Perspectives in Biochemistry

Static and Dynamic Lipid Asymmetry in Cell Membranes[†]

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The asymmetrical organization of phospholipids in the human erythrocyte membrane, discovered by Bretscher (1972) and soon confirmed by other groups (Verkleij et al., 1973; Gordesky et al., 1975), has been followed by 15 years of investigations on the lipid topology in eukaryotic plasma membranes and membranes from organelles, bacteria, and viruses. Although the agreement between the different laboratories at a quantitative level is often unsatisfactory, most biological membranes appear to have a different phospholipid composition in their inner and outer leaflets. At least in plasma membranes, transverse lipid segregation is firmly established. In erythrocytes, the best documented system, phosphatidylserine (PS),[1] phosphatidylethanolamine (PE), and probably phosphatidylinositol (PI) are located mainly in the inner monolayer while phosphatidylcholine (PC) and sphingomyelin (SM) are essentially in the outer monolayer.

For many years, lipid asymmetry was considered as the natural consequence of the asymmetrical environment of all biomembranes. Knowing that transverse diffusion, or lipid flip-flop, is a slow process, lipid asymmetry was thought to be the consequence of asymmetrical membrane biogenesis and asymmetrical lipid turnover by endogenous phospholipases and reacylases, together with the asymmetrical insertion of lipid constituents. The differences in potential and/or pH between the two surfaces could also explain the stability of the asymmetrical distribution. However, in 1984, the existence of an ATP-requiring mechanism responsible for the specific translocation of aminophospholipids (PS and PE) was demonstrated in human red cells (Seigneuret & Devaux, 1984) and later in other plasma membranes [for a review, see Devaux (1988)]. Virtually at the same time the rapid redistribution of PC in rat liver endoplasmic reticulum was attributed to a "PC flippase" by Bishop and Bell (1985). Thus, it appeared that living cells had developed elaborate mechanisms to control the transmembrane distribution of phospholipids. The resulting lipid asymmetry could no longer be considered as a passive and unimportant phenomenon. This paper will focus on the dynamic aspects of lipid asymmetry in eukaryotes. Present ideas on the role of transmembrane lipid traffic will be discussed.

THE BILAYER PARADIGM

A basic assumption is that phospholipids in biomembranes are organized in a continuous bilayer. That this is the case, clearly, has not been demonstrated in all membranes. It is, however, a reasonable assumption. In spite of very extensive work undertaken notably by Cullis, de Kruijff, and co-workers (Cullis & de Kruijff, 1979), to date there is no example of permanent nonbilayer structure in a real biological membrane unambiguously demonstrated by X-ray, ^{31}P NMR, or electron microscopy. Lipids extracted from biomembranes frequently form hexagonal or cubic phases at physiological temperatures, but this by no means demonstrates the occurrence of such structures in the presence of proteins. Thus, I shall postulate that the phospholipids that surround membrane proteins indeed form a bilayer and that nonbilayer structures, if any, are only transient nonequilibrium configurations: topologically, inner and outer leaflets are well-defined.

The bilayer paradigm is not restricted to topological considerations. The description of a bilayer includes important considerations of dynamics. In the early seventies, it was shown in Mc Connell's laboratory that in the liquid-crystalline state (Luzzati's L_α phase) (i) the rate of lateral diffusion of phospholipids is fast, with in-plane exchange rates of the order of 10^7 s^{-1} (Devaux & Mc Connell, 1972), and (ii) the rate of transverse diffusion, or flip-flop, is slow (half-time of exchange of several hours) (Kornberg & Mc Connell, 1971). These

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[1] Abbreviations: PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin; PI, phosphatidylinositol; PG, phosphatidylglycerol; ER, endoplasmic reticulum; LUV, large unilamellar vesicle; TNBS, trinitrobenzenesulfonic acid.

Table I: Quantitative Results on the Phospholipid Asymmetry in the Plasma Membrane of Eukaryotic Cells

cell type	% in outer layer					ref
	PC	SM	PE	PS	PI	
red blood cell (man)	76	82	20	0		Verkleij et al., 1973
	78	79	21	8		Van Meer et al., 1981
red blood cell (rat)	62	100	20	6		Renooij et al., 1976
platelet (man)	62		54	6	34	Wang et al., 1986
	45	93	20	9	16	Perret et al., 1979
platelet (pig)	40	91	34	6		Chap et al., 1977
kidney brush border (rabbit)	34	80	23	15		Venien & Le Grimellec, 1988
intestinal brush border (rabbit)	26		28			Barsukov et al., 1986
heart sarcolemna (rat)	43	93	25	0		Post et al., 1988
embryo fibroblast (chick)			34	17		Session & Horwitz, 1983
embryo myoblast (chick)			66	46		Session & Horwitz, 1983
brain synaptosomes (mouse)			10–15	20		Fontaine et al., 1980
LM fibroblast (mouse)			4–6	5		Fontaine & Schroeder, 1979
hepatocytes (rat)						
bile canalicular surface	85	63	50	0	0	Higgins & Evans, 1978
contiguous surface	82	0	0	14	0	Higgins & Evans, 1978
sinusoidal surface	85	66	55	0	0	Higgins & Evans, 1978
Krebs II ascites (mouse)	51	46	45	20	30	Record et al., 1984

values, obtained with spin-labeled phospholipids in egg lecithin liposomes, were confirmed and refined by many investigators using independent techniques. Thus, one important feature of a pure lipid bilayer is the absence of phospholipid communication between the two opposite leaflets. Note that the rapid lateral diffusion implies that a few transmembrane defects or even very localized nonbilayer structures would suffice to allow the flip-flop of a large fraction of phospholipids. In pure lipid systems, these defects, unavoidable a priori, must have short lifetimes since the transmembrane diffusion is slow. It may not be true in protein-containing bilayers where a few permanent "defects" can modify the transmembrane lipid distribution. The fast lateral diffusion of membrane components extends to small hydrophobic molecules like cholesterol or free fatty acids; on the other hand, the transverse diffusion of the latter molecules, if uncharged, is fast (half-time of exchange of a second or less). Thus, the membrane impermeability is not true for neutral lipids (diacylglycerol, fatty esters, and probably cholesterol) as well as for weak acids and weak bases [see Zachowski and Devaux (1989) and references cited therein].

The third dogma of the bilayer paradigm is that unlike lateral segregation spontaneous transmembrane phospholipid segregation is exceptional. It only happens with vesicles of small radius obtained by sonication (Op den Kamp, 1979). As a consequence, asymmetrical large unilamellar vesicles (LUV), which can be obtained for instance by selective chemical modifications of the lipids or by the addition of charged amphiphilic molecules on one layer, are out of equilibrium and relax toward a random distribution. LUVs that are made asymmetrical by the effect of a pH gradient (Hope & Cullis, 1987) can be considered also as out of equilibrium since the release of this external constrain results in the bilayer symmetrization.

