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ATP-Dependent Formation of Phosphatidylserine-Rich Vesicles from the Endoplasmic Reticulum of Leek Cells

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Leek (*Allium porrum*) plasma membrane is enriched in phosphatidylserine (PS) by the vesicular pathway, in a way similar to that already observed in animal cells (B. Sturbois-Balcerzak, D.J. Morré, O. Loreau, J.P. Noel, P. Moreau, C. Cassagne [1995] Plant Physiol Biochem 33: 625–637). In this paper we document the formation of PS-rich small vesicles from leek endoplasmic reticulum (ER) membranes upon addition of ATP and other factors. The omission of ATP or its replacement by ATPγ-S prevents vesicle formation. These vesicles correspond to small structures (70–80 nm) and their phospholipid composition, characterized by a PS enrichment, is compatible with a role in PS transport. Moreover, the PS enrichment over phosphatidylinositol in the ER-derived vesicles is the first example, to our knowledge, of phospholipid sorting from the ER to ER-derived vesicles in plant cells.

The biosynthesis of most of the phospholipid species of the plasma membrane of plant cells, as in animal cells, takes place primarily in the ER (Moore, 1990). This is the case for PS for which an intracellular transport from the ER to the plasma membrane was postulated and demonstrated in vivo. This transport is inhibited by monensin and low temperatures and follows the ER-Golgi-plasma membrane pathway (Sturbois-Balcerzak et al., 1995; Moreau et al., 1998a). Therefore, this transport is expected to be mediated by carrier vesicles. Such structures can transport membrane and secretory proteins in plant cells (Satiat-Jeunemaitre and Hawes, 1993; Bar-Peled et al., 1996), and we also suspect their involvement in lipid transport (Moreau et al., 1988, 1998a; Bertho et al., 1991; Sturbois-Balcerzak et al., 1995).

Few attempts have concerned the isolation of putative vesicular intermediates involved in the delivery of membrane material from the ER in plant cells (Morré et al., 1989; Hellgren et al., 1993). Recently, a cell-free ATP-dependent transfer of phospholipids was obtained between the ER and the Golgi apparatus of leek (*Allium porrum*) cells (Sturbois et al., 1994). PC, PE, and particularly PS were transferred, mimicking the in vivo situation. On the other hand, PI was not found to be transported (Sturbois et al., 1994; Sturbois-Balcerzak et al., 1995).

Although the characterization of proteins likely to be involved in vesicular transport is in progress in plant cells (Bar-Peled et al., 1996; Gomord and Faye, 1996; Hawes and Satiat-Jeunemaitre, 1996), there is still no specific marker for ER-derived vesicles in plant cells. We have developed another strategy to monitor the isolation of putative vesicular structures involved in the transport of phospholipids and especially PS in leek cells.

It has been observed that ER-derived vesicles are 50- to 80-nm vesicular structures in many eukaryotic organisms (Paulik et al., 1988; Morré et al., 1989; Hellgren et al., 1993; Moreau et al., 1993; Bednarek et al., 1995). Moreover, the transport vesicles isolated from the ER of rat liver show an enrichment in PS (Moreau et al., 1992, 1993), and we have observed a selective transfer of PS in vivo (Sturbois-Balcerzak et al., 1995) and between the ER and the Golgi apparatus of leek cells in vitro (Sturbois et al., 1994).

We incubated an ER-enriched membrane fraction from leek cells with ATP and other factors and observed the formation of small vesicles that were PS enriched. Their partial isolation was performed by sedimentation on Sucdensity gradients and/or filtration through 200- and 100-nm-pore membranes (Anotop, Anotec/Whatman). Our results show for the first time, to our knowledge, in a cell-free system from plant cells that phospholipids can be sorted and targeted from the ER to ER-derived vesicles, as is the case for proteins (Bar-Peled et al., 1996).

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Abbreviations: HPTLC, high-performance TLC; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; VLCFA, very-long-chain fatty acid(s); X:Y, a fatty acyl group containing X carbon atoms and Y *cis* double bonds.

MATERIALS AND METHODS

Leek (*Allium porrum* L.) seeds were purchased from Vilmorin (La Ménitré, France) and stored overnight at 4°C before being hydrated with distilled water for 2 h. The seeds were allowed to germinate in the dark for 7 d at 24°C as described previously (Moreau et al., 1988).

All chemicals were from Sigma. [1-^{4}C]Acetate was obtained from CEA (Saclay, France). [^{14}C]Ser and [^{3}H]inositol were purchased from NEN.

Labeling and Isolation of ER Membranes

Twenty batches of 20 seedlings were incubated, each with 2 μCi of [1-^{4}C]acetate (54 Ci/mol) for 120 min at 24°C. Leek seedlings were homogenized in a mortar in the presence of 10 mM KH_2PO_4, pH 8.2, with 0.5 M sorbitol, 5% (w/v) PVP 40, 0.5% (w/v) BSA, 2 mM salicylhydroxamic acid, and 1 mM PMSF. The homogenate was then filtered through two layers of Miracloth (Calbiochem) and centrifuged at 1,000*g* for 10 min. The supernatant was centrifuged for 10 min at 12,000*g*, and the resulting supernatant was centrifuged at 150,000*g* for 60 min with a rotor (model TST 2838, Kontron, Eching, Germany, or model AH 629, Sorvall). The resulting microsomal pellets were resuspended in 10 mM KH_2PO_4, pH 7.8, with 0.5 M sorbitol and loaded onto a discontinuous Suc-density gradient consisting of 2.5 mL of 37% (w/v) Suc, 3.5 mL of 25% (w/v) Suc, and 3.5 mL of 18% (w/v) Suc. After the sample was centrifuged at 150,000*g* for 150 min, the ER membranes at the 18%/25% Suc interface were collected, diluted in the appropriate buffer (see below), and centrifuged at 150,000*g* for 60 min. The pellets were resuspended in the appropriate buffer for marker enzyme assays (Moreau et al., 1988; Bertho et al., 1991).

Preparation of Golgi and Plasma Membrane-Enriched Fractions

Three to four hundred unlabeled leek seedlings were homogenized as described above. One-half of the microsomal pellet was used to isolate the Golgi membranes at the 25%/37% Suc interface. The membranes were diluted with the appropriate buffer and centrifuged at 150,000*g* for 60 min. The pellet was resuspended in the appropriate buffer for marker enzyme assays (Moreau et al., 1988; Bertho et al., 1991).

Plasma membranes were isolated by phase partition using PEG 4000 and dextran T500. The other half of the microsomal suspension was mixed with a polymer (PEG/dextran mixture) in 0.5 M sorbitol containing 10 mM KH_2PO_4 and 40 mM NaCl, pH 7.8, to obtain final PEG 4000 and dextran T500 concentrations of 6.0% (w/w). The solution (final volume, 28 mL) was centrifuged for 15 min at 1,000*g*, and the PEG-enriched upper phase (12 mL) was recovered without disturbing the interface. Membranes were then recovered after centrifugation at 150,000*g* for 60 min and resuspended in the appropriate buffer for marker enzyme assays (Moreau et al., 1988; Bertho et al., 1991).

