D.E. Vance and J.E. Vance (Eds.) *Biochemistry of Lipids, Lipoproteins and Membranes (4th Edn.)*
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CHAPTER 17

Lipid assembly into cell membranes

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1. Introduction

A fundamental problem of cell biology and biochemistry is the elucidation of the mechanisms by which the specific components of subcellular membranes are assembled into mature organelles. The major components of all cell membranes are lipids and proteins. The presence of discrete structural motifs contained in the primary sequence of proteins directs a large number of post-translational processes that enable their sorting among different membrane compartments [1]. The sorting process for proteins is essentially absolute such that plasma membrane proteins are never found in the mitochondria or vice versa. In contrast, lipid molecules do not contain discrete structural subdomains that exclusively direct their movement to specific membranes. The distribution of lipids among different organelles is heterogeneous, but (with a few exceptions) is not usually absolute. These observations about lipids indicate that specialized sorting and transport machinery must exist for their assembly into different membranes.

2. The diversity of lipids

A multiplicity of individual lipids can contribute to membrane formation. The biological role of this lipid heterogeneity is not completely defined and the list of significant actions continues to grow. Some of the diversity contributes to membrane fluidity. Other roles for lipid diversity are the storage of precursors that are metabolized to potent second messengers (e.g., diacylglycerol, ceramide, sphingosine, inositol trisphosphate and eicosanoids) (see Chapters 12, 13 and 14). In addition, many of the polyphosphoinositide (PI3P, PI4P, PI45P_2) function as membrane recognition and attachment sites for protein complexes involved in protein traffic and membrane fusion events [2]. Multiple anionic lipids (PS and polyphosphoinositides) can also regulate attachment of cytoskeletal proteins to membranes. Segregated domains of cholesterol and sphingolipid form microdomains or 'rafts' with unusual physical properties that contribute to protein sorting and are enriched in specific subsets of membrane proteins (see Chapter 1).

In addition to the large numbers of chemically distinct lipid species that occur within a prokaryotic or eukaryotic cell, there is another level of complexity, i.e., the asymmetric

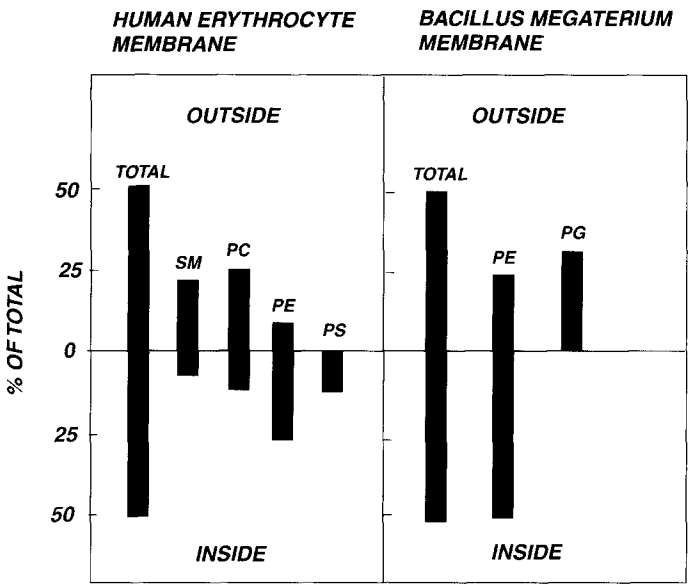


Fig. 1. The asymmetric distribution of lipids across the plane of the cell membrane of the human erythrocytes and *Bacillus megaterium*.

distribution of the lipids across the plane of the bilayer. Two striking examples of membrane lipid asymmetry are found in the red blood cell membrane [3], and the cytoplasmic membrane of *Bacillus megaterium* [4]. The data in Fig. 1 demonstrate that in the red cell membrane the outer leaflet of the lipid bilayer is composed primarily of sphingomyelin (SM) and phosphatidylcholine (PC), and the inner leaflet contains phosphatidylserine (PS) and phosphatidylethanolamine (PE), with lesser amounts of PC and SM. Relatively small amounts of phosphatidylinositol (PI) and its phosphorylated derivatives are also found in the erythrocyte membrane and these anionic lipids are distributed such that the majority is localized to the inner leaflet of the bilayer. In the prokaryote *B. megaterium* the distribution of PE has been shown to be asymmetric, with 30% of this lipid present on the outer leaflet of the bilayer and 70% on the inner leaflet. PE comprises about 70%, and phosphatidylglycerol (PG) about 30%, of the total phospholipid. Thus, nearly all the PG is in the outer leaflet of the bilayer.

Yet another level of complexity is found in cells that possess multiple membrane systems. The Gram-negative bacteria have both an inner and outer membrane system that differ in lipid composition (Chapters 1 and 3). In animal eukaryotes there are numerous membrane systems, the best characterized being endoplasmic reticulum (ER), Golgi membranes, plasma, mitochondrial, lysosomal, peroxisomal and nuclear membranes. In higher plants the eukaryotic organelle repertoire is expanded to include chloroplasts and other plastids, vacuoles and glyoxysomes (Chapter 4). Several of these membrane systems have dramatically different lipid compositions, as shown in Table 1 (A. Colbeau, 1971; T.W. Keenan, 1970). These differences in lipid content raise a variety of interesting questions: How are the different lipid compositions of different

Table 1
Lipid compositions of subcellular organelles from rat liver

Phospholipid [a]	Endoplasmic reticulum		Mitochondrial membranes		Lysosomal membrane	Nuclear membrane	Golgi membrane	Plasma membrane
	Rough	Smooth	Inner	Outer				
Lysophosphatidylcholine	2.9	2.9	0.6	–	2.9	–	5.9	1.8
Sphingomyelin	2.4	6.3	2.0	2.2	16.0	6.3	12.3	23.1
Phosphatidylcholine	59.6	54.4	40.5	49.4	41.9	52.1	45.3	43.1
Phosphatidylinositol	10.1	8.0	1.7	9.2	5.9	4.1	8.7	6.5
Phosphatidylserine	3.5	3.9	1	1	–	5.6	4.2	3.7
Phosphatidylethanolamine	20.0	22.0	38.8	34.9	20.5	25.1	17.0	20.5
Cardiolipin	1.2	2.4	17.0	4.2	–	–	–	–
Phospholipid/protein (μmole P/mg)	0.33	0.47	0.34	0.46	0.21	–	–	0.37
Cholesterol molar ratio Phospholipid	0.07	0.24	0.06	0.12	0.49	–	0.152	0.76

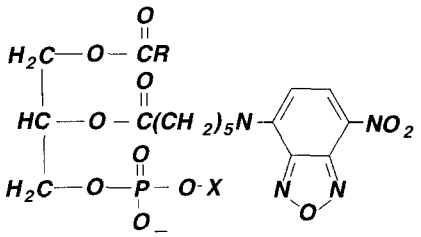
Values for individual lipids are percentage of total phospholipid phosphorus.

organelles established? How are these differences maintained? Are the different lipid compositions essential for organelle function?

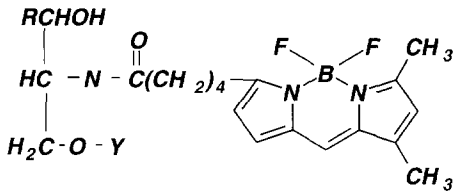
3. *Methods to study intra- and inter-membrane lipid transport*

3.1. *Fluorescent probes*

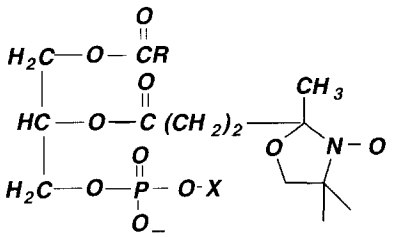
Pagano [5] and coworkers pioneered methods for the rapid insertion of fluorescent phospholipid analogs from liposomes into the plasma membranes of cultured cells. Virtually all of these analogs exhibit slight water solubility and high hydrophobic partitioning coefficients that enable them to be efficiently and reversibly transferred to cell membranes at low temperature from liposomes or albumin complexes containing the fluorescent lipid. A commonly used fluorochrome is nitrobenzoxadiazole (NBD) which is conjugated to short-chain fatty acids in the *sn*-2 position of glycerophospholipids or on the amine of sphingosine. Other analogs such as boron dipyrromethene difluoride (BODIPY) derivatized fatty acids have proved equally effective. Non-fluorescent molecules containing short-chain sphingosines or fatty acids such as diC8 SM or diC8 PC have similar physical properties to the fluorescent molecules, and have also proved to be important probes. The structures of some of these analogs are shown in Fig. 2. Subsequent to the insertion of these lipid analogs into cell membranes, the cells can be washed at low temperature to remove the donor liposomes or albumin complexes. In almost all cases this procedure results in the pulse labeling of the outer leaflet of the plasma membrane with the lipid analog. The lipid analogs can also be removed from the outer leaflet of the plasma membrane at reduced temperature by washing cells with a solution that contains liposomes (e.g., composed of dioleoyl-PC) or albumin. When the lipid analogs are fluorescent their intracellular movement can be observed



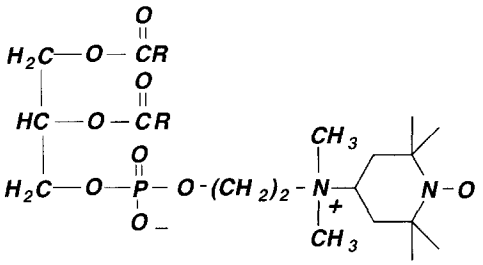
LUORESCENT PHOSPHOLIPIDS
NBD-FLUOROCHROME



FLUORESCENT SPHINGOLIPIDS
BODIPY FLUOROCHROME



SPIN LABELED PHOSPHOLIPIDS
ACYL DERIVATIVE



SPIN LABELED PHOSPHOLIPIDS
HEAD GROUP DERIVATIVE

Fig. 2. General structural features of fluorescent and spin-labeled lipid analogs. The fluorescent lipids contain a short-chain fatty acid, amino-caproic acid, that is derivatized with 4-nitrobenzo-2-oxa-1,3-diazole (NBD), or valeric acid that is derivatized with a boron dipyrromethene difluoride (BODIPY) moiety. For fluorescent phospholipids, X can be hydrogen, or the esterified forms of choline, ethanolamine, serine, or inositol. For fluorescent sphingolipids, Y can be hydrogen, or the esterified forms of phosphocholine or glucose. The spin-labeled lipids modified in the fatty acid portion contain a 4 doxylpentanoyl fatty acid in the *sn*-2 position. Those modified in the polar head group contain a tempocholine moiety in place of choline. The X substituent for the acyl spin-labeled lipids can be hydrogen, or the esterified forms of choline, ethanolamine or serine.

by fluorescence microscopy. A simplified outline of the use of these fluorescent lipids is shown in Fig. 3. In addition to their utility for examining the fluorescence pattern within cells, these lipids can be extracted from cells and their chemical metabolism analyzed using thin-layer chromatography or liquid chromatography/mass spectrometry. The fluorescent sterols dehydroergosterol and NBD-cholesterol are also available for in vivo studies. These sterols can be delivered to cells in reconstituted lipoproteins or from complexes with methyl β-cyclodextrin (E. Kilsdonk, 1995).

