, and 20% glycerol. The whole-cell extract (see Fig. 2) was prepared by lysing cells (carrying *CW15*) under the same conditions. To prepare the chloroplast fractions (Figs. 1 and 2), isolated chloroplasts were osmotically lysed in 2 ml of a hypotonic buffered solution (10 mM tricine, pH 7.8, 10 mM EDTA, 5 mM β-mercaptoethanol) by repeatedly pipetting (10–20 times) with a Pipetman p1000 (Gilson, Omnilab, Switzerland). For the stromal, thylakoid, and low density membranes analyzed in Figs. 1 and 2, this chloroplast lysate was fractionated on two sequential discontinuous sucrose gradients. The first gradient, which had 0.6 and 1.0 M sucrose phases (10 mM tricine pH 7.8, 10 mM EDTA, 5 mM β-mercaptoethanol), was centrifuged for 3 h at 10^5 *g* in an SW40 rotor (Beckman Instruments, Inc., Fullerton, CA). The stromal fraction was the material that did not enter the sucrose. The low buoyant density membranes were collected from the interphase. Thylakoids in the pellet were resuspended in 1.8 M sucrose, 10 mM tricine, pH 7.8, 10 mM EDTA, and a solution containing 1.3 M sucrose, 10 mM tricine, pH 7.8, and 10 mM EDTA was layered on top. This discontinuous sucrose gradient was centrifuged at 10^5 *g* for 3 h, and the thylakoid fraction was collected from the interphase. An equal volume of 10 mM tricine, pH 7.8, 10 mM EDTA was added to the low density and thylakoid membrane fractions to dilute the sucrose so that these membranes could be pelleted by centrifugation at 10^5 *g* for 1 h. Membranes were resuspended in 10 mM tricine, pH 7.8, 0.2 mM EDTA, 60 mM KCl, 5 mM β-mercaptoethanol, and 20% glycerol. The stromal extract was dialyzed against the same buffer, including also 20% (wt/vol) polyethylene glycol 8000 to concentrate it.

For the sucrose density gradients 1 and 3 shown in Fig. 4, osmotically lysed chloroplasts (see above) were fractionated on the basis of buoyant density by centrifugation at 10^5 *g* for 12–16 h on a continuous (0.3–1.8 M) sucrose gradient (with 10 mM tricine, pH 7.8, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol). Fractions were collected from the gradient, and then diluted with an equal volume of 10 mM tricine, pH 7.8, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol. Membranes in these fractions were concentrated by centrifugation at 10^5 *g* for 30 min. The resulting pellet fractions containing thylakoid membranes were resuspended in either 10 mM tricine, pH 7.8, 10 mM EDTA, or 10 mM tricine, pH 7.8, 5 mM $MgCl_2$, and then fractionated on the gradients 2 and 4, respectively, which had 0.3–1.8 M sucrose gradient and either 10 mM tricine, pH 7.8, 10 mM EDTA, 5 mM β-mercaptoethanol (gradient 2), or 10 mM tricine, pH 7.8, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol (gradient 4) for at least 5 h at 10^5 *g*. Fractions were diluted twofold with either 10 mM tricine, pH 7.8, 10 mM EDTA (gradient 2 fractions), or 10 mM tricine, pH 7.8, 5 mM $MgCl_2$ (gradient 4 fractions), and then centrifuged at 10^5 *g* for 30 min to pellet the membranes. The final membrane fractions were resuspended in 10 mM tricine, pH 7.8, 0.2 mM EDTA, 60 mM KCl, 5 mM β-mercaptoethanol, 20% glycerol and stored at −70°C. Each extract was standardized for protein concentration with the assay of Smith et al. (1985). The samples analyzed with the UV cross-linking assay represented equivalent proportions of each fraction. Polyribosomes were prepared by lysing cells carrying the *CW15* mutation in 0.1% TritonX-100 and then immediately fractionating this lysate on a discontinuous sucrose gradient as described previously (Yohn et al., 1996). Chlorophyll and β-carotene were quantified spectrophotometrically (Porra et al., 1989).

To prepare the membranes used in Fig. 3, isolated chloroplasts (see above) were hypotonically lysed in 10 mM tricine, pH 7.8, 10 mM EDTA, 5 mM β-mercaptoethanol, and then fractionated on a discontinuous sucrose gradient, which had 0.3 and 0.8 M sucrose phases (10 mM tricine, pH 7.8, 10 mM EDTA, 5 mM β-mercaptoethanol) and was centrifuged for 3 h at 10^5 *g* in an SW40 rotor (Beckman Instruments, Inc.). The RNA-binding proteins in samples containing 10 μg protein were ^{32}P labeled by UV cross-linking to a ^{32}P-RNA probe (see below). Membranes were pelleted by centrifugation at 10^5 *g* for 60 min at 2°C. The resulting pellets were resuspended in the various solutions described in the text and incubated for 30 min on ice with intermittent vortexing. Membranes were pelleted by centrifugation at 10^5 *g* for 60 min at 2°C, and then resuspended in SDS-PAGE loading buffer (10% glycerol, 1% SDS, 100 mM DTT, 30 mM Tris-HCl, pH 6.8, 0.01% bromophenol blue). The supernatant fractions were extracted with the procedure of Wersel and Flügge (1984) to remove the Triton X-100, NaCl, or urea and concentrate the proteins. Fractions were analyzed by SDS-PAGE and autoradiography or phosphorimaging.

RNA-binding

The *psb*C 5′ UTR RNA probe was transcribed in vitro from pDH245 (Zerges and Rochaix, 1994) in a 10-μl reaction containing 0.5 μg of linear DNA template, 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT, 25 U porcine RNase inhibitor (Biofinex, Praroman, Switzerland), 60 μCi of [α-^{32}P]UTP (800 mCi/mmol; Amersham International, Little Chalfont, UK), 12 μM non-radiolabeled UTP, 0.3 mM each of ATP, CTP, and GTP, and 8 U T7 RNA polymerase (Biolabs, Beverly, MA) for 45 min at 37°C. 1 U of RNase-free DNase was added and the reaction was incubated for an additional 10 min at 37°C. The reactions were extracted once with phenol-chloroform. The RNA probes were separated from the nonincorporated nucleotide-triphosphates on spin columns of G-50 (Sambrook et. al., 1989). Binding reactions (15–20 μl) were performed at 22–25°C for 5 min and contained 30 mM Tris-HCl, pH 7.0, 5.0 mM $MgCl_2$, 5 mM DTT, 50 mM KCl, and 3–80 μg protein (depending on the extract used and as described in the text). Each reaction contained $\sim$2 $\times$ 10^5 cpm of ^{32}P-RNA probe. Subsequent exposure to a 254-nm UV irradiation of 1.0 J/cm^2 and for a $\sim$7 min duration using a Stratalinker UV cross-linker (Stratagene, La Jolla, CA) covalently cross-linked the RNA probe and bound proteins. After irradiation, the nonbound ^{32}P-RNA probe was digested by treatment with 10 μg RNase A (Sigma Chemical Co., St. Louis, MO) for 10 min at 37°C. Samples were incubated at 85°C for 5 min in protein loading buffer (10% glycerol, 1% SDS, 100 mM DTT, 30 mM Tris-HCl, pH 6.8, 0.01% bromophenol blue), fractionated by SDS-PAGE (11% acrylamide), and analyzed by autoradiography or phosphorimaging.