This overview of the bilayer paradigm was meant to be used as a reference for description of biomembranes. Clear departure from the above behavior will demonstrate the influence of proteins and generally imply nonequilibrium situations. It rapidly becomes apparent that phospholipid transmembrane distribution in biomembranes has to be viewed in the context of the lipid traffic within cells; i.e., dynamic considerations are necessary.

Lipid Asymmetry in Biomembranes

The techniques used to determine lipid asymmetry in biomembranes were reviewed by several authors (Op den Kamp, 1979; Etemadi, 1980; Krebs, 1982). These techniques comprise chemical labeling with nonpenetrating agents, for example, with TNBS or fluorescamine, immunological methods, phospholipase digestion of membrane phospholipids, use of phospholipid-exchange proteins, and physicochemical methods such as X-ray diffraction and NMR. When carrying out these experiments, an implicit and sometimes unjustified assumption is that the lipid topology is stable over the reaction time and can resist membrane perturbations such as attack by exogenous phospholipases. Clearly, in the case of fast flip-flop, some of these techniques are no longer valid. Also, if lipid asymmetry is the result of a subtle balance between various lipid fluxes, membrane isolation is likely to lead to erroneous results. This is particularly true for the membranes of organelles.

In the case of the human erythrocyte membrane, different techniques and different laboratories converge to the view that is summarized in Table I. Variations of the order of 10% exist between the different reports dealing with normal erythrocytes. Thus, significant deviations must be larger than 10%. Abnormal distributions have been reported for aged red cells, sickle cells, malaria infected red cells, etc. (Shukla & Hanahan, 1982; Lubin et al., 1981; Gupta & Mishra, 1981). In addition to the asymmetry of the head-group distribution, it has been reported that the average fatty acid composition of PS and PE shows more unsaturation than in the case of PC and SM (Middelkoop, 1989); furthermore, within the same class of phospholipids (SM or PE), acyl chains from the outer layer differ from those of the inner layer (Boegheim et al., 1983; Hullin & Salem, 1989). Cholesterol transmembrane distribution in erythrocytes has been investigated also by several laboratories but without conclusive results. See a discussion in Zachowski and Devaux (1989). Erythrocytes from other mammals have given results similar to those in human, although the proportion of the four main phospholipids varies. The situation is more complex with other eukaryotic cells because the plasma membrane is only a small fraction of the total cell membranes. Nevertheless, membrane purification by differential centrifugation, as well as the analysis of virus membranes that are formed by budding through a plasma membrane, has allowed one to identify the underlying pattern obtained with human erythrocytes. Table I indicates the systems that have been investigated and provides a quantitative summary of the results. Note that chick embryo myoblasts are the only cells for which it is reported that a large fraction of PS is present on the exterior surface.

Many attempts to investigate lipid asymmetry in internal membranes from eukaryotic cells can be found in the literature. Unfortunately, when the same systems have been studied by different laboratories, the results often differ considerably. The difficulty in finding conclusive evidence on lipid topology in organelles may reflect the very rapid lipid redistribution within each membrane and from membrane to membrane within each eukaryotic cell. Massive transfer of phospholipids from the ER to the Golgi and to the mitochondria and from the mitochondria back to the ER and from the Golgi to the plasma membrane takes place continuously (Bishop & Bell, 1988). For example, according to Wieland et al. (1987) about half of the total ER phospholipids is transferred out of the Golgi every 10 min.[2] On the other hand, it is conceivable that specialized organelles in differentiated cells such as granules in chromaffin cells, synaptic vesicles in axons, and disks in retinal rods have a well-defined phospholipid asymmetry because the turnover of the lipids in such systems is not so important. In the above mentioned organelles, the aminophospholipids were generally reported to be exposed on the outer monolayer, which corresponds to the cytosolic surface (Buckland et al., 1978; Michaelson et al., 1983; Litman, 1982).

Very little is known concerning lipid asymmetry in plant cells. Reports have appeared concerning the topology of lipids in bacteria and viruses. The lipid asymmetry in virus most likely reflects the asymmetry of the infected cells and should eventually be randomized after a long incubation at 37 °C [for a review, see Op den Kamp (1979)].

TRANSMEMBRANE MOVEMENT OF LIPIDS IN BIOMEMBRANES

There are many indications in the literature to suggest that lipid topology in biomembranes is not a static situation but rather the result of differential transmembrane movements involving specific lipid–protein interactions. The following paragraphs outline the various mechanisms of transmembrane passage of lipids in biological membranes.

Simple Diffusion. Phospholipid transverse diffusion rates in biomembranes were first measured with PC or PC analogues, and values reported were comparable to those obtained in model systems, i.e., without proteins. Thus, the transmembrane passage of phospholipids in biological membranes was thought to be a passive diffusion process through the lipid bilayer. Half-times of translocation as long as weeks have been reported in virus (Rothman et al., 1976), but in general the transmembrane diffusion of PC in biomembranes was estimated to be of several hours, with a significant variation according to the chain lengths and degrees of unsaturation. For example, in the human red cell membrane, at 37 °C, Middelkoop and collaborators found that 1,2-dipalmitoyl-, 1,2-dioleoyl-, 1-palmitoyl-2-arachidonoyl-, and 1-palmitoyl-2-lineoyl-PCs have half-time values for the rate of their transbilayer equilibration of 26.3, 14.4, 9.7, and 2.9 h respectively (Middelkoop et al., 1986). Thus, phospholipids with at least one unsaturated chain have a more rapid flip-flop. Comparable values were obtained in isolated organelles from eukaryotes. For example, a transmembrane diffusion of several hours was found for spin-labeled PC in the inner mitochondrial membrane from rat liver (Rousselet et al., 1976) and also in adrenal medulla chromaffin granules (Zachowski et al., 1989). However, in some biological membranes different time scales were reported, suggesting that membrane proteins can modify
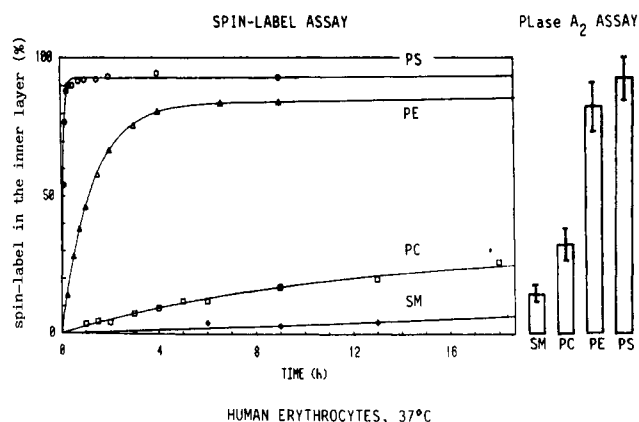

FIGURE 1: Spontaneous passage of spin-labeled phospholipids from the outer to the inner monolayer of human red blood cells at 37 °C. The figure shows the percentage of labels in the inner monolayer as a function of time: PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin. The spin-labels are slightly water soluble because of a short β chain bearing a nitroxide. This property allows their rapid incorporation in the cells' outer monolayer after incubation with erythrocytes. The transmembrane distribution is assayed at different intervals by the back-exchange technique. The right part of the figure shows the percentage of endogenous lipids in the inner monolayer as determined by the phospholipase A2 technique. Data are taken from Morrot et al. (1989) and Verkleij et al. (1973).

drastically the rate of transmembrane diffusion of phospholipids either by facilitated diffusion, by active transport of lipids, or by selective interaction.