3.2. Spin-labeled analogs

Paramagnetic analogs of phospholipids have also been used to investigate lipid transport phenomena in model membrane systems (R.D. Kornberg, 1971) and in biological membranes. Representative structures are shown in Fig. 2. Several of these spin-labeled lipid analogs that are modified in the fatty acid chain can be readily and reversibly transferred from the bulk aqueous phase to biological membranes, in much the same way

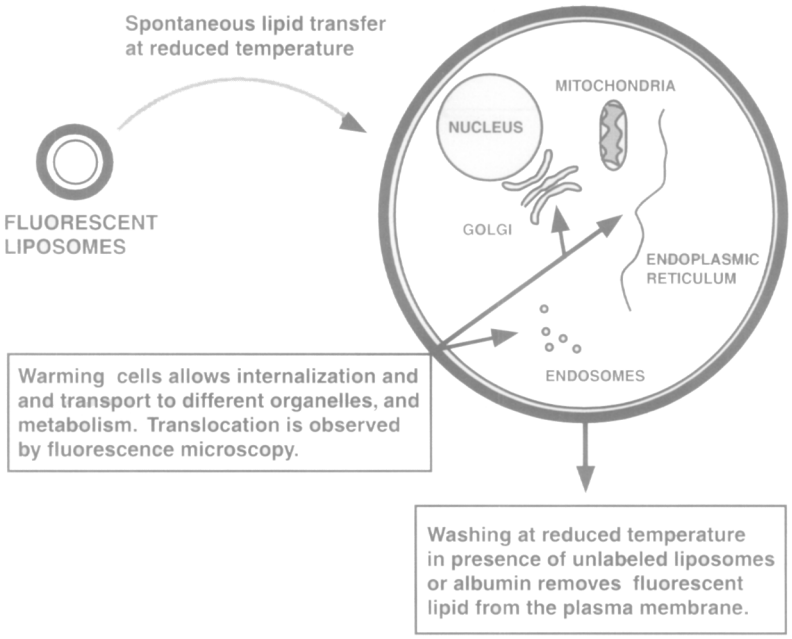


Fig. 3. Fluorescent labeling of living cells with lipid analogs. The heavy line represents the fluorescent phospholipid. Incubation of liposomes containing fluorescent lipid with eukaryotic cells at temperatures of 2–7°C results in the spontaneous transfer of fluorescence to the outer leaflet of the plasma membrane. The fluorescent lipid remains in the plasma membrane at low temperatures and can be reversibly removed by washing the cells with unlabeled liposomes or albumin solutions. Warming the cells to 37°C or intermediate temperature results in the internalization of phospholipid and subsequent labeling of intracellular organelles that can be monitored by fluorescence microscopy.

as the fluorescent lipid analogs. Since the amplitude of the ESR spectra is proportional to the amount of spin-labeled lipid present, these analogs can be used to measure the depletion or retention of the lipids (M. Seigneuret, 1984). In a typical experiment, an intact red cell is incubated with trace amounts of spin-labeled phospholipid at reduced temperature. This treatment effectively pulse labels the outer leaflet of the plasma membrane. Upon warming, the spin-labeled lipids can either remain in the outer leaflet of the plasma membrane or be internalized. If the cells are subsequently cooled and incubated in the presence of ascorbate, the ESR signal of lipid present in the outer leaflet (but not the inner leaflet) of the plasma membrane is quenched and the spectral difference can be used to determine both the rate and extent of transbilayer movement.

3.3. Asymmetric chemical modification of membranes

One method for ascertaining the distribution of lipids across the plane of the membrane bilayer is the use of membrane-impermeant reagents that react with the primary amines of PS and PE on only the external leaflet of the bilayer. Reagents such as trinitrobenzenesulfonate (TNBS) and isethionylacetimidate (IAA) (Fig. 4) are impermeant at reduced temperatures [6] and the chemically modified lipids can be readily identified

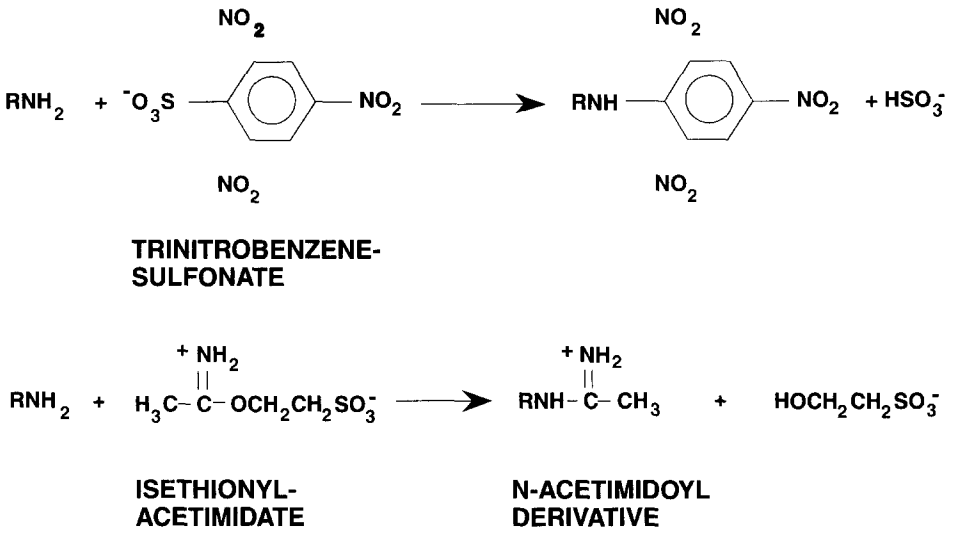


Fig. 4. Primary amine modifying reagents. Phospholipids containing the primary amines PE and PS can be modified by treatment with either trinitrobenzenesulfonate or isethionylacetimidate yielding the *N*-trinitrophenyl derivative or the *N*-acetimidoyl derivative.

by thin-layer chromatography. When such reagents are used in conjunction with in vivo radiolabeling of the lipid, it is possible to discern the temporal and metabolic conditions required for the newly synthesized lipids to reach the compartment that is accessible to the chemical modifying reagents. A useful variation of this approach combines chemical reduction of NBD phospholipids with dithionite to eliminate fluorescence (J.C. McIntyre, 1991). When this latter technique is employed with fluorescence microscopy or spectrofluorometry, it can be extremely informative for resolving questions about transbilayer topology.

Specific pools of lipids on the external surface of cells can also be modified by the action of enzymes such as phospholipases, sphingomyelinases [3], and cholesterol oxidase (Y. Lange, 1985). These enzymes also generate characteristic derivatives of the parental lipids that can be readily identified by thin-layer chromatography and this approach provides another technique for identifying specific pools of lipid on the external surface of the cell membrane. Another means to sample lipids at membrane interfaces relies upon specific chemical desorption. The interaction between sterols and methyl β-cyclodextrin provides a high affinity interaction that can be used to selectively remove (or deliver) cholesterol and dehydroergosterol to membranes (E. Kilsdonk, 1995).

3.4. Phospholipid transfer proteins

In 1968, K.W. Wirtz identified a soluble intracellular protein derived from rat liver that was capable of binding PC and transferring it from one population of (donor) membranes to a second population of (acceptor) membranes (K. Wirtz, 1968). Since

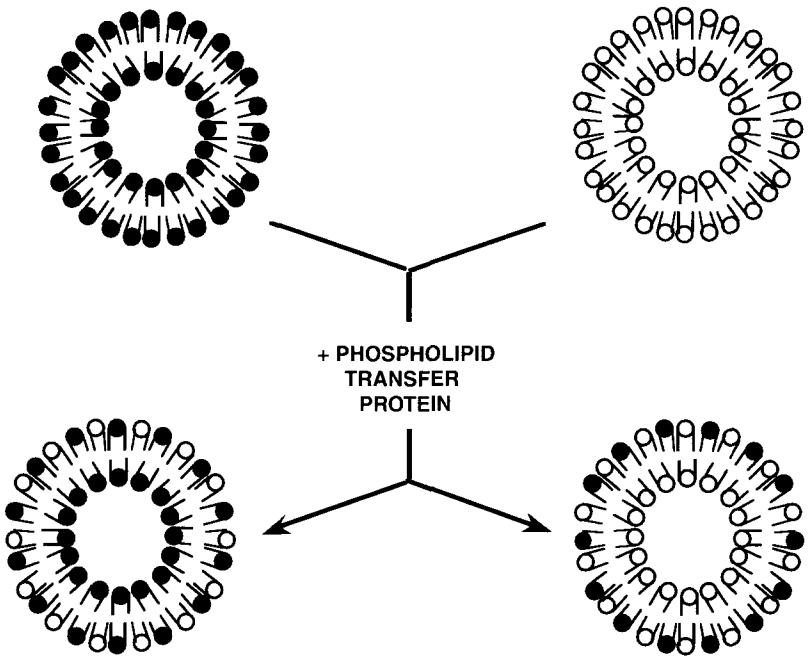


Fig. 5. The action of phospholipid transfer proteins. Mixing of equivalent populations of labeled (denoted in black) and unlabeled liposomes (denoted in white) with phospholipid transfer protein leads to the transfer of lipid between outer leaflets. In the absence of transbilayer movement of the lipid, only the outer leaflets equilibrate with each other.

this initial observation, many of these proteins have been identified in virtually all mammalian tissues, in plants, and in yeast and other microorganisms [7]. The well characterized phospholipid transfer proteins fall into three main categories: (1) those specific for PC; (2) those with high activity for PI and less, but significant, activity with PC, and in some cases SM (J. Westerman, 1995); and (3) those with transfer activity with most phospholipids and cholesterol (this latter protein is referred to as the nonspecific lipid transfer protein). In addition to the phospholipid transfer proteins, there are also intracellular proteins with high transfer activity for sphingolipids (T. Sasaki, 1990). The action of these proteins is typically a one for one exchange of lipid molecules between donor and acceptor membranes. As shown schematically in Fig. 5, the transfer proteins typically equilibrate the lipid present in the outer leaflets of liposomes. The ability of these proteins to transfer lipids from accessible membrane compartments has made them useful tools for inserting lipids into, or removing them from, membranes and probing the transbilayer movement of phospholipids. The role of these proteins in membrane biogenesis will be discussed in Section 4.3.2.6.

3.5. Rapid plasma membrane isolation

One approach to sampling the arrival of newly synthesized lipids at the cell surface utilizes a rapid plasma membrane isolation technique (R.F. DeGrella, 1982). In this

approach, the intact cells are adsorbed onto cationic beads at reduced temperature. After adsorption, the cells are lysed by brief sonication, which liberates the majority of intracellular organelles. The density of the beads containing adsorbed plasma membrane allows them to be separated from the intracellular organelles by low-speed centrifugation. Subsequent to this isolation procedure, the lipids present in the membrane can be extracted and analyzed. When this method is used in combination with radiolabeling of the intracellular pool, the characteristics of the processes required for movement of the lipid from within the cell to the plasma membrane can be determined. A related approach has employed an iron-derivatized wheat germ agglutinin to bind the external surface of the plasma membrane (D. Warnock, 1993) that can be selectively isolated using high-intensity magnetic fields.

3.6. Organelle specific lipid metabolism

For a few lipids, distinct changes in structure also serve to define the arrival at certain organelles or their subcompartments. The enzyme PS decarboxylase is located at the inner mitochondrial membrane of mammalian cells (L.M.G. van Golde, 1974). The synthesis of PS, however, occurs primarily in the ER and related membranes. Thus the decarboxylation of PS can be used as an indicator of the transport of this lipid to the inner mitochondrial membrane [8]. Yeast also contain a mitochondrial PS decarboxylase (PSD1), and in addition, a second enzyme (PSD2) is found in the Golgi and vacuoles. Mutations in the *PSD1 or PSD2* genes of yeast make it possible to use PS metabolism to PE as an index of lipid transport to the locus of either the mitochondria or the Golgi–vacuolar compartment. In yeast, the movement of PE (derived from either PSD1 or PSD2) to the ER can also be followed by measuring its methylation to PC, since the methyltransferases are only present in the ER. Site-specific metabolism also occurs for sphingolipids (Chapter 14). Ceramide, the hydrophobic precursor for all sphingolipids, is synthesized in the ER. The formation of SM from ceramide (Cer) occurs at the luminal surface of the *cis*-medial Golgi (A.H. Futerman, 1990; D. Jeckel, 1990). Thus, SM synthesis from Cer can be used to follow ceramide transport from ER to Golgi. The synthesis of GlcCer also occurs at the Golgi but at the cytosolic side of the membrane. Subsequently, the GlcCer moves into the lumen of the organelle and is converted to LacCer and more complex glycosphingolipids. As with phospholipids, each metabolic step that occurs in a separate organelle or with different topology from the precursor, can serve as an indicator of lipid transport/translocation.

Important elements of sterol metabolism can also be used to elucidate where in the cell a particular precursor has moved [9]. The arrival of cholesteryl esters within lysosomes is revealed by cleavage of the fatty acid to yield free cholesterol. The subsequent transport of cholesterol to the ER can be monitored by the action of acyl CoA : cholesterol acyltransferase (Chapter 15) which results in the formation of new cholesteryl esters. In addition, sphingomyelinase treatment of the cell surface induces cholesterol movement from the plasma membrane to the ER where its arrival can likewise be monitored by acyl CoA : cholesterol acyltransferase action. Import of cholesterol into mitochondria (usually restricted to steroidogenic cells) can be followed by side-chain cleavage reactions that produce pregnenolone. Movement of pregnenolone

out of mitochondria can also be followed by oxidations at positions 3, 17 and 21 which occur in the ER.

4. *Lipid transport processes*

The movement of lipids within the cell can be divided into two different general classes of transport: intramembrane transport, which entails the transbilayer movement of the lipid molecule; and intermembrane transport which is the movement of lipid molecules from one distinct membrane domain to another. Extensive reviews of these processes have been published [8–14].

4.1. Intramembrane lipid translocation and model membranes

The observation that biological membranes can be asymmetric with respect to transbilayer disposition of lipid components (Fig. 1) initially raised basic questions about how such asymmetry was established and maintained. An important issue that needed to be resolved on theoretical grounds, was whether lipids in model membranes could undergo spontaneous transbilayer movement. A simple consideration of the events that occur in the transbilayer movement of a zwitterionic molecule such as PC suggests that at least two energetically unfavorable events must occur. The first is desolvation of the molecule and the second is movement of the charged portion of the lipid through the hydrophobic portion of the bilayer.

