Significance and Implications of Flippase Activity

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Abstract

Lipid bilayers are composed of lipids that are distributed unevenly across the membrane. Membrane asymmetry is maintained by energy-dependent flippase activity that keeps PS from accumulating in the outer (or luminal) leaflet by moving it across to the cytosolic leaflet. This is of crucial importance in membrane trafficking events, particularly those that employ clathrin-coated vesicles. Inhibition of flippase activity is associated with apoptosis and ingestion of apoptotic bodies by phagocytes. Some enveloped viruses can inhibit flippases (and stimulate scramblases) to disguise themselves as apoptotic bodies as they bud away from the cell. Binding of the exposed phosphatidylserine to PtdSer-mediated virus entry enhancing receptors then enhances viral attachment and entry. Drugs that interfer with apoptotic mimicry may be useful as anti-virals for enveloped viruses. Cancer cells, on the other hand, avoid apoptosis and may partially do so by upregulating flippases to prevent phosphatidylserine exposure and apoptotic uptake. Selectively knocking out flippases in cancer cells may be less harsh of a treatment on the body than radiation treatment.

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Introduction

The plasma membrane is a lipid bilayer that separates the cell interior from its exterior environment and regulates the movement of molecules in or out of the cell. Furthermore, in eukaryotic cells, lipid bilayers act additionally to divide the cell into various functional compartments called organelles [1]. These membranes are composed of three classes of phospholipids. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI) constitute the phosphoglyceride class of phospholipids, while other membrane components such as sphingomyelins and cholesterol derive from the sphingolipid and steroid classes respectively [2]. However, these various phospholipids are not distributed uniformly throughout eukaryotic membrane. PE and PS tend to localize on the cytoplasmic leaflet, while PC and sphingomyelins reside on the exoplasmic (or luminal) leaflet [3]. The importance of membrane asymmetry is emphasized by its role in signal transduction [4] and vesicle transport [5] and is further underscored by the loss of asymmetry as a marker for cellular apoptosis [6].

The spontaneous transfer of phospholipids from one leaflet to another is an unfavorable reaction that occurs slowly [7], but still contributes to the disorganization of membrane composition. Thus membrane asymmetry must be maintained by an energy-driven process [8].

Mediators of Membrane Asymmetry

There are three types of proteins that function in trans-membrane lipid transport. Scramblases are unique in that they serve to randomize membrane composition rather than to maintain asymmetry and are implicated in apoptosis as well as the activation of various lymphocytes [9]. Floppases, which include several members of the ATP-binding cassette (ABC) transporter family, nonspecifically move phospholipids from the cytosolic monolayer to the outer (or luminal) monolayer [9, 10, 11]. Flippases, which will be the focus of this review, translocate aminophospholipids (PS and PE) from the outer and luminal leaflets to the cytoplasmic leaflet [12].

P4-ATPases as Flippases

The existence of membrane asymmetry was first proposed by Bretscher in 1972 [3] and he went on to suggest that an enzyme may be responsible for its generation [13]. However, the first direct evidence for the existence of such an enzyme was not published until over a decade later [12]. ATPases were proposed as candidates for the phospholipid translocase [14, 15] and a new subfamily of P-type ATPases (referred to as P4-ATPases) was soon identified as possessing aminophospholipid-translocase (APT) activity and was thought to have diverged from its ion-translocating cousins [16, 17]. Research also showed

that flippases may be active not only in the plasma membrane, but in organelle membranes as well [14].

The first P4-ATPase to be identified and cloned was the mammalian ATPase II (now known as ATP8a1) [14, 16]. Since then, much of the research regarding flippases has been conducted using the yeast, *Saccharomyces cerevisiae,* resulting in the identification of five yeast P4-ATPases: Drs2p, Neo1p, Dnf1p, Dnf2p, and Dnf3p [18]. Research has identified P4-ATPases in other species as well, including the *homo sapiens* ATP8a1, ATP8a2, and ATP8B1 ATPases [19, 16, 20].

