Is lipid translocation involved during endo- and exocytosis?

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Abstract — Stimulation of the aminophospholipid translocase, responsible for the transport of phosphatidylserine and phosphatidylethanolamine from the outer to the inner leaflet of the plasma membrane, provokes endocytic-like vesicles in erythrocytes and stimulates endocytosis in K562 cells. In this article arguments are given which support the idea that the active transport of lipids could be the driving force involved in membrane folding during the early step of endocytosis. The model is sustained by experiments on shape changes of pure lipid vesicles triggered by a change in the proportion of inner and outer lipids. It is shown that the formation of microvesicles with a diameter of 100–200 nm caused by the translocation of plasma membrane lipids implies a surface tension in the whole membrane. It is likely that cytoskeleton proteins and inner organelles prevent a real cell from undergoing overall shape changes of the type seen with giant unilamellar vesicles. Another hypothesis put forward in this article is the possible implication of the phospholipid ‘scramblase’ during exocytosis which could favor the unfolding of microvesicles. © 2000 Société française de biochimie et biologie moléculaire / Éditions scientifiques et médicales Elsevier SAS

aminophospholipid translocase / membrane budding / spontaneous curvature / liposomes / K562 cells

1. Introduction

During the last 10–15 years, a large number of proteins have been recognized as playing central roles during endo-exocytosis phenomena and more generally during vesicle traffic: clathrin, NSF, ARF, dynamin, caveolin, annexins and GTP-binding proteins, have been implicated in the processes of membrane vesiculization (also called budding) or during fission, targeting and fusion of vesicles. A few reconstitution experiments with a limited number of proteins have reproduced, if not quantitatively, at least qualitatively some of the important steps [1, 2] and from these observations, generalized models were proposed. However, the main emphasis of earlier research has been to show which proteins are required, rather than to dissect the molecular mechanisms involved. Membrane folding which is a prerequisite for vesicle formation, is often assumed to be explained by the binding of clathrin or of coat proteins. Yet, endocytosis can exist without clathrin or without clathrin polymerization [3, 4]. Furthermore, increasing the amount of polymerized clathrin does not systematically increase the number of coated pits at the plasma membrane [5]. In fact, it has not been proven yet whether clathrin polymerizes and then pinches off the membrane to form the buds or if polymerization takes place around a pre-formed bud. An alternative explanation proposed for membrane budding during endocytosis is the formation of local invaginations named caveola formed by self association of the hydrophobic protein caveolin with specific lipids [6]. To what extent is ATP necessary for budding is still debated [7]. In summary, the physical origin of the local membrane curvature is still unexplained in spite of the correlation between the presence of specific proteins and the formation of coated pits. Concerning exocytosis, it is believed that the translocation of lipids which a priori is necessary after the fusion of microvesicles to the relatively flat plasma membrane can take place spontaneously, yet it is known that lipid flip-flop requires several hours which is quite incompatible with the time scale of a continuous endocytotic process.

In general, the role of lipids has been largely overlooked in these cell biology investigations. Only recently, in experiments with reconstituted vesicles to which coat proteins were attached, has the importance of lipids been realized. In fact, even pure liposomes enable one to mimic some fundamental steps of biological membrane traffic. To those who believe that the representation of a biological membrane by a mere lipid bilayer is irrelevant, it should be recalled that membrane budding, fission and fusion refer primarily to the status of the lipid bilayer. Thus, a detailed analysis of the physical constraints associated with folding and fusion of a pure lipid bilayer is of primary importance. Of course, investigating liposome behavior would be sterile for biology if one does not...
keep in mind the numerous specificities of biological membranes. Not only the lipid composition of all eukaryotic membranes is complicated, i.e., involves mixtures of many different lipids, the proportion of which seems to be strictly regulated, but in addition the plasma membrane of eukaryotic cells has an asymmetrical transmembrane distribution of phospholipids which is difficult, if not impossible, to reproduce with model membrane [8]. Another important characteristic of in vivo vesicularization is the actual size of the vesicles involved in membrane traffic. Sufficient attention has not been paid to this point in previous investigations on shape change of liposomes by physicists attempting to mimic endo- and/or exo-cytosis in giant vesicles.