Direct experiments to examine the transbilayer movement of phospholipids (R.D. Kornberg, 1971) made use of spin-labeled analogs of PC in which the choline moiety was replaced with the tempocholine probe, *N,N*-dimethyl-*N*-(1′-oxyl-2′,2′,6′,6′-tetramethyl-4′-piperidyl)-ethanolamine (Fig. 2). These workers found that only the ESR signal generated by molecules in the outer leaflet of unilamellar liposomes could be rapidly quenched by ascorbate. The ESR signal from lipid molecules initially residing at the inner leaflet of liposomes was accessible to ascorbate with a $t_{1/2}$ of >6.5 h, indicating slow transbilayer lipid movement (Fig. 6).

Additional evidence for slow transbilayer phospholipid movement in liposomes came from experiments using ^{3}H-PC-labeled liposomes and PC-transfer protein. In the presence of excess unlabeled acceptor membranes, only the PC in the outer leaflet of the liposome membrane was rapidly transferred (J.E. Rothman, 1975). The ^{3}H-PC initially present in the inner leaflet of the membrane moved to the outer leaflet with a $t_{1/2}$ of 11–15 days (Fig. 6). Further evidence demonstrating slow transbilayer movement of phospholipids was obtained from unilamellar liposomes containing 90% PC and 10% PE [6]. In these liposomes the PE initially residing in the outer leaflet of the membrane was rapidly modified by IAA. The PE at the inner leaflet remained refractory to modification by trinitrobenzenesulfonate (i.e., did not undergo transbilayer movement) with a $t_{1/2}$ of >80 days (Fig. 6).

In contrast to phospholipids, non-polar lipids such as diacylglycerol (DG) behave differently. B.R. Ganong (1984) synthesized a structural analog of DG in which the *sn*-3 hydroxyl group was replaced by an SH group that could be detected with dithio-

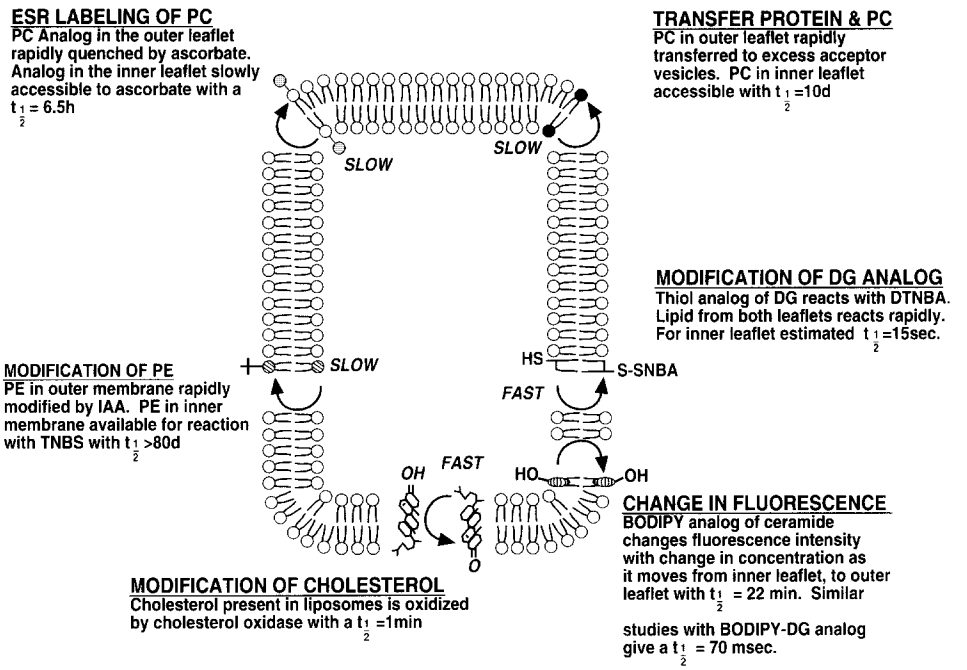


Fig. 6. Summary of key experiments examining transbilayer lipid movement in liposomes. Abbreviations: IAA, isethionylacetimidate; TNBS, trinitrobenzenesulfonate; DTNBA, dithiobisnitrobenzoic acid (Ellman's reagent). Reaction of DTNBA with RSH gives R-S-SNBA. Each of the experiments was specifically designed to initially sample only the outer leaflet of the bilayer and then at subsequent periods detect the movement of lipid from the inner to the outer leaflet of the bilayer.

bisnitrobenzoic acid (Ellman's reagent). When liposomes containing the thiol analog of DG were reacted with Ellman's reagent, the $t_{1/2}$ for transmembrane movement was determined to be 15 s. Concentration-dependent changes in the fluorescent properties of BODIPY lipids have also been used to estimate transbilayer movement of DG and ceramide (J. Bai, 1997). By the fluorescence method the $t_{1/2}$ for transbilayer movement of BODIPY-DG is 70 ms and that of BODIPY-Cer is in 22 min (see Fig. 6). These results strongly suggest that the polar moiety of phospholipids is the portion of the molecule that greatly retards transbilayer movement of these molecules in model membranes. Cholesterol is another non-polar lipid whose transmembrane movement has been examined. Treatment of PC/cholesterol liposomes with cholesterol oxidase demonstrated that the entire cholesterol pool could be readily oxidized with a $t_{1/2}$ of 1 min at 37°C (J.M. Backer, 1981) (see Fig. 6).

Thus, studies with model membranes provide clear evidence that the transbilayer movement of phospholipids is a very slow process in this system, whereas the process appears to be rapid for non-polar lipids. The results imply that if transbilayer movement of phospholipids does occur in biological membranes, it must be a facilitated process.

4.2. Intramembrane lipid translocation and biological membranes

4.2.1. Prokaryotes

The primary consideration in the genesis of any biological membrane is the location of the synthetic apparatus that manufactures the subunits of the membrane and its relationship to the final distribution of its products. In *Escherichia coli*, substantial evidence indicates that the synthesis of phospholipids occurs at the inner (cytoplasmic) membrane by enzymes that have their active sites on the cytoplasmic surface of the inner membrane [13]. Such an orientation allows free access of water-soluble substrates and reaction products to the cytosol.

In experiments performed with *B. megaterium*, Rothman and Kennedy [15] used chemical modification with TNBS, under conditions where the probe did not enter the cell, to distinguish between PE molecules located on the outer and inner sides of the cell membrane. This technique was coupled with pulse-chase experiments with [^{32}P]inorganic phosphate and [^{3}H]glycerol and demonstrated that newly synthesized PE is initially found on the cytoplasmic surface of the cell membrane and is rapidly translocated to the outer leaflet of the membrane with a $t_{1/2}$ of 3 min at 37°C. Although the translocation is rapid, it does not occur coincident with synthesis, but rather, with a significant delay after the molecule is synthesized. In addition, the translocation can continue in the absence of PE synthesis. These findings indicate that lipid synthesis and translocation are two distinct events.

The energetic requirements for transmembrane movement of phospholipids have been investigated (K.E. Langley, 1979). Using *B. megaterium* and a TNBS probe, these studies demonstrated that the transbilayer movement of newly synthesized PE was unaffected by inhibitors of ATP synthesis and protein synthesis. Thus, the driving force for phospholipid translocation in *B. megaterium* is independent of metabolic energy, lipid synthesis, and protein assembly into cell membranes.

More recent work with closed vesicles derived from *B. megaterium* membranes demonstrates that NBD analogs of PE, PG, and PC can translocate across the membrane with a $t_{1/2}$ of 30 s at 37°C (S. Hraffnsdottir, 1997). Similar types of experiments conducted with closed vesicles isolated from *E. coli* inner membrane reveal that NBD phospholipids traverse the bilayer with a $t_{1/2}$ of 7 min at 37°C (R. Huijbregts, 1996). This latter process is insensitive to protease and *N*-ethylmaleimide treatments and does not require ATP. Collectively, the data indicate that transbilayer lipid movement is rapid and does not require metabolic energy in bacterial membranes. The basic characteristics of the lipid translocation in the intact cell appear to be retained in isolated membranes.

4.2.2. Eukaryotes

4.2.2.1. Transbilayer movement of lipid at the endoplasmic reticulum. In eukaryotic systems a detailed pattern of synthetic asymmetry has emerged with respect to the topology of the enzymes of phospholipid synthesis in rat liver microsomal membranes. Protease mapping experiments (R. Bell, 1981) have indicated that the active sites of the phospholipid synthetic enzymes are located on the cytosolic face of the ER. Thus, in both prokaryotic and eukaryotic systems it appears that the site of synthesis of the

bulk of cellular phospholipid is the cytosolic side of the membrane. This asymmetric localization of synthetic enzymes strongly implicates transbilayer movement of phospholipids as a necessary and important event in membrane assembly that is required for the equal expansion of both leaflets of the bilayer (reviewed in A. Zachowski, 1993).

The transbilayer movement of phospholipids in microsomal membranes has been measured using several different approaches. In preparations of liver microsomes that were first radiolabeled with lipid precursors in vivo, the transbilayer movement of lipids was examined using phospholipid transfer proteins (D.B. Zilversmit, 1977). The results from these experiments provided evidence that PC, PE, PS, and PI from both membrane leaflets were exchanged between labeled microsomes and excess acceptor membranes with a maximal $t_{1/2}$ of ~45 min. This value, was set as an upper limit because the amount of lipid transfer protein used could not exchange out the labeled phospholipid with a $t_{1/2}$ of less than 45 min.

In a different approach, a water-soluble, short-chain (dibutyroyl) analog of PC was used to measured the rate of uptake and luminal sequestration by isolated liver microsomes (W. Bishop, 1985). This PC analog was taken up in a time- and temperature-dependent manner. The kinetics of uptake were saturable with respect to substrate concentration and the transport activity was protease sensitive. The transporter was also shown to be stereospecific in its action and it was unaffected by the addition of ATP. Virtually identical properties have also been described for a microsomal transporter that utilizes butyroyl-lyso-PC (Y. Kawashima, 1987).

Additional studies utilized spin-labeled analogs of PC, PE, PS and SM, and the $t_{1/2}$ for the translocation of these lipid analogs from the cytosolic face to the luminal face of the microsomes was calculated to be 20 min. The transport process did not require ATP and the translocation of each class of lipid showed identical sensitivity to inhibition by *N*-ethylmaleimide (Fig. 7). Furthermore, different species of lipid showed transport kinetics that were consistent with mutual competition for a single transporter. These

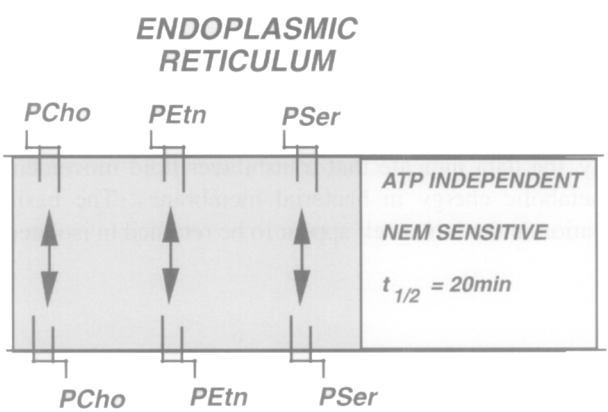


Fig. 7. Transbilayer movement of phospholipids in eukaryotic membranes. The general features of the transmembrane transporters of the ER are shown. The ER transporter does not require ATP and is inhibited by *N*-ethylmaleimide (NEM). The structure (⌙) represents the DG portion of the lipids and PCho, PEtn and PSer are abbreviations for phosphocholine, phosphoethanolamine and phosphoserine, respectively.

results indicate that the ER has a relatively nonspecific, ATP-independent transporter that is capable of translocating multiple species of lipid across the bilayer.

Thus, the data from both bacteria and animal cells demonstrate that transbilayer movement of phospholipid occurs on a time scale of minutes, in an ATP-independent fashion in membranes that contain the majority of the enzymes involved in their biosynthesis. These intramembrane transport properties observed in the major biosynthetic membranes, however, are not generally true for other membrane systems. This is especially true of the plasma membrane.

4.2.2.2. Transbilayer lipid movement at the eukaryotic plasma membrane. The transbilayer movement of lipids at the cell surface of eukaryotes is being understood with increasing molecular and biochemical detail. Three fundamental classes of transport are now recognized and consist of the aminophospholipid translocases (also called flippases) the scramblases and the ATP binding cassette (ABC) pumps. A schematic summary of some of the properties of these proteins is shown in Fig. 8.

