Eur Biophys J (2010) 39:1277–1280
DOI 10.1007/s00249-009-0557-3

BIOPHYSICS LETTER

Asymmetrical stress generated by the erythrocyte lipid flippase triggers multiple bud formation on the surface of spherical giant liposomes

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Received: 23 April 2009 / Revised: 8 September 2009 / Accepted: 19 October 2009 / Published online: 24 November 2009

Abstract Proteo-giant liposomes were electroformed from a mixture of lecithin vesicles and inside-out vesicles from erythrocytes. After addition of Mg-ATP in the vicinity of the proteo-giant liposomes, small buds appeared on the liposome surfaces, which—via an increase in lipids in the outer monolayer—demonstrated the active transport of lipids from the inner to the outer monolayer, indicating flippase activity.

Keywords Phospholipid flippase · Giant vesicles · Membrane bending · Surface tension

Introduction

In 1999, Farge and colleagues demonstrated that the formation of plasma membrane invaginations towards the cytosol is a stimulating factor for endocytosis (Farge et al. 1999; Rauch and Farge 2000). Lipid flipping affects lateral tension in opposing manners in the two monolayers, causing a change in the preferred state of bending. Consequently, it was suggested that the aminophospholipid translocase could be the primary molecular motor of endocytosis in confirmation of a model proposed earlier (Devaux 1991). In confirmation also was the fact that if the proteins that are likely to be responsible for the ATP-dependent phospholipid flippase activity were deleted by a knock out process, endocytosis was cancelled (Pomorski et al. 2003).

An asymmetrical lateral tension inducing membrane curvature and membrane invaginations or budding can be generated in giant liposomes if the distribution of lipids is made asymmetrical, for example by an increase in lipid population in one leaflet only (Farge and Devaux 1992, Traikia et al. 2002). The mismatch can be achieved by addition in one monolayer of a partially soluble lipid such as lyso-PC that stays in the same leaflet or by an ATP-driven stimulated flip-flop of phospholipids that are transported from one leaflet to the other by an ATP-dependent flippase.

In previous articles, we have shown that with a giant unilamellar vesicle (GUV) with a prolate shape, i.e. a liposome with a nonspherical symmetry, a single bud of large dimension is generated by in situ addition in the external leaflet of a small amount, about 1%, of lyso-lecithin per phospholipid molecule (Farge and Devaux 1992; Mathivet et al. 1996; Lopez-Montero et al. 2005). Here the lipid translocation was achieved by activation of the erythrocyte phospholipid flippase triggered by Mg^{2+}ATP injection. In order to monitor a shape change by optical microscopy, the use of giant vesicles was necessary (Fig. 1d–f). For this purpose, non-purified flippase contained in the erythrocyte membrane fragments, but freed from the cytoskeleton, i.e. inside-out vesicles (IOV), was used to swell the proteo-giant liposomes (PGL) via a limited dehydration step followed by a controlled rehydration step. The swelling techniques were inspired by protocols previously used and succintly described by other laboratories (Girard et al. 2004; Doeven et al. 2005). Typically IOV in suspension in the buffer

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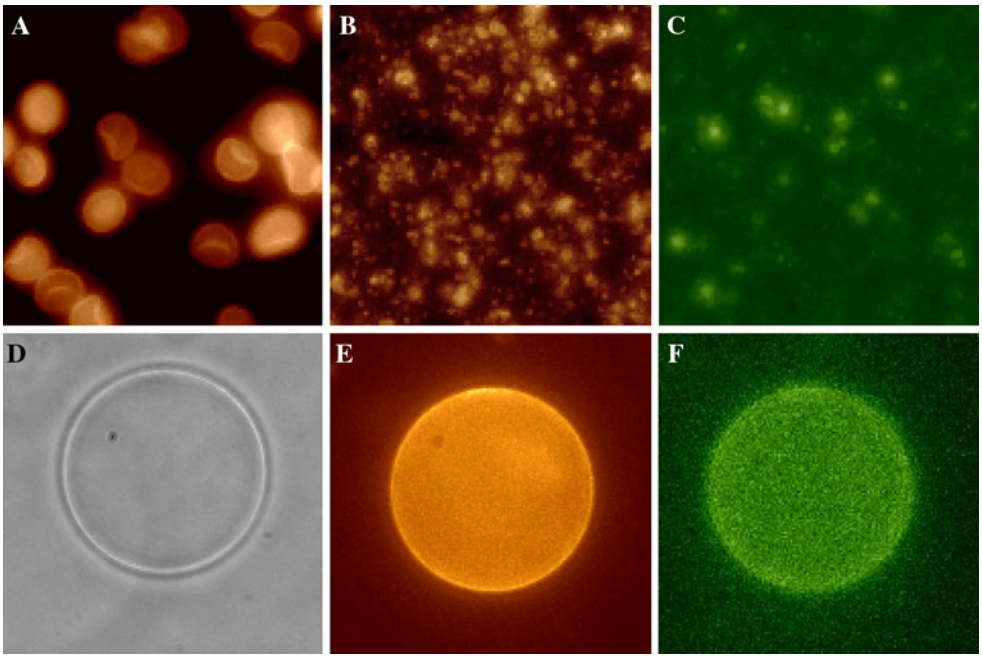

