Phospholipid Scramblases

Supplementary Issue: Cellular Anatomy of Lipid Traffic

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ABSTRACT: The distribution of phospholipid types between the two leaflets of a membrane bilayer is a controlled feature of membrane structure. One of the two membrane catalytic activities governing this distribution randomizes the composition of the two leaflets—the phospholipid scramblases. Two proteins (Xkr8 and TMEM16F) required for the activation of these activities have been identified. One of these proteins (TMEM16F) is quite clearly a scramblase itself and provides insight into the mechanism by which transbilayer phospholipid movement is facilitated.

KEYWORDS: lipid asymmetry, TMEM16, Xkr8, phospholipid transport

Introduction

By nature of the forces that stabilize them, membrane bilayers with a hydrophobic core require that their two leaflets have the same area. In primitive bilayers formed with molecules such as fatty acids,[1] rapid exchange of components between the two leaflets ensured that this condition was always met at the expense of homogenizing the composition of the two leaflets. Modern bilayers are constructed of phospholipids, and phospholipid headgroups are ions. As a result, they do not spontaneously exchange across the membrane bilayer at a physiologically relevant rate. Nevertheless, phospholipids do cross bilayers in all living organisms because membranes contain proteins that induce or facilitate such movement. While there is still much to be learned about phospholipid movements in different organisms and organelles, the mammalian plasma membrane provides an insight into two different modes of transbilayer phospholipid movement. The first kind of phospholipid movement is seen in the plasma membranes of normal growing cells: some types of phospholipids (eg, phosphatidylserine [PS]), when present in the outer leaflet facing the environment, are rapidly captured and transferred to the inner leaflet, while other types (eg, phosphatidylcholine) are not. This type of specific and active transport of phospholipids is catalyzed by ATP-dependent phospholipid translocases, a subfamily of the P-type ATPases that appeared in the earliest eukaryotes.[2,3] The second kind of phospholipid movement is seen in the plasma membranes of apoptotic cells or activated platelets, or in the endoplasmic reticulum (ER) or prokaryote cell membrane: in this case, almost all phospholipids move rapidly across the bilayer from either leaflet to the other. This nonspecific, bidirectional, and energy-independent phospholipid movement is catalyzed by phospholipid scramblase, an activity present in both eukaryotes and prokaryotes.[4,5]

Plasma Membrane Phospholipid Scramblases in Living Cells

A general requirement for a phospholipid scramblase activity arises in the ER in eukaryotes, or the cell membrane in prokaryotes, because the enzymes that fabricate phospholipids and the precursors from which they are constructed are located in the cytoplasm, so that newly formed phospholipids are inserted into the cytoplasmic leaflet of the membrane. The resulting imbalance in leaflet area would be profoundly destabilizing if phospholipids could not exchange freely from the cytoplasmic to the exoplasmic side. Phospholipid scramblase activities that catalyze such exchange have been demonstrated in both eukaryotes[6,7] and prokaryotes.[8]

A second membrane with demonstrated scramblase activity is the plasma membrane in animal and particularly vertebrate cells. There are two prominent physiological functions, blood clotting[9] and the removal of apoptotic cells,[10,11] that depend on these phospholipid rearrangements and have been the source of most of our information about scramblase activities. Studies of scrambling in these cells were the source of the general impression that phospholipid movement is bidirectional and not specific for any particular phospholipid,[12] although there is an upper limit to the size of the headgroups whose transbilayer movement is accelerated.[13] These studies also imply that the trigger for the activation of these scramblases is probably variable. In the ER, no activating signal is

known, as might be expected for an activity that is likely to be constitutive. In the coagulation pathway, scramblase is activated by cytoplasmic Ca^{2+} levels, although other mechanisms can contribute;[14] in addition to platelets, Ca^{2+}-activated scramblase is clearly present in all cells in the hematopoietic family, including lymphocytes and macrophages, but not in cells in other differentiation lineages.[15] Phospholipid scramblase activity is activated in apoptotic cells in order to expose PS on the surface; the latter is a general signal for engulfment of apoptotic cells.[16] The inducing signal in this case is not clear, but is probably not Ca^{2+},[17] and is likely to involve caspase cleavage in some cases,[18,19] but perhaps not always.[20]

Observation of the net scrambling of phospholipids inevitably requires a concentration gradient, so that no energy source for phospholipid movement has ever been needed or identified. In these measurements, the nonspecificity of scramblase activity has posed a mechanistic question: is scramblase activity fundamentally a channel activity, opening a diffusion path between the two leaflets, or is it a transporter, with scrambled phospholipids enclosed in a protein pocket that has alternating access to the two leaflets? One piece of negative evidence for the latter possibility was the absence of any large-scale conductance pathway associated with the opening of the scramblase link between the two sides of the membrane; given the large size and structural heterogeneity of the phospholipid headgroups that can traverse the bilayer by way of the scramblase, it is not easy to envision how small ions might be excluded from an open channel that was available to phospholipids.