Aminophospholipid translocase. Several studies using either short-chain versions of PS, or spin-labeled or fluorescent analogs of PS and PE have established the properties of the translocases on the erythrocyte and nucleated cells [10,11]. The aminophospholipid translocase recognizes PS and PE at the external surface of the plasma membrane and translocates the lipids to the cytoplasmic side. This flipping process requires ATP on the cytosolic side of the membrane. The affinity for PS is about 30-fold higher than PE, and the equilibrium distribution is 95% PS and 90% PE in the inner membrane. The aminophospholipid translocases are susceptible to protease digestion and inactivation with *N*-ethylmaleimide. Additional susceptibilities to inhibition by AlF_4 and Na_2VO_4

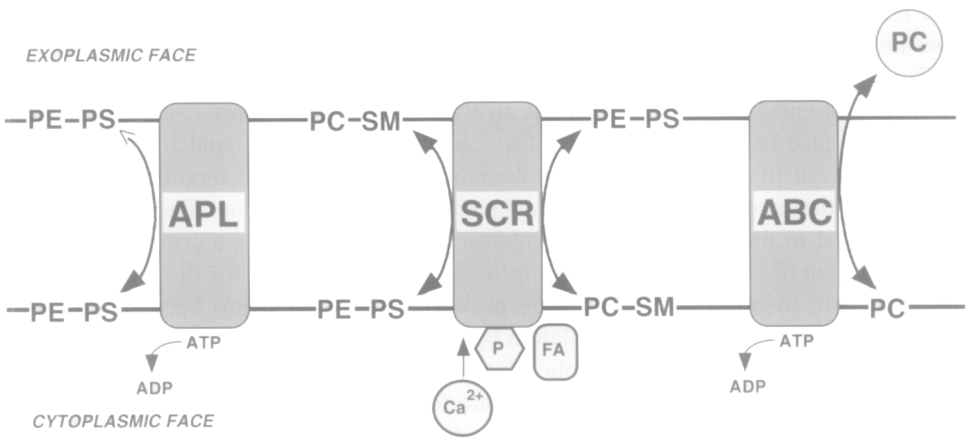


Fig. 8. Summary of the general features of transbilayer transporters found in plasma membranes. APL, amino phospholipid translocase; SCR, bidirectional transporter or scramblase; ABC, ATP binding cassette protein. The APL and ABC transporters utilize cytoplasmic ATP to drive transport. The APL is specific for PS and PE and the reaction greatly favors internalization. The ABC transporter pumps PC out of the plasma membrane. The scramblase is activated by high intracellular Ca^{2+} levels and regulated by phosphorylation and acylation. The scramblase is nonspecific and randomizes the lipid distribution across the bilayer.

suggest that the translocases belong to the P-type ATPase family. The stoichiometry of ATP utilization per phospholipid translocation event is approximately one. All of the characteristics are consistent with the amino phospholipid translocase translocase being a specific plasma membrane ATPase that is activated by aminophospholipids.

An erythrocyte ATPase that is Mg^{2+} and PS dependent, has been partially purified and reconstituted into vesicles. The reconstituted protein preparation is capable of transporting PS across the bilayer, albeit with low efficiency [11]. A second P-type ATPase, designated ATPase II, has been purified to homogeneity and its cDNA cloned. The deduced protein sequence reveals that it contains 3 P-type ATPase consensus sequences (X. Tang, 1996). Definitive reconstitution of the ATPase II into vesicles and demonstration of its aminophospholipid translocase activity has not yet been accomplished.

The cDNA sequence of the ATPase II was used to identify the yeast ortholog, which is the *DRS2* gene, that encodes the Drs2p protein, and for which *drs2* mutants are known, that have defects in ribosomal assembly. Some *drs2* mutant strains appear to have defects in their NBD-PS transport (X. Tang, 1996). However, *drs2*Δ mutants lacking any of the gene product have normal NBD-PS and NBD-PE transport (A. Siegmund, 1998). Thus, the aminophospholipid translocase has yet to be definitively identified in either mammalian or yeast systems.

Bidirectional transporters. The bidirectional transporters at the plasma membrane function to randomize the lipid distribution across the plane of the bilayer, and are commonly referred to as scramblases [10]. The action of the scramblase is similar to that of the previously described transbilayer transporter present in the ER. The protein was first functionally identified in erythrocytes, but is also present in nucleated cells. The scramblase shows no lipid specificity and essentially collapses the asymmetry of lipids at the cell surface. Phospholipids, SM and glycosphingolipids all serve as substrates. The randomizing function of this plasma membrane protein is activated by Ca^{2+} and does not require ATP.

The scramblase protein was purified to homogeneity and its cDNA cloned [16]. The protein is oriented with its N-terminus in the cytosol and a short C-terminus at the external surface of the membrane. A Ca^{2+} binding domain is found in the cytosolic region adjacent to the transmembrane domain. Post-translational modifications to the scramblase that alter activity include acylation and phosphorylation. There is currently much interest in the regulation of scramblase function, as it plays a critical role in the externalization of phosphatidylserine, a process that is important for the recognition of apoptotic cells by phagocytes. Four isoforms of the protein have now been identified (T. Wiedmer, 2000). In addition to regulation by Ca^{2+} and oligomerization, the scramblase activity can be enhanced by phosphorylation directed by protein kinase C-delta (S. Frasch, 2000). The protein is also a substrate for the protein tyrosine kinase c-Abl (J. Sun, 2001). Detailed understanding of the mechanism of scramblase action is now likely to provide important fundamental information about the energetics and maintenance of lipid asymmetry.

ABC transporters. The ABC transporters are a large family of proteins involved in moving molecules across membranes in ATP-dependent reactions (I. Klein, 1999). A subset of this family transports molecules that include xenobiotics, bile acids, and

hydrophobic compounds. In 1993, Smit and coworkers described a transgenic mouse with null allelles for the ABC transporter, *mdr2*, that exhibited a profound defect in transporting PC into bile. These findings led Ruetz and Gros [17] to examine the activity of the *mdr2* protein as a PC translocase. The heterologous expression of the *mdr2* cDNA in yeast leads to incorporation of *mdr2* protein into yeast secretory vesicles and acquisition of the ability to translocate NBD-PC across the bilayer. The translocation process is time-, temperature- and ATP-dependent. These findings indicate that the *mdr2* protein acts as a PC transporter. Unequivocal experiments in transgenic mice establish that the human MDR3 protein and mouse *mdr2* have identical function [18].

Further implication of ABC transporters in lipid translocation comes from work with human ABC A1, ABC R and ABC G5 plus ABC G8. The ABC A1 protein acts on cholesterol export and is described in Chapter 20. The ABC R protein is defective in Stargardt's macular dystrophy (R. Allikmets, 1997). In mice with null alleles for ABC R, retinylidene PE (a conjugate of retinaldehyde and PE) accumulates in the inner membrane of rod outer segment discs, due to failure of translocation by ABC R (J. Weng, 1999). These findings implicate ABC R as a translocase for retinylidene PE. The dimeric ABC G5 and ABC G8 transporter is defective in individuals with sitosterolemia (M. Lee, 2001). Under normal conditions, intestinal absorption of the plant sterol, sitosterol, appears to be minimized by an efflux pumping mechanism that continually translocates the sterol back to the lumen of the gut. In individuals with ABC G5 or ABC G8 defects, this pumping mechanism is lost, and blood and tissue levels of this deleterious sterol increase dramatically.

Data implicating ABC family transporters are growing, and it is likely that additional members will be added to the list. It is important to highlight one fundamental difference observed between the ABC transporters and other translocases (Fig. 8). In each case of the ABC family, the transported substrate either enters an environment that is a different phase from the membrane or the substrate is rapidly moved into another membrane or metabolized. In contrast, the substrate for the aminophospholipid translocases and scramblases remains within the bilayer across which it is transported.

4.3. Intermembrane lipid transport

From a theoretical perspective a number of processes could contribute to the intermembrane transport of lipids. These are outlined in Fig. 9 and include monomer solubility and diffusion (A), soluble carriers such as lipid transfer proteins (B), carrier vesicles (C), membrane apposition and transfer (D) and membrane fusion processes (E). Lipids such as free fatty acids, lysophosphatidic acid and CDP-DG may have sufficient solubility to allow for some monomeric transport but most other lipids are likely to require one of the other potential mechanisms due to their extremely low solubility.

4.3.1. Transport in prokaryotes

The presence of multiple membrane systems in organisms, such as Gram-negative bacteria, photosynthetic bacteria, and the eukaryotes, raise significant questions about the mechanisms of membrane biogenesis. In a 'simple' organism such as *E. coli* there are two membrane systems: the inner or cytoplasmic membrane, and the outer

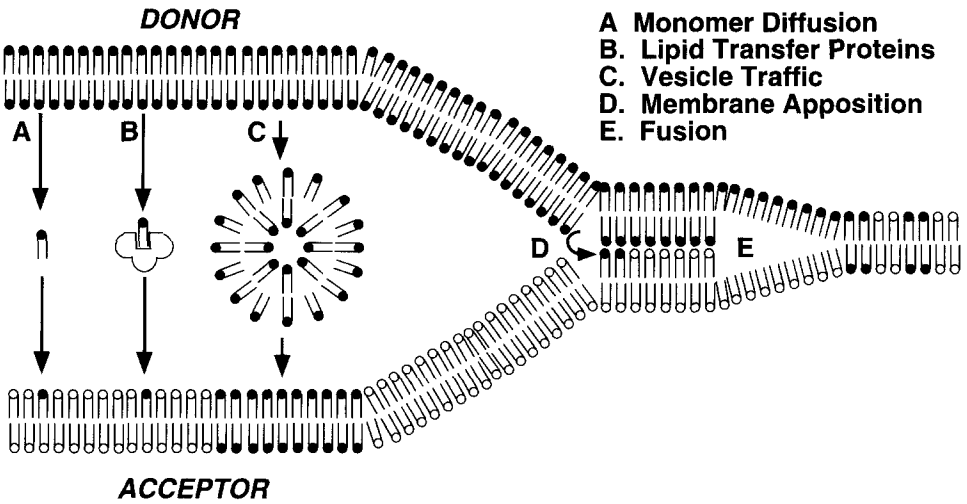


Fig. 9. Theoretical mechanisms for transporting lipids and altering membrane composition.

membrane (Chapter 3) [13]. The entire apparatus for phospholipid synthesis is located at the inner membrane. Consequently, there must exist a mechanism for exporting phospholipids from the inner membrane to the outer membrane.

In some of the earliest studies of phospholipid transport between the inner and outer membranes of *E. coli*, labeling of PE revealed that the specific activity of this lipid was fivefold higher in the inner membrane than the outer membrane, immediately following a 30-s pulse with [^{3}H]glycerol (A.M. Donohue-Rolfe, 1980). During the chase period the specific activity of the outer membrane increased, while that of the inner membrane decreased. After several minutes the specific activities of both membranes asymptotically approached the same value, which indicated radioequilibration between the membranes. The $t_{1/2}$ for the translocation of PE was determined to be 2.8 min. The transport in these studies was independent of protein synthesis, lipid synthesis, and ATP synthesis. It appeared, however, to be dependent upon the cell's protonmotive force.

In recent work, Doerrler et al. [19] have examined the role of an ABC transporter in *E. coli* named *msbA*, in the phenomena of lipid transport between the inner and outer membrane. The *msbA* is an essential gene whose overexpression suppresses growth arrest in *E. coli* strains defective in the final steps of lipid A synthesis. Examination of the mode of rescue effected by high copy *msbA*, in the lipid A synthesis mutants, suggested that the protein acted to transport toxic lipid A precursors from the inner to the outer membrane. The function of *msbA* was examined further by creating a temperature-sensitive allele (*msbA*^{ts}). When cultures of strains with *msbA*^{ts} are shifted to the non-permissive temperature, cell growth arrests after about 60 min. In wild-type cells, subjected to the same treatments, cell growth is unaffected and 40–60% of the phospholipid, lipid A and protein, moved from the inner to the outer membrane. In the *msbA*^{ts} strain, $<10\%$ of the phospholipid and lipid A moved to the outer membrane, whereas protein traffic was unaffected. Furthermore, electron micrographs

reveal significant invagination of the inner membrane consistent with excess lipid accumulation at this site. These experiments provide striking evidence for the role of *msbA* in the export of both phospholipid and lipid A from the inner membrane to the outer membrane of *E. coli*. These findings raise the possibility of other ABC transporters acting at sites of membrane contact or apposition, as a general mechanism for intermembrane lipid transport.

4.3.2. Transport in eukaryotes

Currently, there is a broad understanding of the elements of interorganelle transport for several different lipid classes. In many studies the questions have been narrowly focused to the movement of one lipid class between a donor and an acceptor compartment that are temporally, metabolically and geographically segregated within the cell. The discussion of these processes is organized by class of lipid and then by membrane systems examined.