For the assay of glucuronyltransferase, we followed the procedure of Hobbs et al. (1991) and Baydoun and Brett (1997).

Isolation of ER-Derived Vesicles

The cell-free assay used for the formation of putative carrier vesicles consisted of labeled donor ER membranes (600 μg of protein), cytosol (300 μg of protein), ATP, and an ATP-regenerating system (500 μM ATP, 2.5 mM magnesium acetate, 3 mM UTP, 2 mM phosphocreatine, and 10 units/mL phosphocreatine kinase), a "lipid mixture" composed of 12.5 μM CDP-choline, 12.5 μM CDP-ethanolamine, 2.5 μM palmitic acid, 7.5 μM oleic acid, 500 μM CoA, and 30 mM Hepes buffer (pH 6.8, total volume 1 mL). A lipid mixture was added because we found that an ongoing biosynthesis of lipids stimulated the formation of the ER-derived vesicles.

A cytosolic fraction, stimulating the ER-Golgi transport and prepared as described earlier (Sturbois et al., 1994), was also added since the formation of ER-derived vesicles has been shown to be cytosol dependent in animal and yeast cells (Rothman and Wieland, 1996; Schekman and Orci, 1996).

The ER membranes were incubated in glass vials at 22°C to 24°C for 20 min, either in the absence (−) or in the presence (+) of ATP. ATPγ-S was used at the same concentration as ATP. We determined earlier (Sturbois et al., 1994) that the ATP-stimulated transport of lipids between the ER and the Golgi membranes was dependent on time, temperature, and ATP concentration. The optimal conditions for the formation of the vesicles were chosen accordingly.

After the ER membranes were incubated according to the conditions described above, the reaction mixtures were treated alternatively by the following two methods: (a) The incubation mixture was loaded onto a discontinuous Suc-density gradient consisting of 1.5 mL of 18% (w/v) and 1.5 mL of 37% (w/v) Suc solutions in 30 mM Hepes buffer containing 30 mM KCl, pH 6.8. After centrifugation at 100,000*g* for 45 min, the sample/18% Suc interface and the 18%/37% Suc interface were collected, diluted with distilled water, and centrifuged at 150,000*g* with a rotor (model RT 80, Himac CS 100, Hitachi, Tokyo) for 15 min. The putative ER-derived vesicles and the ER membranes were resuspended in distilled water. (b) The incubation mixture was centrifuged at 10,000*g* for 5 min to discard the bulk of the ER donor membranes. The resulting supernatant was passed successively through 200- and 100-nm filters (Anotop membranes purchased from Anotec/Whatman). The 200-nm filters eliminated large ER fragments that did not sediment after centrifugation. Filters of 100 nm were chosen according to earlier findings showing that transition vesicles arising from the ER generally occur as 50- to 80-nm membrane structures (Paulik et al., 1988; Morré et al., 1989; Hellgren et al., 1993; Moreau et al., 1993; Bednarek et al., 1995). The membrane vesicles recovered after filtration through the 100-nm filters were sedimented at 150,000*g* with a rotor (model RT 80) for 15 min. The pellets were resuspended in an appropriate volume of distilled water.

The membrane material isolated from the ER incubated in the absence of ATP was called TV(−) and the ER-derived vesicles obtained from the ER incubated in the presence of ATP were named TV(+).

Protein Determination, SDS-PAGE, and Immunoblots

The quantity of proteins from the ER and Golgi membranes and from the ER-derived vesicle fractions was determined according to the Bradford (1976) and bicinchoninic acid procedures (Smith et al., 1985) using BSA as a standard.

SDS-PAGE was carried out on 12% polyacrylamide gels (Bio-Rad). A molecular mass kit was used that contained phosphorylase b (94 kD), BSA (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), and soybean trypsin (20 kD). Membrane fractions were mixed with an equal volume of a solution of 0.125 M Tris buffer, pH 6.8, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol and incubated for 5 min at 100°C. After electrophoresis, the gel was subjected to electrophoretic transfer on a PVDF membrane (NEN). Immunostaining was carried out at room temperature. The membrane was sequentially subjected to incubation with (a) a blocking reagent for 1 h, (b) an antiserum for 16 h (both antibodies were used as undiluted culture supernatants), (c) an anti-mouse or anti-rat-immunoglobulin-peroxidase conjugate (Sigma) for 30 min, and (d) a chemiluminescent reagent (Renaissance kit, NEN). The membrane was finally exposed to Reflection film (NEN).

Immunofluorescence

Root apices were treated for immunofluorescence staining as described previously (Satiat-Jeunemaitre et al., 1996a). They were fixed for 1 h in 4% paraformaldehyde in PBS, pH 6.9. A partial cell wall digestion was performed by a 20-min treatment with 1% cellulase (Onazuka R10) and 1% pectinase in PBS. Release of individual cells was achieved by gently squashing the roots on coated Vectabond (Vector Laboratories, London, UK) multiwell slides. Cells were then permeabilized with 0.5% Triton X-100 in PBS for 20 min. For the immunoreaction procedure, cells were incubated in 1% BSA in PBS to block the unspecific reaction sites for 20 min before incubation in primary antibody (anti-HDEL or JIM 84) for 1 h at room temperature. After four washes in buffer containing 1% fish gelatin, cells were stained with an appropriate fluorescein isothiocyanate-conjugated second antibody (anti-mouse or anti-rat, diluted 1:40, Sigma) for 1 h at room temperature. After the preparations were washed thoroughly, they were mounted in Vectashield antifade agent (Vector Laboratories) and viewed with a confocal laser microscope.

The anti-HDEL monoclonal antibody was a generous gift from Richard Napier (Horticulture Research International, Wellesbourne, UK). It was produced in mice against a synthetic peptide corresponding to the C terminus of yeast Bip (Napier et al., 1992). The JIM 84 monoclonal antibody was a generous gift from Chris Hawes (Oxford Brookes University, UK). It was produced in rats and recognizes glycoproteins associated with cisternal membranes (Horsley et al., 1993). Both antibodies were used as undiluted culture supernatants.

Lipid Analyses of the ER-Derived Vesicles and the ER Membranes

Lipids were extracted by chloroform:methanol (1:1, v/v) for 30 min at room temperature. They were then washed three times with distilled water. The solvent was evaporated and the lipids were resuspended in an appropriate volume of chloroform:methanol (1:1, v/v) according to procedures described previously (Moreau et al., 1988; Bertho et al., 1991). Lipids (5–10 μg) were loaded onto HPTLC plates (60F254, Merck, Darmstadt, Germany) and chromatograms were developed by methyl acetate: *n*-propanol:chloroform:methanol:aqueous 0.25% KCl (25:25:25:10:9, v/v), according to the method of Heape et al. (1985).