Facilitated Diffusion. Using a short-chain phospholipid (dibutyroyl-PC), Bishop and Bell discovered a PC translocation saturable and sensitive to proteases and chemical modifications in endoplasmic reticulum vesicles from rat liver (Bishop & Bell, 1985). They postulated the existence of a specific protein, which they called "flippase". The existence of the flippase was confirmed by reconstitution experiments using microsomal proteins (Backer & Dawidowicz, 1987). The existence of distinct transport systems for PC metabolites was also postulated (Kawashima & Bell, 1987). Finally the translocation of amphiphilic spin-labeled phospholipids was assayed in microsomes, and a NEM-sensitive fast diffusion of PC, PE, PS, SM, and lyso derivatives was observed with the same half-time of approximately 20 min (Herrmann et al., 1990). This process, which is a nonenergy-requiring catalysis of phospholipid flip-flop, can be classified as a facilitated diffusion. The same process may exist in sarcoplasmic reticulum and perhaps in bacteria, but this has not been demonstrated so far.

Active Transport: The Lipid Pump. Active translocation of phospholipids in human red blood cells was described for the first time in 1984 (Seigneuret & Devaux, 1984). By use of spin-labeled phospholipids, it was shown that the aminophospholipids (PS and PE) are selectively transported from the outer to the inner leaflet of erythrocytes, providing the cells contained hydrolyzable MgATP ($\sim$1 mM). Figure 1 shows that the half-times of outside–inside movement of PS and PE analogues are 5 and 60 min, respectively, at 37 °C. Thus, the movement is considerably faster than the passive diffusion. Similar observations in red blood cells were made by several groups working with other techniques involving short-chain lipids (Daleke & Huestis, 1985), long-chain radioactive lipids (Tilley et al., 1986), or fluorescent lipids (Connor & Schroit, 1987). This phenomenon cannot be explained by a simple potential effect, since the interior of the red cell is negative and attracts the negatively charged PS molecule. The specific translocation of aminophospholipids would be due to an en-

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[2] This emphasizes by contrast the simplicity of the erythrocyte situation.

zyme, aminophospholipid translocase, which is NEM-sensitive ($\sim$0.3 mM), vanadate-sensitive (50 μM), and Ca^{2+}-sensitive (inhibition by 0.2 μM cytosolic Ca^{2+}). Concentration dependence of the initial rate of translocation was demonstrated as well as the competition between PS and PE: PS has an apparent K_m 10 times lower than that of PE (Zachowski et al., 1986). A systematic investigation of the lipid specificity of this protein-mediated translocation was undertaken with spin-labeled analogues (Morrot et al., 1989). This study revealed that not only the head group is recognized by the aminophospholipid translocase but also the glycerol backbone as well as the ester bonds. At least a short β chain is necessary, since lyso derivatives are not efficiently transported.

As shown in Figure 1, for each category of phospholipid, the percentage of spin-labeled phospholipids inside, at equilibrium is remarkably similar to that of the corresponding endogenous lipid on the inner monolayer. If cells are partially depleted of ATP, the PS plateau reached by the spin-labels remains at the same level as long as the ATP concentration exceeds about 0.2 mM; the PE plateau, on the other hand, is more sensitive to the ATP concentration and decreases from 80% to $\sim$50% inside when the cytosolic ATP concentration falls below $\sim$0.5 mM. The same observation on their respective plateau levels was made when a fraction of the aminophospholipid translocase was inhibited by NEM or Ca^{2+}. These observations suggest that the plateaus, or equilibrium distribution, are in fact steady states corresponding to a balance between inward and outward movements. A kinetic interpretation of the data will be presented later in this review.

To date the aminophospholipid translocase has not been purified. This may be a difficult objective since it must correspond to a minor protein in the red cell membranes. When reasonable guesses on the turnover of this enzyme are made from the comparison with other ATPases, it can be estimated that the number of copies in a red cell is indeed very limited: less than 1000 copies per cell. Radioactive photoactivable PS has been used by two laboratories in an attempt to determine the molecular weight of the protein. Unfortunately, many bands are labeled by these PS analogues (Schroit et al., 1987; Zachowski et al., 1987a). Nevertheless, Connor and Schroit concluded from their experiments that the translocase was a 32-kDa protein that is labeled also by sulfhydryl reagents (Connor & Schroit, 1988). This molecular weight is similar to that of the $Rh_0(D)$ protein. However, Smith and Daleke have shown recently that in Rh_{null} cells depleted in $Rh_0(D)$ protein the translocase activity is not affected (Smith & Daleke, 1990). Alternatively, the aminophospholipid translocase could be a vanadate-sensitive Mg^{2+}-ATPase of molecular mass 115–130 kDa, which is present in red cells and in several plasma membranes of eukaryotes and whose function is yet unknown (Gantzer & Grisham, 1979; Damiani et al., 1987). This ATPase in detergent is selectively reactivated by PS and less efficiently by PE (Morrot et al., 1990). Positive identification of the aminophospholipid translocase awaits successful reconstitution experiments.

The same ATP-dependent translocation of aminophospholipids, susceptible to chemical protein reagents, has been found in plasma membranes from lymphocytes (Zachowski et al., 1987b), platelets (Sune et al., 1987), cultured hamster fibroblasts (Martin & Pagano, 1987), and synaptosomes from Torpedo electric organ (Zachowski & Morot Gaudry-Talarmain, 1990). Recently this activity was found in the membrane of a cell organelle, namely, adrenal chromaffin granules (Zachowski et al., 1989). In the granules, the amino-

phospholipid translocase is oriented in such a way so as to accumulate PS on the external monolayer that is the cytosolic surface of this organelle. Interestingly, chromaffin granules possess a membrane-bound Mg-ATPase of molecular mass 115 kDa, i.e., of the same molecular mass as the erythrocyte Mg-ATPase.

Lipid Segregation by pH or Potential Gradient. The influence of a transmembrane pH gradient on the distribution of phospholipids was thoroughly investigated by Cullis and collaborators in large unilamellar vesicles (LUV) (Hope & Cullis, 1987; Hope et al., 1989; Redelmeier et al., 1990). They showed that lipids which exhibit weak acid or weak base characteristics, such as free fatty acids, rapidly redistribute across LUV membranes in response to a transmembrane pH gradient. Certain phospholipids, such as PG and PA, also cross a lipid bilayer under the influence of a pH gradient, and an asymmetrical distribution can be created. The transmembrane movement of PG occurs via permeation of the uncharged (protonated) dehydrated form, which can exhibit half-times of transbilayer transport of the order of seconds. The same phenomenon is expected to happen in cells. Very likely the trapping of chlorpromazine in the interior of red blood cells can be explained by the slightly acidic interior of erythrocytes. Chlorpromazine probably crosses the cell membrane in its neutral form and is afterward trapped in the inner leaflet in its charged form. The same hypothesis can be made for other amphiphilic drugs like vinblastine and primaquine.