4.3.2.1. Phosphatidylcholine.

Transport of newly synthesized PC from the ER to the plasma membrane. The principal site of PC synthesis is the ER (Chapter 8). The transport of PC from the ER to the plasma membrane has been examined using pulse-chase labeling with a [^{3}H]choline precursor and rapid plasma membrane isolation with cationic beads (M. Kaplan, 1985). These studies reveal that PC transport is an extremely rapid process occurring with a $t_{1/2} \approx 1$ min (Fig. 10). This transport is unaffected by metabolic poisons that deplete cellular ATP levels, disrupt vesicle transport, or alter cytoskeletal arrangement. The mechanism of this transport is presently unknown, but the results are consistent with a soluble carrier mechanism such as PC transfer protein, or zones of apposition that facilitate rapid intermembrane transfer. Recent work by Pichler and colleagues has identified a subfraction of the ER that associates closely with the plasma membrane in yeast (H. Pichler, 2001). Future studies examining the effects of agents or mutations that disrupt these intracellular membrane associations will be critical for determining their role in lipid traffic.

Transport of newly synthesized PC from the ER to the mitochondria. Using conventional subcellular fractionation techniques, the transport of nascent PC to the mitochondria of BHK cells was examined by pulse-chase experiments with a [^{3}H]choline precursor (M.P. Yaffe, 1983). These experiments show that the newly made PC pool equilibrates between the outer mitochondrial membrane and the ER in approximately 5 min (Fig. 10). Similar studies performed in yeast (G. Daum, 1986) revealed that the PC pool rapidly equilibrates between the ER and mitochondria. Addition of metabolic poisons did not eliminate the PC radioequilibration in yeast. Studies with isolated mitochondria demonstrate that PC loaded into the outer mitochondrial membrane can be transported to the inner membrane in an energy-independent manner (M. Lampl, 1994). Consistent with this finding is the observation that PC rapidly moves across the membrane of vesicles derived from mitochondrial outer membranes prepared from either mammalian cells or yeast (D. Dolis, 1996; M. Janssen, 1999).

Transport of exogenous PC analogs from the cell surface to intracellular organelles. Clear evidence for the movement of PC from the plasma membrane to intracellular

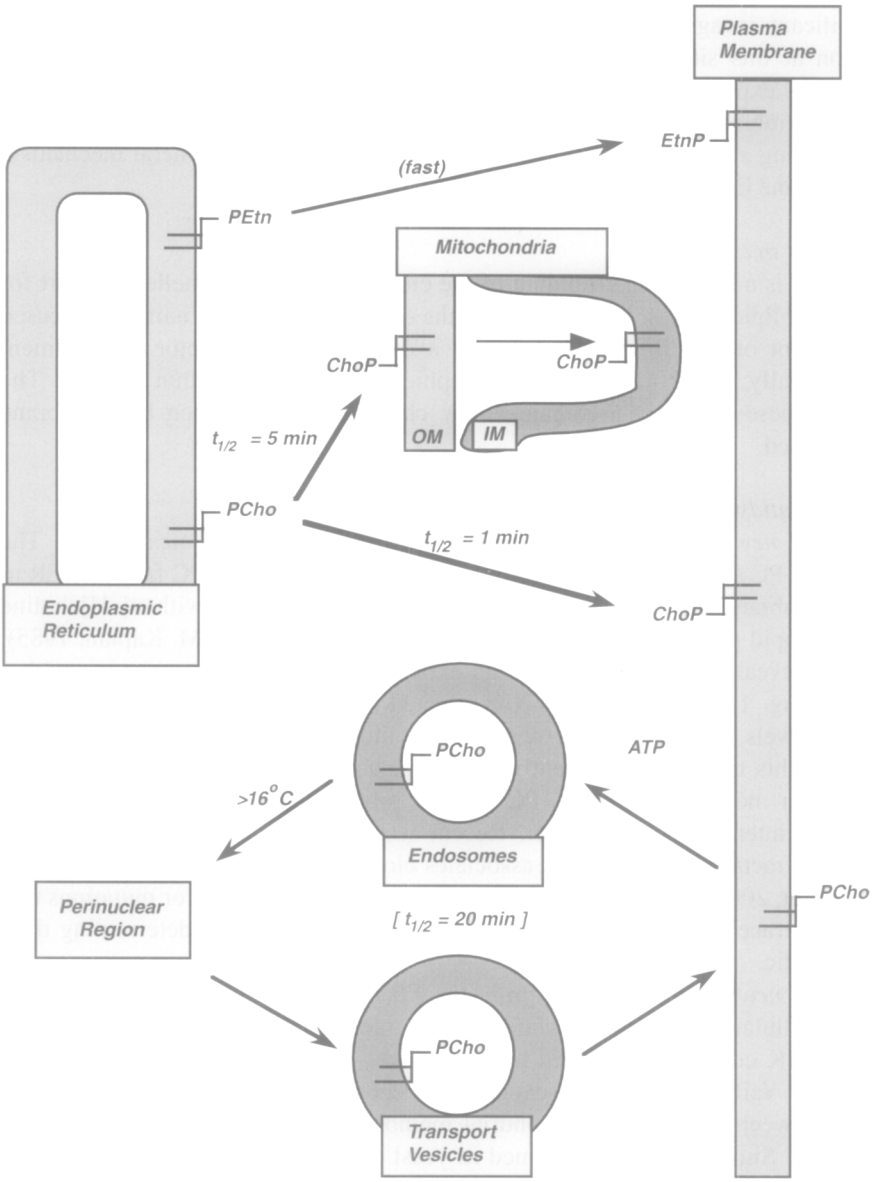


Fig. 10. Interorganelle transport of PC and PE within eukaryotic cells. The structure (⊢) represents the DG portion of the phospholipid, and PCho and PEtn are the abbreviations for phosphocholine and phosphoethanolamine respectively. OM and IM are abbreviations for the outer and inner membranes of the mitochondria. The $t_{1/2}$ for PC transport from the perinuclear region of the cell to the plasma membrane is shown in brackets and estimated to be 20 min.

organelles has been obtained using the fluorescent lipid analog NBD-PC (R. Sleight, 1984). The fluorescent lipid can be pulse-labeled into the outer leaflet of the plasma membrane at 2°C. Upon warming the cells, the fluorescent lipid is transported from the

plasma membrane to the perinuclear region of the cell in the proximity of the Golgi apparatus and the centrioles, via an ATP-dependent process (Fig. 10). The lipid transport occurs by endocytosis and the process can be disrupted by reducing the temperature to 16°C which causes the PC to accumulate in endosomal vesicles. The kinetics for endocytosed NBD-PC transport from intracellular membranes back to the plasma membrane occurs with $t_{1/2} = 20$ min. During the transit cycle the NBD-PC remains restricted to the non-cytosolic face of the respective membranes. The kinetics of this vesicle-based recycling of PC between the cell interior and the plasma membrane are markedly different from those for transport of newly synthesized PC to the cell surface. The disparity between the two transport processes suggests that there is restricted intermixing of nascent and recycling pools of PC.

4.3.2.2. Phosphatidylethanolamine.

Transport of newly synthesized PE to the plasma membrane. When an ethanolamine precursor is used, the primary site of PE synthesis is the ER (Chapter 8). The appearance of newly synthesized PE at the external leaflet of plasma membrane has been determined using chemical modification of the cell surface with TNBS at reduced temperature (R. Sleight, 1983). The results indicate that the initial rate of transport of PE is rapid and proceeds without a lag (Fig. 10). The transport process is insensitive to metabolic poisons that disrupt vesicle transport and cytoskeletal structure. The rapid transport kinetics occur at rates consistent with a soluble carrier-mediated process or transfer at zones of apposition between membranes. Analysis of the kinetics of the process is complicated since only PE at the outer leaflet of the plasma membrane is measured, and the ATP-dependent aminophospholipid transporter activity within the plasma membrane (P. Devaux, 1988; O. Martin, 1987) may be a required step for the lipid to arrive at this location. Despite these complications the results clearly indicate that the initial rate of arrival of PE at the plasma membrane occurs on a time scale that clearly distinguishes it from well characterized vesicle transport phenomena, and is independent of processes involved in protein transport to the cell surface.

PE derived from a PS precursor that is decarboxylated at the mitochondria is also transported to the plasma membrane (J. Vance, 1991) (Fig. 11). This mitochondrial PE is transported to the plasma membrane, with greater efficiency than PE synthesized from an ethanolamine precursor. The mechanism of this translocation remains to be elucidated but the process is unaffected by brefeldin A, a fungal metabolite that alters the structure and function of the Golgi apparatus.

Transport of newly synthesized PE to the mitochondria. Early studies examining the movement of newly synthesized PE from the ER to the mitochondria of hepatocytes demonstrated that the process was markedly slower ($t_{1/2} \approx 2$ h) than that observed for PC (M.P. Yaffe, 1983). These experiments used classical rate sedimentation to isolate the organelles. More recent studies indicate that such mitochondrial fractions are likely to contain another resolvable compartment, the mitochondria-associated membrane (MAM) (J. Vance, 1990). Evidence obtained using CHO-K1 cells [20] indicates that nascent PE (made via CDP-ethanolamine) is transported to the MAM but not to the inner mitochondrial membrane. It remains unclear whether some of this PE is transported to the outer mitochondrial membrane. The results are consistent with little import of PE

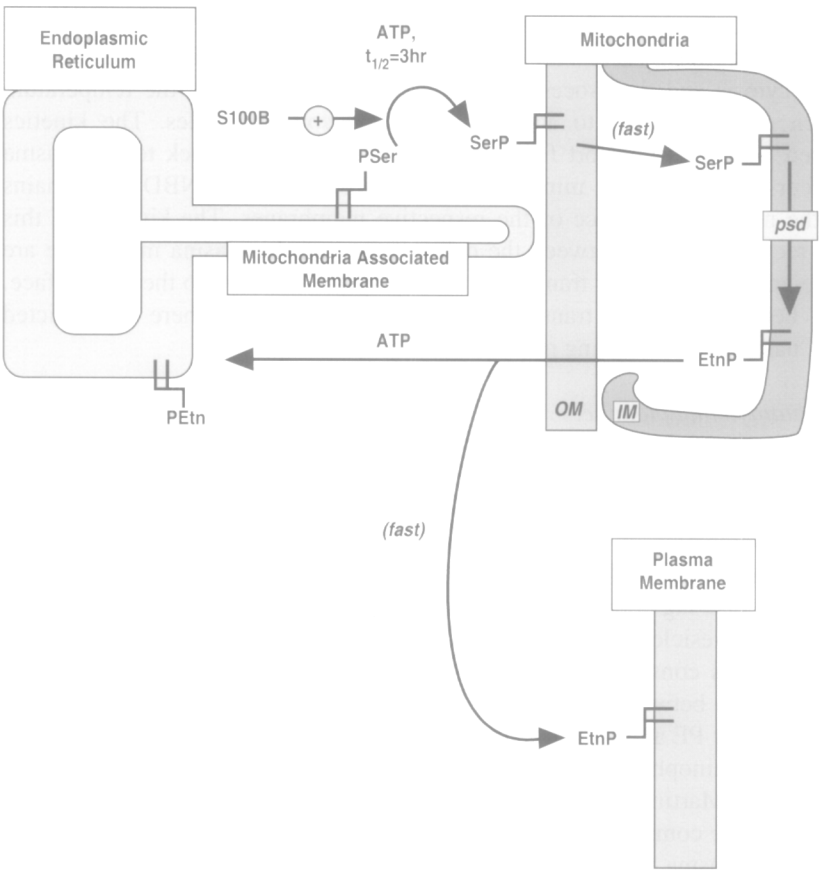


Fig. 11. Interorganelle transport of PS in eukaryotic cells. The structure (⊢) represents the DG portion of the phospholipid. PSer, PEtn and PCho are abbreviations for phosphoserine, phosphoethanolamine and phosphocholine. The term *psd* stands for PS decarboxylase. The rate for the transport of PS between the outer (OM) and inner (IM) mitochondrial membrane has not been determined but appears to be on the order of minutes.

derived from ethanolamine, into the mitochondria. Furthermore, yeast mutants lacking a functional allele for PS decarboxylase 1 are markedly deficient in mitochondrial PE (P.J. Trotter, 1995). The reduced PE in mitochondria cannot be fully restored by PE synthesized in the ER from an ethanolamine precursor, or that made in the Golgi or vacuole by PS decarboxylase 2 (R. Birner, 2001; M. Storey, 2001). These latter findings clearly demonstrate that there is compartmentation and restricted transport of different pools of PE within cells.

