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Laurence Bessueille, Nicolas Sindt, Michel Guichardant, Soraya Djerbi, Tuula T. Teeri, et al.. Plasma membrane microdomains from hybrid aspen cells are involved in cell wall polysaccharide biosynthesis. *Biochemical Journal*, 2009, 420 (1), pp.93-103. 10.1042/BJ20082117 . hal-00479117

HAL Id: hal-00479117

<https://hal.science/hal-00479117>

Submitted on 30 Apr 2010

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Plant glucan synthases are located in plasma membrane microdomains

Plasma membrane microdomains from hybrid aspen cells are involved in cell wall polysaccharide biosynthesis

by

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Running title: Plant glucan synthases are located in plasma membrane microdomains

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Synopsis

Detergent-resistant plasma membrane microdomains (DRMs) were isolated recently from several plant species. As for animal cells, a large range of cellular functions, such as signal transduction, endocytosis, protein trafficking, etc, have been attributed to plant lipid rafts and DRMs. The data available are essentially based on proteomics and more approaches need to be undertaken to elucidate the precise function of individual populations of DRMs in plants. We report here the first isolation of DRMs from purified plasma membranes of a tree species, the hybrid aspen *Populus tremula x tremuloides*, and their biochemical characterization. Plasma membranes were solubilised with Triton X-100 and the resulting DRMs were isolated by flotation in sucrose gradients. The DRMs were enriched in sterols, sphingolipids and glycosylphosphatidylinositol-anchored proteins and thus exhibited similar properties as DRMs from other species. However, they contained key carbohydrate synthases involved in cell wall polysaccharide biosynthesis, namely callose [(1→3)-β-D-glucan] and cellulose synthases. The association of these enzymes with DRMs was demonstrated using specific glucan synthase assays and antibodies, as well as biochemical and chemical approaches for the characterization of the polysaccharides synthesized *in vitro* by the isolated DRMs. More than 70% of the total glucan synthase activities present in the original plasma membranes was associated to the DRM fraction. In addition to shedding light on the lipid environment of callose and cellulose synthases, our data demonstrate the involvement of DRMs in the biosynthesis of important cell wall polysaccharides. This novel concept suggests a function of plant membrane microdomains in cell growth and morphogenesis.

Keywords: callose synthase; cellulose synthase; cell wall biosynthesis; detergent-resistant membranes; hybrid aspen; lipid rafts

Abbreviations used: DRMs, detergent-resistant membranes; GPI, glycosylphosphatidylinositol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIPLC, phosphatidylinositol phospholipase C; PS, phosphatidylserine; TLC, thin layer chromatography.

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INTRODUCTION

Lipid rafts are ordered plasma membrane sub-domains that are enriched in sterols, sphingolipids, glycosylphosphatidylinositol (GPI) anchored proteins and proteins covalently modified by lipids carrying long saturated acyl chains [1-4]. A considerable amount of evidence supports their involvement in many cellular processes such as for instance signal transduction [5], intracellular sorting and exocytotic membrane transport [6]. In addition to their involvement in physiological processes, rafts play a role in cell invasion by pathological micro-organisms and penetration of bacterial toxins [7]. While sterols and (glyco)sphingolipids are the common building blocks of all types of lipid rafts, their relative proportion in membrane microdomains varies, thereby suggesting the occurrence of heterogeneous populations of rafts that reflect different functions [8].

Owing to their specific lipid composition and inherent physical properties, lipid rafts are typically isolated by exploiting their resistance to solubilisation by Triton X-100 at 4°C [9]. The resulting detergent-resistant membranes (DRMs) can subsequently be used for protein identification, thereby providing information on the potential function of lipid rafts *in vivo*. However, despite the usefulness of DRM preparations for biochemical and functional investigations, their relationship to physiological lipid rafts is currently debated [10,11]. A major criticism is the use of non-physiological conditions for DRM isolation, which may artificially lead to the formation of detergent-resistant structures that do not fully represent the original *in vivo* lipid raft structures [10]. Despite these considerations, extractions of DRMs reflect differential affinities of specific sets of membrane proteins to various lipid environments [10]. Thus, the full characterization of DRMs is valuable for understanding the interactions and functions of biologically important proteins that segregate in sterol/sphingolipid-rich environments [10]. For this reason, DRMs from animal cells have been extensively studied during the last decade. More recently, DRMs that exhibit similar biochemical properties as animal DRMs have been isolated from several plants including tobacco [12,13], *Arabidopsis* [14] and *Medicago truncatula* [15]. But despite this progress, plant DRMs are still poorly characterized compared to their animal counterparts.

Based on earlier work in our laboratory on plant (1→3)-β-D-glucan (callose) and cellulose synthases, we hypothesized that these biologically important membrane-bound enzymes are located in DRMs that have a lipid composition that varies between plant species [16,17]. The rationale for making such hypothesis was that cellulose synthase, which is extremely unstable upon detergent extraction, can be kept active *in vitro* only under highly selective conditions that involve a careful selection of the optimal detergent [16,17]. The choice of the detergent seems to be species-specific since an *in vitro* synthesis of cellulose was possible only when solubilising the enzyme from hybrid aspen (*Populus tremula x tremuloides*) with digitonin [17], while taurocholate or Brij 58 were the only efficient detergents for extracting an active enzyme from blackberry (*Rubus fruticosus*) [16]. In addition, detergents from different families have a dramatic effect on the *in vitro* callose synthase activity from different plant species [17-19]. From these observations, it was proposed that callose and cellulose synthases are located in DRMs that present a lipid composition that varies between plant species [16,17]. The occurrence of these glucan synthases in DRMs would explain the difficulty to purify them to homogeneity.

Here, we demonstrate the existence of DRMs in the plasma membrane of cells from the model tree species *P. tremula x tremuloides*, and show that the isolated DRMs are involved in cell wall polysaccharide biosynthesis. Their lipid composition was analyzed to confirm the nature of the isolated structures. Specific assays for (1→3)-β-D-glucan (callose) and cellulose synthases [17] were combined with the use of antibodies to reveal the occurrence of both enzymes in DRMs. These carbohydrate synthases were also found in DRMs from tobacco cells, suggesting that their association with DRMs is a general feature of higher plants. Our

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data provide evidence that the role of lipid rafts as signalling platforms may be extended to plant cell wall polysaccharide biosynthesis and cell growth.

EXPERIMENTAL

Plant cell cultures

Suspension cultures of hybrid aspen cells [17,20] were grown at 24°C in a 12 h light/12 h dark regime in a modified MS medium containing sucrose (3%), 2,4-dichlorophenoxyacetic acid (1 mg/L) and kinetin (0.02 mg/L) [21]. The cells were harvested 25 to 30 days after inoculation of fresh medium [20] and extensively washed with distilled water by vacuum filtration.

Isolation of plasma membranes and assay of specific enzyme markers

In typical experiments, ~150 g of cells were used for the preparation of total membranes as previously described [17]. All following steps were performed at 4°C. Plasma membranes were purified from the total membranes by partitioning in an aqueous two-phase system consisting of 5.8% (w/w) polyethylene glycol 3350 (Sigma), 5.8% (w/w) dextran T-500 (Amersham Pharmacia Biotech), 4 mM KCl and 5 mM potassium phosphate, pH 7.6 [22]. The upper phase containing enriched plasma membranes was diluted 5-fold with resuspension buffer (50 mM Mops/NaOH buffer, pH 7). Plasma membranes were then pelleted (50,000g for 45 min) and resuspended in 1 mL of ice-cold resuspension buffer to obtain a final protein concentration in the range 1-3 mg/mL. Suspensions of intracellular membranes were prepared in a similar manner from the lower phase of the partitioning system. Protein content was measured using the Bradford dye-binding assay (BioRad) [23].