On the other hand, an electric field is not sufficient to pull a charged phospholipid through a bilayer. For example, Hope and Cullis found that PS does not flip through LUVs under the influence of a potential gradient (Hope & Cullis, 1987). Only very high pulsed electric fields that disrupt locally the membrane (electropermeation) seem to be able to scramble partially the phospholipid distribution in red cells (Dressler et al., 1983).

Lipid (Re)distribution Accompanying a Transient Membrane Perturbation. During the cell's lifetime, various events can perturb the bilayer organization and produce a partial lipid redistribution. Some of these events are spontaneous, such as endo- and exocytosis; some are artificial manipulations, for example, drug addition or electropermeation. Fusion of two membranes implies a local and transient departure from the bilayer structure. It is probably irrelevant to try to classify these temporary structures as hexagonal, micellar, or cubic phases, but the net result is that a fraction of the lipids is redistributed. The increase of cytosolic Ca^{2+} (or Ca^{2+} burst) would be responsible for the trigger of such exocytic event. A possible molecular mechanism would be the stimulation by Ca^{2+} of an endogenous phospholipase C, which would form diacylglycerol, a membrane-destabilizing and fusogenic agent (Siegel et al., 1989). Whether this is the general mechanism of exocytosis is still to be demonstrated, but at least in model systems it has been shown that physiological levels of diacylglycerols in phospholipid membranes induce nonbilayer structures and membrane fusion (Siegel et al., 1989). Alternatively, Ca^{2+} could trigger a phospholipid flippase with low phospholipid selectivity.

Lipid scrambling can be caused also by cell lysis. Williamson et al. (1985) have shown that if 1 mM Ca^{2+} is present during red blood cell lysis and resealing, asymmetry is at least partially abolished as judged by the observation that much more PE becomes accessible to digestion by externally added lipase and that merocyanine is able to bind the external leaflet. Similarly, it has been shown by ESR and by photobleaching that after hemolysis the asymmetrical viscosity of the red cell

bilayer is abolished, indicating at least a partial randomization of the lipid layers (Tanaka & Ohnishi, 1976; Morrot et al., 1986).

Addition of membrane binding drugs at high concentration can provoke lipid reorientation. For example, addition of chlorpromazine to red blood cells at sublytic concentration ($\sim$0.5 mM) is accompanied by a redistribution of a small fraction ($\sim$10%) of the four main phospholipids (PS, PE, PC, and SM). After incubation in the presence of the drug, the asymmetry is progressively reestablished at least for PS and PE (Schrier et al., 1983; Rosso et al., 1988). The insertion of proteins in bilayers can be responsible also for a transient redistribution of phospholipids. It was reported that the binding of cytochrome b_5 to model membranes accelerates transmembrane movement of lipids (Greenhut & Roseman, 1985). Finally bacterial cytotoxins, amphotericin B, and local anesthetics such as tetracaine enhance transbilayer mobility of phospholipids in erythrocyte membranes (Schneider et al., 1986).

Electropermeation is a drastic membrane treatment that is accompanied by partial lipid reorientation (Dressler et al., 1983). Electropermeation and electrofusion are achieved by submitting the membranes to high electric fields (transmembrane potential above 250 mV). The exact nature of the holes formed is not known, but it has been reported that the electric pulses induce the formation of transient nonbilayer structures as judged from the ^{31}P NMR line shapes (Lopez et al., 1988).

In conclusion, it appears that although transmembrane phospholipid motion is a slow process in liposomes, there are many ways, often involving specific proteins, by which lipid flip-flop can be accelerated either unidirectionally or bidirectionally.

ORIGIN OF LIPID ASYMMETRY IN BIOMEMBRANES

In the plasma membrane of eukaryotes, the aminophospholipid asymmetry appears to be due to the ATP-dependent aminophospholipid translocase activity that counterbalances the spontaneous lipid randomization by flip-flop. The simplest model to account for the experimental kinetic data obtained with spin-labeled lipids (Figure 1) is a diffusion model in which K_{in} and K_{out} are different (Herrmann & Muller, 1986). At least K_{in} must be ATP-dependent to account for the influence of the carrier protein. This model when applied to the data obtained for internalization of spin-labeled aminophospholipids leads to the hypothesis that there is a relatively rapid outward movement as well, suggesting in turn that the inside–outside motion of aminolipids is also at least partially catalyzed by a protein (Bitbol & Devaux, 1988). By analyzing in the framework of a similar model the data of Seigneuret and Devaux (1984), Williamson and collaborators concluded that the same protein-catalyzed outward motion was ATP-dependent and that the translocase had the same efficiency for the inward and outward movements. To explain the phospholipid asymmetry, they implicated the role of spectrin (Williamson et al., 1987). However, a more elaborated model in which the translocase is described within the classical formalism used for carrier proteins, in particular a model wherein the initial velocity of translocation versus the concentration of labels added on the outer layer obeys the Michaelis–Menten law (i.e., is saturable), enables one to account for the observed plateaus of PS or PE without assuming ATP dependence of the outward movement or trapping by the cytoskeleton (Bitbol and Devaux, unpublished).

In practice, the inward motion of PS, even at low ATP concentration ($\sim$0.2 mM), is so rapid that the outward motion of PS is almost negligible (plateau above 95%). But, because the K_m for PE is higher, the inward movement of that lipid is sensitive to any variation of ATP level in the range 0.5–4 mM, and this affects the level of the plateau. For the same reason, the fraction of PE on the inner monolayer in the steady state is sensitive to many factors, such as the fraction of inhibited aminophospholipid translocase or the nature of the PE acyl chains. Thus, certain classes of PE are more efficiently transported. This can explain a segregation among PE species.[3]

While all laboratories agree about the role of the aminophospholipid translocase in establishing phospholipid asymmetry in eukaryotic plasma membranes, there is some controversy about the involvement of cytoskeleton proteins. Indeed, it was suggested originally by Haest et al. (1978) and later by other laboratories that long-term PS–cytoskeleton interaction could explain partly the stability of the aminophospholipid location in the inner monolayer. In favor of a role for the cytoskeleton, the following arguments were given:

(i) Experiments in model systems showed interactions between PS and spectrin or band 4.1 (Mombers et al., 1979; Cohen et al., 1988; Shiffer et al., 1988). (ii) A reduced asymmetry has been observed in cells where the cytoskeleton had been modified chemically or partially disconnected from the membrane as is believed to happen in deoxy sickle cells (Lubin et al., 1981; Middelkoop et al., 1988). (iii) Lipid asymmetry is only lost slowly when cells are deprived of ATP. However, these points can be critically analyzed. First, the interaction between spectrin and PS is very weak at neutral pH (Mombers et al., 1979), of the order of thermal energy (Maksymiw et al., 1987). NMR experiments show no immobilized components (Bitbol et al., 1989). Thus the exchange must be rapid (time scale $\sim$10^{-5} s). If the lifetime of PS–cytoskeleton interactions is of the order of 10^{-5} s or less, it should have a negligible effect on the average lifetime of each phospholipid in the inner or outer monolayers. Photobleaching experiments with fluorescent phospholipids indicate also a rapid lateral diffusion of all phospholipids on the inner monolayer, with an immobilized fraction, corresponding to less than 10% (Morrot et al., 1986). Finally, there is no report of PE–cytoskeleton interaction. As for the data with the protein-modified system or with sickle cells, their interpretation implies many assumptions that are difficult to verify, in particular those concerning the specificity of the protein modifications. Simpler experiments have been done with heat-induced vesicles or heat-denatured red cells. In these systems, the cytoskeleton is absent or denatured, yet provided the vesicles or the remnant cells contain ATP, the asymmetry is maintained (Calvez et al., 1988; Gudi et al., 1990). Finally, concerning the slow lipid scrambling in ATP-depleted cells, it should be noted that complete ATP depletion is difficult to achieve without cell lysis: at 0.1 mM (i.e., at 1/20 of the normal ATP level), the aminophospholipid translocase is still capable of maintaining PS inside. Furthermore, in the absence of MgATP, the outward diffusion pathway is only through the lipid bilayer; this is a slow process that depends on the nature of the acyl chain and may take 24 h or longer at 37 °C.

In conclusion, the importance of the cytoskeleton for the maintenance of phospholipid asymmetry in red blood cells is yet to be proven. It certainly is not implicated in organelles

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[3] However, the difference in acyl chain composition of PE, located respectively in the inner and outer monolayer, could be explained also by the activity of the endogenous reacylase, an enzyme located in the inner monolayer. Indeed, it should be kept in mind that even in erythrocytes, which are often considered as relatively inert cells, lipids are continuously remodeled. The turnover rates depend on the lipid species and their transmembrane location.

such as chromaffin granules that have no cytoskeleton.

Can the aminophospholipid translocase activity result in the segregation of the choline-containing lipids as well? This can be explained in the framework of the bilayer paradigm in which a basic assumption is that the average number of phospholipids on both sides is equal. Although it is probably improper to use the term "sites" for the lipids in each layer (since the sites have only a statistical meaning unlike a binding site on a protein), it is nevertheless convenient to describe the overall behavior of PC and SM as a simple diffusion between an asymmetrical population of sites due to the amino-phospholipid accumulation on the inner monolayer. One objection is as follows: an unbalanced number of sites between inner and outer leaflets may be compensated by a bilayer bending. Indeed, several laboratories have shown that addition of drugs, or exogenous lipids, on one layer of erythrocytes produces the immediate formation of echinocytes or stomatocytes, depending on the location of the added lipids. This will be further discussed below. I see two answers to this objection: first, it should be noted that approximately 1% of unbalance between inner and outer lipids produces these drastic morphological changes (Ferrell et al., 1985); higher disproportion of lipids between both leaflets, if brutal, produces cell lysis. Thus, to avoid cell lysis, the progressive accumulation of aminophospholipids on one layer should be compensated by the slow diffusion of PC to the other layer.[4] Second, echinocytes will slowly revert to discocytes if the amino-phospholipid translocase is still functional; the time scale of shape reversion may correspond to the diffusion of PC. Another objection to the site model is that cholesterol could perhaps compensate the disappearance of phospholipids on one layer. But obviously cholesterol cannot compensate for a large fraction of phospholipids: one cannot form a monolayer or a large domain of pure cholesterol. So, in conclusion, the diffusion of PC (and SM?) from the inner to the outer monolayer should form an outer layer enriched in the latter lipids. This diffusion could be facilitated by one protein or several proteins but does not require ATP.

A major assumption in the above discussion is that the asymmetry of the plasma membrane of erythrocytes and other eukaryotic cells is not a static situation but rather the result of a subtle balance between different lipid fluxes.

What are the other models proposed to explain lipid asymmetry? Hubbell proposes to explain phospholipid asymmetry in retinal rod disk membranes by the asymmetrical charge distribution on rhodopsin, which constitutes approximately 80% of the disk proteins (Hubbell, 1990). This theory is not fundamentally different from that which suggests that spectrin–PS electrostatic interactions are responsible for lipid asymmetry in red cells, except that Hubbell's model does not imply a direct protein–PS interaction. Rather, the charged proteins would form a mean electric field perpendicular to the membrane and capable of orienting (sequester) the negatively charged lipids. Hubbell's model contains no kinetic considerations. Hope and Cullis have shown experimentally that it is not possible to force PS to flip through a lipid bilayer solely under the influence of an electric field (Hope & Cullis, 1987). Thus, Hubbell's model seems only realistic with a membrane containing a phospholipid flippase that would catalyze the exchange of lipids between the two layers, or perhaps the highly unsaturated phospholipids of disk membranes undergo spontaneously a rapid flip-flop. Alternatively, the disk lipid asymmetry (which

is highly probable but not yet clearly established) may be created in the plasma membrane, from where the disk originates, by an aminophospholipid translocase and subsequently be preserved in the disks because of the electric gradient.

In other eukaryotic organelles, phospholipid asymmetry has been reported. I have indicated already that chromaffin granules possess an aminophospholipid translocase that orients the aminophospholipids toward the cytosolic milieu. Thus, specialized organelles may have an ATP-dependent carrier protein, which could be the ATPase of molecular mass 115 kDa detected in presynaptic vesicles (Diebler & Lazereg, 1985) and in coated vesicles (Xie et al., 1984). In other organelles where lipids are synthesized or remodeled, for example in the endoplasmic reticulum or in the mitochondrial membrane, the phospholipid asymmetry, if it exists, is certainly largely due to the asymmetric synthesis. In principle, in the ER the flippase, first reported by Bishop and Bell, allows lipids to equilibrate between the two layers (Bishop & Bell, 1985). Thus, a priori there should be no or little asymmetry. However, if the phospholipid synthesis is very active, the flippase may be unable to distribute newly synthesized lipids at a sufficient rate.

Similarly, the asymmetry reported in prokaryotes could originate from the asymmetrical synthesis of phospholipids that takes place in the bacterial membranes. However, fast phospholipid translocation was measured in the membrane of *Bacillus megaterium*. Thus, the inner and outer halves of the membrane should rapidly equalize. According to Langley and Kennedy (1979), this does not occur. Cells in which the generation of metabolic energy was completely blocked by suitable inhibitors had, in fact, a somewhat *increased* asymmetry. Perhaps an asymmetric charge distribution on the proteins could be invoked as an explanation as suggested by Hubbell for the disks, in which case PG, which is a charged lipid, might be sequestered in the inner monolayer and PE would diffuse to the outer monolayer via a *flippase* comparable to that existing in microsomes.