The major issue that I will address in this article is the following: is membrane bending and unfolding during endo-exocytosis caused by the transmembrane redistribution of lipids by the aminophospholipid translocase which is a ubiquitous lipid transporter present in the plasma membrane of eukaryotic cells? I shall first try to shed some light on physical constraints which cannot be ignored when trying to understand how membranes invaginate. In view of our knowledge on lipid-protein interactions this will bring up some suggestions on possible mechanisms involved at the early stage of endocytosis and the late stage of exocytosis, in particular the putative role of ‘flippases’ and ‘scramblases’. The possible involvement of the aminophospholipid translocase was presented several years ago by myself as a working hypothesis [9, 10]. Recent studies have given experimental support to this idea [11–14].

2. Membrane budding

Let us consider first a protein-free giant unilamellar vesicle containing one or several types of phospholipids. Unlike a soap bubble, the surface tension of a liposome in the absence of osmotic pressure, is very low and the membrane undergoes visible surface undulations. However, it would be an erroneous conclusion to infer that a bilayer is easy to deform. The budding of a synaptic-like microvesicle out of the surface of a giant liposome imposes locally a high curvature which for a bilayer must be associated with a difference in the area of the inner and outer monolayers. If lipids were free to diffuse from one side of a membrane to the other, invaginations and budding would happen as a manifestation of thermal fluctuations as it happens with surfactant films. But in a lipid bilayer, in general it costs a lot of energy for a phospholipid to traverse the membrane; \( t_{1/2} \) of spontaneous phosphatidylcholine flip-flop is of the order of several hours or even days at physiological temperatures [15]. Furthermore, the surface compressibility of a lipid monolayer is low, so thermal fluctuations only give rise to surface undulations.

In a biological membrane, local deformations can be achieved by applying external forces for example by the contraction of cytoskeleton proteins. In a liposome a protrusion forming a long tether can be formed by sucking the membrane with a micropipette or by pulling it with optical tweezers. Alternatively, the shape can be changed in a more subtle manner by progressive modification of the ratio between inner and outer areas: \( A_i \) and \( A_o \). For example, if the two opposing monolayers react differently to their environment or are selectively modified by addition or depletion of lipids, they eventually have different areas (\( \Delta A = A_i - A_o \neq 0 \)), then within the framework of the so-called bilayer couple hypothesis [16], the membrane bends.

As indicated originally by Helfrich, the bending energy of a liposome can be written in the following way [17]:

\[
E_c = \frac{k_c}{2} \int_A \left( C_1 + C_2 - 2 C_0 \right)^2 dA
\]

Here, the Gaussian curvature term is omitted. As long as vesicle fission (or fusion) has not taken place, we deal with vesicles of spherical topology and the Gaussian curvature term can be omitted. \( k_c \) has the dimension of an energy and is of the order of a few times the thermal energy (\( k_B T \)). The integral extends over the whole area \( A \) of the lipid vesicle. \( C_1 \) and \( C_2 \) are the local curvatures (see figure 1). In practice for a closed vesicle the two leaflets have a slightly different area. Under the condition that the two leaflets are everywhere separated by the same spacing \( h \) which is of the order of 6 nm, \( \Delta A \) is related to the local curvature by the following relation which is correct to order \( h/R \) [18]:

\[
\Delta A = h \int dA (C_1 + C_2)
\]

\( C_0 \) is the spontaneous curvature that can be defined as the curvature that the membrane would take in a relaxed state, i.e., not constrained by the necessity to form a closed vesicle in order to avoid exposing the hydrophobic lipid chains to water. \( C_0 \neq 0 \) can be due to an asymmetry in the lipid composition or lipid environment or simply to a difference in the number of lipids present at each interface. In Helfrich’s formulation \( C_0 \) includes both the local and non-local tendency to bend. A more explicit formalism was proposed recently in the laboratory of Wortis [18]. In this model, the elastic energy has two separate terms, hence the difference in area of the two leaflets is accounted for by a term corresponding to a new elastic stretching energy:

\[
E_c = \frac{k_c}{2} \int_A \left( C_1 + C_2 - 2 C_0 \right)^2 dA + \kappa (\pi/2 Ah^2) \left( \Delta A - \Delta A_0 \right)^2
\]

\( \Delta A \) is the actual difference in area, whereas \( \Delta A_0 \) is the difference in area that would exist in a relaxed state. \( \kappa \) is
a new elastic modulus having also the dimension of an energy. Then \( C_0 \), the spontaneous curvature, reflects solely the local curvature constraints due for example to local binding of proteins or ions or to the chemical nature of the phospholipids which are usually asymmetrically distributed in a biological membrane \([8, 10]\). In principle, the elastic modulus \( \kappa \) is different from \( k_c \). However, it has the same order of magnitude. For example, in the case of homogeneous phosphatidylcholine vesicles Miao et al. \([18]\) have reported that the ratio \( \alpha = \kappa/k_c \approx 1 \). Thus, for the qualitative approach of the present review, I shall continue to use Helfrich’s formulation throughout, and consider that \( C_0 \) reflects either local spontaneous curvature or the area difference term. For a rigorous and more quantitative development of this section the separation between local and non-local spontaneous curvature would be necessary.

