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Review

Phospholipid scramblase: An update

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ABSTRACT

The best understood consequence of the collapse of lipid asymmetry is exposure of phosphatidylserine (PS) in the external leaflet of the plasma membrane bilayer, where it is known to serve at least two major functions: providing a platform for development of the blood coagulation cascade and presenting the signal that induces phagocytosis of apoptotic cells. Lipid asymmetry is collapsed by activation of phospholipid scramblase(s) that catalyze bidirectional transbilayer movement of the major classes of phospholipid. The protein corresponding to this activity is not yet known. Observations on cells from patients with Scott syndrome, a rare hereditary bleeding disorder resulting from impaired lipid scrambling, have shown that there are multiple activation pathways that converge on scramblase activity.

1. Introduction

Phospholipids in the plasma membrane of mammalian cells are not randomly distributed between the two leaflets of the membrane bilayer. Choline-containing phospholipids phosphatidylcholine (PC) and sphingomyelin (Sph) dominate the outer leaflet, while the aminophospholipids phosphatidylethanolamine (PE) and phosphatidylserine (PS) are largely components of the inner leaflet. Although ongoing asymmetric lipid synthesis and chemical modification make some contribution, ATP-dependent directional lipid transport is the primary mechanism for generation and maintenance of lipid asymmetry. The latter transport is catalyzed by an enzyme called the aminophospholipid translocase, a P-type ATPase that specifically and rapidly transports the aminophospholipids, phosphatidylserine and phosphatidylethanolamine, from the outer to the inner leaflet of the plasma membrane [1]. Because uncatalyzed transbilayer movement of lipids is slow, lipid asymmetry is stable in quiescent cells; inactivation of active lipid transporters such as the aminophospholipid translocase does not itself result in rapid loss of membrane lipid asymmetry. Nevertheless, dissipation of membrane lipid asymmetry is an important physiological process, and the machinery for accomplishing it is universal to most eukaryotic cells. At least one, and probably more than one, membrane protein is required to facilitate a rapid loss of lipid asymmetry, yet no protein mediating this function has been identified. The underlying process of lipid randomization can be readily observed, and the unknown protein(s) which catalyze it are called 'phospholipid scramblases'. A search of protein data bases reveals at least 4 'phospholipid scramblases' from different species, but, as will be outlined below, there is good evidence that the protein(s) so labelled in fact have a different function. This review will focus on the most important properties and functions of this elusive protein; we will restrict ourselves to proteins responsible for collapse of lipid asymmetry in the plasma membrane of eukaryotic cells. Similar, but equally elusive proteins facilitating bidirectional lipid transport in biogenic membranes have been reviewed elsewhere [2].

2. Physiological importance of phospholipid scrambling

2.1. Surface exposed PS promotes blood coagulation

Because PS is normally excluded from the external leaflet of the plasma membrane, the most pronounced effect of a collapse of lipid asymmetry is exposure of PS at the cell surface. Rapid loss of lipid asymmetry was first discovered in blood platelets [3]. Upon vessel wall rupture, platelets become activated and accumulate at the site of injury, where they cause a primary arrest of bleeding. When activated by contact with the subendothelial matrix, particularly collagen, and trace amounts of thrombin, platelets lose lipid asymmetry. The surface exposed PS provides a platform for the assembly of highly active protease/cofactor complexes, accelerating two sequential reactions of the coagulation cascade, the activation of factor X and the subsequent formation of

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thrombin by activated factor X. Platelets have the highest rate of scramblase activity known, which guarantees the rapid and localized coagulation of the blood. A compromised lipid scrambling process in platelets will therefore become manifest as a bleeding diathesis as illustrated by the Scott syndrome [4] (vide infra). Alternatively, unwanted PS exposure on circulating blood cells or cells lining the vessel wall presents a risk of thrombosis.