BIOLOGICAL FUNCTION OF THE TRANSMEMBRANE LIPID TRAFFIC

The biological function of the phospholipid flippase in the ER appears clear since it is required in order to rapidly redistribute newly synthesized lipids. As for the amino-phospholipid translocase, it is responsible for the phospholipid asymmetry but what is the role of the lipid asymmetry? It seems logical to infer that phospholipid asymmetry in biomembranes has a physiological function, particularly considering that the cells use ATP to establish and maintain this lipid segregation. In the following paragraphs I shall speculate on possible advantages of this energy-consuming lipid asymmetry in eukaryotes. The erythrocyte membrane is a reference for these speculations. However, erythrocytes are very specialized cells, atypical because of their unique membrane and the absence of membrane traffic within the cell. Thus, it is not certain that the general physiological function of the amino-phospholipid translocase can be inferred from studies in such an unusual cell. Obviously it is necessary to consider also other systems.

Several different roles for lipid asymmetry and, hence, for the aminophospholipid translocase have been proposed in the literature. The first speculation deals with a lipid modulation of membrane–membrane interactions including organelle–plasma membrane fusion, namely, *exocytosis*. Another role, which I see as important for the aminophospholipid translocase, is that of providing the driving force for membrane bending, particularly inward bending, as it would occur in *endocytosis*.

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[4] In practice, it is not certain that the aminophospholipid translocase could generate lipid asymmetry from a totally scrambled membrane without lysis.

Membrane–Membrane Interactions. Membrane–membrane interactions are often evaluated by cell biologists solely in terms of protein recognition pattern. Yet lipids also are involved. In fact, a requisite for the contact between membranes is the proximity of large areas of lipids from two different membranes. Thus, membrane–membrane interaction depends upon the interaction between phospholipids from two contiguous membranes. In the case of PC interfaces in vis-à-vis, the interaction is unfavorable because of the large layer of bound water, which imposes a bilayer spacing of approximately 30 Å (Rand & Parsegian, 1989). PS or PE interfaces can come in closer contact; the bilayer separation imposed by the water molecules is only ~15 Å. Furthermore, membrane fusion, which by definition is the mixing of lipids from two contiguous membranes, can be achieved only if there is at least locally the formation of a nonbilayer structure. This can be triggered by the interaction of Ca^{2+} with PS–PE but not with PC–SM. Thus normally cells are not fusion-competent since their outer layer is composed essentially of PC and SM. An interesting exception is myoblasts (Sessions & Horwitz, 1983), which precisely are cells that normally fuse. On the other hand, the inner monolayer of the plasma membranes contains PS and PE; it can therefore fuse easily with intracellular vesicles containing on their cytoplasmic surface aminophospholipids. Such is in fact the case for granules in chromaffin cells (Buckland et al., 1978) and presynaptic vesicles in Torpedo electric organ (Michaelson et al., 1983) and presumably all vesicles involved in exocytosis. The role of the aminophospholipid translocase in these organelles would be to optimize the fusion with the plasma membrane. Intracellular fusion seems to be triggered by Ca^{2+} liberation, but it probably involves also specific proteins such as annexins (Drust & Creutz, 1988). Binding studies have shown that these proteins also have a specificity for PS interfaces. Other specific proteins may be involved in order to direct the vesicles to their proper target, but addressing proteins is not sufficient to explain all the molecular mechanisms involved in exocytosis. The process of membrane fusion is fundamentally a mechanism whereby lipids interact and mix; thus, an optimization of lipid interaction is essential.

It is tempting to postulate as a generalization that the whole cell interior is polarized by the transmembrane orientation of phospholipids of each organelle. Some organelles would be fusion-competent (endosomes, Golgi vesicles, lysosomes) and others not (mitochondria, nucleus). The actual fusion of two organelles or the fusion of an organelle with the plasma membrane probably involves other mechanisms (see below), but the present hypothesis at least simplifies the problem and provides us with a preliminary sorting rule.

Phospholipids can influence membrane–membrane interactions in other ways. Indeed, proteins that are involved in cell–cell recognition or aggregation can be modified by specific phospholipid interactions. For example, the appearance of PS on the outer monolayer of a cell membrane can stimulate a protein activity. A typical example is that of the prothrombinase stimulation by the exposure of PS on the outer layer of platelets. It has been shown that after platelet stimulation, possibly because of increased cytosolic free calcium, PS exposure on the outer layer increases (Bevers et al., 1983). The presence of PS produces more than a millionfold increase in the rate of thrombin formation. The conversion of prothrombin into thrombin is then followed by the formation of fibrin strands and of the clot. The main function of the platelet membrane in this process is to provide a catalytic surface on which the coagulation factors interact, thereby increasing their

local concentration (Zwaal, 1988). In fact, intravascular clotting can be triggered by PS exposure on the outer surface of red cells as well; this may happen in vivo in the crisis phase of sickle cell disease (Lubin et al., 1981). In in vitro experiments, it was shown that PS on the outer layer of erythrocytes or of erythrocyte ghosts has another effect: it increases the adhesion of the cell to macrophages and to endothelial cells (Tanaka & Schroit, 1983; Schlegel et al., 1985). In addition, Allen et al. (1988) have shown PS-dependent uptake of liposome models of erythrocytes by macrophages in vivo. From these observations, it was proposed that the loss of phospholipid asymmetry in erythrocytes could be the message for cell elimination from the blood stream (Schroit et al., 1985; Mc Evoy et al., 1986). The mechanism of macrophage binding to red cells following PS exposure is not elucidated. It cannot be a simple charge effect since the red cell surface bears many other negative charges. One may speculate that PS on the outer monolayer of red cells interacts with band 3 and provokes the exposure of the senescent antigen, which is thought by some investigators to be the main message for red cell elimination (Kay, 1984; Lutz et al., 1988). Alternatively, it is conceivable that a PS receptor exists on the macrophage outer layer. Anti-PS antibodies were made, showing that the PS head group can be antigenic (Maneta-Peyret et al., 1988).

The physiological importance of lipid scrambling as a trigger for cell elimination is still debated. The fact is that treatment of red cells by H_2O_2 or malonyldialdehyde, which are artificial ways of mimicking cell ageing, decreases the aminophospholipid translocase activity and partially scrambles the phospholipid distribution (Herrmann & Devaux, 1990; Jain, 1984). Similarly, aged red cells separated by density centrifugation have a less pronounced lipid asymmetry than young cells (Shukla & Hanahan, 1982).