The relative enrichment of plasma and intracellular membranes was evaluated by assaying specific enzyme markers. Cytochrome-c oxidase and antimycin A-insensitive NADH-cytochrome-c reductase were used as mitochondrial and endoplasmic reticulum markers, respectively, and assayed as described earlier [24]. The activity of Triton-dependent inosine diphosphatase (IDPase) was determined in the absence and presence of 0.01% (v/v) Triton X-100 [25]. The Golgi IDPase activity was obtained by subtracting the activity measured in the absence of Triton X-100 to that measured in the presence of the detergent [26]. The released inorganic phosphate was assayed [27] in the presence of 0.075% SDS. The activity of vanadate-sensitive ATPase, a marker for plasma membrane [28], was measured as described elsewhere [29] using 200 µM vanadate. The assays contained 1 mM sodium azide, 1 mM sodium molybdate and 50 mM potassium nitrate as inhibitors of mitochondrial ATPase, acid phosphatase and tonoplast ATPase, respectively. The release of inorganic phosphate was determined as indicated above for the Golgi IDPase assay.

Preparation of membrane microdomains

Triton X-100 in resuspension buffer was added to the plasma membranes to a final concentration of 1% (detergent-to-protein ratio=15 (w/w)). After 30 min at 4°C, a sucrose solution was added to reach a final concentration of 46% sucrose. The preparation was fractionated by flotation in linear 15-45% sucrose gradients (centrifugation at 250,000g and 4°C for 20 h). Fractions of 1 mL were collected from the bottom of the ultracentrifuge tubes.

Assay conditions for callose and cellulose synthases

Callose and cellulose synthase assays [17] were performed in a final volume of 200 µL, using 20 µL of a membrane preparation or sucrose gradient fraction as a source of enzymes. The mixture for callose synthase assay consisted of 50 mM Mops/NaOH buffer (pH 7.0), 20 mM cellobiose, 8 mM CaCl₂, 1 mM UDP-glucose and 0.16 µM UDP-D-[U-¹⁴C]glucose (Perkin-Elmer, 7.4 MBq.mmol⁻¹) (final concentrations). For cellulose synthase assay, the reaction

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mixture was supplemented with 8 mM MgCl₂ and the CaCl₂ concentration was decreased to 1 mM. After 45 min at 25°C, the reactions were stopped by the addition of 400 µL ethanol and the amount of radioactive glucose incorporated into ethanol-insoluble polysaccharides were determined by liquid scintillation as described earlier [17].

The proportion of callose and cellulose synthesized *in vitro* was determined by using enzymes that specifically hydrolyze (1→3)-β-D-glucans or cellulose [16,17,19,30]. *In vitro* synthesis reactions performed in triplicates were run for 4 h in the presence of radioactive substrate, by using 200 µL of the DRM fraction as a source of enzyme. The radioactive polysaccharides recovered by centrifugation (10,000g for 10 min) were washed with distilled water and resuspended in 400 µL of 50 mM acetate buffer (pH 5). They were then incubated for 8 h at 40°C with 0.16 unit of exo-(1→3)-β-D-glucanase from *Trichoderma reesei* (Megazyme) or at 55°C with a mixture of recombinant cellulases from *Thermobifida fusca* expressed in *Escherichia coli* (Cel 6A and Cel 6B, 1 unit each; generous gift from D. B. Wilson, Cornell University). An enzymatic unit was defined as the amount of enzyme releasing 1 µmol of reducing sugar per min. Controls were performed in the same conditions by substituting the hydrolytic enzyme solutions by acetate buffer. The radioactivity in non-hydrolyzed ethanol-insoluble polysaccharides was measured by liquid scintillation [17,19] and the extent of hydrolysis was calculated by comparison with the controls where no hydrolytic enzyme was used.

Structural characterization of callose by ¹³C-NMR spectroscopy

A sensitive ¹³C-NMR method was used for structural analysis of the (1→3)-β-D-glucan synthesized *in vitro* in the presence of UDP-[U-¹³C]glucose [31]. The latter substrate was synthesized from [U-¹³C]glucose (Cambridge Isotopes Laboratories) using a cocktail of commercial enzymes [32]. *In vitro* synthesis of callose was performed as for a typical assay by using 8 mL of the DRM fraction as a source of enzyme and scaling up the reaction mixture proportionally. The (1→3)-β-D-glucan synthesized *in vitro* was then purified [19], dissolved in deuterated dimethylsulfoxide and analyzed by ¹³C-NMR spectroscopy [31]. The central peak of the dimethylsulfoxide multiplet was used as a reference.

Morphological characterization of callose by transmission electron microscopy

The (1→3)-β-D-glucan synthesized *in vitro* by the DRMs was purified and analyzed by transmission electron microscopy at the “Centre Technologique des Microstructures” (University of Lyon, France) exactly as described in Pelosi *et al.* [19].

Isolation of GPI-anchored proteins

Plasma membrane proteins were biotinylated using the ECL Protein Biotinylation Module from Amersham Biosciences. DRMs were subjected to two-phase partitioning in the presence of Triton X-114 to extract all types of membrane-bound proteins [33]. The latter were subsequently incubated in the presence of the specific phosphatidylinositol phospholipase C (PIPLC) from *Bacillus cereus* (Molecular Probes) to demonstrate the occurrence of GPI-anchored proteins. DRMs (ca 200 µL) were mixed at 0-4°C with a minimum volume of Triton X-114 (Sigma) in extraction buffer (10 mM Tris-HCl pH 7.4 containing 150 mM NaCl) to reach a final detergent concentration of 1%. The sample was kept on ice for 5 min before being layered onto a sucrose cushion (6% sucrose in extraction buffer containing 0.06% Triton X-114). After 10 min at 30°C and centrifugation (3 min at 300g and room temperature) for phase separation, the Triton X-114 extraction was repeated on the pellet containing the extracted membrane-bound proteins. The latter were resuspended in 200 µL extraction buffer containing 1 unit of PIPLC. The mixture was incubated for 1 h at 37°C

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under continuous stirring and subjected to phase separation in Triton X-114 as described above. The biotinylated proteins released by PIPLC in the aqueous phase were lyophilized, analyzed by SDS-PAGE, transferred to nitrocellulose and detected using peroxidase-conjugated streptavidin and the corresponding chemiluminescent substrate (ECL Western Blotting Detection Reagents, Amersham Biosciences).

Production of anti-cellulose synthase antibodies

Antibodies were raised against a recombinant polypeptide corresponding to a fragment of the *PttCesA2* cellulose synthase gene (N582 to L861; accession # AY573572). The fragment fused to a dual affinity tag consisting of His₆ and albumin binding protein (ABP) was cloned in the inducible vector pAff8c [34] and expressed in *E. coli* XL1 Blue MRF' cells. A 3C-site was introduced to allow cleavage of the tags after protein purification. The cells were grown in Tryptic Soya Broth (TSB, 30g/L, Difco, Detroit, MI) supplemented with 5g/L yeast extract, 50 µg/mL kanamycin and 34 µg/mL chloramphenicol, and the recombinant polypeptide was obtained as intracellular inclusion bodies upon induction with 1 mM isopropyl-β-D-thiogalactopyranoside (Apollo Scientific Ltd., Whaley Bridge, UK) at an OD₆₀₀ of ca 1. The cells harvested by centrifugation after 4 additional h of growth at 37°C were washed in 50 mM Hepes buffer pH 7.5 and resuspended in the same buffer for storage at -80°C. For the preparation of the recombinant polypeptide, the cells were thawed and inclusion bodies were isolated by sonication, centrifugation and solubilization in 6 M guanidinium-HCl pH 7.2. Cell debris were removed by centrifugation and the recombinant polypeptide was purified by immobilized metal affinity chromatography according to the manufacturer's instructions (TALON Metal Affinity Resins, CLONTECH Laboratories Inc.). After Coomassie-blue staining of the purified recombinant polypeptide separated by SDS-PAGE, the bands were excised and used for production of polyclonal antibodies in rabbits following standard immunization protocols (Agrisera AB, Sweden).