If a liposome is formed by gentle swelling of a lipid film in water, in general both leaflets are identical in composition and density of lipids, hence: \( C_0 \approx 0 \). However, the necessity to close the bilayer in order to avoid exposing hydrophobic surfaces forces the bilayer to bend and the total energy is minimize for \( C_1 \) and \( C_2 \approx 0 \), i.e., for an average vesicle radius, \( < R > \), very large. In practice, giant vesicles are formed with a radius of several microns, that is exceeding by far the thickness of the bilayer and corresponding to a low average curvature.

Theoreticians have predicted shapes of liposomes for different values of \( C_0 \) by minimalization of Helfrich’s formula or of formula derived from this formula \([18-21]\). Phase diagrams of vesicle shapes were drawn from these computations and typical shapes corresponding to the budding of a small vesicle from a ‘mother vesicle’ of larger size can be recognized. Figure 2 shows some

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**Figure 1.** Principal radii of curvature of a bilayer of thickness \( h \). Locally any continuous surface can be approximated by an hyperboloid (or a paraboloid) which can be written: 
\[
Z = -\frac{1}{2} \left( \frac{x^2}{R_1^2} + \frac{y^2}{R_2^2} \right),
\]
where \( z \) is the direction of the normal to the surface; the axes \( x \) and \( y \) are chosen so as to obtain the symmetrical quadratic form of the surface equation. This defines the two principal axes. By definition \( C_1 = \frac{1}{R_1} \) and \( C_2 = \frac{1}{R_2} \) are the radii of curvature: they correspond to local parameters. See the text for the definition of the spontaneous curvature \( C_0 \).

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**Figure 2.** Schematic ‘phase diagram’ of vesicle shapes. This ‘phase’ diagram shows the shapes of lowest bending energy for given reduced volume \( v \) and equilibrium differential area \( \Delta A_o \). \( \Delta A_o \) in this diagram is a dimension-less or scaled area difference which is effectively proportional to \( \Delta A_o \) defined in the text. With the exception of the shapes indicated with a dashed contour, the shapes calculated have an axis of rotational symmetry along the vertical (reproduced from \([32]\)).
predicted shapes. Such shape changes in giant vesicles were observed by several groups who found ways to manipulate $\Delta A$.

The contraction or expansion of one of the two leaflets of a liposome or of a biological membrane, which I shall call a $C_0$ modification, can be achieved by chemical modifications in situ resulting, for example, from phospholipase or sphingomyelinase degradation [7, 22–24]. Phosphorylation of phosphonositides on the cytosolic interface of an erythrocyte has been reported to be involved in shape changes in the case of red cells [25]. $C_0$ modification can be triggered by ions and/or proteins interacting with specific lipids of one of the leaflets, causing the formation of rigid domains or ‘rafts’ with a condensed area and therefore leading to asymmetrical membranes [6].

Alternatively, $C_0$ can be modified by the insertion or depletion of lipids from one leaflet. A non-physiological way to enrich the lipid composition of one bilayer leaflet consists in adding amphiphilic molecules such as lyso-phosphatidylcholine (L-PC) to pre-formed giant liposomes or to erythrocytes. L-PC molecules penetrate the outer leaflet where they remain and expand selectively the external surface since the flip-flop rate of L-PC is extremely slow even in phosphatidylcholine liposomes [26]. In vivo a net translocation of phospholipids can be achieved by the ‘phospholipid flippases’ which are able to catalyze the translocation of phospholipids or to transport phospholipids at the expenses of ATP consumption. Modulation of the transmembrane asymmetry of certain lipids, such as phosphatidic acid or phosphatidylglycerol, can be achieved in liposomes if a transmembrane pH gradient is applied [27]. These various techniques which enable one to modulate the spontaneous curvature of a membrane by the formation of asymmetrical bilayers induce membrane invaginations.