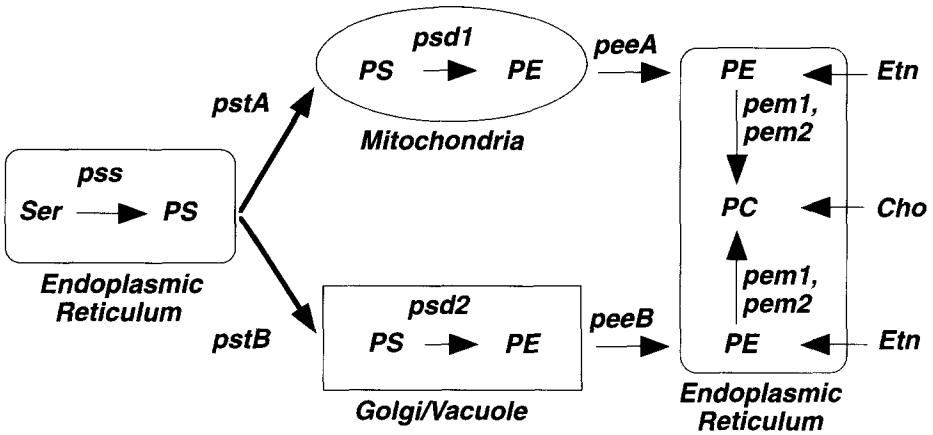
4.3.2.3. Phosphatidylserine.

Transport of newly synthesized PS to the mitochondria. The location of PS decarboxylase at the inner mitochondrial membrane [8] provides a convenient method for determining the arrival of PS at this cellular location. The extremely low steady

state level of PS at the mitochondrial inner membrane (Table 1) coupled with kinetic considerations, indicates that PS is rapidly decarboxylated upon its arrival at the inner membrane. The general features of nascent PS transport are outlined in Fig. 11. The initial studies with intact mammalian cells that used PS decarboxylation as an indicator for lipid transport identified a clear ATP requirement for the transport process. Subsequent reconstitution studies, with isolated organelles, established that mitochondria could take up PS in an ATP-independent process. These findings indicated a requirement for ATP at a stage earlier than the presence of PS at the outer mitochondrial membrane. Additional studies with isolated organelles provided evidence for a tight association between specialized elements of the ER and the mitochondria. These in vitro associations were also shown to have in vivo counterparts evidenced by electron microscopy (D. Ardail, 1993). Successful isolation of these specialized ER structures, now called the mitochondria-associated membrane (MAM), indicated that they are selectively enriched in a subset of lipid synthetic enzymes especially PS synthase (J. Vance, 1990). A MAM structure has also been identified and isolated from yeast cells [21]. Pulse-chase experiments coupled with subcellular fractionation have now established the PS destined for the mitochondria must transit through the MAM and that exit from the MAM requires ATP [20].

The synthesis of PtdSer and transport to the mitochondria have been successfully reconstituted using permeabilized cells [8]. Permeabilized cells retain cellular organelles and cytoarchitecture but are depleted of soluble cellular components. The compromised plasma membrane enables the addition of defined soluble components under controlled conditions to reconstitute the transport processes. The transport of PS to the mitochondria in permeabilized cells occurs in the absence of cytosol, displays an absolute requirement for ATP and occurs with a $t_{1/2}$ of approximately 3 h at 37°C. This transport does not require ongoing synthesis of PS, and 45 fold dilution of the permeabilized cells does not alter the rate or extent of transport. These results are consistent with a membrane bound transport intermediate that utilizes zones of close membrane apposition or fusion between the ER and mitochondria. Although there is not an absolute requirement for cytosol in the transport reaction, a soluble 9 kDa Ca^{2+} binding protein, named S100B, that is highly conserved across mammalian species can enhance the transport several fold (O. Kuge, 2001). Permeabilized yeast have also been used to examine PS transport (G. Achleitner, 1995). Unlike mammalian cells, the transport of PS to yeast mitochondria does not require ATP.

Genetic approaches to identifying components involved in interorganelle aminophospholipid transport. Genetic tools constitute a powerful approach for identifying the components involved in lipid transport. The ability to isolate mutant strains defective in a given transport step, and then clone the genes by complementation of the mutation, can lead to clear molecular and mechanistic resolution of complex processes. The genetic aspects of PS and PE transport in eukaryotic systems are shown in Fig. 12. Understanding the genetic approach to examining aminophospholipid transport requires an appreciation that the synthesis and decarboxylation of PS and the methylation of PE are all geographically separate events within a cell. A basic hypothesis of the genetic approach is that there are specific genes, designated *PST* for (*PS t*ransport), and *PEE* (for *PE e*xport), that either regulate or directly participate in the transfer process. Prominent methods for identifying mutant strains defective in these processes rely on



MUTANT	SOURCE	GENE	PROTEIN
pstA	*CHO-R41*	-	-
pstB	*Yeast, pstB1* *Yeast, pstB2*	*STT4* *PSTB2*	*PI-4K* *PITP*

Fig. 12. Genetic analysis of aminoglycerophospholipid transport in eukaryotes. The transport of PS synthesized in the ER is hypothesized to be regulated by *PSTA* and *PSTB* genes. The acronym stands for *PS t*ransport (either A or B pathways). Likewise, the transport of PE synthesized in the mitochondria or Golgi/vacuole is proposed to be regulated by *PEEA* and *PEEB* genes. The acronyms stand for *PE e*xport (either A or B pathways). Both known and proposed mutations along the metabolic and transport pathways appear in lower case italics. The table summarizes the mutants, genes and proteins that have been identified. Other abbreviations: pss, PS synthase; psd, PS decarboxylase, pem, PE methyltransferase; PI-4K, PI-4-kinase; PITP, PI transfer protein; Cho, choline; Etn, ethanolamine; Ser, serine.

the identification of drug-resistant mammalian cells [22] or isolation of Etn auxotrophs (described in more detail in [8]) in suitable genetic backgrounds in yeast.

Studies with mammalian systems have identified a mutant line of CHO cells, designated R-41, that is resistant to an antibiotic that recognizes PE and causes cytolysis [22]. The cells have normal enzyme activity for PS synthases and decarboxylase, but labeling with [^{14}C]serine reveals a defect in PE formation. Further analyses with isolated mitochondria demonstrate that the rate of import of PS from the outer to the inner mitochondrial membrane occurs at approximately 40% of the rate found for wild-type cells. By the scheme shown in Fig. 12 this cell line belongs to the *pst A* class of mutants. Although the mutant line has a lesion in PS import into mitochondria, protein import into the inner membrane is unaffected. This cell line now provides an important tool for cloning the complementing cDNA that encodes an element required for PS import into the mitochondria.

In the yeast system, selection for Etn auxotrophs has yielded a number of mutant strains and genes that are involved in aminophospholipid transport [8]. Currently, the majority of the work has provided information about the transport of nascent PS from the ER to the Golgi/vacuole compartment in the *pstB* arm of the pathway. One

mutant strain, *pstB1*, accumulates PS and shows reduced formation of PE. The gene complementing the defect, *STT4*, encodes a PI-4-kinase (P. Trotter, 1998). The results suggest that PI-4P or perhaps $PI4,5P_2$ may serve as a recognition motif on either the donor or the acceptor membranes participating in PS transport. Polyphosphoinositides are known to form recognition sites for the assembly of multiple protein components involved in membrane fusion machines [2].

A second mutant strain identified in the yeast system, *pstB2*, displays a 50% accumulation of PS and a 70% decrease in PE formation relative to wild-type strains when labeled with [^{3}H]serine. The gene that complements the defect, named *PSTB2*, is related to the PI transfer protein in yeast encoded by the *SEC14* gene. The protein encoded by the *PSTB2* gene, denoted PstB2p, is a PI transfer protein, but does not transfer PS (W. Wu, 2000). PstB2p is not a cofactor for the PS decarboxylase 2 but a protein that appears to regulate the access of PS to the enzyme. The site of action of PstB2p has now been shown to be at the acceptor membranes (W. Wu, 2001). The mechanism of action of PstB2p may be to dock donor and acceptor membranes, or it may act to regulate PI kinase activity in donor membranes.

4.3.2.4. Sphingolipids.

Ceramide transport from ER to Golgi. Ceramide is synthesized in the ER and the majority of this lipid is subsequently transported to the Golgi apparatus where it is metabolized to sphingomyelin and glycosphingolipids such as GlcCer and LacCer (Chapter 14) [14]. Measurement of conversion of ceramide to either sphingomyelin or glycosphingolipids can serve as an indicator of ceramide transport (Fig. 13). Hanada and coworkers have isolated mutant strains of CHO cells that are resistant to a toxin,

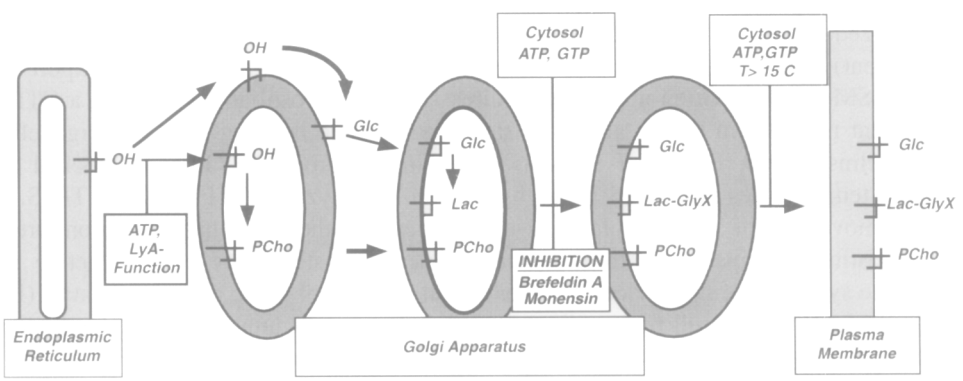


Fig. 13. Interorganelle transport of sphingolipids in eukaryotic cells from ER to plasma membrane. The structure (⊣) represents the Cer portion of sphingolipids. PCho, Glc and Lac are the abbreviations for phosphocholine, glucose, and lactose. Cer is transported to the Golgi in an ATP-dependent reaction. GlcCer is synthesized on the cytosolic face of the Golgi. SM is synthesized on the luminal face. The LyA mutation selectively affects the access of Cer to the site of SM synthesis. GlcCer must reach the luminal face of the Golgi for conversion to LacCer and more complex glycosphingolipids, denoted by GlyX moieties. Movement of the sphingolipids through the Golgi requires cytosol, ATP, and GTP and is inhibited by brefeldin A, monensin, GTPγS and reduced temperature. The $t_{1/2}$ for sphingolipid transport from the Golgi to the plasma membrane is 20 min.

lysenin, that binds cell surface SM and causes cytolysis [23]. One class of mutants (LyA) is selectively defective in SM synthesis, but not glycosphingolipid synthesis, despite normal activity of SM synthase. These findings suggest that the routing of Cer to SM synthase and GlcCer synthases is different and regulated by different gene products.

Ceramide transport to the locus of SM synthase demonstrates that the process can be reconstituted in permeabilized cells (T. Funakoshi, 2000) and requires the addition of ATP and cytosol. Depletion of ATP in intact cells also yields arrest of ceramide (and BODIPY-ceramide) trafficking to SM synthase. When wild-type permeabilized cells are reconstituted with cytosol from the LyA cells, ceramide transport-dependent SM synthesis does not occur. Thus, the LyA lesion resides in a soluble protein that participates in transport of nascent Cer to the locus of SM synthase. In contrast to these findings the GlcCer synthesis failed to exhibit a clear requirement for cytosol for ceramide transport to the enzyme.

Transport of newly synthesized sphingolipids from the Golgi to the plasma membrane. The synthesis and intracellular trafficking of SM and glucosylceramide (GlcCer) has been examined using several different fluorescent ceramides and short-chain radiolabeled ceramides [14]. When fibroblasts are incubated with NBD-Cer at 2°C, it is rapidly taken up and distributed randomly among all cell membranes (N. Lipsky, 1985). Upon warming the cells to 37°C, the fluorescent lipid concentrates in the Golgi apparatus as it is converted to NBD-SM and NBD-GlcCer. These sphingolipids are subsequently exported from the Golgi apparatus to the plasma membrane by a process that is partially monensin-sensitive and brefeldin-A-sensitive in most cells and occurs with a $t_{1/2}$ of 20 min, a time similar to that required for the transport of proteins from the Golgi to the plasma membrane (Fig. 13). However, there appears to be a pool of Glc–Cer that can be transported by routes insensitive to inhibitors of vesicle trafficking [14]. Vesicle-based protein transport is arrested in mitotic cells as is the transport of newly synthesized NBD-SM and NBD-GlcCer (Kobayashi, 1989). Experiments using (non-fluorescent) short-chain analogs of Cer in permeabilized cells indicate that the export of nascent SM from the Golgi apparatus requires ATP and cytosol and occurs via a GTP-dependent mechanism that is also consistent with vesicle budding from the organelle (J.B. Helms, 1990). Export of nascent sphingomyelin from the Golgi is blocked at reduced temperatures such as 15°C and by the non-hydrolyzable GTP analog, GTPγS.