SDS-PAGE and western blot analyses

Proteins in the sucrose gradient fractions were analyzed by SDS-PAGE and stained with silver [35]. For western blot analyses, proteins separated by SDS-PAGE were transferred to ECL nitrocellulose membranes (0.45 µm, Amersham Biosciences), stained with Ponceau red (0.2% in 3% trichloroacetic acid) and destained in water. The membranes were blocked for 1 h in Tris Buffer Saline (TBS: 20 mM Tris-HCl pH 7.4, NaCl 150 mM) containing 5% non-fat milk. After washing in TBS containing 0.05% Tween 20, the membranes were probed overnight at 4°C with the anti-cellulose synthase antibodies (1/1000 in TBS-Tween). The membranes were washed twice in TBS-Tween and incubated for 2 h at room temperature with goat anti-rabbit antibodies conjugated to peroxidase (1/5000 in TBS-Tween). Detection of antibody binding was performed using the same chemiluminescent substrate as for the detection of biotinylated GPI-anchored proteins. The relative amount of cellulose synthase in the different gradient fractions was estimated by measuring the intensity of the signals on the western blot obtained using anti-*PttCesA2* antibodies. Semi-quantitative densitometric analyses were performed using the Molecular Imager Gel Doc XR System and the Quantity One-1-D Analysis software from Biorad.

Lipid analyses

Total lipids were extracted from purified plasma membranes or DRMs according to Bligh and Dyer [36]. One mL of either of the two membrane samples containing approximately 400 µg protein were mixed with 3.75 mL methanol/chloroform (2/1, v/v), vigorously shaken for 1 min and centrifuged for 10 min at 450g. The pellet containing precipitated proteins was discarded. Chloroform (1 mL) and water (0.8 mL) were added to the supernatant and phase

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separation was performed by centrifugation at 450g for 10 min. The organic phase was collected and dried under a stream of N₂. Total lipids were re-dissolved in 200 µL of chloroform and either analyzed by thin layer chromatography (TLC) or used for various assays. Total phospholipids were assayed using the ammonium ferrothiocyanate method [37] or the Enzymatic Colorimetric kit from Wako Chemicals (Germany). The enzymatic Amplex Red Cholesterol Assay kit from Molecular Probes was used to quantify sterols. The presence of sphingolipids was evidenced by subjecting an aliquot of the lipid extract to mild alkaline methanolysis, which hydrolyzes all phospholipids but preserves sphingolipids. The sample was successively incubated for 1 h at 60°C in 2.5 M KOH in methanol, neutralized with HCl, extracted in chloroform, concentrated and analyzed by TLC. The identification of the sphingolipids was completed by a specific staining with orcinol (0.3% orcinol in 3 M H₂SO₄). The excess of solvent was dried and the plates were heated at 180°C for 20 min.

Major lipid classes were separated by TLC on silica gel 60 plates (Merck), using two-solvent systems. Lipids were quantitatively applied to TLC plates and development was carried out in ethyl acetate/propanol/chloroform/methanol/0.25% aqueous KCl (25/25/25/10/9, by vol.). After the solvent had reached 10 cm from the bottom of the plates, the latter were dried and further developed in hexane/diethyl ether/acetic acid (75/21/4, v/v/v) for an additional 6 cm. Lipids were stained by dipping the TLC plates in a mixture of 10% cupric sulphate (w/v) and 8% phosphoric acid (v/v). After 20 min at 180°C the plates were scanned from the origin to the solvent front using a Camag II TLC scanning densitometer. Quantification was by *in situ* densitometry with absolute amounts of the lipid classes determined from co-chromatographed standards [38] using the CATS software analysis package from Camag. Lipid standards (0.5-10 µg) were dissolved in chloroform (phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS) and cholesterol from Sigma; glucosylceramide from Avanti Polar lipids).

For qualitative and quantitative GC/MS analyses of sterols, the total lipid fraction was supplemented with 20 µg of an internal standard, namely 7-OH-cholesterol (Sigma), which was not present in the original samples. Lipids were separated by TLC as described above and visualized by spraying the plates with 0.02% dichlorofluorescein in 95% methanol. The sterols were scrapped off the plate and eluted with chloroform/methanol. Samples were dried under vacuum, treated at 60°C for 30 min with 100 µL of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and diluted in isooctane for GC/MS analysis. A Hewlett Packard (HP) quadrupole mass spectrometer interfaced with an HP gas chromatograph (Les Ulis, France) was used for the analysis. The DB-17MS fused-silica capillary column (60 m x 0.25 mm (i.d.), 0.25 µm film thickness, Agilent Technologies) was held at 57°C and the following oven temperature program was used: 5 min at 57°C, 57-200°C at 40°C/min, 200-310°C at 10°C/min, and 310°C for 20 min. The interface, injector and ion source were at 280, 280 and 150°C, respectively. Electron energy was set at 70 eV and helium and methane were used as carrier and reagent gas, respectively. The electron multiplier voltage was set at 1400 V and mass spectra were acquired from 100 to 600 Da using positive chemical ionization (PCI) mode. Sterols were identified on the basis of their retention times and mass spectra that were compared to those of standards (ergosterol, stigmasterol, dihydroxy-cholesterol from Sigma, campesterol from LGC Promochem) derivatized as described above (BSTFA). They were quantified using the selected-ion monitoring PCI mode and their corresponding characteristic fragments ([M+H]⁺-TMSOH) at m/z of 369, 379, 383, 397, 395 for trimethylsilylether derivatives of cholesterol, ergosterol, campesterol, β-sitosterol and stigmasterol, respectively. The ion at an m/z value of 367, which corresponds to [M+H]⁺-TMSOH-H₂O was used as the internal standard.

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For fatty acid analysis by GC, total lipids were separated by TLC as described above and stained with 0.02% (w/v) dichlorofluorescein in 95% methanol. The glycerophospholipids were scraped and transesterified in 250 μL toluene/methanol (40/60) and 250 μL BF_3 in 14% methanol at 100°C for 90 min. The reaction was stopped in ice by adding 1.5 mL of 10% K_2CO_3 . Fatty acid methyl esters were extracted with 2 mL isooctane and analyzed by GC (Agilent Technologies, model 6890) using a BPX 70 fused silica capillary column (60 mm x 0.25 mm i.d., 0.25 μm film thickness; SGE Europe Ltd, France). The oven temperature was set at 80°C for 1.5 min and increased to 150°C at 20°C.min⁻¹ and then to 250°C at 2°C.min⁻¹. The latter temperature was maintained for 10 min before returning to the initial conditions. Helium was used as the carrier gas (1 mL.min⁻¹) and the split/splitless injector and flame ionisation detector were set at 230°C and 280°C, respectively.

RESULTS

Isolation of detergent-resistant membrane microdomains from hybrid aspen cells

Enriched plasma membranes were prepared from suspension cultures of hybrid aspen cells by two-phase partitioning. The relative specific activities of callose synthase and vanadate-sensitive ATPase, which are markers for plasma membranes [28, 39], were much higher in the upper phase than in the lower phase and the total membranes (Figure 1A). Conversely, the lower phase was enriched in intracellular membranes, as expected. This is evidenced by its significantly higher levels of antimycin A-insensitive NADH cytochrome-c reductase and Triton-dependent IDPase specific activities, which are markers of endoplasmic reticulum and Golgi compartments, respectively [24,26]. The mitochondrial marker cytochrome-c oxidase [24] was also detected in higher amounts in the lower phase, however the difference was not as pronounced as for the endoplasmic reticulum and Golgi markers. This indicates that the plasma membrane fraction was slightly contaminated by mitochondrial membranes and traces of endoplasmic reticulum and Golgi membranes. No better enrichment of plasma membranes could be obtained with any of the conditions screened for cell disruption and two-phase partitioning. Thus, the type of upper phase illustrated in Figure 1A was considered as the best source of enriched plasma membranes for the isolation of DRMs.