Addition of less than 1% of lipids to the external leaflet suffices to modify a GUV with a discoid or obloid shape into a eight-shape vesicle formed by a small spherical vesicle connected to a larger one; similarly a very small lipid transfer of lipids from the inner to the outer leaflet triggers the formation of a budded vesicle with a typical diameter of the order of a few µm (see figure 3). In some instances the small vesicle is separated from the large vesicle by a thin tether difficult to detect unless fluorescent lipids have been incorporated [28]. But in general, the single budded vesicle does not shed off. If more than 1% of L-PC is added, the overall shape of the GUV can take more complicated forms with several connected spheres (figure 4). If the external leaflet of a liposome is depleted of a fraction of its lipids, for example by removing L-PC from the outer leaflet with bovine serum albumin, a single invagination is formed which resembles membrane invaginations during endocytosis [29]. In Sackmann’s laboratory the same sequences of shape change were observed with GUVs when the temperature was varied, the interpretation being that even a very small differential thermal expansion of the two monolayers can induce a change of $C_0$ [30, 31].

3. The scaling effect

How much asymmetrical has the membrane to be in order to induce a detectable shape change or more precisely to generate budding of a small lipid vesicle out of a larger one that I shall call the ‘mother vesicle’? In other words, if $C_0$ modification is caused by a transfer of lipids, what proportion of lipids has to be transported from one leaflet to the other to obtain vesiculization?

The result from quantitative experiments with GUVs, discussed in the above paragraphs, is rather astonishing: 0.1% of lipids in excess on the external leaflet suffice to modify a GUV with a typical size of 20–50 µm. Theoretical calculations had even predicted that 0.01% asymmetry would be sufficient [20]. Simple geometrical arguments allow one to predict a scaling effect: namely the fraction of lipids that has to be reoriented or added selectively depends on the vesicle size. The scaling parameter is essentially the ratio $h/\text{Ro}$ where $h$ is the thickness of the membrane and $\text{Ro}$ is the radius of the vesicle. Obviously, if the thickness $h$ is close to the radius of the vesicle $\text{Ro}$, then membrane folding is difficult. If $N$ is the average number of lipids per monolayer, the asymmetry in lipid distribution between both leaflets (δ N/N) must be of the
order of \( h/R_0 \) for ‘vesicularization’. In the case of a GUV with a typical diameter of about 50 \( \mu \)m, the experiments show that the ‘small’ vesicles which are generated by lipid asymmetry have a size of 5–10 \( \mu \)m. Since the bilayer thickness is around 6 nm, a very small asymmetry, below 1\%, is sufficient in principle. In the case of a 200 \( \mu \)m LUV and if one neglects temporarily the lateral compressibility, pure geometrical considerations show that the excess of lipids on the outer layer of a budded vesicle must be between 5 and 10\%, meaning that a proportion of phospholipids of the order of 5\% may have to be translocated. In the latter case, a significant surface tension is generated by the mismatch between both layers and the process requires more energy than for giant vesicles.

Indeed, surface asymmetry not only leads to bending but also to surface tension \( T \). By definition: \( T = K \alpha \), where \( K \) is the lateral compressibility coefficient and \( \alpha \) the surface strain which is the relative area variation of one leaflet due to lateral compressibility. In the initial state, the surface tension of both leaflets is practically zero. If one adds new molecules to the outer monolayer, because of the coupling with the inner monolayer which resists to this expansion, a surface tension is generated. Eventually a torque is exerted on the membrane. But it is important to note that bending does not relax completely the tension. Because of the conditions of closure (fixed volume), the membrane cannot bend so as to completely relax the tension. In fact the curvature adopted eventually by the membrane equilibrates the difference in surface constraints between the two leaflets, so that the torque is canceled but not the surface tension.

In practice, the addition of 0.1\% lipids on the external leaflet of a GUV generates a negligible surface tension. However, an asymmetry of 5–10\% between the two leaflets of a GUV or of a LUV generates a significant surface tension. One manifestation of the tension is the inhibition of surface undulations which are partially or totally inhibited by addition of lyso-PC to their external surface of vesicles [29]. Recently the symmetrical surface tension generated in liposomes by the asymmetrical addition of L-PC was demonstrated by Traikia using NMR \(^{31}\)P-chemical shift as a way to detect the molecular packing at the level of the phospholipid head groups of each monolayer in an experiment involving magic angle spinning of lipid vesicles [26]. The surface tension which is equivalent to an increased lipid packing, manifests itself also by an increased resistance to transmembrane lipid diffusion. Thus, while the formation of a 5 \( \mu \)m vesicle budding out of a 50 \( \mu \)m giant unilamellar vesicle can be achieved in a few minutes at room temperature when the membrane (containing a PC/PG mixture) is submitted to a pH gradient [29], the same overall shape with 100 \( \mu \)m LUVs required 60 mn of incubation at 60 °C [32]. The latter observation reported by the Cullis’ group is in accordance with previously published theoretical predictions [33, 34].