2.2. Surface exposed PS induces recognition of apoptotic cells

In 1992, a more general role for PS exposure was uncovered when it was reported that apoptosis-induced PS exposure in lymphocytes [5], a phenomenon which is now a generally accepted hallmark of cells in apoptosis. PS exposure results from the same inhibition of aminophospholipid translocase activity and activation of scramblase activity that accounts for PS exposure in activated platelets [6,7]. Surface exposure of PS on apoptotic cells presents a recognition and engulfment signal for removal by phagocytosis competent cells even before the morphological changes usually associated with cell death occur [8]. As a variant on this process, removal of senescent hematopoietic cells from the circulation may also be controlled by surface exposed PS as signal for the reticulo-endothelial system. Increased PS exposure on the surface of erythrocytes may explain drug-induced or disease-related anemia [9]. Normal engulfment of PS exposing cells does not stimulate an immune response. Consequently, anything that compromises recognition of PS exposing cells by the reticulo-endothelial system may evoke an autoimmune syndrome caused by presentation of self-antigens to the immune system [10,11].

2.3. Surface exposed PS in parasite and development

PS-dependent recognition and engulfment is a potential pathway for pathogenic invasion of phagocytic cells; Leishmania parasites seem to use apoptotic mimicry to invade macrophages [12] and there is evidence that infection of monocytes by HIV is dependent on PS exposure [13]. Also, surface exposed PS in the membrane of vaccinia virus appeared critical for host infection [14]. It is not known what role scramblase plays in the exposure of PS on these membrane surfaces.

Less well documented examples of physiological PS exposure, presumable related to scramblase activity, are fusion related processes. Transient PS exposure appears to play a role in the formation of myotubes in muscle cell differentiation [15] and also in the formation of the placental syncytiotrophoblast layer from fusing trophoblasts [16]. A potentially related phenomenon is the scramblase mediated collapse of lipid asymmetry in the sperm membrane, an event which seems to be essential to the capacitation reaction [17].

3. Characteristics of the lipid scrambling process

3.1. Lipid specificity

Spin-labelled and fluorescent (NBD)-labelled phospholipid probes have been used to estimate the rates of in- and outwardly directed movement for each of the major phospholipid classes. One early study of activated platelets reported that the rate of externalisation of PS and PE greatly exceeded the rate of inward movement of PC [18], suggesting that PS exposure might be an active transport process. However, subsequent measurements found that the rates of in- and outwardly movement were similar for PS and PC following scramblase activation in platelets and erythrocytes [19]. Dekkers et al. reported that the rate of inward movement of NBD-PE exceeds that of NBD-PC, but that the outward rate was slower [20], differences that disappeared upon methylation of the aminogroup of PE. Although this result might suggest that the rate of lipid scrambling is influenced by the presence of an amino group, no such differences were found for NBD-PS. It has also been suggested that internalization of NBD-sphingomyelin is appreciably slower than its outward movement, which in turn appeared indistinguishable from that of NBD-PC [19,21,22]. This result might suggest that a lower rate of inward movement of ceramide-derived lipids reflects their high affinity for lipid rafts, and a resulting decrease in their accessibility to the scramblase. It should be noted that high resolution measurements of NBD-Sph inward movement in activated platelets showed no difference between the rates of Sph and PC movement [23], suggesting that the scramblase itself does not distinguish between inward and outward Sph movement.

Scrambling is not restricted to natural phospholipids or their spin-labelled or fluorescent-labelled derivatives; also lipid-like molecules such as platelet activating factor (PAF) [24], lyso-PC [25], fatty-acid carnitine esters [25] and trimethylammonium–diphenylhexatriene (TMA–DPH) [26] participate in the scrambling process. One important study used headgroup modified NBD-phospholipids to investigate the relationship between rate of scrambling and the size of the polar headgroup. Scrambling rates were found to decrease successively as the headgroup increased in size in the sequence of phosphatidylgalactose, phosphatidylmaltose and phosphatidylmaltotriose [20]. This finding suggests that the scramblase creates a proteinaceous aqueous pore that facilitates migration of the polar headgroup of the lipids across the hydrophobic core of the bilayer, while keeping the acyl chain moieties in the core of the bilayer.

3.2. Rate of lipid scrambling depends on cell type

As mentioned above, the cell with the highest rate of lipid scrambling is the platelet, as expected since PS exposure in these cells is needed to provide a procoagulant surface at sites of vascular injury. Quantitative measurements of the scramblase rate in different cells revealed that in comparison with the highest rate constant found for platelets ($78\cdot \times 10^{-3}\ s^{-1}$), ionophore activation of scramblase in T-cells and EBV-transformed B-cells gave rate constants over a range from 1.0 to $24 \times 10^{-3}\ s^{-1}$. Of all the cells in which scramblase is activated by Ca^{2+} and ionophore, erythrocytes have the slowest scrambling rate (rate constant, $0.45 \times 10^{-3}\ s^{-1}$) [27].