Immunoblot Analyses

Samples of the various extracts (each containing 20 μg of protein) were incubated in 10% glycerol, 1% SDS, 100 mM DTT, 30 mM Tris-HCl, pH 6.8, and 0.01% bromophenol blue for 1–2 h at room temperature, electrophoresed through an 11% polyacrylamide gel (Sambrook et al., 1989), and then electroblotted to nitrocellulose membranes (Protran, 0.45 μm; Schleicher and Schuell, Inc., Keene, NH). Protein transfer was verified by staining the filter with Ponceau S (Sambrook et al., 1989). Filters were blocked in 5% nonfat dry milk, 0.02% Tween-20, PBS (Sambrook et al., 1989), and then incubated with primary antisera for 1 h, washed three times for 10 min in PBS (0.02% Tween-20), reacted with peroxidase-linked anti–rabbit Ig or anti–mouse Ig (Amersham International) for 1 h, and washed three times for 10 min with PBS containing 0.02% Tween-20. Signals were revealed with a chemiluminescence detection system (Durrant, 1990).

Acyl Lipid Analysis

Lipids were extracted with the procedure of Wersel and Flügge (1984)

from fractions. Samples were all derived from an equal number of cells ($\sim 10^{10}$). The final organic phase was dried under vacuum, and the remaining lipids were resuspended in 40 μl of chloroform/methanol (53:37). These samples were applied to silica gel 10 thin layer plates (Merck, Darmstadt, Germany). Chromatograms were developed with the solvent system; chloroform/methanol/glacial acidic acid/water (85:15:10:3; Goldberg and Ohad, 1970). The galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) were revealed by spraying the chromatogram with 50% (vol/vol) H_2SO_4 followed by a 10-min incubation at 120°C. Phosphatidylinositol (PI) and phosphatidylglycerol (PG) were revealed on the same thin layer chromatogram with phosphomolybdic acid spray (Sigma Chemical Co.), which is specific for phospholipids (Ryu and MacCoss, 1979). Acyl lipid classes were also identified from their reported R_f values (Goldberg and Ohad, 1970)

Results

RNA-binding Proteins Cofractionate with the Low Density Chloroplast Membranes

Isolated chloroplasts were fractionated by sucrose density gradient centrifugation as described in Materials and Methods. When the resulting fractions were tested with an in vitro, UV cross-linking assay (see Materials and Methods for experimental details), we detected several proteins that bind to a ^{32}P-RNA probe derived from the *psb*C 5′ UTR (Fig. 1). All of these proteins appear to be localized to the chloroplast because none were detected in a 20-μg sample (on the basis of protein content) of whole cells (data not shown). In addition, five of these proteins (of 30, 32, 40, 46, and 80 kD) were detected in a 20-μg sample of isolated chloroplasts (Fig. 1, lane *1*). Enrichment of at least four RNA-binding proteins (of 32, 46, 47, and 60 kD) in the preparations of low density chloroplast membranes can be seen by comparing the binding signals of these proteins produced by a 20-μg sample of these membranes (Fig. 1, lane *4*) and 20-μg samples of the stroma (Fig. 1, lane *2*) or thylakoid membranes (Fig. 1, lane *3*). In several similar experiments, in which the levels of binding of these proteins to the ^{32}P-RNA probe were compared for 20-μg samples of stroma or thylakoids and 1-μg samples of the low density membranes, we found that these proteins are enriched over 100-fold in the low density membranes (data not shown). Although, these low density membranes have been reported to be derived from the chloroplast envelope (Douce et al., 1973; Clemetson et al., 1992), for reasons presented in the Discussion they will not be referred to as such in this report. An 80-kD RNA-binding protein was detected in both the stroma and the low density membrane fractions. Binding of a 30-kD protein to the ^{32}P-RNA probe was detected in the stromal subfraction. The binding of one or more 31-kD protein(s) to the ^{32}P-RNA probe was detected in the stroma and low density membranes.

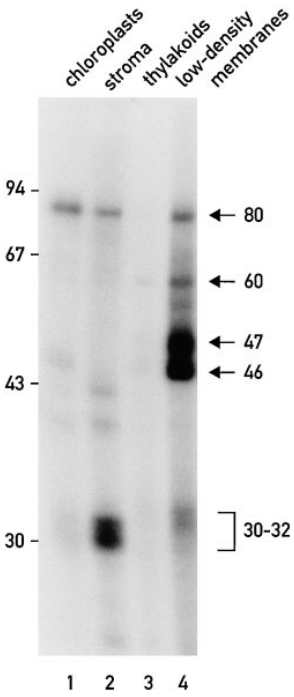

Figure 1. RNA-binding proteins (of ~30, 32, 46, 47, 60, and 80 kD) cofractionate with the chloroplast low density membranes. UV light was used to cross-link the ^{32}P-RNA probe derived from the *psb*C 5′ UTR to proteins in extracts from isolated chloroplasts (lane *1*), chloroplast stroma (lane *2*), thylakoids (lane *3*), and the low density chloroplast membranes (lane *4*). Each sample contained 20 μg of protein.

To ascertain the purity of the stroma and thylakoid membrane fractions, each was tested for the enrichment of one or more marker protein(s) from the desired compartment and the depletion of marker protein(s) from the other. Separation of the proteins in these fractions by SDS-PAGE, followed by immunoblot analyses (Fig. 2 *A*), revealed that the stromal fraction was highly enriched for the small subunit of ribulose bisphosphate carboxylase (Rubisco; Fig. 2 *A*, lane *3*). This stromal fraction lacked detectable levels of two marker proteins of thylakoid membranes, PsaA and D1, core subunits of photosystem I (PSI) and photosystem II (PSII), respectively. Similarly, the thylakoid membrane fraction was highly enriched for PsaA and D1, but contained neither of the Rubisco subunits (Fig. 2 *A*, lane *4*; only the small subunit is shown). In the absence of reliable marker proteins for the chloroplast envelope of *C. reinhardtii*, we were unable to determine by immunoblot analyses whether the low density membranes are enriched for envelope membranes. That we detected neither Rubisco nor the PSI and PSII core subunits in this fraction demonstrates that the low density membranes are not significantly contaminated with stroma or thylakoids, including the stroma lamellae thylakoid membranes (see Discussion).