Calibration curves for phospholipids were established with standard lipids of PC, PS, PI, and PE and by using monogalactosyldiacylglycerol (2 μg) as an external standard (Heape et al., 1985). After separation on HPTLC plates, the lipids were charred using the technique of Fewster et al. (1969), as modified by Macala et al. (1983). The plates were scanned within 1 h using a TLC/HPTLC densitometer (model 76510, Camag, Muttenz, Switzerland) coupled with a computing integrator (model SP 4100, Spectra Physics, Mountain View, CA). The scans were carried out at 366 nm (mercury lamp) at a speed of 0.5 mm/s. The quantities of the various phospholipids of the different membrane fractions were deduced from the calibration curves (Heape et al., 1985).

The radioactivity of the phospholipids was determined as follows. After the different phospholipids were identified by comparison with standards, they were scraped off directly into scintillation vials, and radioactivity was determined by liquid-scintillation counting in a scintillation counter (model 2000 CA, Packard).

Radioactivity of the phospholipids was also determined after autoradiography of the HPTLC plates (Hyperfilm MP RPN 1675, Amersham) and scanning with a densitometer (model 76510, Camag). Both methods gave similar results and were used alternatively.

Fatty Acid Analyses of the Phospholipids

The fatty acid compositions of the phospholipids of the ER and ER-derived vesicle fractions were determined as follows. Lipid extracts were heated in screw-capped tubes at 80°C in 1 mL of 2.5% (v/v) H_2SO_4 in methanol for 60 min. After the addition of 1.5 mL of water and 300 μL of hexane, fatty acid methyl esters were extracted by shaking, and the tubes were centrifuged at low speed. Samples of the organic phase were separated by GLC on a 15-m × 0.53-mm Carbowax column (Alltech, Deerfield, IL) and quantified using a flame-ionization detector. The gas chromatograph was programmed for an initial temperature of 160°C for 1 min and then a 20°C/min ramp to 190°C, a secondary ramp of 5°C/min to 230°C, and a third ramp of 20°C/min to 240°C; this final temperature was maintained

Table I. *Purity of membrane fractions according to marker enzymes*

Activities of CDP-choline phosphotransferase, NADPH-Cyt *c* reductase, IDPase, glucane synthetase II, and the K^+-stimulated Mg^+ ATPase were measured as previously described (Moreau et al., 1988; Bertho et al., 1991). Activity of succinate dehydrogenase was followed as reported previously (Moreau, 1986). Glucuronyl transferase activity was assayed according to the methods of Hobbs et al. (1991) and Baydoun and Brett (1997). Activities of succinate dehydrogenase in the 1,000*g* pellet were 9.4 μmol/h and 0.2 μmol h^{-1} mg^{-1} and those of the 12,000*g* pellet were 127.2 μmol/h and 4.8 μmol h^{-1} mg^{-1}. Numbers in parentheses represent the enrichment factors of the ER, Golgi, and plasma membrane markers in the corresponding membranes. These values were calculated taking the specific activities of the homogenate as equal to 1. The microsomal pellet contained only 2% and 4% of the total chlorophyll and carotenoids, respectively (Moreau, 1986). Those molecules were not detected in the ER, Golgi, or plasma membrane fractions but only in the heavier fraction of the discontinuous gradient and the lower (dextran-enriched) phase after phase partition. In addition, a low contamination of the ER and Golgi fractions by plastid envelope was determined by the presence of small amounts of galactolipids (<10% of the total glycerolipids). Enzyme activities were determined from at least three different fractionations. ND, Not detected.

	Protein	CDP-Choline Phosphotransferase		NADPH-Cyt *c* Reductase		IDPase		Glucuronyl Transferase	Glucane Synthetase II		K^+-Stimulated Mg^{2+} ATPase		Succinate Dehydrogenase	
	mg	*nmol/h*	*nmol h^{-1} mg^{-1}*	*nmol/h*	*nmol h^{-1} mg^{-1}*	*μmol Pi/h*	*μmol Pi h^{-1} mg^{-1}*	*nmol/h*	*nmol h^{-1} mg^{-1}*	*μmol Pi/h*	*μmol Pi h^{-1} mg^{-1}*	*μmol Pi/h*	*μmol Pi/h*	*μmol Pi h^{-1} mg^{-1}*
Homogenate	208	83.2	0.40	68.6	0.33	89.4	0.43	13.5	0.065	15.2	0.07	9.92	0.048	149.7	0.72
Microsomal pellet	58.5	46.2	0.79	34.7	0.59	93.6	1.60	4	0.068	9.13	0.16	5.70	0.097	3	0.05
ER	2.1	3.7	1.77 (4.4)	6.12	2.91 (8.8)	2.29	1.09	0.19	0.092	0.017	0.008	0.009	0.004	ND	ND
Golgi	6.9	1.4	0.20	3.17	0.46	27.5	3.98 (9.2)	1.26	0.182 (2.8)	0.28	0.04	0.092	0.013	ND	ND
Plasma membrane	1.5	0.2	0.12	0.07	0.05	1.95	1.30	0.054	0.036	0.95	0.64 (9.1)	2.31	1.54 (32.1)	ND	ND

for 6 min. Peak identities were determined by comparison with fatty acid methyl ester standards.

Electron Microscopy and Morphometry

ER membranes and ER-derived vesicles were fixed at 4°C by recovering the pellets with a fixative solution containing 2.5% glutaraldehyde in 0.1 M sodium cacodylate at pH 7.5. Fixed pellets were then washed and postfixed in 1% osmium tetroxide in the same buffer. After the samples were washed with distilled water, they were treated in an aqueous solution of 1% uranyl acetate for 30 min at room temperature, washed again, dehydrated, and embedded in Epon. Each pellet was divided into several blocks that were cut into thin sections. The section plane was random but constant in thickness. Sections were contrasted with a uranyl acetate solution and then with lead citrate. They were observed and photographed using an electron microscope (model EM 210, Philips, Cambridge, UK).

Measurement of vesicle sizes and the determination of the relative abundance of the different vesicles were performed as follows. At least 20 photographs were taken at random from the thin sections obtained from several blocks. Two magnifications (×8,900 and ×20,000) were used for the analyses. Vesicle sizes and relative proportions were determined and calculated with an analyzer (model IBAS1, Kontron).