Molecular Mechanisms

P-type ATPases constitute a family of membrane pumps whose name originates from the conserved aspartic residue that is transiently phosphorylated during the reaction cycle [17]. Although each subfamily has a different substrate specificity, the structure of P-type ATPases is highly conserved throughout the five subfamilies [21]. The pump contains five functionally and structurally distinct domains. The three cytoplasmic domains include the actuator (A) domain, nucleotide-binding (N) domain, and the phosphorylation (P) domain, while the remaining membrane-embedded domains comprise of the transport (T) and support (S) domains. The N domain has kinase activity and serves to phosphorylate the Asp residue present in the P domain during the reaction cycle. The A domain has phosphatase activity and functions to dephosphorylate the P domain. The T and S domains bind and move molecules across the membrane with the S domain providing structural support to the T domain throughout the reaction cycle [17].

Multiple models exist to explain the reaction cycle of P-type ATPases. The traditional E1E2 model claimed the existence of only two conformational states referred to as E1 and E2 [22, 23]. The current model, however, states that there are two main and two intermediate conformational states of the pump, known as E1/E2 and E1-p/E2-p respectively [24, 25, 26]. In the E1 conformation, high-affinity binding sites are exposed and bind substrate. Binding allows for phosphorylation of the P domain and causes a conformational change that opens the pump on the other side of the membrane. In this new conformational state termed E2, the high affinity binding sites are lost and the substrate will diffuse away from the low-affinity binding sites. Dephosphorylation and the binding of a counter-ion allows the pump to return to its original conformation [17, 18].

Association with CDC50

Many P-type ATPases also form a complex with a ß-subunit and this association is often required for protein maturation and transport out of the endoplasmic reticulum to its respective location [27, 28, 29]. These Cdc50 family proteins were first discovered in yeast as Lem3p, Ros3p, and Cdc50p [30, 31, 27]. In mammals, three proteins homologous to those in yeast were identified and termed Cdc50A-C. Cells with mutated copies of Cdc50 demonstrate dysfunctional flippase activity and maintenance of membrane asymmetry similar to cells with mutated P4-ATPases [32, 27, 30]. Other research shows that lack of the ß-subunit prevents the P4-ATPase from forming its phosphorylated intermediates and that the affinity for Cdc50 changes throughout the reaction cycle [33, 29]. These results suggest that association with Cdc50 family proteins may be essential for the catalytic function of APTs and similar roles have been found for ß-subunits of other P-type ATPase subfamilies [34].

Regulation

In addition to the five structural domains mentioned above, many P-type ATPases possess a regulatory (R) domain at the C-terminal tail [35]. Often, these R domains possess auto-inhibitory activity and phosphorylation allows certain proteins to bind and displace the R domain to stimulate translocase activity [35, 36, 37, 38, 39]. In yeast, guanine nucleotide exchange factor (Gea2p) was found to bind to the C-terminal tail of Drs2p and to activate its flippase activity [40]. Thus, like the H^+ and Ca^{2+} P-type ATPases, the C-terminal tail of Drs2p may possess an R domain with auto-inhibitory function. Furthermore, the effectors that control regulation of P4-ATPases seem to differ based on the localization of the flippase [41]. Drs2p in yeast, for example, functions in the transgolgi network (TGN) where it is presumably involved in membrane trafficking [42]. In general, phosphoinositides reside in cellular membranes where they act as markers of organelle identity [43] and play roles in cell signaling [44]. Binding of particular phosphatidylinositols has been implicated in flippase activation. Phosphatidylinositol-4-phosphate (PI4P) is localized to the TGN where it recruits various proteins that are involved in clathrin-coated vesicle budding [45, 46, 47]. Binding of PI4P at the Drs2p R domain activates its translocase activity [48]. TGN-specific function of PI4P and localization of Drs2p to the TGN suggests that activation of flippase activity by PI4P may be particular to TGN membranes. In addition, the Ypt31p/32p-Rcy1p pathway is involved in the endocytic and exocytic recycling path-