Stability of the Membrane Association of the RNA-binding Proteins

To further test whether these RNA-binding proteins are associated with membranes, and to determine the stability of these associations, we incubated samples of the low density membranes (corresponding to 1 μg of protein) under various conditions, and then tested whether the RNA-binding proteins still fractionated with the membranes. The RNA-binding proteins in a sample of low density membranes were labeled with ^{32}P by UV cross-linking to the ^{32}P-RNA probe derived from the *psb*C 5′ UTR (see Materials and Methods). These membranes and their associated ^{32}P-RNA–binding proteins were incubated under various conditions for 30 min at 0°C, with intermittent vortexing. After solubilization of membranes with 0.1% (wt/vol) Triton X-100 and centrifugation at 10^5 *g* for 30 min, the 46-, 47-, and 60-kD RNA-binding proteins were detected predominantly in the supernatant fraction (Fig. 3 *A*, lane *2*), and only a small fraction of each protein remained

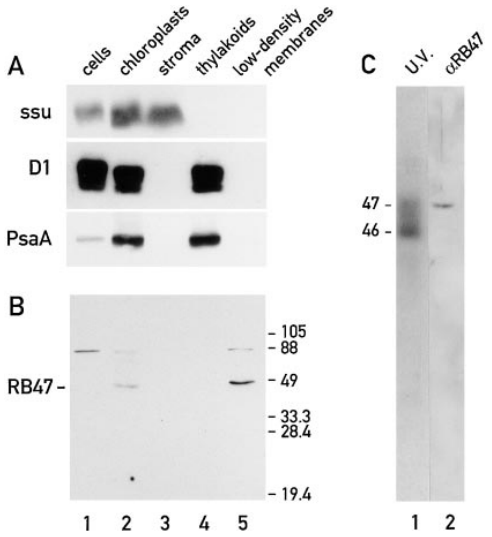

Figure 2. The RNA-binding protein, RB47, cofractionates with low density chloroplast membranes. (*A* and *B*) The immunoblots analyze 20-μg samples of protein extracts from total cells (lane *1*) isolated chloroplasts (lane *2*), chloroplast stroma (lane *3*), thylakoids (lane *4*), and the low density chloroplast membranes (lane *5*). The immunoblot in *A* was reacted with antisera against marker proteins for the chloroplast stroma, the small subunit of Rubisco (*ssu*); for the stacked (appressed) thylakoid membranes, the D1 subunit of PSII (*D1*), and for the nonstacked or stroma lamellae of thylakoid membranes, the PsaA subunit of PSI (*PsaA*). The immunoblot in *B* was reacted with antisera against the RNA-binding protein RB47 (Danon and Mayfield, 1991, 1994). (*C*) The 47-kD RNA-binding protein (radiolabeled by UV cross-linked to the ^{32}P-RNA probe) comigrates during SDS-PAGE (and in the same lane) with RB47 detected with the antiserum against it.

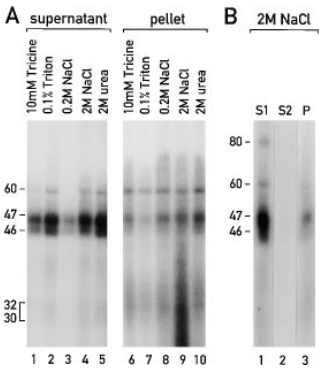

Figure 3. Membrane association of the RNA-binding proteins. (*A*) Samples (1.0 μg protein) of low density chloroplast membranes containing the RNA-binding proteins (which had been radiolabeled by the UV cross-linking to the ^{32}P-RNA probe) were treated with 10 mM tricine, pH 7.8, 0.1% Triton X-100, 0.2 M NaCl, 2 M NaCl and 2 M urea. The samples were incubated 30 min at 0°C. After centrifugation of the extracts at 10^5 *g* for 60 min, supernatant and pellet fractions were analyzed by SDS-PAGE and autoradiography. (*B*) A sample of low density membranes with the ^{32}P-RNA–binding proteins was treated for 30 min at 0°C with 2.0 M NaCl, and then centrifuged at 10^5 *g* for 60 min. The membrane pellet was resuspended in 2.0 M NaCl, and again incubated for 30 min at 0°C and then centrifuged at 10^5 *g* for 60 min. The first and second supernatant fractions (*S1* and *S2*) and the final membrane pellet fraction (*P*) were analyzed by SDS-PAGE and autoradiography. The UV crosslinking of the 80-kD protein was often not detected for unknown reasons (see *A* and *B*). However, in other experiments, extraction of this protein paralleled the extraction of the 46-, 47- and 60-kD proteins.

in the pellet (Fig. 3 *A*, lane *7*). To examine further this protein–membrane association we incubated samples of the low density membranes and their associated ^{32}P-RNA–binding proteins (again containing 1.0 μg total protein) in the presence of various reagents including, as a control, 10 mM tricine, 0.2 M NaCl, 2 M NaCl, and 2 M urea. Peripheral membrane proteins should be extracted by some of these treatments, whereas integral membrane proteins remain membrane associated (Hatefi and Hanstein, 1974; Fujiki et al., 1982). After incubation, the samples were centrifuged at 10^5 *g* for 60 min, and the ^{32}P-RNA–binding proteins in the pellet fractions were analyzed with SDS-PAGE followed by autoradiography and phosphorimaging. Membranes treated with Tricine retained ~75% or more of the 60-kD protein and ~25% of the 46- and 47-kD ^{32}P-RNA–binding proteins (Fig. 3 *A*, lanes *1* and *6*). A higher fraction of these proteins was retained on the membranes after treatment with 0.2 M NaCl (Fig. 3 *A*, lanes *3* and *8*). Incubation in 2 M NaCl or 2 M urea resulted in an increased dissociation of the ^{32}P-RNA–binding proteins from the membranes (Fig. 3 *A*, lanes 4, 5, 9, and 10). The retention of ^{32}P-RNA–binding proteins by the low density membranes after the treatment with 2.0 M NaCl probably does not result from an inaccessibility of these proteins because to be ^{32}P labeled they had to be accessible to the ^{32}P-RNA probe. Most of the RNA-binding proteins in the 30–32-kD molecular mass range remained membrane associated after each of these treatments, suggesting a greater stability of these associations than we observed for the larger RNA-binding proteins. The 80-kD protein could not be identified reproducibly in these UV cross-linking experiments. However, in other experiments, extraction of this protein paralleled the extraction of the 46-, 47- and 60-kD proteins.