RESULTS

Characterization of the ER-Enriched Membrane Fraction

The various membrane fractions were first characterized by several enzyme markers (Table I). CDP-choline phosphotransferase and NADPH-Cyt *c* reductase were used as ER markers (Moreau et al., 1988; Bertho et al., 1991). IDPase (which can be considered a nucleotide diphosphatase) was taken for a Golgi marker (Goff, 1973; Morré et al., 1977; Quail, 1979). However, several nucleotide diphosphatases have also been observed to be present in other membranes (Goff, 1973). Therefore, we also measured the activity of glucuronyltransferase, which was found with the Golgi apparatus in pea epicotyls (Hobbs et al., 1991; Baydoun and Brett, 1997). For the plasma membrane, we measured the K^+-stimulated Mg^{2+} ATPase and the glucan synthase II (Quail, 1979). The ATPase activity measured in the plasma membrane fraction (Table I) presented a 93% inhibition by vanadate (Moreau et al., 1988), which corresponds well to its plasma membrane origin (Sze, 1985). The relative enrichments of the various activities in the different membrane fractions (ER, Golgi, and plasma membrane) indicate the extent of purification achieved. Contamination of the membrane fractions by plastidial membranes was considered acceptable according to the levels of chlorophyll, carotenoids, and galactolipids (Table I, legend). The ER fraction, which will be used to form the small vesicles in vitro, has further been characterized by ER-specific antibodies raised against the C terminus (including the HDEL sequence) of yeast Bip (Napier et al., 1992). JIM84 antibodies,

which are more specific for Golgi proteins (Horsley et al., 1993), were also used.

Immunofluorescence microscopy of leek root cells stained with the anti-HDEL antibody revealed a fine reticulate network of ER throughout the cells (Fig. 1, top). The nuclear envelope was often stained as well. When leek cells were stained with the JIM 84 antibody, a characteristic punctuated pattern was seen that corresponded to hundreds of Golgi stacks dispersed throughout the cytoplasm (Fig. 1, bottom). The immunofluorescent staining patterns of leek cells observed with anti-HDEL and JIM84 antibodies corresponded very well to the ER and Golgi staining previously reported for other plant cells (Satiat-Jeunemaitre and Hawes, 1992; Horsley et al., 1993; Henderson et al., 1994; Satiat-Jeunemaitre et al., 1996a).

Immunoblot analyses revealed a strong labeling of the ER fraction with the anti-HDEL antibody (Fig. 2), whereas only a weak band was detected in the Golgi fraction. As expected, JIM84 labeled the Golgi fraction (Fig. 2), but no significant label was observed for the ER fraction. The fact that several bands were revealed in the ER fraction is a result of the presence of several proteins carrying the C terminus HDEL sequence, which is an ER-targeting sequence (Napier et al., 1992; Bar-Peled et al., 1996). Together with enzyme markers (Table I), the immunoblot staining patterns support the obtaining of an ER-enriched membrane fraction from leek cells. This ER fraction was then used to form ER-derived vesicles in vitro and undertake their isolation.

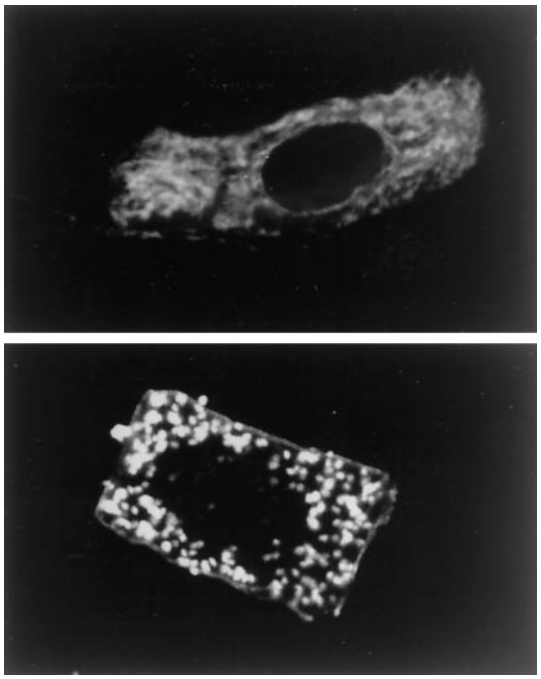

Figure 1. Confocal laser scanning micrographs of the Golgi apparatus and ER in leek root cells. Projection of optical sections from confocal data sets. Magnification, ×1400. Top, Immunolocalization of the ER stained with anti-HDEL. The ER appears as a fine network, often radiating out from the nucleus through the cell. There is often staining in the nuclear membrane as well. Bottom, Immunolocalization of the Golgi apparatus in leek root cells stained with JIM 84. Golgi stacks appear scattered throughout the cytoplasm.

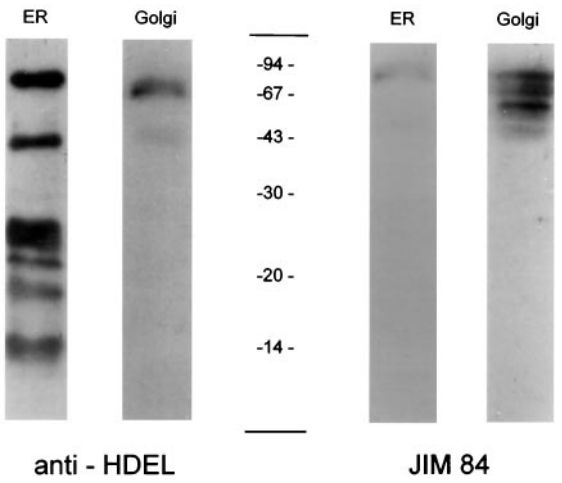

Figure 2. Immunoblots of ER and Golgi fractions with anti-HDEL and JIM 84 antibodies. SDS-PAGE and immunostaining were done as explained in "Materials and Methods." Lanes corresponding to the membranes of the ER and the Golgi-enriched membrane fraction are indicated, respectively, as ER and Golgi. M_rs (in thousands) of classic standards are also indicated. For each lane, 5 μg of proteins was loaded.

Isolation of ER-Derived Vesicles on Discontinuous Suc-Density Gradients

The buoyant density of lipid-rich vesicles involved in lipid transport from the ER to the Golgi apparatus may differ from that of ER from which they are derived. We used this as a first attempt to isolate an ER-derived vesicle fraction. ER membranes were incubated in the presence of ATP, as described in "Materials and Methods," and the incubation medium was loaded onto a discontinuous Suc-density gradient comprising two layers, 18% and 37% Suc. As a control, one-half of the ER membranes was incubated in the same incubation medium deprived of ATP. In the absence of ATP, the amount of membrane material obtained at the sample/18% Suc interface (TV[−]) represented 5.5% ± 0.7% (32 ± 4 μg) of the amount of total ER membranes.

In the presence of ATP, the quantity of membranes recovered at the sample/18% Suc interface (TV[+]) accounted for 10% ± 3.3% (57 ± 11 μg) of the ER starting material. The amounts of vesicles recovered in the absence or presence of ATP were significantly different ($P < 0.01$). The membrane material recovered from the sample/18% Suc interface (likely to contain the ER-derived vesicles) and the ER membranes (recovered at the 18%/37% Suc interface) were sedimented by ultracentrifugation.