ways. The TGN, where Drs2p resides, plays important roles in these membrane trafficking pathways. The Ypt31p/32p-Rcy1p pathway has been shown to interact with and perhaps regulate the Drs2p-Cdc50p complex [49] and may be another instance of location-specific regulation. Further occurrences of location-specific regulation come from the yeast P4-ATPases Dnf1p and Dnf2p which are active in the plasma membrane [50]. These flippases do not interact with the Ypt31p/32p-Rcy1p pathway [49] nor has an association between PI4P or Gea2p and Dnf1p or Dnf2p been found. Rather, the kinases Fpk1p and Fpk2p have been identified as upstream regulators of the plasma membrane flippases [51]. However, regulation by Fpk1p/Fpk2p phosphorylation is not specific to Dnf1p/Dnf2p but is also implicated in Drs2p activity [51]. Because Dnf1p is involved in both the endo- and exocytic pathways, it seems to take up transient residency in the TGN [42]. It is possible that that even though Drs2p is primarily localized at the TGN [42], it may exist transiently in the plasma membrane due to the cycling of membranes from membrane trafficking events. If this is the case, it would indicate that even though regulation by Fpk1p/Fpk2p phosphorylation is not specific to particular P4-ATPases, it is still specific to flippase activity at the plasma membrane.

Significance of Flippases

Membrane Trafficking

Membrane trafficking in a crucial part of many cellular processes such as the secretory pathway and endocytosis. Trafficking usually involves the formation vesicles that transport cargo from one part of the cell to another. Three types of coated vesicles carry out these processes: COPII vesicles, COPI vesicles, and clathrin vesicles [44]. P4-ATPases have been implicated in many membrane trafficking roles involving clathrin coated vesicles. In yeast, Dnf1p and Dnf2p are involved in the formation of endocytic vesicles from the plasma membrane [50], while Drs2p functions in the formation of clathrin-coated vesicles from the TGN [42, 52]. Furthermore, Neo1p has been associated with receptor-mediated endocytosis, formation of vacuoles, and retrograde transport from the TGN to the ER [53, 54]. It is not yet known how flippases facilitate vesicle budding but various theories have been proposed. Adaptor and coat proteins such as AP complexes and clathrin are crucial to vesicle formation [44]. Researchers have theorized that P4-ATPases can recruit these proteins to the site of budding based on the observation that Drs2p has a binding site for Gea2p at its C-terminal tail [40]. However, it has been shown that Drs2p, although important, is not necessarily essential for protein recruitment to the TGN [55]. Alternatively, it has been proposed that PS accumulation in one leaflet via flippase activity causes membrane bending that is recognized by coat and adaptor proteins and thus facilitates budding [56, 41]. One more theory states that coat and adaptor proteins recognize a specific phospholipid and its accumulation from flippase activity could recruit these proteins needed to stimulate membrane invagination [41].

Viral Entry and Replication

It has long been known that the presence of PS on the outer membrane of eukaryotic cells is a marker of apoptosis. These aminophospholipids typically serve as an "eat me" signal to circulating macrophages [6]. Flippases are present in the membranes of healthy, normal cells and constitutively translocate PS back to the inner membrane. Therefore, for PS to accumulate on the outer leaflet during apoptosis, these translocase mechanisms need to be inhibited. Caspases are proteases with roles in many apoptotic signaling pathways. They cleave various substrates within the cell and the proteolysis of these target proteins eventually leads to cell death [44]. Recent evidence suggests that flippase molecules may be a target for caspase activity [57]. Cleavage of the ATP11C flippase in human cells inactivated translocase activity. Furthermore, mutations at the caspase recognition sites located on the flippase resulted in a caspase-resistant ATP11C and cells with these mutated flippases did not accumulate PS on the outer membrane during apoptosis and thus were not ingested by macrophages. This research provides direct evidence that flippase inactivation is required during the apoptotic process.

Apoptotic cells are not the only entities to overexpress PS on their outer membrane. Several groups of enveloped viruses employ a mechanism called "apoptotic mimicry" that enhances their attachment and entry into cells [58]. These include flaviviruses, filoviruses, alphaviruses, baculoviruses, and New World arenaviruses [59, 60, 61]. These viruses mask themselves as apoptotic entities by PS exposure on the outer membrane of their envelope and thus trick cells such as macrophages into ingesting them. It has not yet been elucidated how viruses increase their PS concentrations on the outer portion of the envelope. Because their membranes do not possess flippases to maintain phospholipid distribution, it is possible that such accumulation is the result of spontaneous flip-flop and randomization of the envelope membrane [62]. Since enveloped viruses bud away

from the host cell membrane, it may also be that they are taking advantage of natural cell stress and apoptotic responses that are leading to PS exposure. Infection with influenza A or some flaviviruses results in cell stress leading to apoptosis [63, 64, 65, 66]. Budding away from the membrane of an apoptotic cell would result in an envelope with the same sort of PS distribution. Alternatively, rather than passively exploiting the cell's responses, the viruses may themselves be inducing the deregulation of membrane asymmetry. Some viruses, such as Chikungunya and West Nile Virus seem to actively induce apoptosis [67, 68].