Fig. 1 Labelling samples with fluorescent lipids. **a** Erythrocyte ghosts labelled with rhodamine-PE (excitation at 555 nm; detection at 570 nm). **b** IOVs labelled with 2% rhodamine-PE. **c** Suspension of lecithin sonicated in the presence of C_{5-10} NBD-PC, excitation at 450 nm; detection at 520 nm (5 min sonication with a bath). **d–f** GUVs obtained with the previous lipid mixtures by electroformation (Mathivet et al. 1996)

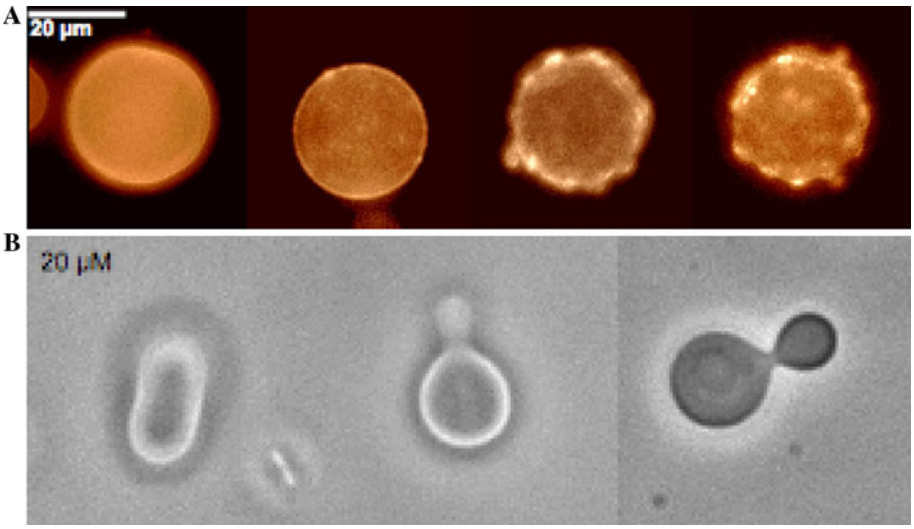

Fig. 2 **a** Shape change of a spherical vesicle, labelled with rhodamine-PE observed by fluorescence microscopy. **b** Shape change after Mg-ATP addition near a prolate shape vesicle showing a single large bud formation. t_0 is the time when Mg-ATP was added

(5 mM NaH_2PO_4, 10 µM EDTA) and liposomes made from azolectine or egg lecithin—1 mg of lipids total—were briefly sonicated (a few minutes with a bath sonifier), mixed with 10–15 fluxes using a pipetman in an Eppendorf tube and deposited upon an ITO or teflon support. A dehydration step (i.e. 5 min vacuum pumping) followed and then a controlled rehydration step, leading to PGL.

An injection with a micropipette, in approximately 15 s, of Mg-ATP (2 mM final) near the membrane containing the flippase molecules, PGL, at a distance of about 2 mm (Devaux et al. 2006; Papadopulos et al. 2007) gave rise to small local buds (Fig. 2a), whereas $MgSO_4$ (Fig. 3) or Na_2-ATP (not shown) at the same concentration, which were used in control experiments, had no effect on the vesicle surface. The bud formation with Mg-ATP showed the influence on the membrane of Mg-ATP injected near the surface and, at the same time, proved that the nonpurified flippases were still active after the transfer to the PGL, i.e. after the partial dehydration step.