These results suggested that there are two populations of the 46-, 47- and 60-kD RNA-binding proteins; one that is weakly associated with the low density membranes and is extracted by treatment with tricine, NaCl, or urea, and another population that is stably associated and is not extracted by these treatments. To test this hypothesis, we asked whether any additional ^{32}P-RNA–binding proteins could be extracted from the low density membranes by a second incubation with 2.0 M NaCl. After the first treatment with 2.0 M NaCl and centrifugation at 10^5 *g*, the pellet fraction was again treated with 2.0 M NaCl, and the membranes were pelleted by a second centrifugation at 10^5 *g* for 60 min. The first and second supernatant fractions and the final pellet fraction were tested for the ^{32}P-RNA–binding proteins by SDS-PAGE and autoradiography (Fig. 3 *B*). Whereas the first treatment with 2.0 M NaCl extracted a fraction of the 46-, 47-, and 60-kD ^{32}P-RNA–binding proteins from the low density membranes (Fig. 3 *B*, *S1*), only trace amounts of these proteins were extracted by the second treatment (Fig. 3 *B*, *S2*). The presence of these RNA-binding proteins in the pellet fraction after two extractions with 2.0 M NaCl strongly suggests that they are stably associated with the low density membranes. The majority of the 80-kD protein was extracted by the first treatment with 2.0 M NaCl in this experiment.

In other trials, a subfraction of this protein appeared to be stably associated with the low density membranes.

The RNA-binding Protein RB47 Cofractionates with the Low Density Chloroplast Membranes

To determine whether the RNA-binding protein RB47 is associated with chloroplast membranes (Danon and Mayfield, 1991, 1994*a*,*b*; Yohn et al., 1996; see Introduction), an immunoblot with equal mass amounts (20 μg) of extracts of whole cells, isolated chloroplasts, and the three chloroplast subfractions (described above and in Materials and Methods) was reacted with a polyclonal antiserum raised against this protein (Fig. 2 *B*). Although RB47 was not detected in an extract derived from whole cells (Fig. 2 *B*, lane 1), it was detected in an extract of isolated chloroplasts (Fig. 2 *B*, lane *2*). This provides the first direct evidence that this protein is located in the chloroplast. Comparison of the levels of RB47 in the three chloroplast subfractions on this immunoblot revealed an enrichment of this protein in the low density membrane fraction (Fig. 2 *B*, lane 5) and that it was not detected in the stroma (Fig. 2 *B*, lane *3*) or thylakoid fractions (Fig. 2 *B*, lane *4*). Therefore, RB47 appears to be associated with the low density membranes.

To determine whether the RNA-binding protein RB47 (Danon and Mayfield, 1991, 1994*a*,*b*) could be one of the RNA-binding proteins in the 46–47 kD molecular mass range detected in preparations of the low density chloroplast membranes with the ^{32}P-RNA probe derived from the *psb*C 5′ UTR, we asked whether it comigrates with either of these proteins during SDS-PAGE. The 46- and 47-kD RNA-binding proteins in a sample of low density membranes were ^{32}P labeled by UV cross-linking to the *psb*C 5′ UTR ^{32}P-RNA probe, subjected to SDS-PAGE, and then transferred to a nitrocellulose filter. As seen in Fig. 2 *C*, the 47-kD RNA-binding protein, but not the 46-kD species, comigrated (in the same lane) with the RB47 detected with the antiserum against it. (The exposure time of this autoradiograph was too short to reveal the weaker signals from the 31-, 32-, 60- and 80-kD RNA-binding proteins.) These results are consistent with RB47 being the 47-kD protein that binds to the *psb*C 5′ UTR ^{32}P-RNA probe. The 46-kD RNA-binding protein associated with the low density membranes comigrates with RB46 (Zerges and Rochaix, 1994) and the 47-kD RNA-binding protein described by Nickelsen et al. 1994 (data not shown).

A protein of ~80 kD, which cross-reacted with the antiserum against RB47, is more abundant in the extract of whole cells (Fig.2 *B*, lane *1*) than in the extract of isolated chloroplasts (Fig. 2 *B*, lane *2*) and therefore appears not to be a chloroplast protein. This protein was not detected in either the extracts of stroma (Fig. 2 *B*, lane *3*) or thylakoids (Fig. 2 *B*, lane *4*). The presence of trace amounts of this protein in the low density membrane extract (Fig. 2 *B*, lane 5) may be because of a low level of contamination of this fraction by extra-chloroplast proteins. The RNA-binding protein with a similar molecular mass was detected in stroma extracts (Fig. 1, lane 2), and not in extracts of whole cells (data not shown), as well as in the extract of low density membranes. Therefore, these two proteins are probably distinct.

The Low Density Membranes with the RNA-binding Proteins Are Physically Associated with Thylakoid Membranes

The composition of RNA-binding proteins in different preparations of low density membranes purified by discontinuous sucrose gradient centrifugation appeared to be highly dependent on the sucrose concentrations used, whether or not $MgCl_2$ was present during the purification of chloroplasts and membrane fractionation steps, and/or other factors (data not shown). When chloroplast membranes were fractionated on the continuous sucrose gradient 1 in Fig. 4 *A* and Mg^{2+} ions were present throughout the preparative procedures, the dark green thylakoid membranes (Fig. 4 *A*, in fractions *4–6*) separated from orange membranes of lower density (Fig. 4 *A*, fractions *1–3*), which have been shown to be derived from the chloroplast envelope (Douce et al., 1973; Clemetson et al., 1992; see below). The presence of thylakoids in fractions 4–6 is evidenced by the presence of all the detectable chlorophyll in these fractions (Fig. 4 *C*). Similarly, the orange color of the low density membranes is because of the absence of chlorophyll, which reveals the β-carotene present throughout the gradient (Fig. 4 *C*, fractions *1–3*). When the fractions of this gradient were tested with the UV cross-linking assay, the RNA-binding proteins of 31, 32, 46, 47, 60, and 80 kD were detected in the fractions containing thylakoid membranes (fractions *4–6*). A 40-kD RNA-binding protein was detected predominantly in fraction *3*, which contains low density membranes. Although these results would seem to contradict the cofractionation of the 31-, 32-, 46-, 47-, 60- and 80-kD RNA-binding proteins with the low density membranes seen in Figs. 1 and 2, these differences appear to have resulted from an association of the low density membranes with thylakoids, which is stabilized by Mg^{2+} ions. For example, when the RNA-binding proteins cofractionated with the low density membranes, and not with thylakoid membranes (Figs. 1 and 2), the membranes had been isolated in the absence of Mg^{2+} ions (see Materials and Methods). This issue was clarified when the membranes in fractions 5 and 6 from gradient 1 were depleted of Mg^{2+} ions by centrifugation at 10^5 *g* followed by vigorous resuspension in a solution lacking $MgCl_2$ (and containing 10 mM EDTA). When these membranes were centrifuged on a sucrose density gradient 2 (lacking Mg^{2+} ions), orange low density membranes were observed that appeared to have been released from the thylakoids obtained from the first gradient. This was indicated by the presence of β-carotene and, to a lesser extent, chlorophyll in fractions 1–3 (Fig. 4 *C*). In addition, some of these low density membranes formed a discrete band (*asterisk*) that was not detected in gradient 1. In addition, the thylakoids were less dense than in gradient 1, due to their being unstacked in the absence of Mg^{2+} ions (for review see Barber, 1980). Fractions 2 and 3 of gradient 2, which are above the thylakoid membranes in fractions 4–6, contained the majority of the RNA-binding proteins of 31-, 32-, 46-, 47- and 80-kD. The majority of the 60-kD RNA-binding protein was detected in fractions 3 and 4, also above the thylakoid membranes. In other trials of this dual-gradient fractionation scheme, the 60-kD protein fractionated with thylakoids on gradient 1 and with low density membranes on