Mass quantitation of the four major phospholipids, PC, PS, PI, and PE, was performed for the three membrane fractions (ER before incubation, vesicles isolated in the absence of ATP, and vesicles isolated in the presence of ATP). For the vesicles isolated from the ER membranes incubated with ATP (TV[+]), there was four times as much

Table II. *Phospholipid composition of the ER and the putative ER-derived vesicles isolated on discontinuous Suc-density gradients*

In the absence of ATP, the amount of membrane material obtained at the sample/18% Suc interface (TV[−]) represented 5.5% ± 0.7% (32 ± 4 μg) of the amount of total ER membranes (as starting material). In the presence of ATP, the quantity of membranes recovered at the sample/18% Suc interface (TV[+]) accounted for 10% ± 3.3% (57 ± 11 μg) of the ER starting material. Lipids were extracted, separated on HPTLC plates, and revealed as mentioned in "Materials and Methods." Then, HPTLC plates were scanned, and the amount of each phospholipid was determined from the calibration curves. PS increase in the vesicles TV(+) compared with the ER was significant ($P < 0.01$, according to Student's *t* test). *n* indicates the number of fractionations and lipid analyses performed.

Membrane Fraction	Phospholipid Composition			
	PC	PS	PI	PE
	% of total			
ER, $n = 10$ (±SD)	75.9 ± 5.8	1.7 ± 1.2	3.2 ± 1.3	19.2 ± 4
TV(−), $n = 3$ (±SD)	67.1 ± 2.9	2.9 ± 0.5	3.7 ± 1.8	26.3 ± 4.5
TV(+), $n = 6$ (±SD)	69.1 ± 3.6	6.9 ± 2.6	3.5 ± 1.9	20.5 ± 3.2

PS (Table II). Similarly, after a 120-min labeling of phospholipids with [^{14}C]acetate, we determined the radioactivity of the phospholipids in the ER and ER-derived vesicles (Table III). There were two major differences between the vesicles isolated from ER membranes incubated in the presence of ATP (TV[+]) and those isolated from ER membranes incubated without ATP (TV[−]). First, we found an increase in labeled PS in the vesicles produced from the ER membranes incubated with ATP. Concomitantly, a significant decrease in labeled PI was obtained. Therefore, the stimulation of the formation of the ER-derived vesicles by ATP resulted in the isolation of PS-rich vesicles that were decreased in their labeled PI content.

Isolation of ER-Derived Vesicles by Filtration

Vesicle size was taken as another advantage for the purification of ER-derived vesicles. For this, the incubation medium containing the ER membranes incubated in the absence or presence of ATP was filtered through successive Anotop filters of 200- and 100-nm-pore sizes. The putative ER-derived vesicles were then sedimented at 200,000*g* for 15 min.

In the absence of ATP, the amount of membrane material recovered in the vesicle fraction (TV[−]) represented 6.1% ± 1% (39 ± 6 μg) of the amount of total ER starting material. In the presence of ATP, the amount of membranes recovered in the vesicle fraction (TV[+]) accounted for 10.3% ± 2.7% (67 ± 10 μg) of the initial ER membranes. The amounts of vesicles recovered in the absence or presence of ATP were significantly different ($P < 0.01$).

After sedimentation of the respective vesicle fractions, the lipids were extracted and analyzed according to the experimental procedures described in "Materials and Methods."

The results concerning the mass proportions of the four phospholipids PC, PS, PI, and PE are given in Table IV. As for the isolation on Suc-density gradients, we observed an increase in PS in the ER-derived vesicles.

Similarly, after a 120-min de novo labeling of phospholipids with [^{14}C]acetate, we determined the radioactivity of the phospholipids in the ER and ER-derived vesicles (Table V). PS labeling was also greatly increased in the ER-derived vesicles obtained from the ER incubated in the presence of ATP (TV[+] fraction) and the amount of labeled PI was slightly decreased.

The proportion of labeled PS in the ER-derived vesicles (TV[+] fraction) was increased 4 and 7 times compared with the TV[−]) fraction (control) and the ER membranes, respectively.

Both methods (Suc-density gradient and filtration) gave similar results, i.e. the isolation of an ER-derived vesicle fraction that is PS enriched and has a decreased amount of labeled PI.

Table III. *Labeling of the various phospholipids of the ER and the putative ER-derived carrier vesicles isolated on discontinuous Suc-density gradients*

Yield of vesicles was as in Table II. Lipids were extracted and separated as already described. The spots corresponding to the phospholipids were scraped off the HPTLC plates and their radioactivity was determined by liquid-scintillation counting. The increase of PS labeling in the vesicles TV(+) compared with the ER was significant ($P < 0.02$, according to Student's *t* test). *n* indicates the number of fractionations and lipid analyses performed.

Membrane Fraction	Phospholipid Radioactivity			
	PC	PS	PI	PE
	% of total			
ER, $n = 10$ (±SD)	56.5 ± 9	6.5 ± 3.7	9 ± 4.2	28 ± 6.2
TV(−), $n = 3$ (±SD)	53.2 ± 14	7.2 ± 2.5	10.9 ± 4	28.7 ± 9.5
TV(+), $n = 6$ (±SD)	52.3 ± 5.3	13.1 ± 3.8	5.4 ± 2.9	29.2 ± 4.3

Table IV. *Phospholipid composition of the ER and the putative ER-derived carrier vesicles isolated by filtration through Anotop filters*

In the absence of ATP, the amount of membrane material recovered in the vesicle fraction (TV[−]) represented 6.1% ± 1% (39 ± 6 μg) of the amount of total ER starting material. In the presence of ATP, the amount of membranes recovered in the vesicle fraction (TV[+]) accounted for 10.3% ± 2.7% (67 ± 10 μg) of the initial ER membranes. Lipids were extracted, separated, and revealed as already described. Then, the HPTLC plates were scanned, and the amount of each phospholipid was determined from the calibration curves. PS increase in the vesicles TV(+) compared with the ER was significant ($P < 0.01$, according to Student's *t* test). *n* indicates the number of fractionations and lipid analyses performed.

Membrane Fraction	Phospholipid Composition			
	PC	PS	PI	PE
	% of total			
ER, $n = 7$ (±SD)	72.3 ± 4.8	2.3 ± 1	3.7 ± 1.3	21.7 ± 4.1
TV(−), $n = 3$ (±SD)	72.1 ± 3.3	2.6 ± 0.5	4.1 ± 0.7	21.2 ± 2.4
TV(+), $n = 6$ (±SD)	73 ± 4.8	5.7 ± 1.8	2.2 ± 1.4	19.1 ± 3.5

To determine whether the formation of ER-derived vesicles required ATP hydrolysis, we also carried out these experiments in the presence of ATPγ-S and compared it with that of ATP. In the presence of ATPγ-S, the amount of membrane material recovered at the sample/18% Suc interface was only 33 ± 6 μg ($n = 4$). In the presence of ATP, the amount of proteins recovered in the ER-derived vesicles was 72 ± 14 μg ($n = 4$). The amounts of vesicles recovered in the presence of ATPγ-S or ATP were significantly different ($P < 0.01$). The results obtained in the presence of ATPγ-S were similar to those reported in the absence of ATP (Tables II and IV). Similarly, incubating ER membranes at 4°C in the presence of ATP did not result in the formation of ER-derived vesicles.