The DRMs prepared by solubilisation of the plasma membranes with Triton X-100 and flotation on a sucrose gradient were visible as a white and rather sharp band at a density of 1.16-1.18 (Figure 1B). Protein assay revealed 2 peaks in the gradient, one corresponding to the solubilised membrane proteins in the high-density fractions (bottom of the tube, fractions 1-3; $d=1.20$), the other to DRMs (fractions 8-10; $d=1.16-1.18$) (Figure 1B). The DRM peak contained ~50% of the total protein loaded at the bottom of the gradient prior to centrifugation. Phospholipids were rather broadly distributed in the lower half of the gradient (fractions 1-13), with the highest concentrations (200-250 $\mu\text{g.mL}^{-1}$) measured at a density of 1.18-1.20 (Figure 1B). The amount of phospholipids decreased progressively in the fractions of a lower density (fractions 6-13), including those that contained the DRMs (140-160 $\mu\text{g.mL}^{-1}$; fractions 7-10). In addition, higher amounts of sterols were detected in fractions 8-10 (Figure 1B). Altogether these data strongly suggest that the membrane structures in fractions 7-10 correspond to DRMs. Their composition was analyzed in greater detail as described in the following sections.

DRMs from hybrid aspen cells contain GPI-anchored proteins

In order to investigate whether GPI-anchored proteins are associated to the hybrid aspen DRMs, all types of membrane-bound proteins were extracted from the DRM fractions using a Triton X-114 phase partitioning system. The proteins were subsequently subjected to the

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action of a highly specific PIPLC, which cleaves off GPI anchors from GPI proteins. As a consequence of this treatment, the protein moieties of GPI proteins are specifically shifted from the Triton X-114 phase to the aqueous phase when repeating the phase partitioning procedure. When this approach was applied to the DRMs from hybrid aspen cells, at least 5 protein bands were clearly detected in the aqueous phase after the PIPLC treatment, indicating that the corresponding proteins were originally linked to GPI anchors (Figure 2). The proteins were hardly visible in SDS-PAGE gels after silver staining and became easily detectable only after having been preliminarily subjected to biotinylation (Figure 2). Their recovery in sufficient amount for sequencing was challenging and their identity remains to be determined. Interestingly, a putative GPI protein from *A. thaliana* designated as COBRA is involved in cellulose microfibril orientation and the corresponding mutants exhibit reduced amounts of crystalline cellulose in the cell walls of the root growth zone [40]. The use of antibodies produced against peptides corresponding to the hybrid aspen ortholog of COBRA suggests that this protein is not present in DRMs (data not shown).

Lipid analyses of DRMs from hybrid aspen

Lipid analysis revealed that plasma membranes (Figure 1A) and DRM (Figure 1B) fractions exhibit a similar lipid pattern consisting of sterols and more polar lipids, such as PC, PE, PA and PI (Figure S1A in Supplementary Material). The occurrence of glucosylsphingolipids in both fractions was demonstrated by alkaline methanolysis and orcinol staining, and by comparing their mobility with that of commercially available standards (Figure S1A). In spite of a similar general lipid profile, plasma membranes and DRMs exhibited clear differences in terms of relative abundance of the different classes of lipids (Table 1). In particular, PC and PE were the two major phospholipids in the plasma membrane fraction (15.7% and 27.4% of the total lipids, respectively) while PC accounted for only 9.5% of the total lipids in the DRMs. The proportion of total phospholipids (*i.e.* PC + PI + PA + PE) was significantly lower in the DRMs than in the enriched plasma membrane fraction (~45% of the total lipids for the DRMs versus ~65% for the plasma membrane fraction). This was accompanied by a substantially higher proportion of free sterols and sphingolipids in the DRMs (29.6% and 28.3% of the total lipids respectively, compared to 13.5% and 19.9% for the plasma membranes) (Table 1). This higher proportion was also observed with respect to the protein content: the sphingolipid-to-protein and sterol-to-protein ratios were respectively 3 times and 4 times higher in the DRMs (Figure S1B). Altogether these data demonstrate that the isolated DRMs exhibit a composition that is quantitatively clearly distinct from that of the starting plasma membranes, with a characteristic enrichment in sterols and sphingolipids.

Total sterols from the plasma membrane and DRM preparations were analyzed as acetate derivatives by GC/MS. The most prominent sterol in both fractions was β -sitosterol, representing respectively 97.9% and 91.6% of the total sterols in plasma membranes and DRMs. Four minor sterols were also present in both types of membranes, namely campesterol, cholesterol, stigmasterol and an unidentified compound that possibly corresponds to an isoform of sitosterol. Campesterol and the non-identified sterol occurred in slightly higher proportions in the DRMs (5.4% and 3.5% respectively) than in the enriched plasma membranes (3% and 2.5% respectively). But overall, the relative proportion of each sterol within the sterol family was similar in the plasma membrane and DRM fractions. Thus, despite a higher proportion of total sterols in DRMs with respect to the total lipid content, there was no obvious selective partitioning of any of the analyzed sterol species in the DRMs.

Analysis of the fatty acids from total phospholipids revealed a slightly higher proportion of saturated fatty acids (palmitic and stearic acids) in the DRMs, while the plasma membrane fraction contained a higher proportion of unsaturated fatty acids (essentially linoleic and linolenic acids) (Figure 3). Overall, the saturated to unsaturated fatty acid ratio was of about

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0.62 for the DRMs compared to 0.45 for the enriched plasma membranes. Such differences are in agreement with the general observation that phospholipids from DRMs are enriched in saturated fatty acids compared to overall plasma membranes, and the differences observed here for individual fatty acids are comparable to those reported for DRMs from other plants [12,41].

Glucan synthases from higher plants are associated to DRMs

Callose is the only polysaccharide synthesized *in vitro* when membranes or detergent extracts of membranes from hybrid aspen cells are incubated in the presence of 1 mM UDP-glucose and 8 mM CaCl₂, while cellulose and callose are co-synthesized in the presence of 1 mM UDP-glucose, 1 mM CaCl₂ and 8 mM MgCl₂ [17]. These 2 reaction mixtures were used to assay glucan synthase activities in each fraction of the sucrose gradient presented in Figure 1. The assays revealed that the peaks of glucan synthase activities systematically coincided with the peaks of protein and sterols that correspond to the DRMs (Figures 1B and 4A, fractions 7-10). The specific glucan synthase activities reached a maximum of 57.9 nmol of glucose incorporated per min per mg of protein in the presence of 8 mM CaCl₂, and 72.1 nmol.min⁻¹.mg⁻¹ in the presence of 1 mM CaCl₂ and 8 mM MgCl₂, which represents a purification fold with respect to the total plasma membranes of 11.3 and 6.6, respectively. In addition, an average of 73% of the total glucan synthase activities originally present in the enriched plasma membranes was recovered in the DRMs, whichever assay condition was used. Highly reproducible levels of activities were obtained from a total of 5 independent experiments. Analyses of the polysaccharides synthesized *in vitro* by the DRMs revealed that 100% of the polysaccharide synthesized in the presence of 8 mM CaCl₂ corresponded to callose (not shown), while an average of ~10% cellulose and ~90% callose were synthesized by the DRMs in the presence of 1 mM CaCl₂ and 8 mM MgCl₂ (see Table 2 and following section on *in vitro* product characterization). These data are in agreement with those previously obtained using identical reaction mixtures and detergent extracts of plasma membranes from hybrid aspen cells [17]. However, in the case of the DRM preparation, the level of cellulose synthase activity was rather low. This may be explained by an inactivation of this highly unstable activity upon the Triton X-100 treatment. Indeed, of the total cellulose synthase activity present in the plasma membranes a fraction only is still detectable after the sucrose gradient step, all of which occurs in the DRM fraction (Figure 4A and Table 2). Altogether these data show a clear enrichment (73%) in DRMs of the glucan synthase activities originally present in the total plasma membranes. Similar results were obtained from tobacco BY2 cell suspension cultures (data not shown), suggesting that the occurrence of callose and cellulose synthases in DRMs is a common feature of higher plants.