Proteins that do Not Mediate Plasma Membrane Phospholipid Scrambling

Such detailed mechanistic questions were irrelevant in the absence of information about the protein machinery underlying the phospholipid scrambling activity that is so readily measurable in intact cells. Progress toward the identification of relevant proteins has encountered a variety of pitfalls. The relevant polypeptides were initially sought using membrane protein isolation and reconstitution, with several quite different outcomes. In the case of the ER scramblase, this strategy has substantially narrowed the collection of potential candidates without identifying a single molecule yet.[21] While promising, other results from this approach have generated very interesting, if perhaps not as promising, conclusions. For example, the reconstitution of membrane proteins from human erythrocytes (which contain the Ca^{2+}-activated scramblase activity) identified a protein (PLSCR1), which accelerated transbilayer phospholipid movements in vesicles in the presence of Ca^{2+}.[22] In fact, the elimination of this protein (still labeled as phospholipid scramblase in the NCBI Protein database) has no effect on phospholipid scrambling in hematopoietic cells,[23] and it may not even be a membrane protein.[24] The important lesson from this experience is that proteins may enhance phospholipid movement in the reconstituted vesicles, even though

that activity has no part in the protein's physiological function, and may even be unlikely to scramble phospholipids in vivo. An even more dramatic case of such behavior has been observed in the case of G protein coupled receptors, beginning with opsin.[25] Purified opsin substantially enhances transbilayer phospholipid movement when reconstituted into vesicles, and a similar behavior is observed with β1-adrenergic receptors.[25] While these interesting experiments have great promise as potential systems for studying the mechanisms of protein-enhanced phospholipid scrambling, there is no evidence (or any reason to think) that the G protein coupled receptors in plasma membranes of normal healthy cells support constitutive phospholipid scrambling. However, there is one important lesson from these experiments that should be emphasized—the ability to promote transbilayer movement of phospholipids seems to be an activity present, at least in embryonic form, in structurally very distinct proteins. In addition to the cautionary implications of this fact for interpreting experiments in simple biochemical and biophysical systems, these findings suggest that natural selection can find multiple materials ready to hand in physiological cases where the transbilayer movement of phospholipids is advantageous.

Xkr Family Proteins

In recent years, the confusing fog about the protein basis of phospholipid scramblase activity has been substantially lifted by the identification of two proteins that are involved in phospholipid scrambling at the plasma membrane of animal cells, one of which is clearly a scramblase itself. One of these plays a critical role in the scrambling of membrane phospholipids in animal cells as they enter apoptosis. The loss of lipid asymmetry in apoptotic cells results in the exposure of PS, which is the basic signal for the engulfment of apoptotic cells by their neighbors or by professional phagocytes.[5] Many years ago, a gene in nematodes, *CED-8*, was identified as an important component of the apoptotic machinery,[26] and subsequent careful examination showed that mutations in this gene result in the persistence of apoptotic cells because of the failure to expose PS.[18,19] The overexpression of a protein from the same membrane protein family, Xkr8, was shown to increase PS exposure in apoptotic mammalian cells.[18] This protein family is specific to the animal lineage, and its first member was originally identified as a membrane protein component of red cells, where it occurs as part of a heterodimer together with a membrane protease.[27] The presence of multiple transmembrane domains in this protein has long suggested that it is a transport protein of some kind, but there is not yet any experimental evidence that it transports anything, including phospholipids. On the other hand, its connection to apoptosis is clear. Both CED-8 and Xkr8 contain caspase cleavage sites, and the ability of these proteins to promote PS exposure is linked to their cleavage by caspases.[18,19] Several other members of the family (but not Xkr1, the red cell protein) can replace Xkr8, and those members of the family also contain caspase

cleavage sites.[28] The critical question is what exactly do these proteins do? One obvious possibility is that they are caspase-activated phospholipid scramblases; an alternative is that they sense the onset of apoptosis and transduce the caspase signal by direct or indirect interaction with some other protein that is the actual scramblase. Future investigations should soon resolve this issue.

TMEM16 Proteins

It has long been known that a Ca^{2+}-induced phospholipid scramblase activity catalyzes the exposure of PS on the platelet surface, where it serves as the platform for the assembly of protease complexes that underlay clot formation.[29] Moreover, a rare human mutation gives rise to Scott syndrome, a bleeding disorder resulting from the disappearance of this Ca^{2+}-induced scramblase from platelets,[30] as well as other hematopoietic cells, including erythrocytes and lymphocytes.[5] Scott syndrome mutations are defects in TMEM16F, a member of the TMEM16 or anoctamin family of membrane proteins.[31,32] At the time of this discovery, it was already clear that the TMEM16A member of this protein family is a Ca^{2+}-activated chloride channel,[33] showing that these proteins do mediate transbilayer ion movements. Remarkably, two fungal members of this family show robust Ca^{2+}-activated scramblase activity when reconstituted into vesicles,[34,35] and for one of these, a structure of the Ca^{2+}-bound form at atomic resolution has been determined.[35] This fungal phospholipid scramblase is a homodimer, with 10 transmembrane helices and a conserved Ca^{2+}-binding site in each of the two monomers. A striking feature of the structure is the presence of a relatively hydrophilic trench in each