Fatty Acid Composition of the Phospholipids of the ER and the ER-Derived Vesicles

To complete the lipid analysis of the ER-derived vesicles, we determined the fatty acid composition of the major phospholipids PC, PE, and PS of the vesicles by GLC and compared it with that of the phospholipids of the ER membranes.

The fatty acid composition of PC and PE was similar in the ER membranes and the ER-derived vesicles (Table VI). However, a large increase in VLCFA-containing PS was observed in the ER-derived vesicles (Table VI). VLCFA were a mixture of saturated and unsaturated fatty acids with 20 to 24 carbon atoms, the major species being the saturated 22 and 24 fatty acids. No or few VLCFA molecules were observed to be esterified to PI in both membranes.

Since PS was enriched compared with the other phospholipids in the ER-derived vesicles (Tables II, IV, and VIII), VLCFA-containing PS was therefore greatly increased in the vesicles, and we calculated that its amount (percentage of total) was 6 times higher than in the ER membranes. Therefore, PS molecules and particularly VLCFA-containing PS are targeted to the ER-derived vesicles, supporting the fact that these molecules could be transported via these small vesicles.

Characterization of the ER-Derived Vesicles by Electron Microscopy

Figure 3A shows ER-derived vesicles formed from the ER membranes incubated with ATP and isolated on Suc-density gradients. Most of the vesicles were small (<100 nm), but larger structures could be observed. The pictures of the ER-derived vesicles isolated by filtration appeared similar by electron microscopy. The vesicle fraction obtained after incubation of ER membranes with (B) or without ATP (C) is shown with higher magnification in Figure 3, B and C.

The mean sizes of the different vesicles and their proportion were analyzed and determined with a Kontron IBAS 1 analyzer. The results are shown in Table VII. Two

Table V. *Labeling of the various phospholipids of the ER and the putative ER-derived carrier vesicles isolated by filtration through Anotop filters*

Yield of vesicles was as in Table IV. Lipids were extracted and separated as already described. The spots corresponding to the phospholipids were scraped off the HPTLC plates and their radioactivity was determined by liquid-scintillation counting. The increase of PS labeling in the vesicles TV(+) compared with the ER was significant ($P < 0.01$ according to Student's *t* test). *n* indicates the number of fractionations and lipid analyses performed.

Membrane Fraction	Phospholipid Radioactivity			
	PC	PS	PI	PE
	% of total			
ER, $n = 7$ (±SD)	57.1 ± 7.9	4.2 ± 1.4	7.9 ± 3.1	30.8 ± 6
TV(−), $n = 3$ (±SD)	55.1 ± 0.2	2.6 ± 1.4	6.7 ± 3.2	35.6 ± 4.3
TV(+), $n = 6$ (±SD)	47.2 ± 8.2	15.8 ± 4.8	5.4 ± 3.2	31.6 ± 5.7

Table VI. *Fatty acid composition of the three major phospholipids of the putative ER-derived carrier vesicles (TV[+]) and the ER membranes*

For these analyses, the ER-derived carrier vesicles were either isolated on density-Suc gradients or by filtration. The data are mean values ± SD of nine experiments. For each phospholipid, 16 is a fatty acid with 16 carbon atoms (more than 97% of 16:0); 18 is a fatty acid with 18 carbon atoms (>75% of 18:2, the rest corresponding to 18:1 and 18:3); VLCFA is a fatty acid with more than 18 carbon atoms (major fatty acids representing >75% are 22:0, 24:0, and 26:0; the rest correspond to 20:0, 20:1, and 22:1).

Phospholipid and Fatty Acid (Carbon Chain Length)	ER	TV(+)
	% of total	
PC		
16	22.7 ± 0.7	24.5 ± 3.6
18	74.0 ± 1.4	71.6 ± 4.4
VLCFA	3.3 ± 0.8	3.9 ± 2.4
PE		
16	34.1 ± 1.3	37.0 ± 2.3
18	59.7 ± 1.4	58.1 ± 2.8
VLCFA	6.2 ± 2.4	4.9 ± 3.3
PS		
16	28.0 ± 4.2	20.8 ± 2.5
18	58.4 ± 3.5	45.1 ± 3.4
VLCFA	13.6 ± 4.1	34.1 ± 4.4

major membrane populations are present in the ER fraction, one having a mean size greater than 300 nm and a second of about 200 nm. Very few small vesicles (<100 nm) were observed in the ER fraction (Table VII).

The TV(−) fraction contains chiefly a vesicle population of about 250 nm, accounting for 67% of the total number of membrane vesicles and 94% of the total (estimated) membrane surface. Smaller vesicles (mean size of 90 nm) were also present (33% of the total number of membrane vesicles and only 6% of the total membrane surface).

The TV(+) fraction, which corresponds to vesicles isolated from the ER membranes incubated in the presence of ATP, also contains two membrane populations but is characterized by a high proportion of small vesicles (with a mean size of 76 nm). The small vesicles accounted for 74% of the total number of vesicles and 40% of the total membrane surface (Table VII). The other population of vesicles had a mean size of about 190 nm and represented only 26% of the total number of membrane structures (60% of the total membrane surface). A simplified calculation (detailed in the legend of Table VII) showed that the amount of small ER-derived vesicles in the TV(+) fractions was 12 times that found in the control (TV[−]).

DISCUSSION

PS was demonstrated to be transported to the plasma membrane of leek cells through the vesicular pathway (Sturbois-Balcerzak et al., 1995; Moreau et al., 1998a). A first attempt to reconstitute in vitro an ATP-dependent transfer of phospholipids and especially PS from the ER to the Golgi apparatus of leek cells was performed previously (Sturbois et al., 1994). However, no carrier vesicles were isolated and shown as intermediate structures. The aim of this study was to create a cell-free system from leek cells, making it possible to reconstitute the formation of ER-derived vesicles in vitro, and to address the question of phospholipid sorting and targeting (here PS) to the ER-derived vesicles.

In this paper we have shown that an ER-enriched fraction from leek cells (Table I; Fig. 2) is capable of producing small vesicles in vitro when incubated with ATP and other factors. We observed that ATP addition resulted in a significant increase and, therefore, formation of small vesicles from the ER membranes (Table VIII).