The movement of sphingolipids between elements of the Golgi has been monitored in reconstituted preparations from mutant Chinese hamster ovary cells defective in either the synthesis of lactosylceramide or the attachment of sialic acid to the latter (B. Wattenberg, 1990). In cell free systems, donor Golgi that accumulate lactosylceramide transfer this lipid to acceptor Golgi that are devoid of the substrate. The acceptor Golgi add sialic acid to the lactosyl ceramide to make GM_3. The lipid transfer reaction between Golgi compartments requires ATP and cytosol and is inhibited by GTPγS. The properties of glycosphingolipid transport between Golgi compartments are thus identical to those found for vesicular protein transport.

Import of exogenous sphingolipids. The NBD and BODIPY analogs of SM, GlcCer, and LacCer can be readily inserted into the outer leaflet of the plasma membrane of fibroblasts at reduced temperature (see Fig. 3). When fibroblasts treated in such a manner are warmed to 37°C the fluorescent sphingolipids are internalized and accumulate in the

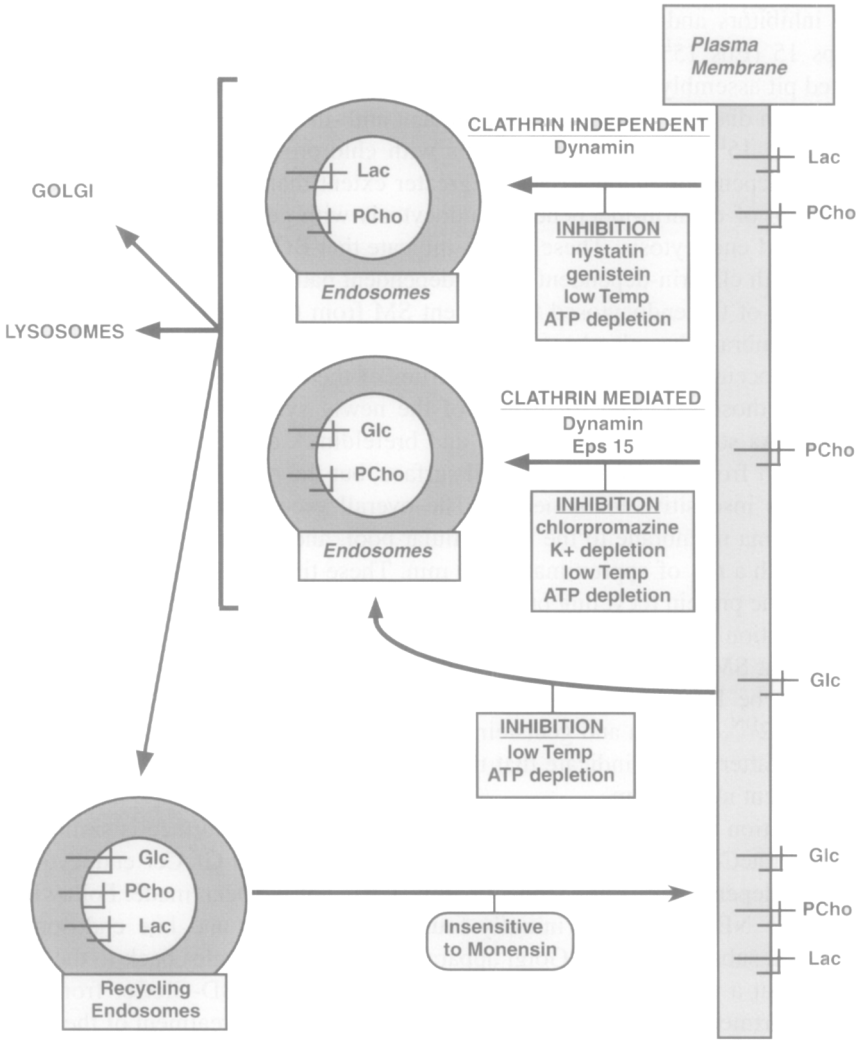


Fig. 14. Interorganelle transport of exogenously supplied sphingolipids. The structure ($\perp\!\!\!+$) represents the Cer portion of sphingolipids. PCho, Lac and Glc are the abbreviations for phosphocholine lactose and glucose, respectively. Following insertion into the plasma membrane at reduced temperature, SM is internalized to the endosomal compartments by both clathrin-dependent and -independent pathways. LacCer is endocytosed primarily by the clathrin-independent pathways. The endocytic process can be generally inhibited by ATP depletion and reduced temperature. The endocytosed lipid can recycle back to the plasma membrane and this recycling is insensitive to monensin. The endocytosed lipid can also be transported to the Golgi apparatus or lysosomes.

endosomal compartments of the cell. General inhibition of endocytosis by ATP depletion, or maintenance at low temperature, effectively prevents any internalization of the polar sphingolipids. Internalized NBD-SM subsequently accumulates in the perinuclear region of the cell containing the centrioles (M. Koval, 1989) and the Golgi apparatus (see Fig. 14). The initial steps of BODIPY-SM internalization have been dissected using

a variety of inhibitors and dominant negative structural variants of dynamin 2 (Dyn 2^{DN}) and Eps 15 (Eps 15^{DN}) [24]. The Eps 15 protein has a regulatory function in clathrin coated pit assembly. The internalization of BODIPY-SM is completely arrested by Dyn 2^{DN} which disrupts both clathrin-dependent and -independent endocytosis. The expression of Eps 15^{DN}, or treatment of cells with chlorpromazine or K^+ depletion, inhibits clathrin-dependent endocytosis to a greater extent than SM endocytosis. Conversely, inhibition of clathrin-independent endocytosis with genistein or nystatin does not fully block SM endocytosis. These results indicate that BODIPY-SM is likely to be internalized by both clathrin-dependent and -independent pathways.

The movement of the endocytosed fluorescent SM from the internalized pool, back to the plasma membrane has also been examined in fibroblasts (M. Koval, 1989). This transport process occurs via vesicles. The properties of the recycling pool of NBD-SM are distinct from those observed for export of the newly synthesized SM out of the Golgi (Fig. 13). As stated above, monensin and brefeldin A arrest newly synthesized NBD-SM transport from the Golgi to the cell surface, but the recycling of endocytosed fluorescent SM is insensitive to monensin. The overall process of internalization of SM from the plasma membrane to the intracellular pool, and transport back to the cell surface occurs with a $t_{1/2}$ of approximately 40 min. These time constants are similar to those for membrane protein recycling processes from the plasma membrane.

The internalization of BODIPY-LacCer follows a route that partially overlaps with that for fluorescent SM [24]. The BODIPY-LacCer is internalized into endosomes and subsequently can be localized to the Golgi. The endocytosis of BODIPY-LacCer is inhibited by Dyn 2^{DN}, nystatin and genistein but not Eps 15^{DN}, chlorpromazine, or K^+ depletion. These latter results indicate that the fluorescent LacCer is endocytosed by a clathrin-independent mechanism.

The internalization and recycling of NBD-GlcCer from the cell surface is similar to that for SM and LacCer analogs [14,25]. The assignment of NBD-GlcCer endocytosis to either clathrin-dependent or -independent pathway has not yet been made. Following internalization the NBD-GlcCer is initially found in both early and late endosomal compartments and subsequently the Golgi apparatus. The lipid recycles back to the cell surface (Fig. 14) at a rate similar to that for SM. Transport of NBD-GlcCer from the endosomal compartment to the plasma membrane is insensitive to treatment of the cells with either monensin or brefeldin A.

4.3.2.5. Cholesterol.

Transport of cholesterol to and from the plasma membrane. Following its synthesis at the ER, cholesterol is transported throughout the cell and becomes enriched in the plasma membrane [9]. The transport of newly synthesized cholesterol to the plasma membrane has been examined in tissue culture cells using pulse-chase experiments with either the rapid plasma membrane isolation procedure (M. Kaplan, 1985), caveolae isolation (A. Uittenbogaard, 1998), oxidation of accessible cholesterol by cholesterol oxidase (Y. Lange, 1985), or desorption of newly labeled cholesterol with methyl-β-cyclodextrin (S. Heino, 2000). These lines of experimentation have revealed that the minimum transport time for cholesterol to the plasma membrane is 10 min at 37°C (Fig. 15). The transport process can be completely blocked by reducing the temperature

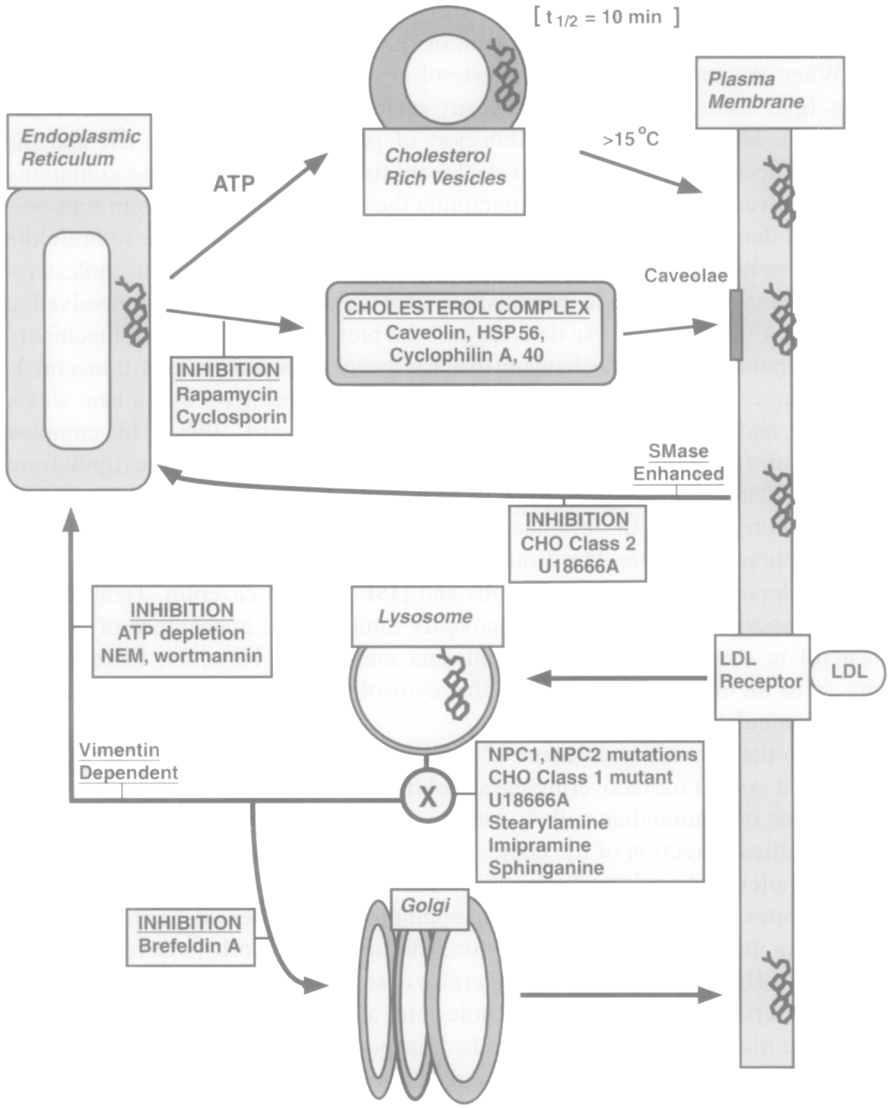


Fig. 15. Interorganelle transport of cholesterol. Newly synthesized cholesterol is transported from the ER to the plasma membrane in an ATP- and temperature-dependent process. One intermediate identified in this transport is a low-density cholesterol-rich fraction believed to be comprised of vesicles. A second proposed intermediate consists of a soluble cholesterol/protein complex. It is not clear if the vesicle fraction and soluble complex are the same. Cholesterol present in the plasma membrane can be induced to move to the ER by SMase treatment of the cell surface. This latter process is inhibited by hydrophobic amines and class 2 mutations in CHO cell lines. Low-density lipoprotein (LDL) derived cholesteryl ester enters the lysosome and is cleaved to form free cholesterol. The lysosomal cholesterol is exported from the lysosomes by a process regulated by NPC1 and NPC2 gene products that is susceptible to inhibition by hydrophobic amines. The cholesterol exported from the lysosome also traverses the Golgi en route to the plasma membrane, or travels directly to the ER via a process that exhibits partial dependence on intermediate filaments and requires ATP.

to 15°C or depleting cellular ATP levels with metabolic poisons. The transport of nascent cholesterol is unaffected by treatment of the cells with cytoskeletal poisons or monensin. When the translocation of cholesterol is inhibited by maintaining the cells at 15°C, this lipid accumulates in a low-density membrane fraction (M. Kaplan, 1985; Y. Lange, 1985). Intermediates in the transport of proteins between the ER and the Golgi apparatus accumulate at 15°C in vesicles of similar density to those containing cholesterol. However, the compartment containing the intermediates in protein transport is different from that containing cholesterol because the former is sensitive to brefeldin A treatment, whereas the latter is not [26]. This result demonstrates that cholesterol travels to the plasma membrane via intermediates that are distinct from those involved in protein transport. Collectively, these data suggest the presence of specialized machinery for cholesterol transport. One mechanism that has been proposed for this transport is non-vesicular and consists of a complex of caveolin with cholesterol and a heat shock protein, HSP 56, and the cyclophilins A and 40 (A. Uittenbogaard, 1998). This complex is believed to form a cytosolic cholesterol carrier that can transport the lipid from the ER to a caveolae-rich fraction of plasma membrane. Pulse-chase experiments with [^{3}H]acetate are consistent with caveolae serving as an entry point for cholesterol at the plasma membrane (Chapter 11). Both cyclosporin A and rapamycin are predicted to disrupt the interactions of the cyclophilins and HSP 56 with caveolin. Treatment of cells with cyclosporin A and rapamycin markedly inhibited the appearance of nascent [^{3}H]cholesterol in caveolae and the total plasma membrane. However, there is still uncertainty as to the relationship between this cytosolic complex and the low-density fraction that accumulates at 15°C.