Finally, I would like to emphasize as a conclusion of this section that the formation of one or several microvesicles with a diameter of about 20 nm is always accompanied by a surface tension. Because of the continuity between a budded vesicle and the rest of the liposome this tension must be the same in the whole

![Figure 4. Shape transformation of an egg phosphatidylcholine unilamellar vesicle containing fluorescent dextran (0.1 mM) after L-PC addition in proximity of the vesicle with a micropipette. A. \( t = 0 \). B. \( t = 4 \) min. C. \( t = 7 \) min. The bar corresponds to 10 \( \mu \)m (reproduced from [28]).](image)
vesicle membrane, even in a GUV. In other words, although local fluctuations of the surface density in a GUV could give birth to a microvesicle of the size encountered in vivo during endocytosis or vesicle shedding, this vesicle would promptly disappear because of the instability of a local surface tension. Thus a stable budded vesicle connected to the rest of the plasma membrane is only possible if the whole plasma membrane is constrained.

4. Is microvesicle formation in liposomes and red cells relevant to endocytosis?

Typical diameters of the vesicles involved in endocytosis-exocytosis, and more generally vesicle traffic within eukaryotic cells, are around 200 nm. They result from local membrane curvature, either of the plasma membrane itself or of organelles such as the ER or the Golgi system, the typical size of which is in the range of one or several microns. Although lipid vesicle experiments demonstrate a close relationship between vesicle shape and lipid transmembrane distribution and although they show that endocytic-like invaginations may be triggered in GUVs by the redistribution of a small proportion of lipids, they do not satisfactorily mimic endocytosis. Indeed, endocytosis implies the formation of one or several microvesicles from the invaginations of a cell membrane which has a typical radius of several μm. This is not what is observed in giant unilamellar vesicles when for example 1% of L-PC is added externally. We have mentioned above that the formation of very small spherical vesicles requires a lipid asymmetry of the order of 5–10% and because these vesicles (or invaginations) remain at least temporarily connected to the rest of the membrane, the same lipid asymmetry must exist all along the cell surface and will cause an important surface tension. The so-called large unilamellar vesicles (LUVs) with a diameter of 100–200 nm can sustain surface tension without collapse and form budded vesicles or protrusions with a diameter of about 50 μm or less. On the other hand, a giant vesicle of the size of an eukaryotic cell will not form at its surface a series of small vesicles with a diameter which is $\approx 10^{-3}$ times the average diameter of this ‘mother vesicle’ without a complete shape change unless some scaffolding, analogous to the cytoskeleton meshwork in a biological cell prevents the transformation of this large vesicle. Figure 4 shows that if ΔA is increased by the addition of a large amount of L-PC into the external leaflet of a GUV, the shape undergoes a dramatic change that does not correspond to a mere ‘decoration’ of a large obloid vesicle by a series of microvesicles. Of course, if the giant vesicle is initially a perfect sphere and if the internal volume remains approximately constant, there is no possibility of any other shape. In the latter case, the additional surface on the outer layer will generate a tension that will inhibit surface undulations and eventually the surface of the sphere may become covered by microvesicles.

Figure 5 shows an experiment where a large number of relatively small vesicles appear at the external surface of a giant vesicle. This figure was obtained with multilamellar vesicles (MLVs) on the surface of which a large excess of L-PC was added. The inner membranes probably prevent the liposome from an overall deformation or collapse. The large excess of lipids on the external leaflet of the external membrane can only form series of small vesicles which eventually shed off, thereby pealing the external bilayer of this multilamellar vesicle. But even in this example the size of the small vesicles (= 1 μm) was much larger than the size of endo-exo-cytic vesicles. The above example is reminiscent of the situation obtained with red blood cells to which a large excess of L-PC has been added. In the latter case, very small membrane protrusions cover the cell surface which seems to shrink and form a uniform sphere (spherocyte) if viewed with an optical microscope. In reality, the erythrocyte surface is covered by small vesicles with a radius below resolution of the optical microscope. The cell resists because of the cytoskeleton and eventually lyses if more L-PC is added (≥ 5%).