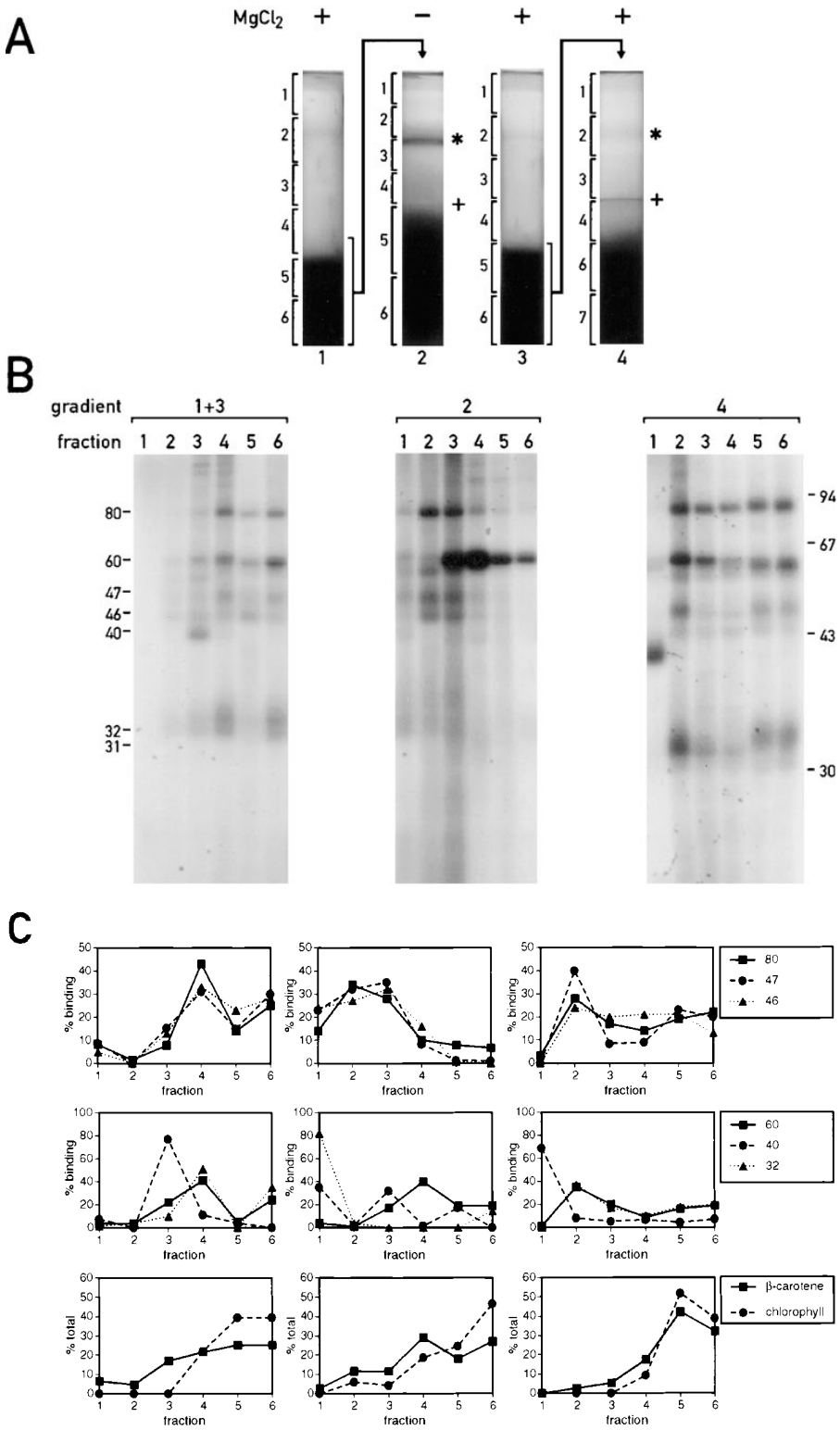

Figure 4. The low density membranes (with the RNA-binding proteins) are associated with thylakoid membranes. (*A*) Sequential sucrose density gradients were used to fractionate chloroplast membranes. On gradient 1 total chloroplast membranes were fractionated in the presence of 5 mM $MgCl_2$. Thylakoids from this gradient (the *dark band* at the bottom) and the low density membranes associated with them were separated on gradient 2, which lacked $MgCl_2$. The band of orange membranes observed on gradient 2 is indicated with an asterisk. When both gradients contained $MgCl_2$ (gradients *3* and *4*) considerably fewer low density membranes were observed above the thylakoid membranes. The lower density of the thylakoids in the absence of Mg^{2+} (indicated by their upward shift in gradient *2* and the +) reflects the destacking of the grana. (*B*) Binding of proteins to the ^{32}P-RNA derived from the *psb*C 5′ UTR in the fractions from the gradients shown in *A* was measured with the UV cross-linking assay. Samples represented equivalent proportions of each fraction (i.e., were analyzed on a per chloroplast basis) and contained between 8 and 30 μg protein. (*C*) The binding signals for each protein were determined by phosphorimaging and graphed as percentage of the total signal for that protein in each gradient. Chlorophyll concentrations throughout each gradient are also graphed to indicate the distribution of thylakoid membranes.

gradient 2 (data not shown). In summary, the difference in the buoyant densities of the membranes associated with the RNA-binding proteins in the presence and absence of Mg^{2+} ions appears to result from an association of these membranes with the denser thylakoid membranes.