Phospholipid flippases, which are ubiquitous proteins of eukaryotes, normally translocate lipids from the outer leaflet to the inner one. However, because the proteo-giant

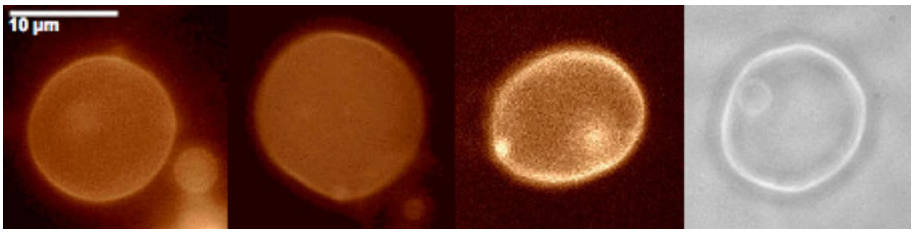

Fig. 3 Control experiments: absence of budding after injection of Na_2-ATP instead of Mg-ATP near a vesicle. Images were obtained with fluorescence microscopy except the last image, which was obtained by phase contrast microscopy

liposomes (PGL) were swollen from erythrocyte IOVs, and moreover, because ATP was added to the external leaflet, only the flippases with an inside-out orientation were activated, and an excess of lipids appeared in the outer monolayer of the vesicles (approximately 5 min after incubation). The difference in lipid population generated between the two leaflets explains the mechanical tension, which was caused by the mismatch between the two monolayers and gave rise to a bud.

Discussion and speculations

In no instances have we seen invaginations formed after such treatment, i.e. membrane bending towards the cytosol. This is an indication that the excess of lipids was always on the liposome's outer leaflet. Thus at least a large fraction of the ATPase molecules were oriented with the ATP binding site exposed towards the external environment of the vesicles. Furthermore the data indicated without ambiguity that the lipid transport activity of the red cell flippase was preserved during the various stages of the membrane manipulation. A priori a purification step of the flippase would seem necessary but such purification is difficult to reproduce systematically (Dolis et al. 1997), furthermore, it does not seem to be indispensable for investigating the possible roles played by the lipid transporters, at least in model systems.

The very fact that the flippase activity led to membrane curvature was an indication that the ATP-dependent flippase can provoke an increase in lateral tension within at least one side of a membrane. Hence we speculate that this increase in lateral tension can be "used" in a cell for other purposes than endocytosis, for example if the conformation of membrane protein is modified even slightly by the lateral pressure associated with the increase in lipids on one side (Martinac et al. 1990). This physical modification of a membrane may be a property of the flippase proteins and could be an important regulatory mechanism in membranes in general. For example lateral pressure may inhibit specific enzyme activities, including that of the flippase itself or of enzymes that synthesize new lipids. In other words, we suggest that the lateral pressure could be used as a regulatory mechanism to stop the activity of specific proteins and avoid "overcrowding" of lipids in a membrane.

In a recent publication, we discussed the size of buds as a function of the lateral tension of the giant vesicle (Devaux et al. 2008). In the present experiments, the lateral tension was not measured nor imposed, but the quasi-spherical shape indicated that the GUVs were initially under tension. In principle, the ratio r/R (which is the ratio of the bud radius to the giant vesicle radius) should be linked to the surface tension (see Fig. 4 in Devaux et al. 2008). A precise value of r is practically impossible to obtain with our images, which are at the limit of optical resolution, however, the last two photos in Fig. 2a of the present article allow one to estimate $r \sim 1$ µm, which would lead to a ratio r/R on the order of 0.1, hence to a tension of about $\gamma \sim 0.06$ mN/m according to the scheme drawn in Fig. 4 of the article by Devaux et al. (2008). This value of the local tension suffers from a large uncertainty but it is worth noting that (1) this value is possible and (2) (more interesting perhaps) if the tension is reduced, the size of the buds increases considerably (Fig. 2b), hence following the prediction of Devaux et al. (2008). We have attempted to modulate the tension with a micropipette. However, higher tension led to nonvisible buds, if any (below 1 µm, i.e. near the limit of the resolution of optical microscopy). Thus, the experiment was not very convincing because it could not allow us to measure with any precision the actual size of the buds (or radius of bending). Nevertheless one can conclude something that is intuitive because bending forces the system in one restrained configuration, hence is counterproductive in terms of entropy. Indeed entropy would tend to increase the number of configurations.