Figure 6a–f shows in an (over)simplified way the shape transformations that can undergo an obloid GUV (figure 6a), by adding to the external surface molecules such as L-PC [28, 29] or by increasing area to volume ratio by heating [30, 31] Shapes in figure 6b, c are the canonical axial symmetrical shapes calculated by theoreticians (see figure 2). The radius R of the spherical ‘budded’ vesicle(s) is large. Such transformation does not resemble in vivo budding. Shapes in figure 6d, e correspond to the formation of relatively small vesicles. They require first a transformation of the ‘mother vesicle’ into a sphere. Again, overall this is not what is seen with real cells. In the case of figure 6f, which represents a multilamellar vesicle (MLV), the obloid shape is maintained by the inner bilayers. In vivo the cell shape is maintained by the cytoskeleton and by the presence of organelles.

I infer that the activity of a lipid pump transporting lipids from the outer monolayer to the inner monolayer (or a selective fraction of the outer leaflet lipids) can induce invaginations of the plasma membrane and form endocytic vesicles of the size observed in vivo, provided the cell surface is reinforced by a cytoskeleton. This cytoskeleton and tubulin meshwork is represented schematically in figure 6g,h.

5. Experimental observations in favor of the role of lipid translocation during endocytosis

The aminophospholipid translocase is present in the plasma membrane of all animal cells [10, 35–37]; recent results suggest that it also exists in the plasma membrane
Figure 5. Shape transformation observed by phase contrast microscopy of a multilamellar egg phosphatidylcholine vesicle after addition of L-PC with a micropipette. A. $t = 0$, B. $t = 8$ s, C. $t = 30$ s, D. $t = 1$ min, E. 2 min. The bar corresponds to 20 µm. Note that the scale is different in E (reproduced from [29]).
of plant cells [38]. This protein transports phosphatidylyserine (PS) and to a lesser extent phosphatidylethanolamine (PE) from the outer to the inner leaflet where these aminophospholipids are predominantly located. Cytosolic ATP is necessary and must be hydrolyzed for this transport to take place. By controlling the level of cytosolic ATP, which in vivo is normally of the order of $2–3$ mM, it is possible to modify the equilibrium distribution of the aminophospholipids and in turn to modify erythrocyte shapes by shifting a fraction of the lipids from the inner to the outer leaflet or conversely. Indeed, unpaired lipid transport is accompanied by echinocyte formation while artificial ATP enrichment leads to stomatocytes. When the level of ATP is of the order of $5–6$ mM endocytic vesicles form at the cytosolic interface of erythrocytes. They are characterized by the formation of a few relatively large

Figure 6. Schematic representation of examples of shape transformations of an obloid giant vesicle when a difference in area $\Delta A$ between inner and outer leaflet is imposed. When the external area is increased by $5–10\%$ very small microvesicles will bud at the surface of the ‘mother vesicle’ only if the latter has a spherical shape (d, e) or if the overall shape of the ‘mother vesicle’ is protected by inner lipid vesicles (f) or by a cytoskeleton meshwork (g, h). h. The external surface is depleted. See text for more comments.
vesicles and can be quantified by monitoring the acetylcholine esterase activity which normally takes place on the outer surface of red cells [12, 39]. The decrease in esterase activity is an indication of the amount of surface becoming non-accessible. If phosphatidylcholine (PC) is added to the outer surface of red cells this vesicularization process is affected because the plasma membrane would rather tend to fold in the opposite direction (see figure 7).

**Figure 7.** Modulation of endocytosis activity in resealed erythrocyte ghosts as determined by the acetylcholinesterase activity. The reduction of the esterase activity which is normally exposed on the external surface of the plasma membrane is an indication of the formation of endocytic vesicles. A. Effect of ATP concentration in the resealed ghosts. ATP stimulates the aminophospholipid translocase. B. Inhibition of endocytosis which normally takes place with 5 mM ATP by addition of phosphatidylcholine to the outer leaflet. C. Inhibition followed by stimulation after addition of phosphatidylserine. This lipid is initially incorporated in the outer leaflet and therefore slows down the inward folding of the plasma membrane. The translocation to towards the inner leaflet by the aminophospholipid translocase reverses the situation and favors membrane invagination. The exogenous lipids added have a short β chain which permits their rapid incorporation (from [12]).
and [12]). Actually, the difference in the shape change scenarios which are triggered by the addition of various phospholipids to the external surface of erythrocytes were noticed already in 1984 by Seigneuret and Devaux [40] and used later by Daleke and Huestis to monitor the activity of the aminophospholipid translocase [41].