To determine the extent to which depletion of Mg^{2+} ions caused the release of the low density membranes associated with the RNA-binding proteins from thylakoids on gradient 2, we fractionated thylakoid membranes obtained from a gradient containing $MgCl_2$ (Fig. 4 *A*, gradient *3*) on a second gradient that also contained $MgCl_2$ (Fig. 4 *A*, gradient *4*). Note that gradients 1 and 3 are identical, whereas gradients 2 and 4 differ only by the absence and presence of $MgCl_2$, respectively. After centrifugation, gra-

dients 2 and 4 differed drastically in appearance; on gradient 4 only a minor fraction of the thylakoids became unstacked, as indicated by the presence of green material (+) above the denser band of stacked thylakoids. No chlorophyll and low amounts of β-carotene (2–5% total) were detected in the first three fractions. In addition, the band of low density membranes seen in gradient 2 was only faintly visible in gradient 4 (*asterisk*). Analysis of the gradient 4 fractions with the UV cross-linking assay revealed that the membranes associated with the RNA-binding proteins of 30, 32, 46, 47, and 80 kD were retained by thylakoids to a much greater extent than they were in the absence of Mg^{2+} ions on gradient 2. The partial release of the low density membranes associated with the RNA-binding proteins from the thylakoid membranes may have resulted from mechanical forces produced by repeated pipetting that was used to resuspend these membranes before they were loaded on gradient 4. Consistent with this possibility, we observed that when thylakoid membranes from gradients similar to gradients 1 and 3 were gently resuspended (with a paint brush), all of the RNA-binding proteins cofractionated again with thylakoid membranes both in the presence and absence of Mg^{2+} ions. Therefore, whereas the removal of Mg^{2+} ions appears to be required for the release of all of these low density membranes from thylakoid membranes, mechanical forces also appear to facilitate this dissociation.

The fractionation behavior of the RNA-binding proteins shown in Fig. 4 is not because of their association with ribosomes because these proteins were not detected in several preparations of polyribosomes (data not shown). Also, chloroplast ribosomes (detected by RNA-gel blot analysis using a probe complementary to the chloroplast 16-S rRNA) did not cofractionate with the RNA-binding proteins in the gradients 1 and 2 shown in Fig. 4 *A,* they were detected only in fractions 5 and 6 of both gradients (data not shown).

Fractions of sequential sucrose density gradients similar to gradients 1 and 2 in Fig. 4, were assayed for the 46- and 47-kD RNA-binding proteins with the UV cross-linking assay, and for RB47 and various thylakoid proteins by immunoblot analyses (Fig. 5). RB47 fractionated with the 46- and 47-kD RNA-binding proteins. The enrichment of thylakoid membranes at the bottom of the gradients is evidenced by the abundance of the known thylakoid proteins (the D1 subunit of the PSII reaction center, cytochrome f) in fractions 5 and 6. Trace amounts of these proteins were detected in the fractions containing the low density membranes (fractions 2 and 3 of gradient 2). The distributions of P6, the PSII subunit encoded by *psb*C, and the PsaA subunit of PSI were similar to those of D1 and cytochrome f (data not shown).

A Sub-fraction of the Chloroplast Envelope is Associated with Thylakoids

To test the hypothesis that low density membranes and the associated RNA-binding proteins are released from thylakoids upon the removal of magnesium ions, the acyl lipid compositions of fractions from gradients similar to those shown in Fig. 4 *A* were determined by thin layer chromatography. Inspection of the thin layer chromatogram in Fig. 6 revealed that the distributions of the various acyl lipid classes in gradients 1 and 2 are similar. The thylakoid membranes on both gradients (fractions 4 and 5) are rich in the galactolipids DGDG and MGDG, and also contain the phospholipids PG and PI (see Materials and Methods for experimental details). The membranes above the thylakoids in gradient 1 have been reported to be derived from the chloroplast envelope (Douce et al., 1973; for review see Joyard et al., 1991; Clemetson et al., 1992). The outer envelope membrane is less dense than the inner membrane, enriched in PI, but also contains low amounts of MGDG, DGDG, and PG (Joyard et al., 1991). On the thin layer chromatogram in Fig. 6, the least dense fractions from both gradients (i.e., fractions 1) contain predominantly PI. Therefore, these fractions appear to be enriched for the outer envelope membrane. In buoyant density, the inner envelope membrane is intermediate to the outer envelope and thylakoid membranes and therefore, should be found in fractions 2 and 3 of each gradient. The inner envelope membrane has an acyl lipid composition that is close to that of thylakoid membranes; both are rich in MGDG and DGDG and contain lower amounts (1%) of PI than does the outer envelope membrane (for review see Joyard et al., 1991). In fractions 2 and 3, the presence of MGDG and DGDG, and the lack of chlorophyll are consistent with an enrichment in the inner envelope membrane.

The most important conclusion that can be drawn from these data is that membranes were released from the thylakoid containing fractions from gradient 1 (Fig. 4) after the removal of Mg^{2+} and vigorous resuspension. This is evidenced by the presence of acyl lipids in fractions 1–3 of gradient 2. The 46- and 47-kD RNA-binding proteins were detected in fractions 2 and 3 of gradient 2, and not in the corresponding fractions of gradient 1 (data not shown; but see Fig. 4 *B* and *C*). Thus, the fractions from gradient 2 that contain the RNA-binding proteins also contain membranes that are similar to the inner envelope membrane in their acyl lipid composition and buoyant density.

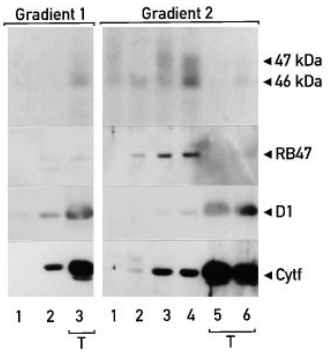

Figure 5. Immunoblot and UV cross-linking analyses of fractions from gradients 1 and 2. The 46-, 47-, and 60-kD RNA-binding proteins, which were found to bind to the ^{32}P-RNA probe in the UV cross-linking assay, cofractionate with thylakoids (*T*) in the presence of magnesium ions (*Gradient 1*). In the absence of magnesium ions (*Gradient 2*), these proteins and the low density membranes associated with them were separated from thylakoid membranes (*T*). Thylakoid membranes were followed by their enrichment for the PSII reaction center subunit D1, and cytochrome f detected by immunoblot analysis. RB47 (detected by immunoblot analysis) also cofractionates with the low density membranes that are associated with thylakoid membranes in the presence of magnesium ions. Samples contained 20 μg of protein.