These rate constants represent maximal rates, under conditions of strong activation with ionophore and Ca^{2+}. Care should be taken, though, when comparing these rates to bulk measures of PS exposure, such as procoagulant activity or internalization of lipid analogs in cell suspensions. For instance, procoagulant activity in platelets activated with Ca^{2+}-ionophore develops within a minute, whereas 10–15 min are required before an end stage of procoagulant activity is reached after stimulation with physiological agonists. Flow cytometric analysis revealed that with physiological agonists, only a fraction of the platelets shows PS exposure comparable to the maximum observed after ionomycin treatment, but that the size of this fraction increases in time [28,29]. These data indicate that the difference between the two cases does not reflect conflicting measures of scramblase rate, but rather differences in the efficiency of scramblase activation.

4. Proposed candidate proteins

4.1. Phospholipid scramblase, PLSCR

Initial attempts to identify a protein responsible for lipid scrambling used reconstitution experiments with membrane protein

fractions from platelets and erythrocytes [30,31]. From erythrocytes (but not platelets), a rather homogeneous fraction with a prominent band at 36 kDa was isolated; identification of peptide sequences from this purified protein led to cloning of the cDNA and identification of a gene, referred to as 'PLSCR', for phospholipid scramblase [32]. The human genome contains four members of this family (hPLSCR1-4), and orthologs are present in mouse, rat, yeast, *Drosophila* and *Caenorhabditis* elegans genomes [33]. It was found that levels of PLSCR1 mRNA correlated reasonably well with scramblase activity in a variety of hematological and non-hematological cells and tissues [34]. Estimation of PLSCR1 protein expression using specific antibodies showed that platelets have a 10-fold excess of this protein relative to erythrocytes, in line with the observed rates of lipid scrambling between these cells [32]. Stable transfection of PLSCR1 in Raji cells, which show a low constitutive expression of this protein, resulted in an increased Ca^{2+}-inducible scrambling activity [34].

In spite of this and other circumstantial evidence correlating PLSCR1 and scramblase activity, follow-up studies have shown that identification of PLSCR as the scramblase was incorrect. Early results showed that transcriptional upregulation of hPLSCR by interferon had no effect on PS exposure [35] and no correlation was found in six different cell lines between expression levels of this protein and apoptosis-induced PS exposure [36]. Overexpression of PLSCR1 in Raji cells failed to confer apoptosis induced PS exposure in these cells [36], contrasting with a previous report by Zhao et al. looking at Ca^{2+}-ionophore induced scrambling [34]. Most importantly, platelets from PLSCR$^{-/-}$ knockout mice have no hemostatic defects and show normal PS exposure upon activation [37]. In addition, single or double deletion of two PLSCR homologs in *Drosophila melanogaster* or RNAi mediated knock down of 10 annotated homologs of hPLSCR1 in *C. elegans* did not compromise PS-mediated phagocytic clearance or apoptosis induced PS exposure, respectively [38,39]. Together, these data show that PLSCR is not the scramblase, nor is it required for scramblase activation or activity. Recent data suggest that it plays a role in cell signalling (for review on PLSCR, consult [33].

4.2. Abca1

ABCA1, a member of the family of ATP-binding cassette (ABC) transporters, is required for the conversion of apoA1 to HDL [40]. Mutations in this protein cause Tangier disease, an autosomal recessive disorder characterized by the virtual absence of circulating HDL and accumulation of cholesterol in peripheral tissues. A role for ABCA1 in Ca^{2+}-induced PS exposure was suggested from in vivo loss of function and in vitro gain of function experiments [41]. Engulfment of apoptotic cells is impaired in ABCA1$^{-/-}$ mutants and it was reported that fibroblasts from these knock out mice show a decreased Ca^{2+}-induced scramblase activity. Experiments with erythrocytes from ABCA1$^{-/-}$ mice suggested that Ca^{2+}-induced PS exposure is impaired in these cells, as judged by a decreased potency in formation of a procoagulant lipid surface and a decreased externalisation of spin-labelled PS. It was also observed that generation of ABCA1 null mice was associated with high lethality during the first weeks after birth; autopsies revealed deep perivisceral hemorrhages suggesting that the mutation reduced the effectiveness of the coagulation process [41]. Support for a role of ABCA1 in scramblase-mediated PS exposure came from Albrecht et al who found a single missense mutation in the ABCA1 gene of a patient with Scott syndrome in which lipid scrambling is compromised [42]. Together, these data suggested that ABCA1 might be the scramblase, or a component of the machinery that activates it.