Aminophospholipid Translocase and Membrane Bending. The ratio of phospholipids on the inner and outer leaflets of a bent membrane deviates significantly from 1. The extreme case corresponds to sonicated vesicles where the ratio of inner to outer lipids is ~35:65. In erythrocytes, the ratio of phospholipids between the two leaflets can vary in a more limited range: the addition of 2% or 3% of phospholipids on one layer of erythrocytes produces shape changes from discocytes to echinocytes or from discocytes to stomatocytes; higher enrichment produces cell lysis. The shape transformations, which are triggered in erythrocytes by the addition of amphiphiles and were explained by Sheetz and Singer in the framework of the bilayer couple hypothesis, are not restricted to erythrocytes (Sheetz & Singer, 1974). Recent experiments in Paris have shown that if a small amount of lipids is selectively added to, or subtracted from, the outer surface of a large-size unilamellar liposome, the shape is immediately transformed with the formation of one or several protrusions or the formation of a stomatocyte-like vesicle; sometimes fragmentation of the liposome happens (Farge and Devaux, unpublished). Thus proteins, in particular cytoskeleton proteins, are not necessary for shape changes. In biological cells, the actual morphology after addition of exogenous lipids on one layer depends *also* upon cytoskeleton proteins, which have different characteristics in different cells. For example, the addition of amphiphiles to red cells generates echinocytes with many spicules, whereas in platelets the same treatment generates a few pseudopodia. In both instances, these protrusions revert if the extra lipids flip to the inner monolayer. In red cells, the addition of exogenous PS ($\simeq 2\%$) to the outer monolayer produces echinocytes, but later discocytes and stomatocytes are formed when the amino-

phospholipids are pumped inside by the aminophospholipid translocase (Seigneuret & Devaux, 1984; Daleke & Huestis, 1985, 1989).

The erythrocyte shape can be modified also without addition of exogenous lipids. As is well-known, erythrocytes depleted of ATP become echinocytic; regeneration of ATP enables them to recover their discocytic shape, and excess ATP leads to stomatocytes (Nakao et al., 1960; Ben Bassat et al., 1972), while in ghosts, endocytic vacuoles are formed (Schrier et al, 1975). These observations can be explained by a redistribution of a small percentage of endogenous phospholipids between the two monolayers, mediated by the ATP-dependent aminophospholipid translocase. The basic assumption is that the resting state is the echinocytic shape which must correspond to a bilayer with a random, or almost random, lipid distribution but constrained by the cytoskeleton meshwork. The activity of the aminolipid pump, which depends upon the cytosolic MgATP concentration, modifies the ratio of inner and outer lipids and, hence, according to the bilayer couple hypothesis, the cell shape. It is conceivable that the steady state imposed by the continuous action of the aminophospholipid pump in the normal physiological state imposes a small excess of phospholipid on the inner half of the membrane. In support of this statement, Verkleij and collaborators demonstrated that red cell membrane phospholipid distribution slightly favors the inner monolayer (Verkleij et al., 1973). In the relaxed or equilibrium state (i.e., the pump being blocked) a net transfer of lipids to the outer monolayer would take place. Only a few percent of phospholipid net transfer from the inner to the outer monolayer is enough to generate the shape change.

From the model of the erythrocytes, one can extrapolate to a putative cell with a higher aminophospholipid translocase activity. A priori, such a cell where the inward movement of the lipids would be much more efficient than the outward movement would continuously form invaginations that may lead to endocytosis (Figure 2). This would not mean the cells' destruction provided the plasma membrane losses were compensated by an equivalent gain by the fusion of organelles i.e., by exocytosis. It is necessary to postulate that during the two processes (endo- and exocytosis) a partial lipid scrambling takes place in order to provide the outer layer with the aminophospholipids. Both processes involve the fusion of membranes, and since fusion is a transient but drastic perturbation, partial redistribution may take place, indeed, during these events. Interestingly, there is evidence to suggest that the translocase activity is higher in cells where endocytosis is important. In particular, fluorescent analogues of PS and PE, which are very slowly transported in the plasma membrane of erythrocytes at 37 °C (Colleau et al., 1990), are rapidly translocated through the plasma membrane of fibroblasts at 7 °C (Martin & Pagano, 1987). Also the transmembrane movement of spin-labeled PS and PE is faster in erythroblasts than in erythrocytes (Cribier, unpublished). Thus, I propose that the aminophospholipid translocase is a necessary protein in the triggering process of endocytosis. The translocase does not have to be located at, or near, the site of invagination. The lateral pressure will propagate the physical constraints in the plane of each monolayer, and the rapid lateral diffusion would permit a homogeneous in-plane distribution of phospholipids that do not have to accumulate near the translocase. Probably the cytoskeleton meshwork imposes specific locations for the bending. Alternatively, it is quite conceivable that an aggregation of membrane proteins (receptors) constitutes a domain for the initiation of the membrane invagination. The former case would correspond to fluid-phase endocytosis and
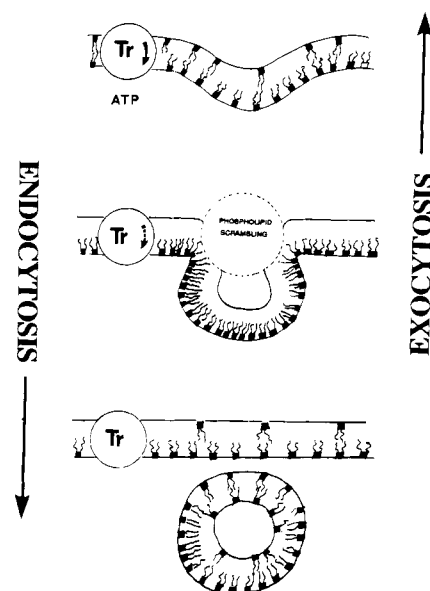

FIGURE 2: Schematic representation of a possible role of the aminophospholipid translocase (Tr) in the triggering of endocytosis and/or membrane budding (from top to bottom). Only the aminophospholipids have been represented. The accumulation of aminophospholipids on one side of the membrane due to the activity of the translocase is accompanied by membrane bending. At a certain threshold, endocytosis (or budding) takes place with possibly partial lipid scrambling during the separation of the two membranes (i.e., between steps 2 and 3). The curvature does not necessarily take place near the translocase. Either the underlaying cytoskeleton proteins or the clustering of receptors within the membrane determines the initiation sites for bending. Exocytosis may be visualized as the opposite scenario (from bottom to top). Partial lipid scrambling during endocytosis and exocytosis will supply the outer layer with the necessary aminophospholipids for a new endocytic process. Hence, a continuous ATP-driven lipid flow can take place.

the latter to receptor-mediated endocytosis. Clathrin coating could be initiated by the membrane invagination rather than causing it. The function of clathrin would then be to protect and label the "coated" vesicles for proper delivery within the cell.

In this model, the division of the phospholipids into two classes (the aminophospholipids and the others) is advantageous. Only one class is transported by the translocase and, hence, is involved in triggering the membrane traffic; in the absence of lipid returning from the cytosol (by exocytosis) endocytosis should stop, without cell lysis, hereby protecting the plasma membrane integrity. In fact, subtle tuning of the transmembrane lipid flow can be achieved by having subclasses of phospholipids, in particular, with PS and PE having different affinities for the aminophospholipid translocase.