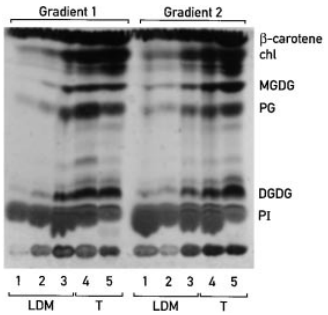

Figure 6. The low density membranes that are associated with thylakoids in the presence of magnesium have acyl lipid compositions that are similar to the chloroplast envelope membranes. The thin layer chromatogram resolves acyl lipid classes in fractions from gradients similar to those shown in Fig. 4. Indicated are the pigments β-carotene and chlorophyll (*chl*), the galactolipids monogalactosyldiacylglycerol (*MGDG*) and digalactosyldiacylglycerol (*DGDG*), and the phospholipids phosphatidylglycerol (*PG*) and phosphatidylinositol (*PI*). *LDM,* the low density membranes. *T,* thylakoid membranes. Samples represent equivalent fractions of each fraction (i.e., were analyzed on a per chloroplast basis).

Discussion

This study has revealed that RNA-binding proteins are associated with chloroplast membranes in *C. reinhardtii*. From our data, low density membranes in the chloroplast can be subdivided into two general classes. The first class separates from the thylakoids in the presence of magnesium ions and cofractionates with an RNA-binding protein of 40 kD (Fig. 4, gradient *1*). The second class is associated with thylakoids and six RNA-binding proteins of 31, 32, 46, 47, 60, and 80 kD (Fig. 4 and data not shown), and RB47 (Fig. 5). The stability of the association of the second class of membranes with thylakoid membranes is evidenced by their cofractionation during a 16 h sucrose density gradient centrifugation (Fig. 4, gradient *1*). The association of these RNA-binding proteins with the low density membranes was only revealed when thylakoid membranes were pelleted by centrifugation and harshly resuspended in the absence of Mg^{2+} ions. After these steps, the RNA-binding proteins and the low density membranes separated from thylakoid membranes on a second sucrose density gradient lacking Mg^{2+} ions (Fig. 4, gradient *2*). On a second sucrose density gradient containing Mg^{2+} ions (Fig. 4, gradient *4*), considerably greater amounts of low density membranes with the RNA-binding proteins remained associated with thylakoids than was observed on gradient 2 (i.e. in the presence of Mg^{2+} ions). As the stacking of thylakoids requires divalent cations (for review see Barber, 1980), these low density membranes may be retained by thylakoid grana. However, the Mg-dependent association between thylakoids in grana differs from the association of the low density membranes with thylakoid membranes because the latter association is maintained in the absence of Mg^{2+} ions if the membranes are gently resuspended (data not shown).

A fraction of each of the RNA-binding proteins, comprising ~25% of the total, were found to be stably associated with the second class of low density membranes because they were not extracted with 2 M NaCl or 2 M urea (Fig. 3). NaCl concentrations >0.7 M have been shown to remove the OE23 subunit from the oxygen-evolving complex of PSII, which is embedded in thylakoid membranes (Ghanotakis and Yocum, 1990; Hashimoto et al., 1997). However, another fraction of the 46- and 47-kD RNA-binding proteins, comprising ~75% of the total amount, was extracted by vortexing several times in tricine and, therefore, is weakly associated with membranes (Fig. 3 *A*). As these RNA-binding proteins did not appear to have been liberated to the chloroplast stroma during the preparation of the low density membranes analyzed in Figs. 1 and 2, we assume that the extraction of the low density membrane from thylakoids, binding of the ^{32}P-RNA probe, or vortexing weakened the association of the RNA-binding proteins with the low density membranes. The distinction between these different subpopulations of the RNA-binding proteins on the basis of the strength of their membrane association was supported by the observation that a second incubation in 2.0 M NaCl did not result in a further release of the tightly associated ^{32}P-RNA–binding proteins (Fig, 3*B*, S*2*). Thus, these tightly associated ^{32}P-RNA–binding proteins were detected in the pellet fraction (P) after two sequential treatments with 2 M NaCl.

The buoyant densities and the acyl lipid compositions of both classes of low density membranes are compatible with their origin being the inner chloroplast envelope membrane (Fig. 5). However, at present we cannot exclude the possibility that the second class of low density membranes, which has not been described previously, is derived from an intra-chloroplast compartment. The unavailability of a reliable marker protein for the inner envelope of *C. reinhardtii* chloroplasts has impeded the identification of these low density membranes. An antiserum against the 37-kD inner envelope protein E37 from spinach chloroplasts (Block et al., 1983) detected a 33-kD protein in the middle fractions of both gradients 1 and 2 (Fig. 4 *A*), which contain the RNA-binding proteins in gradient 2 (unpublished data). However, as this *C. reinhardtii* protein has not been shown to be homologous to E37 or localized at the inner chloroplast envelope membrane, it cannot be used as a definite marker for this compartment. Similarly, an antiserum raised against total envelope membranes from *C. reinhardtii* chloroplasts detected proteins with both classes of low density membranes, many of which were specific to one class. However, the membranes against which this antiserum was raised were prepared by similar fractionation procedures to those used in this study. Therefore, to use the proteins detected by this antiserum as criteria for chloroplast envelope would entail a circular argument. It is clear that the low density membranes were not derived from the stroma lamellae (non stacked) subfraction of thylakoid membranes because they contain only trace amounts of PSI (Fig. 2; and unpublished data) and the stroma lamellae have been shown to be enriched in PSI complexes (Andreasson et al., 1988).