SDS-PAGE analysis of the DRMs (fraction 8 of the gradient) revealed an enrichment of proteins of 24, 25, 30, 35, 58, 67 and 90 kDa compared to the plasma membranes and fraction 2 of the gradient, which corresponds to the peak of proteins solubilised by Triton X-100 (Figure 4B). The identification of these proteins is in progress in our laboratory. Interestingly, specific polyclonal antibodies produced against the putative catalytic site of one of the cellulose synthases of hybrid aspen (*PttCesA2*) recognized a protein of ~120 kDa in the high-density fractions of the sucrose gradient (fractions 2-5), while a protein of ~57 kDa was recognized in the DRM fractions (fractions 7-10, Figure 4C). No signal was obtained when replacing the polyclonal antibody solution by the pre-immune serum (not shown). The 120-kDa protein exhibits an apparent molecular weight similar to that of the full-length *PttCesA2* gene product. However, the signal obtained in western blots at 120 kDa (Figure 4C) is present in fractions devoid of glucan synthase activities, while the 57-kDa protein recognized by the antibodies occurs in the DRM fractions in which both callose and cellulose synthase activities

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were detected (Figure 4 and Table 2). These results suggest that the active form of *PttCesA2* may correspond to a 57-kDa proteolytic fragment of the full-length *PttCesA2* gene product. In addition, the intensity of the signal obtained with the anti-*PttCesA2* antibodies was significantly higher for the 57-kDa band associated to DRMs than for the 120-kDa band (Figure 4C). Semi-quantitative densitometric analysis of the signals from the western blot revealed that the intensity attributable to the 57-kDa band in the DRM fractions represents 85% of the cumulated intensities arising from the 57-kDa and 120-kDa bands. Thus, based on antibody detection, about 85% of the cellulose synthase loaded at the bottom of the gradient and originally present in the plasma membranes is recovered in the DRMs in an active form of 57 kDa. Conversely, 15% of the enzyme is recovered in the high-density fractions of the gradient as a Triton-solubilised inactive protein of 120 kDa. Altogether these data strongly support an enrichment of cellulose synthase in DRMs.

Characterization of the polysaccharides synthesized *in vitro* by the DRMs

The proportion of cellulose and (1→3)-β-D-glucan synthesized *in vitro* by the plasma membrane and DRM fractions was determined by measuring the extent of hydrolysis of the radioactive glucans by specific glycoside hydrolases (Table 2). The specificity of the hydrolases was verified on well-characterized polysaccharides (not shown) and each hydrolytic enzyme was active only on its specific substrate as reported recently [30]. The glucans synthesized by the plasma membranes in the presence of 1 mM CaCl₂ and 8 mM MgCl₂ consisted of an average of ~65% cellulose and ~35% (1→3)-β-D-glucan, while a larger proportion of (1→3)-β-D-glucan (~90%) was synthesized by the DRMs together with ~10% cellulose (Table 2). Comparable data were obtained with tobacco cells, with however a higher proportion of cellulose synthesized *in vitro* by the DRMs (~20% cellulose versus ~80% callose). As mentioned above, the lower proportion of cellulose synthesized by the DRMs may be attributable to a partial inactivation of the cellulose synthase during the Triton X-100 treatment. The fact that the cellulose synthase activity detected in the sucrose gradient occurs only in the DRM fractions (Figure 4A), together with the western blot data (Figure 4C) and the corresponding densitometric analysis, further support an enrichment of cellulose synthase in DRMs.

The callose synthesized by the DRMs in the presence of UDP-[U-¹³C]glucose was solubilised in dimethylsulfoxide and characterized by ¹³C-NMR spectroscopy. The spectrum revealed six resonance signals that appeared as doublets or triplets due to ¹³C-¹³C coupling interactions (Figure 5). The presence of these multiplets confirmed the *de novo* synthesis of the glucan since the molecule was fully enriched in ¹³C. The signals at 61, 68.5, 73.2, 76.7, 86.6 and 103.5 ppm can be assigned to C6, C4, C2, C5, C3 and C1 of a strictly linear (1→3)-β-D-glucan [42,43]. The spectrum is comparable to those obtained for other (1→3)-β-D-glucans synthesized *in vitro* [16,18,19,31,44]. This data confirms the ability of DRMs to synthesize callose and provides a complementary chemical analysis to the biochemical approach based on the use of radioactive UDP-glucose and specific glycoside hydrolases (Table 2). The amount of cellulose synthesized *in vitro* by the DRMs was too low to allow a similar NMR analysis. Indeed, for cellulose - which unlike callose is insoluble in dimethylsulfoxide - the NMR analysis would need to be performed in the solid state. This typically requires the synthesis of at least several mg of ¹³C-enriched cellulose, which was not possible given the rather low cellulose synthase activity in the DRMs (Table 2).

The callose synthesized by the isolated DRMs consisted of elementary structures of 2–3 nm in width connected through their tips to form longer microfibrillar strings of a few μm in length (Figure 6). This morphology was similar to that of other (1→3)-β-D-glucans synthesized *in vitro* by detergent extracts of plant plasma membranes [18].

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DISCUSSION

While animal DRMs have been intensively studied in the last decade, a lot less information is available on the organization and function of equivalent structures from plant cells. Here we have employed a selective method for the purification of DRMs from a cell suspension culture of a model tree species, the hybrid aspen *P. tremula x tremuloides*. A highly enriched plasma membrane fraction was first isolated to avoid lipid interferences from sub-cellular membrane compartments and prepare DRMs that reflect as closely as possible the composition of physiological lipid rafts. As expected, the DRMs from hybrid aspen cells were enriched in sterols, sphingolipids, GPI proteins and phospholipids carrying saturated fatty acids. However, as already observed for other plant species, the apparent density of the hybrid aspen DRMs was higher than that of animal and yeast DRMs [12,14]. The lower lipid/protein ratio in plant DRMs is most likely responsible for their higher density. In addition to proteins and lipids, intercalated detergent molecules may influence the apparent density of DRMs, and the removal of weakly associated proteins and lipids by Triton X-100 may alter the protein/lipid ratio of DRMs. Therefore, DRMs exhibiting different apparent densities do not necessarily reflect different original populations of plasma membrane microdomains.

Although our data represent the first demonstration of the occurrence of DRMs in cells from a tree species, similar structures have been isolated from other plants [12-15]. Most interestingly, our data show that the purified DRMs are enriched in biologically important carbohydrate synthases, namely callose and cellulose synthases. In particular, 73% of the total glucan synthase activities originally present in the plasma membranes is detected in the DRMs, as opposed to only 50% for protein. The remaining of the glucan synthase activities (27%) was not detectable in the gradient, most likely because of a partial inactivation of the enzymes by the Triton X-100 treatment. Part or all of the glucan synthase molecules corresponding to the 27% loss of activity may end up at the bottom of the gradient, *i.e.* in fractions devoid of activity and containing the proteins that were solubilized by the detergent (fractions 1-5, Figure 4A). In this first hypothesis, 73% at least of the plasma membrane glucan synthases would be located in DRMs. A second possibility is that the enzyme molecules corresponding to the 27% loss of activity all end up in the DRMs, together with the glucan synthase molecules responsible for the detected activity (73%). In this second possibility, 100% of the glucan synthases would be located in the DRMs. In conclusion, whichever of the two possibilities envisaged above is right, it can be concluded that at least 73% of the total glucan synthases originally present in the plasma membranes are present in the DRMs. Such high enrichment is further supported by the semi-quantitative analysis based on the use of anti-cellulose synthase antibodies. Indeed, the analysis revealed that about 85% of the cellulose synthase catalytic subunit segregates in the DRM fractions. The glucans synthesized *in vitro* by the DRMs were characterized using biochemical and chemical methods, which firmly confirmed the occurrence of both callose and cellulose synthases in DRMs.