Several observations, however, argue against a role for ABCA1 as lipid scramblase. The original reports suggested that Ca^{2+}-induced transbilayer movement of spin-labelled PC was unchanged in ABCA1$^{-/-}$ erythrocytes [41], while, as indicated above, rates of scramblase-mediated PS and PC movement in various blood cells are normally quite similar [22,23]. It might be thought that externalisation of PS requires one activity (i.e., ABCA1) while PC movement depends on another (scramblase), but then both activities must be impaired in cells from patients with Scott syndrome. In fact, platelets from patients with Tangier disease, though having abnormal hemostatic function, show normal PS exposure upon activation with collagen or Ca^{2+}-ionophore [43] and Ca^{2+}-induced PS exposure in murine B-cells was found to be independent of ABCA1 [44]. Similarly, studies with ABCA1 null mouse and human cells (Tangier disease) showed that Ca^{2+}-induced and apoptosis induced scramblase activity is still present in lymphocytes, fibroblasts and macrophages [45]. The alternative possibility that ABCA1 modulates the activity of the scramblase was rejected since no appreciable disturbance was observed in the onset of the scramblase-mediated phospholipid movement in ABCA1-deficient cells. In conclusion, it is unlikely that either PLSCR or ABCA1 are responsible for Ca^{2+}-induced loss of lipid asymmetry.

5. Scramblase activation

Even if the protein that mediates transbilayer phospholipid scrambling were known, it is not certain that the mechanism by which this protein is activated would be known as well. In fact, there is evidence that there is more than one way to activate this plasma membrane activity, and, less certainly, that these activation mechanisms converge on a common scramblase, and may operate in parallel in the same cell.

5.1. Ca^{2+} activation

Considering the importance of cytoplasmic Ca^{2+} as regulatory mechanism, it is not a shock that this bivalent cation can play a role in the regulation of lipid scrambling. The clearest demonstration of its capabilities comes from studies of resealed erythrocyte ghosts, which retain their normal transbilayer lipid distribution when prepared in the absence of Ca^{2+}, but lose lipid asymmetry when as little as 10 μM Ca^{2+} is present in the lysis buffer. Ca^{2+} can be replaced by Sr^{2+} to evoke lipid scrambling, but other bivalent cations fail to do so or even inhibit lipid scrambling in cases of Mg^{2+} and Mn^{2+} [25]. In erythrocytes, once activated by Ca^{2+}, the scrambling pathway remains active for at least 2 h [22]. Elevation of intracellular Ca^{2+} using selective ionophores also induces collapse of lipid asymmetry in a variety of other hematopoietic cell types, including lymphocytes [27] and macrophages [45]. A continuous fluorescence-based assay in platelets demonstrated that intracellular Ca^{2+} acts as a switch for the scrambling machinery: ionomycin mediated influx and efflux of Ca^{2+} in platelets allow multiple cycles of activation and inactivation, respectively, of scramblase activity [23] (see Fig. 1). This assay also suggested that the rate of ionomycin-induced PS externalization increases over a Ca^{2+} concentration range from 50 μM to 1 mM [23]. PS exposed at the surface as the result of lipid scrambling in platelets and erythrocytes can be pumped back to the cytoplasmic leaflet by reactivation of the aminophospholipid translocase following removal of Ca^{2+} [46,47].