The ability of the membrane-associated proteins to bind to RNA in vitro suggests that they function in chloroplast mRNA metabolism or translation. Previous studies have supported translational roles of 46- and 47-kD RNA-binding proteins (Danon and Mayfield, 1991, 1994*a*,*b*; Hauser et al., 1996; Yohn et al., 1996; Zerges and Rochaix, 1994). We show here that RB47, an RNA-binding protein that has been proposed to activate the translation of the chloroplast *psbA* mRNA (RB47; Danon and Mayfield, 1991, 1994*a*,*b*; Yohn et al., 1996), is associated with the second

class of low density membranes. Although RB47 and the 47-kD RNA-binding protein detected in our UV cross-linking assay comigrate during SDS-PAGE (Fig. 2 *C*) and cofractionate on the two sequential sucrose density gradients shown in Fig. 5, the binding specificities of these proteins differ. The RNA-binding proteins described here bind to A- and U-rich RNAs; non-radiolabeled 5′ UTRs of various chloroplast mRNAs, including *psb*A, compete equally well (on a μg basis) for binding of the membrane-associated RNA-binding proteins to the *psb*C ^{32}P-UTR probe, whereas no competition was observed by an RNA with a normal base composition (Zerges and Rochaix, 1994; and unpublished data). In addition, poly-A and poly-U competed for binding, whereas no competition was observed for poly-C or poly-G (Zerges and Rochaix, 1994; and unpublished data). In contrast, RB47 has been reported to bind specifically to the 5′ UTR of the *psb*A mRNA, based on the results of similar competition experiments (Danon and Mayfield, 1991; Yohn et al., 1996). Thus, the possibility remains that RB47 and the 47-kD RNA-binding protein described here are distinct. Alternatively, the *psb*A 5′ UTR affinity chromatography used in the purification of the multiprotein complex containing RB47 (Danon and Mayfield, 1991) may have selected for a subpopulation of the 47-kD RNA-binding protein described here that binds specifically to the *psb*A 5′ UTR. Consistent with this possibility, the binding of this protein to the *psb*C ^{32}P-RNA probe, like the binding or RB47 to the *psb*A 5′ UTR (Danon and Mayfield, 1991), is light regulated and inhibited by ADP, and to a lesser extent by ATP, but not by other nucleotide-triphosphates (Zerges, W., and J-D. Rochaix, unpublished data).

In the mitochondria of *S. cerevisiae*, five gene-specific translational activator proteins (Michaelis et al., 1991; McMulin and Fox, 1993; for review see Fox, 1996), and other proteins involved in RNA processing and turnover (Dake et al., 1988; Wiesenberger and Fox, 1997) are associated with the inner mitochondrial membrane. This association has been postulated to reflect a compartmentalization of processing, translation, and turnover of mitochondrial RNAs at the inner mitochondrial membrane, where the proteins encoded by these RNAs function in the respiratory electron transport and the synthesis of ATP (McMulin and Fox, 1993). Whereas there is substantial genetic evidence that the activator proteins of COX3 mRNA translation physically interact with the COX3 5′ UTR (for review see Fox, 1996), the translational activator proteins of *S. cerevisiae* mitochondria have yet to be shown to interact with RNA in vitro. In chloroplasts, however, proteins known to function in RNA metabolism or translation have not yet been shown to be associated with membranes. Similar to the proposed roles of the translational activator proteins in *S. cerevisiae* mitochondria, the association of RNA-binding proteins with low density membranes in chloroplasts may reflect a compartmentalization of chloroplast RNA metabolism and translation.

Several previous observations suggest a role of the inner envelope membrane in chloroplast gene expression and thylakoid biogenesis. The galactolipids of thylakoid membranes are synthesized at the chloroplast envelope (Rawyler et al., 1995). Consequently, the acyl lipid compositions of the inner envelope and the thylakoid membranes are nearly identical (for review see Joyard et al., 1991). Extensions of the inner envelope membrane and membrane vesicles in the stroma have been observed by electron microscopy in the chloroplasts of *C. reinhardtii* (Hoober et al., 1991), tobacco, pea, soybean, and spinach (Morré et al., 1991), and in the chromoplasts of red pepper fruits (Hugueney et al., 1995). Moreover, steps in chlorophyll synthesis after Mg-protoporphyrin IX occur in the chloroplast envelope (for review see Joyard et al., 1991). How chlorophyll is transported to the thylakoids is unknown (for review see Reinbothe and Reinbothe, 1996). If the chlorophyll-binding proteins encoded by chloroplast mRNAs are synthesized on the inner envelope membrane, newly synthesized chlorophyll could bind directly to them there. Moreover, cocompartmentalization of the synthesis of chlorophyll and chloroplast genome–encoded, chlorophyll-binding proteins could facilitate a coordination of the these two processes. The plastid genome in the rapidly dividing chloroplasts of young pea leaves has been shown to be associated with the envelope (Sato et al., 1993). Lastly, a recent study has demonstrated the presence of a homologue of the *Escherichia coli* ribosome releasing factor associated with the chloroplast envelope in spinach. This protein was not detected in purified thylakoid membranes (Rolland, N., and J. Joyard, personal communication). Thus, many lines of evidence suggest that thylakoid biogenesis, and possibly translation of chloroplast mRNAs, occurs at the chloroplast inner envelope membrane. We should point out that other hypotheses are also compatible with our data. For example, the RNA-binding proteins might hold a pool of nontranslated or "silent" mRNAs at the chloroplast envelope as a reserve to sustain protein synthesis during conditions of limiting transcription.

A role of the low density membranes in chloroplast mRNA metabolism and translation would seem to be contradicted by many previous studies that have demonstrated that the synthesis of thylakoid membrane proteins occurs on thylakoid-associated ribosomes (Herrin et al., 1981; for review see Harris et al., 1994). However, magnesium ions were always present in the thylakoid preparations used in these studies. We find that low density membranes are associated with thylakoids (Fig. 4). Thus, these previous studies did not determine whether polyribosomes are associated with thylakoids directly, or with the membranes of lower buoyant density. The observation that, after the removal of magnesium ions, thylakoid membranes can be separated from the low density membranes and their associated RNA-binding proteins on the basis of buoyant density (Fig. 4) demonstrates that these membranes are distinct membrane compartments.

The hypothesis that a subfraction of the inner envelope membrane, or a previously uncharacterized internal chloroplast membrane compartment of similar buoyant density, is the site of thylakoid biogenesis necessitates a means by which the newly synthesized thylakoid proteins move from this membrane compartment to thylakoids. As mentioned above, some reports have suggested vesicular trafficking between the inner envelope and thylakoids (Hugueney et al., 1995; Hoober et al., 1991). However, it is also possible that this low density membrane compartment is contiguous with thylakoid membranes. Movement of the newly synthesized proteins between the two compart-

ments, in this case, would occur laterally. We have made several attempts to detect the newly synthesized thylakoid proteins in the low density, thylakoid-associated membranes during short pulse-labeling experiments. These experiments showed that the proteins synthesized during the shortest possible pulse (1 min) were in thylakoid membranes (Zerges, W., and J.-D. Rochaix, unpublished data). However, this does not exclude the possibility that these proteins were synthesized in the low density membranes and then transported to thylakoids because known secretory pathways are typically too rapid to detect proteins in intermediate stages in the absence of inhibitors or mutations that result in a specific block in the pathway (for review see Gruenberg and Clague, 1992).

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