The western blot analysis with the anti-cellulose synthase antibodies and the enzymatic assays on the gradient fractions suggest that cellulose synthase subunits are originally incorporated in the plasma membrane in a catalytically inactive form of ~120 kDa, which corresponds to the theoretical mass of cellulose synthase gene products. The results suggest that this inactive form of the enzyme can be readily solubilised by Triton X-100 since it occurs exclusively in fractions of the gradient that contain solubilised proteins (fractions 1-5; Figure 4A). From this experiment, it can be proposed that the 120-kDa protein is subsequently proteolytically processed to yield a catalytically active form of ~57-kDa that is associated to

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DRMs. Interestingly, proteolytic processes have been proposed to be involved in the turnover and/or degradation of cellulose synthase complexes of the cotton fiber [45] and *A. thaliana* [46]. In the case of cotton fiber, the proteolysis involved in the turnover of the enzyme seems to be controlled by the state of dimerization of the cellulose synthase catalytic subunits, which is due to interactions between the N-terminal zinc-binding domains of the individual catalytic subunits [45]. In *A. thaliana*, phosphorylation of two serine residues seems to play an important role in the proteolytic regulation of the cellulose synthase complex involved in secondary cell wall biosynthesis [46].

To date, no glucan synthase activity has been reported in plant DRMs. This is certainly due to the fact that the authors of the available reports have focused their work on the general proteomic and lipid characterization of the DRMs rather than on the specific search of associated carbohydrate synthases [12,14,15,41,47]. However, it is noteworthy that peptides arising from a putative callose synthase have been reported in DRMs from tobacco, even though the percentage of sequence coverage did not exceed 10% and the function of the corresponding protein was not demonstrated [47]. Proteomic analyses of DRMs from tobacco [47] and *M. truncatula* [15] allowed the identification of a total of 145 and 270 proteins respectively. Besides conventional raft markers such as receptor kinases and proteins related to signalling, proteins involved in cellular trafficking and cytoskeleton assembly were identified [15,47]. Such proteins could be indirectly involved in the targeting and turnover of carbohydrate synthases between sub-cellular compartments and the plasma membrane.

The formation of lipid rafts is considered to be dependent on specific interactions between (glyco)sphingolipids, sterols and specific proteins. As in the case of *A. thaliana* [14], β -sitosterol was the major sterol of the hybrid aspen plasma membranes and DRMs. DRMs from hybrid aspen were also enriched in glucosylceramides. Due to its high abundance, sitosterol most likely plays a structural role in DRMs, although it cannot be excluded that it serves as a primer for cellulose biosynthesis as proposed earlier for the cotton cellulose synthase [48]. Interestingly, several *Arabidopsis* mutants affected in sterol biosynthesis showed a deficiency of cellulose in embryonic and post-embryonic tissues [49]. However the observed reduction of cellulose could not be directly attributed to a lack of structural sterols in the plasma membrane. Another interesting observation is the demonstrated involvement of GPI proteins in the regulation of cell wall biosynthesis or deposition [40,50-52]. In addition, specialized lipid domains in plants are involved in the polarized growth of pollen tube and root hair, and the asymmetric growth of plant cells is in general due to the asymmetric distribution of membrane components [53]. Altogether these observations and our data point towards a link between cell wall biosynthesis and lipid rafts or DRM components, such as sterols, glycosphingolipids and GPI proteins. Thus we propose that membrane microdomains play a key role in cell growth and morphogenesis in plants by acting as dynamic platforms involved in the turnover of structural and functional membrane components, such as sterols and cell wall carbohydrate synthases. It will now be important to dissect the molecular events that control the dynamics of plant membrane microdomains in relation with cell elongation and morphogenesis.

Acknowledgments

This work was supported by grants to V.B. from CNRS (France) and the European Union [contract no. QLK5-CT-2001-00443], and by the Swedish Centre for Biomimetic Fibre Engineering. We thank A. Ohlsson and T. Berglund (KTH, Stockholm, Sweden) for the generous gift of the hybrid aspen cell suspension cultures [17,20] and D.B. Wilson (Cornell University, Ithaca, New York) for generously providing the Cel 6A and Cel 6B cellulases.

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Table 1 Relative proportions of different classes of lipids in the enriched plasma membrane (PM) and DRM fractions (expressed as percentage of the total lipid analyzed for each sample). Standard deviations were calculated from 3 independent experiments.

| | PM | DRM |
|---------------|------------|------------|
| PC | 15.7 ± 1.6 | 9.5 ± 1.4 |
| PI | 7.8 ± 1.8 | 5.9 ± 1.9 |
| PA | 14.8 ± 2.9 | 11.7 ± 1.3 |
| PE | 27.4 ± 1.3 | 18.2 ± 2.4 |
| Sphingolipids | 19.9 ± 3.9 | 28.3 ± 4.1 |
| Sterols | 13.5 ± 1.4 | 29.6 ± 1.6 |

Table 2 Enzymatic hydrolysis of the radioactive β -D-glucans synthesized *in vitro* by the purified plasma membranes and DRMs (the values are expressed as percentage of hydrolysis with respect to the total β -glucans synthesized *in vitro* by each type of membrane preparation). The *in vitro* synthesis experiments were performed using 200 μ L of membrane preparation, in the presence of 1 mM UDP-glucose, 8 mM Mg^{2+} and 1 mM Ca^{2+} . The different conditions of hydrolysis are described in the Experimental section. Standard deviations were calculated from 3 independent experiments.

| Hydrolytic enzyme | Extent of hydrolysis of the β -glucans synthesized <i>in vitro</i> by | |
|---|---|------------|
| | Plasma membranes | DRMs |
| exo-(1 \rightarrow 3)- β -D-glucanase | 38.1 ± 9.0 | 90.6 ± 3.7 |
| cellulase | 65.1 ± 14.5 | 9.5 ± 6.8 |

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Figure legends

Figure 1 Purification of plasma membranes and DRMs

- (A) Plasma membranes were purified by two-phase partitioning in an aqueous system consisting of polyethylene glycol and dextran. Enzymatic markers specific for plasma membrane (callose synthase and ATPase), Golgi apparatus (IDPase), endoplasmic reticulum (antimycin-A insensitive NADH cytochrome-c reductase) and mitochondria (cytochrome-c oxidase) were assayed in the total membrane fraction and in the lower and upper phases obtained by two-phase partitioning. The activities in the phases are presented relative to the activities in the total membrane fraction, which were arbitrarily set at a value of 100. Results represent the mean \pm SD of 5 separate experiments.
- (B) Distribution of total proteins, phospholipids and sterols in the sucrose gradient obtained after flotation of the whole plasma membrane extract (upper phase from Figure 1A) treated with Triton X-100. Fraction 1: bottom of the gradient (45 % sucrose); fraction 29: top of the gradient (15% sucrose). The protein, phospholipid and sterol contents are expressed as μg per mL of fraction. A picture of the gradient recovered immediately after ultracentrifugation shows a white band that corresponds to the peak of protein in fractions 8-10 (DRMs).

Figure 2 Detection of GPI-anchored proteins in DRMs

Proteins from the DRM fractions were biotinylated and extracted with Triton X-114. The membrane-bound proteins recovered in the insoluble fraction after centrifugation on a 6% sucrose cushion were incubated in the presence (+) or absence (-) of PIPLC and the Triton X-114 phase partitioning step was repeated. A control was performed in the same conditions using plasma membranes (PM) incubated in the absence of PIPLC. Proteins released in the aqueous phase were separated in 12% SDS-PAGE gels and the biotinylated proteins were transferred to a nitrocellulose membrane before detection with streptavidin conjugated to peroxidase and the corresponding chemiluminescent substrate.

Figure 3 Gas chromatography analysis of methylester derivatives of fatty acids from phospholipids

Proportion of individual saturated and unsaturated fatty acids in total phospholipids extracted from purified plasma membranes (grey bars) and DRMs (white bars). The values are expressed as percentage of the total fatty acids in each membrane preparation. Typical data representative of several independent analyses are presented.