Erythrocytes cannot be considered as the best system to study endocytosis since they do not naturally endocytose. It is interesting to point out that concomitantly the aminophospholipid translocase activity (or ‘flippase’ activity) is rather weak in erythrocytes compared to its activity in cells having a high endocytic activity. Cribier and collaborators have compared the translocase activity in some of the cells of the red cell lineage: erythroblasts, reticulocytes and erythrocytes and found that the highest activity was in the cells provided with the highest endocytic activity, namely in K562 cells which are transformed cells derived from human erythroblasts [11]. Similarly, Pomorski et al. [42] have compared the translocase activity in human erythrocytes and in human fibroblasts. It was found that the initial rate of PS translocation was two orders of magnitude larger in fibroblasts than in erythrocytes. In 1995, Farge has measured endocytosis in K562 cells by measuring the uptake of spin-labeled PC added externally and which can only be internalized by endocytosis [13]. He has found that the concomitant addition of PS stimulates PC internalization which suggests an enhanced endocytosis activity. This can be explained by the fact that PS is a substrate of the translocase and that increasing PS should allow more rapid turnover. The interpretation of Farge’s experiments raised some questions because of the instability of the short β chain bearing the nitroxide: these slightly water-soluble lipids were found to be good substrates for the endogenous phospholipase A2 present in such cells as the K562 cells. Nevertheless, the increase in initial rate of PC uptake is probably associated with an increased endocytosis activity. In 1999, a different technique was used to measure endocytosis in K562 cells [14]. The internalization of fluoresceinated membrane proteins was either watched directly or monitored by the decrease of fluorescence which is quenched by the acidity of early endocytic vesicles. It was found that the addition of 4% PS on the external side of the plasma membrane resulted in a five-fold increase in fluorescence quenching in 10 min at 37 °C. Similarly, PE addition stimulated endocytosis but lyso-PS, which is not transported by the aminophospholipid translocase [10], inhibited the residual endocytosis (figure 8).

In a recent investigation aimed at trying to find the aminophospholipid translocase protein, Marx et al. [43] tested the translocation of PS in endocytosis-deficient yeast cells and found very little specific transport of the aminophospholipid analogues and essentially no ATP dependence. These results could suggest that the cells lacking endocytosis activity have also no aminophospholipid transporter. However, it is surprising that these cells seem to have normal lipid asymmetry.

Actually, the doubt concerning the proper identification of the aminophospholipid translocase is the most severe limitation in any experimental proof of its direct implication in endocytosis. Although the sequence of an ATPase with a molecular mass of 170 kDa has been published by Tang et al. [44] and identified as the aminophospholipid

![Figure 8.](image-url)
translocase, there is still a controversy in the literature [43, 45]. One will probably have to wait until this protein can be expressed. Recently, a preliminary report was given about a successful complementation of an aminophospholipid translocase activity in translocase deficient yeast cells (DRS2) using RNA obtained from plants [38].

6. Exocytosis and lipid scramblase

The same geometrical argument that led us to conclude that the formation of microvesicles by bending of the plasma membrane requires the translocation of lipids from the outer to the inner leaflet, forces us to conclude that fusion of small vesicles to the plasma membrane, i.e., exocytosis, requires the opposing transfer of lipids (see figure 9). A priori the two processes (endocytosis and exocytosis) are not exactly symmetrical. Indeed a single small vesicle, with a high surface tension and an asymmetrical repartition of phospholipids can fuse with the plasma membrane without perturbing it because the latter can be considered as a sink of infinite size. The small perturbation brought by fusion of one vesicle is a local perturbation that will eventually be canceled out by the slow spontaneous flip-flop of lipids. However, in a living cell exocytosis is a continuous process that permits the whole plasma membrane to be renewed within a short period, and fusion of synaptic vesicles or granules is a synchronized event which concerns a large number of vesicles. It is therefore apparent that independently from the process that enables the two bilayers to come in close contact and to merge into a single bilayer, it would be useful for the efficiency of this process that some mechanism accelerates lipid flip-flop in order to facilitate the randomization of the transmembrane lipid distribution. Additionally, if this lipid scrambling process brings to the outer leaflet some aminophospholipids (PS and PE), it should facilitate later the formation of inward invaginations (i.e., endocytosis) by the aminophospholipid translocase. In this regard, it is quite interesting to note that most cells do have in their plasma membrane a protein called lipid ‘scramblase’ which is activated by cytosolic calcium. This scramblase plays an important role in specialized cells such as platelets which expose PS on their outer leaflet when they are stimulated, but it is also functional in all cells of the blood circulation during apoptosis [46].