There is little doubt that the Ca^{2+} activation pathway in hematopoietic cells is physiologically relevant, since loss of lipid asymmetry following platelet activation with physiological agonists results in elevation of intracellular Ca^{2+}, and lowering the Ca^{2+} levels causes the scrambling process to cease. For physiological agonists, a persistent elevation of Ca^{2+} seems to be most important [48]. Keuren et al. determined that a sustained intracellular

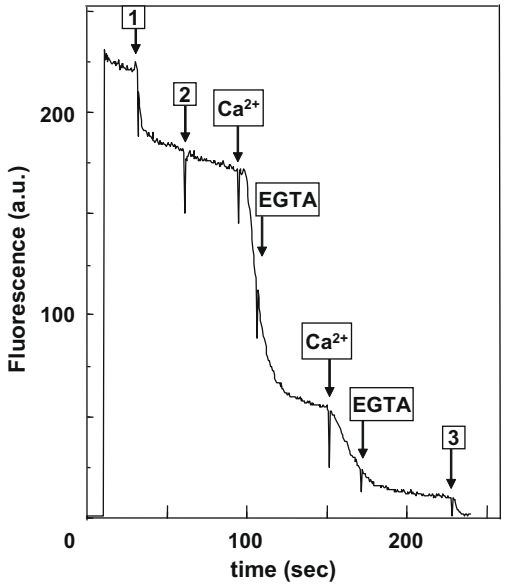

Fig. 1. High intracellular Ca^{2+} acts as a switch for lipid scrambling in platelets. Platelets loaded for 60 min with fluorescent NBD-PS were suspended in a Ca^{2+}-free buffer (containing 0.1 mM EGTA). Only a small fraction of the NBD-PS is quenched at first addition of sodium dithionite (arrow 1) because most of the probe has been transported to the inner leaflet by the aminophospholipid translocase. Addition of ionomycin alone (arrow 2) is without effect. Ionomycin-mediated internalization of added extracellular Ca^{2+} (arrow 'Ca^{2+}') results in scramblase mediated externalization of previously internal probe, and quenching of the newly externalized NBD-PS by the dithionite in the medium results in rapid decay of the fluorescence signal. Chelation of the Ca^{2+} (arrow 'EGTA') causes the process to come to a stop. Scrambling can be resumed by readdition of Ca^{2+} and arrested again by its removal. Triton X-100 addition (arrow 3) demonstrates that there is still sufficient dithionite to quench all remaining NBD-PS.

Ca^{2+} concentration of approximately 400 nM is required for the combined action of collagen and thrombin to evoke collapse of lipid asymmetry [49]. For erythrocytes and lymphocytes the minimal extracellular Ca^{2+}-concentration required for lipid scrambling ranges from 25 to 100 μM [22,27,50]; external Ca^{2+} concentrations above 1 mM appear to be slightly inhibitory [27,51]. Interestingly, the Ca^{2+} concentration required for activating lipid scrambling is considerably higher than that required for half maximal inhibition of the aminophospholipid translocase (IC50 ~0.2–2 μM) [52], indicating that loss of lipid asymmetry is not simply a matter of inhibition of the inward transport of aminophospholipids.

5.2. Apoptotic activation

The discovery that exposure of PS was a characteristic of apoptotic cells [5] led naturally to two questions. The first, quickly answered [6,7], was whether PS exposure in apoptotic cells resulted from scramblase activation, as was known to occur in erythrocytes and platelets. These experiments not only confirmed that the mechanism was similar, but also showed that the basic features of the scramblase activity (non-specificity and bidirectionality) were the same. The next question was the mechanism by which scramblase activation (and aminophospholipid translocase inactivation) occurred. It was quickly shown that, in mammalian cells, PS exposure was a hallmark of apoptosis in a variety of cells subjected to a variety of apoptotic stimuli [53], and that agents that blocked apoptosis in any particular case, including caspase inhibitors, Bcl-2, or inhibitors of transcription or translation, would also prevent PS exposure. While these results show that PS exposure is downstream of the apoptotic signalling process, they do not cast much light on the proximal mechanism of scramblase activation. For example, it is clear that caspases do not act directly on the scramblase, since in some cells scramblase activation proceeds efficiently in the presence of broad spectrum caspase inhibitors that block downstream apoptotic events such as DNA degradation [6]. Time course experiments show that PS exposure occurs relatively early, after the onset of membrane blebbing but before DNA degradation or loss of membrane integrity.

Early experiments suggested that the role of Ca^{2+} in apoptosis induced PS exposure was problematic. Removal of extracellular Ca^{2+} with EGTA prevented PS exposure in apoptotic Jurkat cells, but chelation of intracellular Ca^{2+} by BAPTA-AM did not [54]. Similarly, Bratton et al. suggested [7] that Ca^{2+} flux, but not a net change in intracellular Ca^{2+} is essential, but not sufficient, for activation of the scramblase in apoptotic cells. This conclusion seems inconsistent with the idea that PS exposure induced in platelets or other hematopoietic cells requires a persistent elevation of intracellular Ca^{2+}.