Figure 4 Distribution of the callose and cellulose synthase activities in the flotation gradient used to isolate the DRMs

- (A) Four mL of the plasma membrane fraction (1.2 mg of proteins) incubated with Triton X-100 were loaded at the bottom of a continuous sucrose gradient. Fraction 1: bottom of the gradient (45 % sucrose); fraction 29: top of the gradient (15% sucrose). Results are expressed as total glucan synthase activities measured in each fraction in the presence of 8 mM CaCl_2 (\bullet) or in the presence of 1 mM CaCl_2 and 8 mM MgCl_2 (\blacksquare). The protein content is expressed as μg of total protein per mL of fraction.
- (B) SDS-PAGE analysis of the total purified plasma membrane fraction before the treatment with Triton X-100 (PM) and of fractions 2 and 8 of the gradient presented in Figure 4A. 15 μg protein was loaded in each lane and separated on a 10% SDS-PAGE gel (silver staining).

Plant glucan synthases are located in plasma membrane microdomains

(C) Western blot analysis of each fraction from the gradient presented in Figure 4A using anti cellulose synthase antibodies directed against the putative catalytic site of Cesa2 from hybrid aspen. The proteins loaded in each lane (15 μ g) were separated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane prior to immunodetection.

Figure 5 ^{13}C -NMR analysis of the (1 \rightarrow 3)- β -D-glucan synthesized *in vitro* by the DRM fraction

The *in vitro* synthesis reaction was performed using UDP-[U- ^{13}C]glucose as a substrate. The ^{13}C -enriched glucans were then solubilised in $(\text{CD}_3)_2\text{SO}$ and analysed by NMR spectroscopy as described in the Experimental section, using the central peak of the dimethylsulfoxide multiplet (39.5 ppm) as a reference. The spectrum is characteristic of a strictly linear (1 \rightarrow 3)- β -D-glucan.

Figure 6 Transmission electron micrograph of the (1 \rightarrow 3)- β -D-glucan synthesized *in vitro* by the DRM fraction

The (1 \rightarrow 3)- β -D-glucan synthesized *in vitro* by the DRM fraction was deposited on carbon-coated grids and negatively stained (2% uranyl acetate). Observations were performed under an accelerating voltage of 80 kV using a Philips CM 120 electron microscope.

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Figure 1

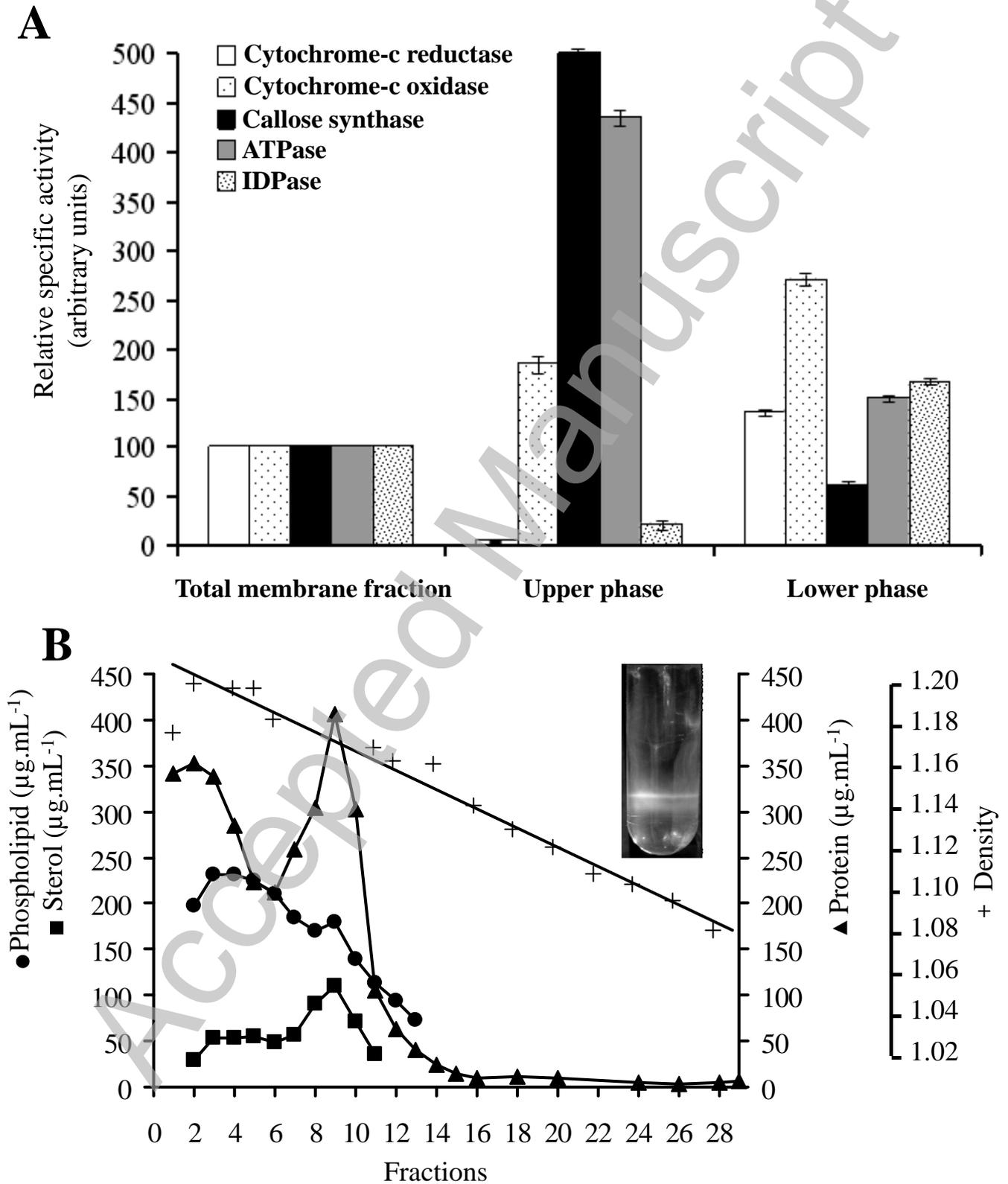
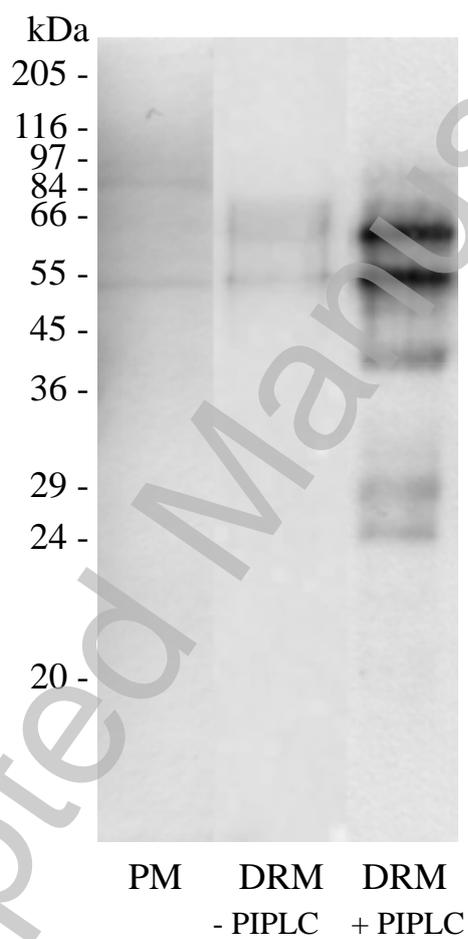
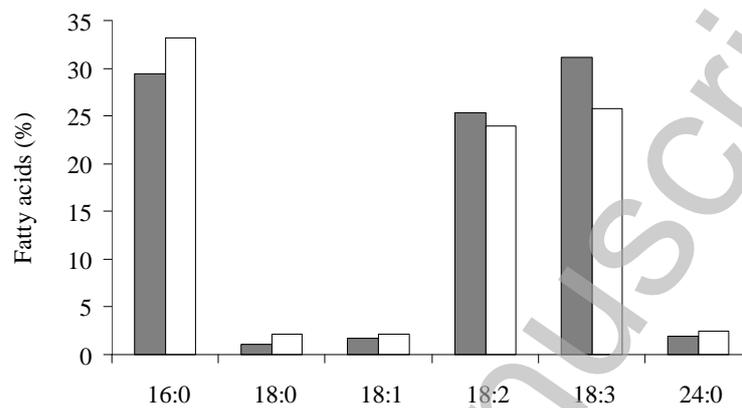