The small vesicles were isolated and partially purified by two different methods (ultracentrifugation on Suc-density gradients and filtration on Anotop membranes). The small vesicles obtained using both methods were similar according to their morphology (mean size of 70–80 nm) and phospholipid composition (Table VIII) and were significantly different from the initial ER membranes.

These vesicles were characterized by a PS enrichment as shown by the amount of the lipids (Tables II and IV) and their labeling (Tables III and V). PS enrichment in the ER-derived vesicles (6.2% of the total phospholipids compared with 1.8% in the ER, Table VIII) corresponds to previous findings obtained for ER-derived vesicles isolated from rat liver in which the amount of PS (among total phospholipids) was 7.2% and only 2.5% to 3% in the ER membranes (Moreau et al., 1992, 1993; Moreau and Cassagne, 1994).

However, it is still possible that PS, enriched in the small vesicles, could have been synthesized locally (i.e. by enzymes located in the small vesicles). In fact, whereas isolated ER membranes exhibited high activities of PS (0.23 nmol mg^{-1} protein min^{-1}) and PI (0.15 nmol mg^{-1} protein min^{-1}) synthesis from [^{14}C]Ser and [^{3}H]inositol, ER-derived vesicles were practically devoid of these activities (< 2% of the total activity of the ER). Moreover, the specific activity (per milligram of proteins) of PS synthesis in the ER-derived vesicles was 5 to 10 times lower than that in the ER. The residual activity in the vesicles does not account for their PS enrichment. Isolated ER membranes (labeled with [^{14}C]Ser and [^{3}H]inositol) were also used to form and isolate small vesicles. These were labeled and their PS:PI label ratio was 2.0 ± 0.3 (n = 5) and of the same order of magnitude as those calculated from Table VIII (2.6 ± 0.6). Similar results were obtained whatever the labeling pathway of the phospholipids, i.e. from the de novo synthesis of the fatty acids (Table VIII) or from the incorporation of [^{14}C]Ser and [^{3}H]inositol by the isolated ER membranes (see above).

It could be argued that the small vesicles do not originate from the ER membranes but from contaminant plastid envelope or outer mitochondrial membranes. However, it has been shown that the plastid envelope is totally devoid of PS and PE (Maréchal et al., 1997) and that the outer mitochondrial membrane is also poor in PS (Douce, 1985; Douce and Joyard, 1990; Guillot-Salomon et al., 1997). Moreover, PS is not synthesized by these membranes (Moore, 1990). Therefore, because ER membranes were the major site of synthesis of PS, the labeled PS that accumu-

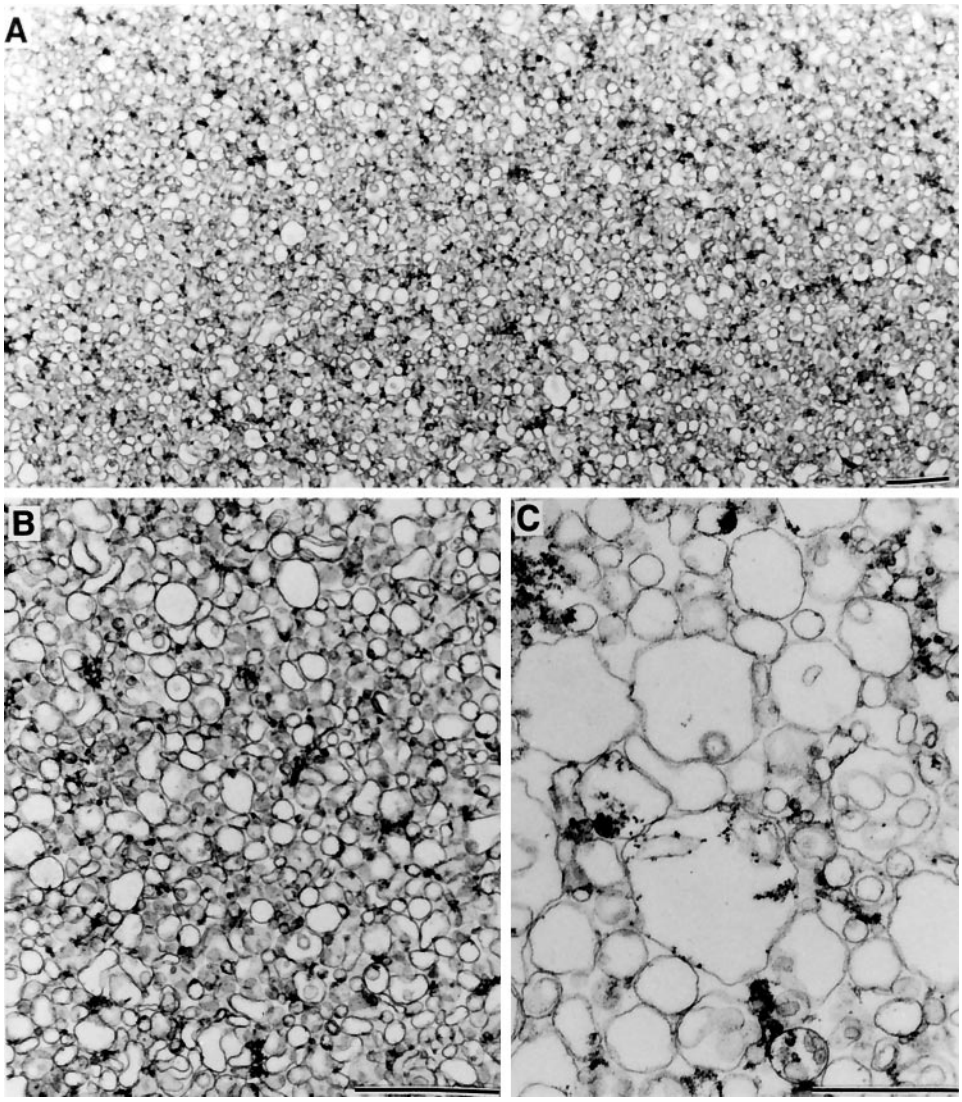

Figure 3. Electron micrograph of the ER-derived vesicles isolated on discontinuous Suc-density gradients. Incubation of ER membranes with or without ATP and isolation of ER-derived vesicles were performed as described in "Materials and Methods." Membranes were fixed and prepared for electron microscopy, as described in "Materials and Methods." A, ER-derived vesicles (TV[+]) obtained from the ER membranes incubated in the presence of ATP. B, The same fraction as in A but at a higher magnification. C, Membrane fraction (TV[−]) isolated from the ER membranes incubated in the absence of ATP. Bars = 1 μm.

lated in the vesicles was likely to be ER derived. Our data strongly support the conclusion that the labeled small vesicles were formed from the ER membranes.