However, not all viruses that employ apoptotic mimicry induce apoptosis, actively or passively. Ebola virus, for example, enhances viral entry via apoptotic mimicry, but does not induce apoptosis in its host [69, 60, 61, 70]. Other viruses, like vaccinia virus, even produce proteins with anti-apoptotic functions [71, 72]. It follows then, that there are other mechanisms of inactivating flippases and altering the cell membrane phospholipid distribution besides induction of apoptosis. Researchers have shown that an increase in cytosolic calcium levels is associated with disruption of membrane asymmetry and scrambles the phospholipid composition [73]. It was previously mentioned that phosphatidylinositols may play a role in activating flippase translocase activity [48]. The degree of calcium-induced phospholipid scrambling has been shown to have a relationship with phosphatidylinositol 4,6- bisphosphate (PIP2) levels [74]. During cell signaling, PIP2 is cleaved into second messengers that initiate the IP3/DAG pathway ultimately triggering elevations in calcium concentrations [44]. It follows that more PIP2 present in the plasma membrane would lead to higher calcium levels following signal transduction. If PIP2 serves as an upstream regulator for flippase activity, it is possible that downstream effectors from the IP3/DAG pathway may inhibit these enzymes and could account for the observations seen by Sulpicess et al [74]. However, another research group found that complexing PIP2 with calcium was not sufficient to account for the rapid redistribution of phospholipids in the membrane [75]. They concluded that there must be other cellular processes involved. This is likely because once flippases are inactivated, the spontaneous flip-flop of phospholipids is energetically unfavorable and does not occur rapidly [7, 8, 76]. Therefore, while flippase inactivation seems to be required for PS accumulation on the outer membrane by either apoptotic or calcium-mediated methods, another enzyme must be required for its rapid redistribution [77**?**]. This protein, termed a scramblase, was isolated and reconstituted in 1996 [78, 77] and has also been implicated in the scrambling of apoptotic membranes [9]. It exhibits non-specific, bidirectional, energy-independent translocase activity. Thus, whether through apoptotic or non-apoptotic means, some enveloped viruses induce (either passively or actively) flippase inhibition and scramblase activation. This alters the membrane composition, allowing the virus to take advantage of it as it buds away from the cell with its own PS-enriched membrane.

There is a group of receptors on cell surfaces that bind the PS in viral envelopes that has been termed PtdSer-mediated virus entry enhancing receptors (PVEERs). A recent review covers PVEERs in depth [62], but their mechanism will be briefly outlined here. As already mentioned, these receptors bind exposed PS in membranes but their primary context is not that of viral envelopes. Rather, they chiefly serve as engulfment receptors to bind and mediate clearing of apoptotic cells [79]. The apoptotic mimicry used by these viruses allows them to disguise themselves as apoptotic bodies and hijack these receptors to find their way into the cell. Thus far, six engulfment receptors are known to act as PVEERs, and are expounded upon elsewhere [62]. Engulfment receptors bind PS in one of two ways. TIM-1 and TIM-4 are two well studied PVEERs that bind PS directly [80, 81, 82], while another set of PVEERs known as the TAM receptors bind PS indirectly through the use of a bridging molecule such as Gas6 or Protein S [83, 84]. Furthermore, engulfment receptors may also function in one of two ways. They may be directly involved in the cell signaling processes that mediate uptake, or they may only mediate adhesion, allowing other receptors to engage their ligands and facilitate uptake [79]. Evidence shows that TIM-4 is not directly involved in signaling [85] and therefore may only be involved in adhesion. This is consistent with the lack of evidence supporting a direct internalization mechanism, and that PVEERs seem to enhance uptake by other means such as macropinocytosis or endocytosis [79]. Although PVEERs may not be strictly required for internalization, facilitation by binding to these receptors may affect the tropism for some enveloped viruses [79].