Note that a PC translocator in the plane of the plasma membrane pumping out the choline-containing phospholipids from the inner to the outer monolayer would diminish or even cancel the bending capacity of the aminophospholipid translocase. Eventually this putative PC translocator would cause budding of the plasma membrane toward the cell exterior, resulting in a possible loss of cell membrane. Thus, this PC translocator would be dangerous for the cell integrity.

Within a cell, budding appears to be a obligatory step in membrane–membrane communication. At least specialized organelles like Golgi apparatus generate vesicles that are directed toward the cytosol and never conversely. To elaborate these vesicles, the Golgi could use an aminophospholipid translocase oriented differently than it is in the plasma membrane. It is remarkable that secretory granules, which are fabricated in specialized cells from the Golgi, do contain such

a protein with the proper orientation (Zachowski et al., 1989).

The formation of small vesicles followed by the fusion of these vesicles with either the membrane of organelles or with the plasma membrane appears to be the general pathway for membrane traffic within a cell. That is, direct fusion of the membranes from two organelles does not happen. This is generally explained by the presence of actin–tubulin filaments that can form spacers between organelles. In the holes of this meshwork, only small vesicles can travel. However, there are also theoretical reasons to believe that fusion between two flat membranes is an unlikely event. Indeed, hydration forces as well as thermal undulations have been shown to induce repulsive steric interactions (Helfrich, 1985). This explains in particular why multilayers do not stick to each other. On the other hand, unilamellar vesicles do cohere and can fuse when the amplitude of the undulations and the hydration repulsive forces are limited by vesicle finite size. We therefore see that membrane budding, vesiculation, and fusion of the vesicle with a target membrane provide the cell with unidirectional pathways. Of course the final intracellular sorting has to be due to a targeting by proteins or oligosaccharides. But the flow direction can be provided by the bending orientation imposed by a lipid translocator.

In retrospect, budding of the ER may appear to be paradoxical because of the existence of the Bishop and Bell flippase, which should cancel the formation of any lipid gradient that we inferred as being necessary for the formation of local membrane bending. It should be pointed out that vesiculation of the ER seems less frequent than in the Golgi. Furthermore, the lateral homogeneity of the ER is not established, so that the budding region of the ER could be separated from the region where the nonspecific phospholipid flippase randomizes the phospholipid distribution. But even with a protein-accelerated lipid flip-flop, it is still possible to envisage conceptually budding as due to a net accumulation of phospholipids on one side: it will depend upon the relative fluxes of the accumulation of lipids on one side (for example, due to lipid synthesis) and phospholipid redistribution due to the flippase. It so happens that the active sites of enzymes involved in lipid biosynthesis are located on the cytoplasmic face of the ER membrane and, thus, would give rise to budding toward the cytosol if the flippase were locally inefficient or temporarily inhibited.

Conclusion

Transmembrane asymmetry provides each membrane with a vectorial character that may be important for membrane–membrane contacts. Only that side of biomembranes which contain aminophospholipids is fusion competent. But, in reality, lipid asymmetry is the consequence of a dynamic process, and in my opinion the asymmetry of the lipid transport may in fact be more important than the asymmetry of the resulting steady-state distribution. The transfer of phospholipids from one leaflet to another by the aminophospholipid translocase, because it is not immediately counterbalanced by an equivalent transfer of choline-containing phospholipids, can cause membrane bending, leading to local invaginations or budding and eventually to vesiculation. Thus the asymmetry of the lipid transport may be an important element in the molecular mechanism of endocytosis and vesiculation, which is a key phenomenon in cell traffic. Nonspecific phospholipid flippases, because they shuttle lipids in both directions and cancel lipid gradients, should prevent or slow down the formation of membrane invaginations. Therefore, a provocative conclusion of the present paper is that the phospholipid asymmetry may be a secondary effect of the activity of specific phospholipid pumps designed to trigger local membrane invaginations by the transfer of a subpopulation of lipids from one monolayer to the other.

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Accelerated Publications

Experimentally Verifying Molecular Dynamics Simulations through Fluorescence Anisotropy Measurements[†]

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ABSTRACT: The fluorescence anisotropy decay of the single tryptophan residue in phospholipase A_2 was studied by use of differential polarized phase fluorometry and computer simulations of protein dynamics. The results enable the verification of a simulated dynamic event by direct experimental measurement on the same time scale. When all hydrogen atoms are modeled explicitly, the simulations agree well with the experimental measurements. However, the measurements contradict simulations in which nonpolar hydrogens are incorporated into "extended" or "united" atoms. These simulations predict an anisotropy decay in excess of measured values and appear to seriously underestimate the electrostatic interactions occurring between water and aromatic side chains. The results support the general validity of studying protein dynamics with the molecular-mechanics approach and illustrate a potentially serious deficiency of simulations which do not explicitly model all hydrogen atoms.

The experimental verification of molecular dynamics simulations of specific protein motions has not been generally feasible due to the limited overlap between experimentally and computationally accessible time scales. Simulations can predict results which are impressively consistent with experimental data (e.g., free energy differences; Ghosh & McCammon, 1987; Bash et al., 1987), but differences in time scale between laboratory measurement and simulation mark such comparisons as precarious extrapolations. Recently, there have been significant advances in the theory and technology of frequency domain fluorometers which enable the direct measurement of subnanosecond photophysical events (Lakowicz et al., 1986; Gratton and vandeVen, submitted for publication). This, along with concomitant increases in available computational power, now make it feasible to extend simulations into an experimentally accessible time scale.

From a theoretical perspective, a particularly straightforward way to compare simulation and experiment is to simulate and measure the anisotropy decay of tryptophan (TRP) fluorescence in proteins. Detailed derivations of the decay function in computer simulations (Ichiye & Karplus, 1983) and in phase fluorometry (Weber, 1977) are available. In the case of ribonuclease T_1 (RNAse-T_1), we have shown that experimental data can falsify simulations of fluorescence anisotropy decay in which solvent is neglected (Axelsen et al., 1988). This comparison was of limited rigor, however, because it involved "limiting anisotropy" data, implicitly requiring an extrapolation from the experimental data into the time scale of the computer simulation. Furthermore, the single Trp at position 59 is largely buried within the protein matrix, effectively precluding extensive rotational activity.

By use of gigahertz-domain differential polarized phase fluorometry and computers capable of propagating molecular dynamics simulations into a comparable time scale, a much more rigorous comparison is possible. Phospholipase A_2 (PLA2) was chosen for study because the single Trp residue at position 3 is in an altogether different circumstance than Trp-59 in RNAse-T_1; in PLA2, Trp-3 is fully exposed to solvent and has no obvious steric basis for hindrance to side-chain rotation. Yet, NMR (Allegrini et al., 1985) and