In addition, the phospholipid composition (percentage of total labeling) of the ER-derived vesicles (Table VIII) showed a very good correlation with the distribution (percentage) of the phospholipid transferred in vitro to the Golgi apparatus (Sturbois et al., 1994, Table VIII). For the ER-derived vesicles, we calculated PS:PI ratios of 2.1 (for the amount of lipids) and 2.6 (for the radioactivity of the lipids), whereas these ratios were only 0.51 and 0.68 for the ER membranes (Table VIII). These calculations illustrate a PS enrichment over PI that was observed in the ER-derived vesicles. However, the PS enrichment over PI was more easily observed for the labeled molecules than for their total amount (Table VIII). Our data are consistent with a targeting of PS to the ER-derived vesicles. In addition, we observed a high amount of VLCFA in PS (Table VI). This result clearly indicates that there is a preferential accumulation of VLCFA-PS over the bulk PS. This observation is highly consistent with well-known observations that plasma membrane PS has a high VLCFA content in many plant tissues (Murata et al., 1984) and suggests that the sorting of these PS species starts at the level of ER budding and confirms the vesicular pathway proposed for the VLCFA (Bertho et al., 1991; Moreau and Cassagne, 1994; Moreau et al., 1998a).

In addition, we recently showed that plant sterols are likely to be transported from the ER to the plasma membrane through the vesicular pathway with kinetics similar

Table VII. *Diameter and relative proportions of the different membrane structures present in the ER, the membrane fraction TV(−), and the putative ER-derived carrier vesicles TV(+)*

The amount of proteins recovered for the fractions TV(−) was 34 ± 4 μg (n = 8) and that obtained for the fraction TV(+) was 63 ± 11 μg (n = 14). Therefore, we estimated per experiment an average quantity of 2 μg of proteins corresponding to small vesicles recovered in the absence of ATP and as much as 25 μg of proteins for the small vesicles that were formed in the presence of ATP. Surface areas were estimated according to perfect spherical structures. Although this calculation is not accurate, it allows a comparison between TV(−) and TV(+) that clearly highlights the stimulation by ATP of the formation of small vesicles. a, Very few small vesicles (<100 nm) were detectable; b, very few structures were observed with a diameter ≥1 μm (Fig. 3C). These structures were omitted from the statistics not to artificially decrease the number of small structures in the TV(−) fraction as compared with the TV(+) fraction.

	Large Structures			Intermediate Structures			Small Vesicles		
	Diameter	No.	Area	Diameter	No.	Area	Diameter	No.	Area
	nm	*% of total*		*nm*	*% of total*		*nm*	*% of total*	
ER	314 ± 82	49b	71	197 ± 30	51	29		a	
TV(−)		b		248 ± 77	67	94	90 ± 37	33	6
TV(+)		—		158 ± 31	26	60	76 ± 15	74	40

to that of PS transport (Moreau et al., 1998b). Therefore, it is tempting to speculate that these lipids could be associated within the same membrane domains, as is the case in animal cells for glycosphingolipids, cholesterol, and some specific proteins that are concentrated in structures called "glycolipid rafts" (Fiedler et al., 1993; Simons and Ikonen, 1997). The cell-free reconstitution of phospholipid sorting from the ER to ER-derived vesicles in leek cells suggests that the formation of membrane lipid domains also exists in plant cells.

A goal and challenge of this study was to perform an in vitro assay, allowing the formation and subsequently the isolation of ER-derived vesicles. It will now be possible to analyze the protein content of these ER-derived vesicles and to investigate the presence of specific "cargo" proteins and/or proteins of the "vesicular transport machinery" (Rothman and Wieland, 1996; Scheckman and Orci, 1996). It will be of great interest to look for proteins of the "snare family" that are involved in the targeting and fusion of transport vesicles (Söllner et al., 1993; Kaiser and Ferro-Novick, 1998). In addition to these perspectives, the cell-free system could also be used to investigate some of the molecular mechanisms involved in the budding of the vesicles from the ER membrane. Phosphoinositides (Alb et al., 1996; De Camilli et al., 1996; Toker, 1998), as well as phospholipid transfer proteins (Alb et al., 1996; Kearns et al., 1998; Paul et al., 1998) and phospholipase D (Tüscher et al., 1997; Siddhanta and Shields, 1998), have been shown to participate in the regulation of vesicle budding from the Golgi apparatus in animal and yeast cells. It will be of particular interest to investigate whether specific lipids (H_{II}-phase-forming lipids, diacylglycerol, phosphatidic acid, lysophospholipids, or phosphoinositides) and enzymes of lipid metabolism (synthesizing or degrading lipids) are active components of the transport process. For example, a PI 3-kinase was recently suggested to be implicated in the targeting of plant vacuolar proteins (Welters et al., 1994). Lipid-binding proteins such as annexin are also expected to play critical roles in membrane traffic in plant cells (Clark and Roux, 1995; Carroll et al., 1998).

Although most of the phospholipid transfer proteins described so far are secreted in plant cells, some can have an intracellular location (Kader, 1996). We can therefore question whether such proteins are involved in the regulation of membrane traffic in plant cells. Recently, a cDNA from Arabidopsis corresponding to Sec 14p was found to complement the Sec 14 mutant of the yeast *Saccharomyces cerevisiae* (Jouannic, 1998). Sec 14p is involved in the regulation of vesicle budding from the Golgi apparatus in yeast (Alb et al., 1996; Kearns et al., 1998).

Therefore, future studies are expected to reveal many proteins, enzymes, and lipids likely to be required in the

Table VIII. *Comparison between the distribution of the phospholipids transferred in vitro and the phospholipid composition of the putative ER-derived carrier vesicles*

For this comparison, the phospholipid compositions of the vesicles isolated by both methods were combined. PS enrichment and the increase of PS labeling in the transition vesicles compared with the ER were significant with P values < 0.001. PS:PI ratios were also significantly different with P values < 0.001; n = 12.

ATP Stimulation of Phospholipid Transfer from the ER to the Golgi[a]	Distribution of the Phospholipid Transferred[a]	Phospholipid Composition of the Transition Vesicles (TV[+])		Phospholipid Composition of the ER	
		Amount	Radioactivity	Amount	Radioactivity
%	%			%	
PC 79	49	70.5 ± 5	49.7 ± 7.5	74.3 ± 5.9	56.8 ± 8
PS 123	16	6.2 ± 2	14.5 ± 4	1.8 ± 1.0	5.8 ± 3.2
PI 20	2	3.0 ± 1.6	5.5 ± 3	3.5 ± 1.4	8.5 ± 4
PE 67	33	20.3 ± 3.4	30.3 ± 4.3	20.4 ± 4.3	28.9 ± 5.9

[a] Calculated from Sturbois et al. (1994).

molecular mechanisms governing vesicular trafficking in plant cells; few proteins have begun to be identified (Bar-Peled et al., 1996, 1997; Gomord and Faye, 1996; Hawes and Satiat-Jeunemaitre, 1996; Robinson et al., 1998).

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