In sum, some enveloped viruses inhibit flippase activity and activate scramblase activity to alter the cell membrane composition and thus mask themselves as apoptotic bodies following budding. One question that still needs answering is whether by altering the viral envelope composition, is it possible to prevent or mitigate infection. Thus, by interfering with apop-

totic mimicry (i.e. preventing inhibition of flippases), it may be possible to inhibit viral entry. Further research is needed to elucidate a safe targeting mechanism, but targeting flippases may be useful as an anti-viral mechanism for enveloped viruses.

Cancer

One of the hallmarks of cancer is evasion of apoptosis [86]. Normal cells expose PS on the outer surface of their membranes during apoptosis and research shows that the presence of PS is a sufficient "eat me" signal [6]. It is possible that one mechanism of avoiding apoptosis may be to prevent the accumulation of PS in the outer leaflet (i.e. by flippases). The first evidence of enhanced phospholipid translocase activity in cancer was in 1983 [87]. Although these results were confirmed by others [88, 89], surprisingly little research on the role of phospholipid translocases in cancer has been done since. Triggering apoptosis in cancer cells results in cell signaling events that inhibit flippase activity and the appearance of PS in the outer membrane [90, 91]. The possibility of stimulating phagocytic removal of cancer cells by inducing PS exposure was proposed by Chang et al [92]. Levano et al have demonstrated the effective use of a genetic therapeutic strategy that selectively targets cancer cells in vitro [93]. In the study, they used tagged mutant forms of the mammalian flippases ATP8a1 and ATP8a2 that efficiently disrupted flippase activity in transfected cells. To selectively target cancer cells, the cells were co-transfected with a promoter that was more highly expressed in cancer cells (GntV). Transfected cells displayed an increased accumulation of PS. Further research is necessary to elucidate whether this method can be used to selectively induce phagocytosis of cancerous cells without unintentionally causing ingestion of normal cells. If selective phagocytosis by inhibiting flippase activity can be demonstrated in vivo, this strategy may be useful as an alternative, less toxic therapeutic for cancer.

Conclusion

Lipid bilayers are selectively permeable membranes that separate the cell interior from the exterior and divide the interior into compartments. They are composed of lipids that are distributed unevenly across the membrane. Because the organization of the membrane composition deteriorates over time, membrane asymmetry must be retained by energy-dependent flippase activity. This keeps PS from accumulating in the outer (or luminal) leaflet by moving it across to the cytosolic leaflet. Membrane asymmetry plays a significant role in membrane trafficking events and are important in the formation of clathrin-coated vesicles. When translocase activity is inactivated, this allows PS to be exposed on the outer cell membrane. This exposure is associated with apoptosis and the phagocytosis of apoptotic cells. Some viruses take advantage of these mechanisms by which phagocytes ingest apoptotic cells. During infection with some enveloped viruses, flippase activity is inhibited, either through induction of apoptosis or other means. They also activate scramblases to more rapidly deorganize the phospholipid distribution, which allows PS to build up just like during apoptosis. Then as viruses bud away from the cell, they have a membrane that mimics that of an apoptotic cell, a strategy called apoptotic mimicry. Binding of the exposed PS in viral membranes to PVEERs on phagocytes then enhances viral attachment and entry. It may be possible to inhibit the ability of the virus to disguise itself with an apoptotic-like membrane by preventing the inhibition of flippase activity. Drugs that interfere with these processes may be useful to treat some infections with enveloped viruses. On a different note, cancer cells have disrupted cell processes that allow them to prevent apoptosis. One mechanism by which they could do this might be by up-regulating translocase activity to prevent PS exposure on the outer membrane and thus apoptotic uptake. It may be possible to redirect these processes and inhibit flippase activity specifically in cancerous cells. This would cause a loss of membrane asymmetry and allow the cancer to be cleared by the body's own immune system. Further research is necessary to test these ideas in vivo, but if successful, it could prevent the necessity for harsh chemotherapeutic treatments.

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