Finally these experiments may be considered, from a more general perspective, as an indication of a putative regulatory mechanism in membranes carried out by the flippase, similar to that of temperature, pH, or viscosity, caused again by the lipid environment of a membrane protein. In conclusion, we suggest that, by increasing the lateral tension, the flippase can modify the conformation of membrane proteins and not only that of specialized mechano-sensitive channels.

Acknowledgments The authors thank Dr. Bertrand (INSERM U665, Paris) for his continuous help through out this work. We thank also Dr. Ivan López-Montero for useful discussions. This work was supported by grants from the Université Paris 7-Denis Diderot, from the Centre National de la Recherche Scientifique, Unité mixte de recherche 7099, from the Research Training Network on Phospholipid

Flippases from the European Community MRTNCT-2004-005330, and from the Agence Nationale de la Recherche 2003-2-313.

References

Devaux PF (1991) Static and dynamic lipid asymmetry in cell membranes. Biochemistry 30:1163–1173

Devaux PF, Lopez-Montero I, Bryde S (2006) Proteins involved in lipid translocation in eukaryotic cells. Chem Phys Lipids 141:119–132

Devaux PF, Herrmann A, Ohlwein N, Kozlov M (2008) How lipid flippases can modulate membrane structure. Biochim Biophys Acta 1778:1591–1600

Doeven MK, Folgering JH, Krasnikov V, Geertsma ER, Van Den Bogaart G, Poolman B (2005) Distribution, lateral mobility and function of membrane proteins incorporated into giant unilamellar vesicles. Biophys J 88:1134–1142

Dolis D, Moreau C, Zachowski A, Devaux PF (1997) Aminophospholipid translocase and proteins involved in transmembrane phospholipid traffic. Biophys Chem J 68:221–231

Farge E, Devaux PF (1992) Shape changes of giant liposomes induced by an asymmetric transmembrane distribution of phospholipids. Biophys J 61:347–357

Farge E, Ojcius DM, Subtil A, Dautry-Varsat A (1999) Enhancement of endocytosis due to aminophospholipid transport across the plasma membrane of living cells. Am J Physiol 276(pt 1):C725–C733

Girard P, Pécréaux J, Lenoir G, Falson P, Rigaud J-L, Bassereau P (2004) A new method for the reconstitution of membrane proteins into giant unilamellar vesicles. Biophys J 87:419–429

Lopez-Montero I, Rodriguez N, Cribier S, Pohl A, Vélez M, Devaux PF (2005) Rapid transbilayer movement of ceramides in phospholipid vesicles and in human erythrocytes. J Biol Chem 280(27):25811–25819

Martinac B, Adler J, Kung C (1990) Mechanosensitive ion channels of E. coli activated by amphipaths. Nature 348:261–263

Mathivet L, Cribier S, Devaux PF (1996) Shape change and physical properties of giant phospholipid vesicles prepared in the presence of an AC electric field. Biophys J 70(3):1112–1121

Papadopulos A, Vehring S, Lopez-Montero I, Kutschenko L, Sockl M, Devaux PF, Kozlov MM, Pomorski T, Herrmann A (2007) Flippase activity detected with unlabeled lipids by shape change of giant unilamellar vesicles. J Biol Chem 282(21):15559–15568

Pomorski T, Lombardi R, Riezman H, Devaux PF, van Meer G, Holthuis JCM (2003) Drs2p-related P-type ATPases. Dnf1p and Dnf2p are required for phospholipid translocation across the yeast plasma membrane and serve a role in endocytosis. Mol Biol Cell 14:1240–1254

Rauch C, Farge E (2000) Endocytosis switch controlled by transmembrane osmotic pressure and phospholipid number asymmetry. Biophys J 78:3036–3047

Traikia M, Warschawski DE, Lambert O, Rigaud JL, Devaux PF (2002) Asymmetrical membranes and surface tension. Biophys J 83:1443–1454