monomer that faces the hydrophobic membrane core and extends from one side of the membrane to the other (Fig. 1). Domain swapping experiments have shown that the substitution of a helix that lines this trench into a Ca^{2+}-activated chloride channel member of the family can convert the latter into a phospholipid scramblase.[36] These data provide relatively direct evidence that this trench forms the pathway for phospholipid headgroup transfer across the membrane core. The dimensions of this trench imply that as phospholipids exchange between the two leaflets, the hydrophobic fatty acid side chains of those phospholipids probably never leave the hydrophobic core of the membrane. The general hydrophilic character of the trench, combined with the absence of a phospholipid bound anywhere along the trench, is consistent with the possibility that phospholipid scrambling does not depend on high affinity binding between a phospholipid and the scramblase. On the other hand, the absence of a Ca^{2+}-free structure makes it difficult to see how Ca^{2+} binding activates the pathway for phospholipid exchange.

The structure described above seems to settle the question of whether scramblases act as channels or as transporters in favor of the former: the TMEM16 family, at least, act as phospholipid channels. The issue of ion conductance through this protein has been the subject of a substantial number of investigations with very heterogeneous results.[37–39] The structure of the fungal protein suggests that the relatively small and variable currents observed in the reconstitutes of TMEM16 proteins may represent a low level of leakage along the same pathway taken by phospholipids.[35]

Remarkably, this protein family is found throughout the eukaryote lineage, including in unicellular organisms
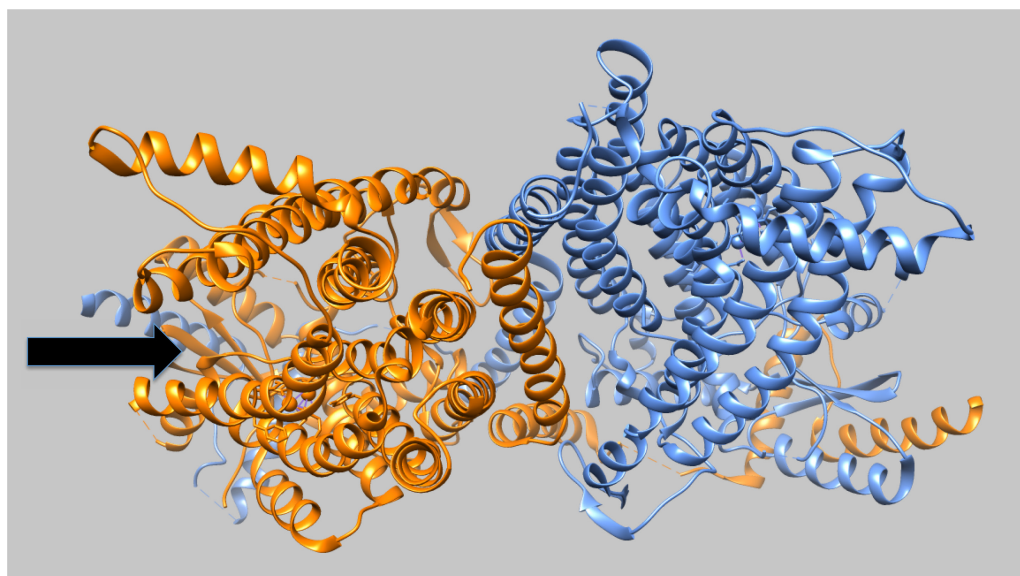

Figure 1. TMEM16 structure. The structure of the *Nectria haematococca* TMEM16 dimer is shown as viewed from the membrane outer surface. The symmetry axis is tilted slightly toward the top of this figure to reveal the hydrophilic trench (arrow) on the protein surface that extends through the membrane core and forms the likely path taken by the phospholipid headgroups in this Ca^{2+}-activated form of the scramblase. A similar groove is present in the other subunit but is obscured by the tilting of the viewing axis. Image created from PDB 4WIS[35] using Chimera (https://www.cgl.ucsf.edu/chimera/).

for which blood coagulation is an irrelevant function. Moreover, phospholipid scrambling may have been the original function of these membrane proteins.[36,40] It is not clear what might be the general physiological role played by Ca^{2+}-activated phospholipid scrambling; one intriguing clue may be provided by the observation that the Scott defect not only impairs blood clotting but also interferes with bone formation.[41] The mechanistic link between these two functions is currently obscure, but light shown on this problem may provide insight into the general function of this ancient activity.

Author Contributions

Wrote the first draft of the manuscript: PW. Contributed to the writing of the manuscript: PW. Jointly developed the structure and arguments for the paper: PW. Made critical revisions and approved final version: PW. Author reviewed and approved of the final manuscript.

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