In principle this ‘scramblase’ has been purified and cloned [47]. There is also a rare and very severe disease called Scott syndrome which was thought to correspond to a lack of scramblase; at least it is associated with an impairment of calcium triggered lipid redistribution in platelets. However the reconstituted vesicles with the ‘purified’ scramblase have a very low scrambling efficiency [48] and this protein is present in the blood cells of the Scott patients [49]. So the identification of the protein may yet have to be confirmed.

7. Discussion and conclusions

It has been demonstrated in giant unilamellar vesicles that a small area increase of one of the two opposing monolayers triggers budding or invaginations. In various eukaryotic cells, a positive correlation between aminophospholipid translocase activity and the endocytic activity was demonstrated. There is also strong evidence that the stimulation of phospholipid translocation from the outer to the inner monolayer of the plasma membrane in K562 cells accelerates endocytosis while the addition of phospholipids that are not transported has an inhibitory effect. Therefore, one can infer that the active transport of
lipids may serve as a driving force for membrane folding during endocytosis. It is an ATP driven process which acts on the whole membrane by generating a non-localized surface tension; this tension is at least partly relaxed by the formation of membrane budding and invaginations. The cytoskeleton is necessary to prevent whole cell deformation but organelles which are present in eukaryotic cells may help also in a way comparable to inner vesicles in MLVs (figures 5, 6). The localization of these invaginations has to be random in a large unilamellar vesicle which has an homogeneous surface, but in a biological membrane which contains heterogeneous domains, it is quite conceivable that rigid domains or localized region with a non-zero spontaneous curvature imposed by the binding of peripheral proteins such as clathrin, or associated with the clustering of caveolin, serve as a point of emergence. Note the model of domain-induced budding of fluid membranes proposed by Lipowsky is based on a different physical property, namely the competition between the minimization of the bending energy and the energy of the line tension between two domains [50]. The applicability of the latter model to biological membranes is still very speculative.

The scramblase hypothesis which suggests a role of the calcium triggered scramblase during exocytosis is more speculative at this stage than the hypothesis about the role of the aminophospholipid translocase during endocytosis. It clearly needs experimental confirmation. One aspect that I have not discussed yet is the synchronization between endocytosis and exocytosis. Clearly in this model the absence of exocytosis should eventually stop endocytosis because there is a need of substrates for the lipid pump, namely if no PS and/or PE returns to the exoplasmic leaflet, the pump cannot continue to work. Therefore the system is safe. If, on the other hand, the pump had no lipid selectivity, it would continue to pump the whole cell plasma membrane regardless of what happens within the cell. This would eventually be a suicide activity. In the framework of the aminophospholipid translocase model an evolutionary advantage of lipid heterogeneity becomes therefore apparent.

There are reports of protein catalyzed outward movement for example by MRP proteins that would return PC to the outer leaflet in erythrocytes [51]. Although the data in the literature do not allow one to compare quantitatively the activity of the aminophospholipid translocase and that of the MRP protein, the very fact that if the level of ATP is raised in the erythrocyte cytosol, the membrane bends inwardly suggests that, if there is competition between the two transports, the inward movement wins. Thus, again this is a safe situation. There is one example in nature of cells shedding off pieces of their own plasma membrane. Indeed, stimulated platelets send in the blood circulation microvesicles formed by plasma membrane budding. However, platelets do not recover from this process. Whether this vesicle shedding is due to a calcium triggered pump transporting an excess of lipids outwardly, or to the activity of cytoskeleton proteins, is a question not settled yet [52].

In the framework of this model, clathrin binding could be necessary to determine which part of the membrane will invaginate rather than clathrin binding being the driving force. On the other hand the coat proteins identified as proteins involved in budding of the Golgi system [1] may have a similar effect than the addition of L-PC added externally to a GUV. Because there is now compelling evidence that lipid flip-flop in inner membranes is much more rapid than in the plasma membrane which is rich in cholesterol [53], it is unlikely than the same mechanism could be used in the ER or Golgi membranes to generate microvesicles. Of course several mechanisms of budding may exist within a cell.

As a final conclusion, the models proposed here have many implications that can be verified. Reconstitution in giant vesicles of the purified proteins involved in lipid traffic appears to be the most significant type of experiments to perform in the future to test the above hypothesis.

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References

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