In addition to the outward movement of cholesterol to the plasma membrane, cells display a retrieval system for recovering the sterol from the plasma membrane. When the plasma membrane of mammalian cells is rapidly depleted of SM by sphingomyelinase treatment, a significant fraction of the cholesterol is transported to the ER and esterified by acyl CoA : cholesterol acyltransferase [27]. The cholesterol retrieval is blocked by hydrophobic amines including U18666A, and sphingosine, and steroids such as progesterone [27] but is insensitive to ATP depletion. Liscum and coworkers have isolated a cell line denoted CHO 3–6 (N. Jacobs, 1997) (also described as a Class 2 mutant) that is defective in recovering plasma membrane cholesterol after sphingomyelinase treatment, that should prove most useful for dissecting the transport mechanism.

Recycling of exogenous cholesterol. Exogenous cholesterol imported into the cell via the low-density lipoprotein (LDL) receptor can be utilized for membrane biogenesis and regulation of sterol metabolism (Chapter 15). The mechanisms whereby lipoprotein-derived cholesterol (generated from cholesteryl esters within lysosomes) is disseminated throughout the cell is being understood with increasing detail (Liscum, 1999; E. Blanchette-Mackie, 2000). The current view indicates that approximately 70% of the lysosomal cholesterol pool is directed to the plasma membrane, whereas 30% is directed to the ER by a separate pathway. One set of inhibitors or mutations appears to affect the export of cholesterol from the lysosomes before the bifurcation of the transport between the plasma membrane and the ER. These early acting conditions include U18666A, imipramine, Niemann–Pick C mutations and CHO class 1 mutants. Subsequent to the bifurcation in the pathway the routing to the plasma membrane is sensitive to brefeldin

A disruption of the Golgi. After the bifurcation the transport to the ER is sensitive to disruption of intermediate filaments, ATP depletion, *N*-ethylmaleimide treatment, and (weakly) wortmannin intoxication.

Important insights into the mechanism of cholesterol transport have come from LDL metabolism in cells from individuals with Niemann–Pick type C (NPC) disease. In NPC fibroblasts, cholesterol transport from the lysosomal compartment to the plasma membrane is markedly retarded compared to that in normal fibroblasts (Liscum, 1999; E. Blanchette-Mackie, 2000). The NPC cells also have impaired regulation of acyl CoA : cholesterol acyl transferase, 3 hydroxy-3-methyl glutaryl CoA reductase and LDL receptor levels, in response to LDL. In addition, free cholesterol accumulates in the lysosomal compartment. In contrast, the transport of newly synthesized cholesterol from the endoplasmic reticulum to the plasma membrane of NPC fibroblasts is essentially identical to that found for normal cells. These findings localize one abnormality of NPC disease to cholesterol export from the lysosomes to other organelles.

Two genes, NPC1 and NPC2 are now known to be responsible for the NPC phenotype. The NPC1 gene product shows significant homology to the morphogen receptor, *Patched*, to a protein of unknown function, NPCL1, and to members of the RND (resistance–nodulation–division) family of prokaryotic permeases. NPC1 has sequence homology to proteins containing sterol sensing domains (3-hydroxy-3-methylglutaryl CoA reductase and SREBP cleavage activating protein, Chapter 15). NPC1 is also closely related to bacterial permeases that transport hydrophobic compounds including acriflavine [28,29]. Consistent with the proposed permease function, NPC1-defective cells cannot efficiently export acriflavine out of their lysosomes. The characteristics of NPC1 protein structure most closely resemble those of fatty acid permeases, and expression of human NPC1 protein in *E. coli* markedly enhances the uptake of oleic acid by the bacteria. Thus a specific biochemical function assignable to NPC1 is modulation of fatty acid transport. The relationship between this defined function and the export of cholesterol remains to be clarified.

The NPC2 gene has also been identified [29]. The gene product is a soluble lysosomal protein that was identified in a global proteomics screen of lysosomal constituents. The protein is found in secreted and intralysosomal forms, with retrieval of the secreted protein mediated by the mannose-6-P receptor. Incubation of NPC2-defective cells with medium containing the secreted form of wild-type NPC2 leads to protein uptake and rectification of the cholesterol accumulation seen in the mutant cells. In addition, individuals with the NPC2 phenotype (which is identical to that for NPC1) show specific mutations in the NPC2 gene, thereby confirming the gene-mutation–disease relationship. The emerging picture is that soluble NPC2 and membrane-bound NPC1 must cooperate in the recognition and translocation of cholesterol out of the lysosome.

Cholesterol import into mitochondria. In steroidogenic tissues cholesteryl esters are hydrolyzed in response to hormonal stimuli, and cholesterol is imported into mitochondria for the synthesis of pregnenolone, the precursor of all steroid hormones. The transit of cholesterol between the outer and inner mitochondrial membrane is regulated by steroidogenic acute regulatory protein (StAR) [30]. StAR is rapidly synthesized in response to hormonal stimuli and targeted to the mitochondria by N-terminal sequences. The StAR protein is imported into the mitochondria and becomes

associated with the matrix and inner membrane. Initially, transit intermediates of StAR were proposed to be the cholesterol carriers between the outer and inner membranes. However, current data demonstrate that import of StAR is not essential and that the association of the C-terminus of StAR with the outer mitochondrial membrane may be the critical interaction required for promoting cholesterol transfer. The mechanism of StAR action is not understood, but interaction of the C-terminus with cholesterol and outer membrane proteins may play a role in assembling a transport complex that moves the lipid to the inner membrane. Individuals with lipoid congenital adrenal hyperplasia lack functional StAR and are unable to make pregnenolone. Analysis of the StAR sequences in these individuals reveals that the mutations accumulate in the carboxy terminal region of the protein. The carboxy terminus of StAR and a structural homolog MLN-64 that binds cholesterol are now recognized to define a large protein family capable of binding hydrophobic molecules (L.M. Iyer, 2001).

4.3.2.6. Phospholipid transfer proteins and membrane biogenesis. Since their discovery in the early 1960s, the PLTPs have been attractive candidates for soluble lipid carriers between membranes in vivo (K. Wirtz, 1968). However, there have been two major points of debate about a lipid trafficking role for the PLTPs. The first point has remained that the proteins effect exchange of lipid between model membranes and do not yield a net transfer of mass. Such an action by the proteins in vivo might be able to change lipid composition of membranes but would not be able to cause net synthesis and accumulation of new lipid mass. However, to be circumspect one cannot rule out the possibility that either protein modification or protein–protein interactions in vivo, may enable the proteins to accomplish net transfer. A second point of concern has been whether the lipid binding and exchange of the proteins simply reflects a lipid binding property that has other functions.

Genetic approaches addressing the role of PLTPs have provided significant insight into their function [31]. The identification of the *SEC14* gene and its corresponding Sec14p protein, as the major PI/PC transfer protein in yeast enabled critical tests of in vivo function. The *SEC14* gene was shown to be essential and play an important role in regulating protein traffic through the Golgi. Tests of the required function of Sec14p demonstrate that the ability of the protein to effect transfer of PI is completely dispensable (S. Phillips, 1999). The PC bound form of Sec14p is now thought to be crucial to its function. In the PC bound form, Sec14p is proposed to be a negative regulator of phosphocholine cytidylyltransferase (Chapter 8) (H.B. Skinner, 1995). The consequences of this regulation of the cytidylyltransferase are thought to be steady state maintenance of a Golgi diacylglycerol pool required for secretory vesicle formation. Other functions of Sec14p have also been proposed including the regulation of polyphosphoinositide pools that are required for operation of the secretory pathway. This latter idea finds support from data in other systems that show a requirement for PI transfer proteins in either exocytosis or signal transduction that appear related to modulation of polyphosphoinositide pools [32].

Another prominent test of in vivo lipid transfer protein function has focused upon the nonspecific lipid transfer protein known as SCP2. Early studies suggested that SCP2 played an important role in intracellular cholesterol traffic. However, antibodies

raised to the protein localized the antigen primarily in peroxisomes, rather than the predicted cytosolic compartment. More detailed analysis revealed that SCP2 arose as a proteolytic fragment of the peroxisomal enzyme 3-ketoacyl-CoA thiolase (Chapter 5). These findings were more consistent with a role for SCP2 in peroxisomal β-oxidation than cholesterol traffic. The development of gene-targeted mice with defects in SCP2 expression revealed a lesion in pristanic acid oxidation and in cholesterol side-chain oxidation [33] but not in intracellular lipid traffic. Collectively, these data fail to define any role for SCP2 in cholesterol transport.

The in vivo role of PC transfer protein has also been examined by genetic deletion in mice (A. van Helvoort, 1999). Animals with homozygous null alleles for the PC transfer protein had no identifiable phenotype. Examination of tissues with high rates of synthesis and secretion of PC, such as liver and lung, also failed to show any perturbation in lipid traffic or metabolism, indicating that the PC transfer protein cannot be essential for these processes.

Thus far, critical biochemical and genetic studies of lipid transfer proteins do not demonstrate the proteins act as lipid carriers that directly function in the transport reactions for membrane biogenesis. However, the issue of a role for such proteins remains open, in light of the observations that some lipids (e.g., PE) move with surprising rapidity between membranes in reactions that are not demonstrably ATP-dependent. Certainly, other mechanisms are possible for such rapid lipid transport reactions, but additional data are required before a role lipid for transfer proteins can be ruled out.

5. Future directions

Lipid transport is a fundamental process essential to all cell growth, division and differentiation. Our understanding of lipid transport has changed markedly in the last five years, and the pace of change is now increasing. Most notably, the identification of mammalian cell lines, yeast, and bacterial strains with defects in lipid traffic is a major advance. The identification of human diseases with lesions in lipid traffic makes additional important tools, and in some cases cell lines, available. Advances in reconstitution of lipid traffic in permeabilized cells now allow for more precise and critical tests of protein function in the processes. The application of fluorescent probes continues to provide new insights and real time images of selected aspects of lipid transport. As the examination of these processes now begins to enter the realm of the manipulation of mutant cells, genes, and gene products, there remains much to be accomplished. Future studies need continued focus on the development of new genetic tools. For many of the lipid trafficking processes described in this chapter there are still no mutants available, and a concerted effort must be made to develop novel selections and screens that attack the voids in our understanding. The current expansion in genomic information and the ease of manipulating genes in heterologous systems now also allows for approaches in which educated guesses can be used for targeting candidate sequences.

Any candidate sequence that appears in yeast can now be obtained in a hemizygous null strain from commercial sources. Straightforward manipulation allows for the

recovery of strains harboring null alleles that are covered by a plasmid-borne copy of the wild-type gene under inducible or repressible promoters. Such tools allow for rapid critical testing of gene product function in lipid traffic. In addition, phylogenetic jumping among databases can also permit the rapid identification, isolation, and testing of specific gene products in suitable in vivo and in vitro assay systems. The mechanisms of intracellular lipid traffic in membrane assembly have thus far been difficult to elucidate, but recent advances are grounds for much optimism. The current molecular tools, combined with new genetic strategies are likely to provide both new research opportunities and rewards for those who tackle this long-standing problem of cell biology.

Abbreviations

BODIPY	boron dipyrromethene difluoride
Cer	ceramide
DG	diacylglycerol
diC8-	dioctanoyl-
ER	endoplasmic reticulum
ESR	electron spin resonance
GlcCer	glucosylceramide
GTP	guanosine triphosphate
GTPγS	guanosine 5′-*O*-(-3-thiotriphosphate)
IAA	isethionylacetimidate
LDL	low-density lipoprotein
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PS	phosphatidylserine
PSD	phosphatidylserine decarboxylase
MAM	mitochondria-associated membrane
mdr	multidrug resistance
NBD	*N*-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]-6-aminocaproyl
NPC	Niemann–Pick type C
SM	sphingomyelin
TNBS	trinitrobenzenesulfonate

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