Figure 2



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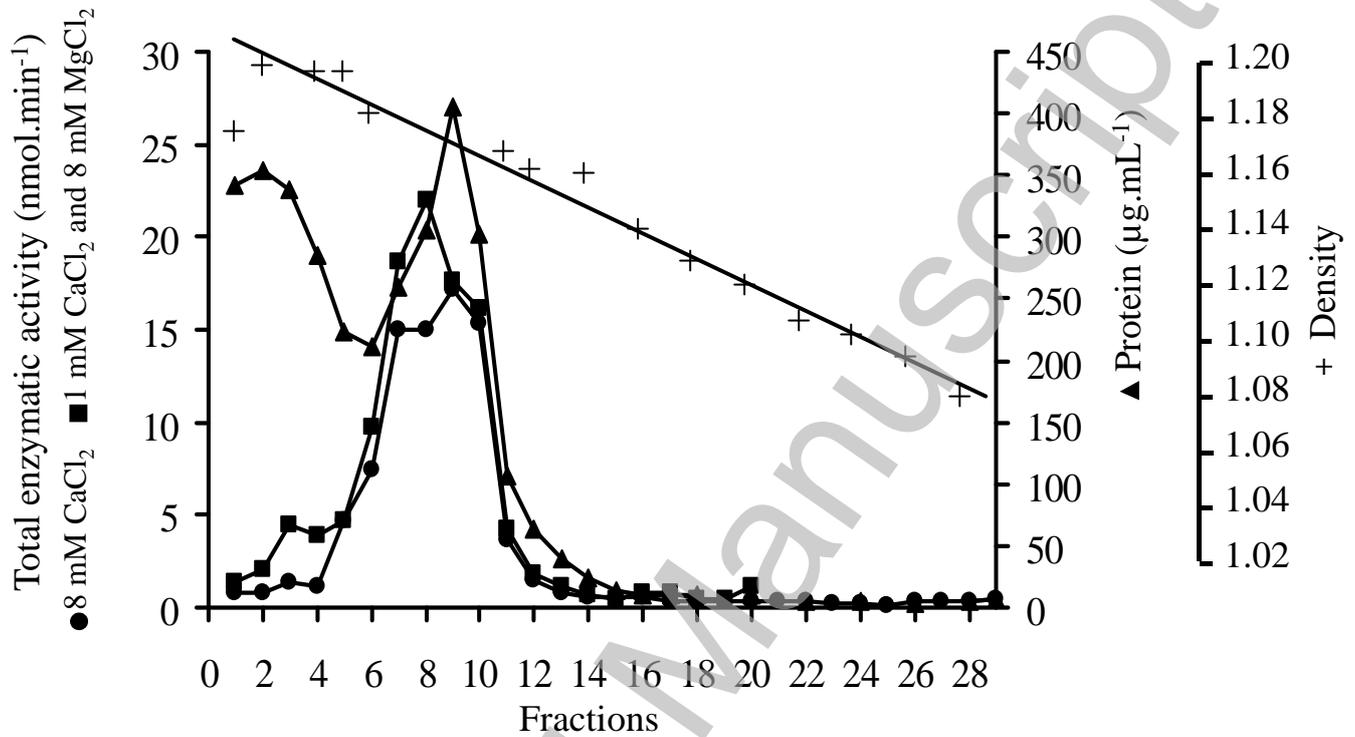
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Figure 3

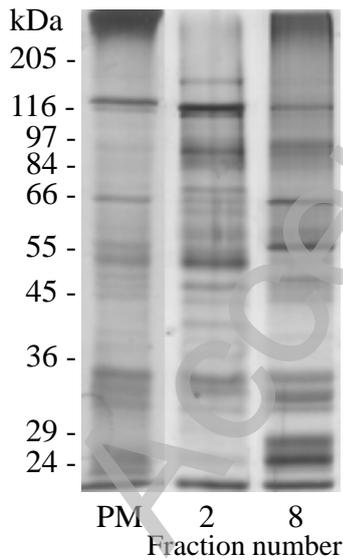
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Figure 4

A



B



C

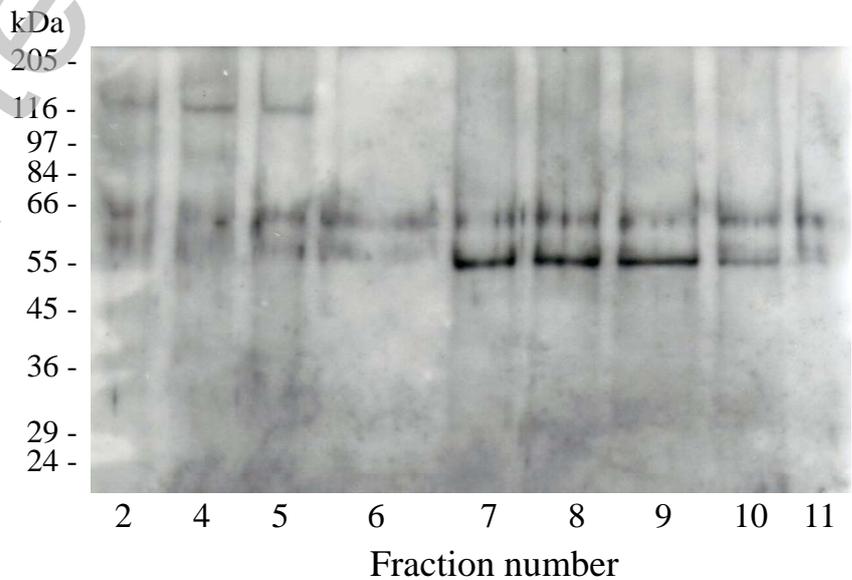
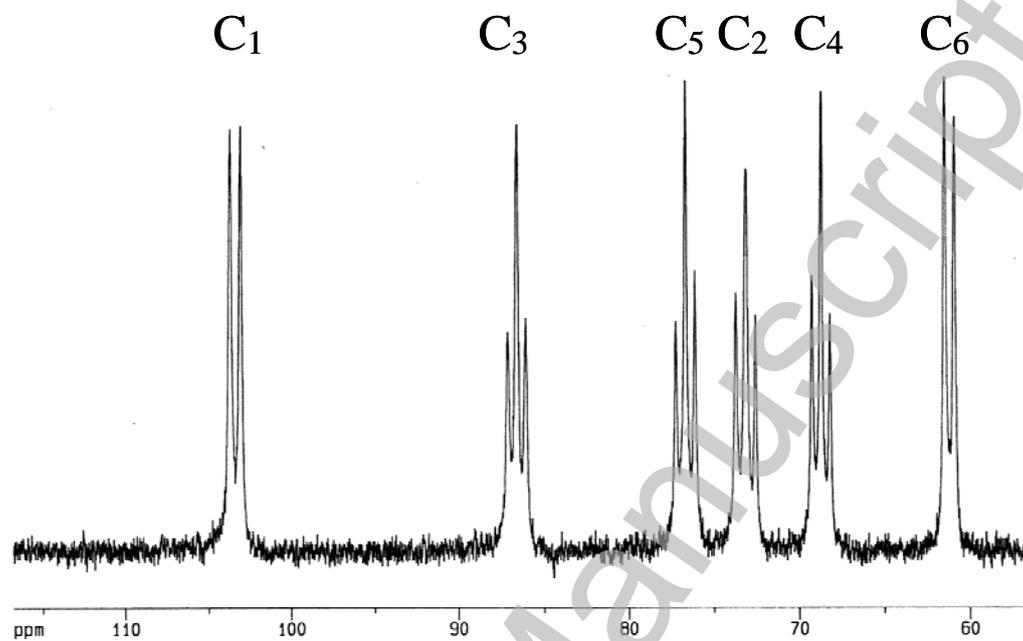


Figure 5**Figure 6**