One important point is the whether the scramblase induced by apoptosis is the same as the one activated by elevation of cytoplasmic Ca^{2+}. As indicated above, the basic properties of the scramblase in the two cases are the same – lack of lipid specificity and bidirectional phospholipid movement. Other evidence supports the idea that the same scramblase is activated in both cases. The rate of ionophore induced lipid scrambling differs between different cell types, and where it can be measured, the rates of lipid scrambling activated by apoptosis vary in the same way. Moreover, simultaneous activation of scramblase activity by both stimuli does not result in an additive effect on the rate of lipid scrambling [27]. Together, these data suggest that there is one scramblase activity at the plasma membrane in cells, but do not settle the question of whether there is one mechanism for its activation.

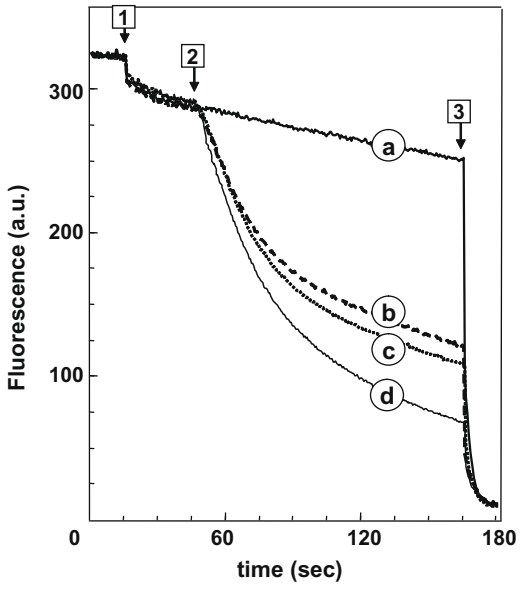

Fig. 2. Defective lipid scrambling in Scott syndrome. The continuous assay described in Fig. 1 was used to monitor lipid scrambling in platelets from a patient with Scott syndrome and two of her children. After loading the platelets with NBD-PS for 60 min, dithionite (arrow 1) was added, quenching residual probe in the outer leaflet. Activation of scramblase by addition of ionomycin (arrow 2) in the presence of 3 mM extracellular Ca^{2+} induces a rapid egress of NBD-PS in control cells as shown by the rapid decay of the fluorescence signal (curve *d*). No appreciable scramblase activation is observed with Scott platelets from the affected mother (curve *a*). In platelets from both children, an intermediate rate of decay of the fluorescence signal is observed (curves *b* and *c*).

5.3. Lessons learned from the Scott syndrome

In 1979, Weiss et al. described a patient with symptoms of moderate to severe bleeding caused by an impaired ability of platelets to form a procoagulant lipid surface [55]. This extremely rare bleeding disorder is called Scott syndrome (OMIM: 262890), after the first patient (Mrs. M.A. Scott, 1939–1996); it has only been recognized in three female patients, and its clinical features and laboratory findings have been reviewed extensively elsewhere [4]. Platelets from Scott patients, when activated with collagen plus thrombin, show a procoagulant response of only 30–50% compared to healthy controls. Importantly, the impairment of PS exposure triggered by Ca^{2+}-ionophore is virtually complete. The elimination of Ca^{2+}-induced lipid scrambling is not restricted to platelets; treatment of erythrocytes and B- and T lymphocytes from patients with Scott syndrome with Ca^{2+}-ionophore does not result in appreciable PS exposure [56]. Children of a Scott patient have no clinically important bleeding history, but they show an intermediate procoagulant response [57] (see Fig. 2) [23], suggesting that the defect is transmitted as an autosomal recessive trait. The Scott syndrome phenotype has been studied in an inbred colony of German Shepherd dogs [58], and recently, a genome-wide linkage scan has localized the canine Scott deficiency to chromosome 27, a region syntenic with the centromeric region of human chromosome 12 [59]. These studies make it likely that the molecular nature of this defect will soon be known.

Not surprisingly, EBV-transformed B-lymphocytes from Scott patients are defective in Ca^{2+}-ionophore induced lipid scrambling [27]. However, these cells display normal PS exposure and rates of lipid scrambling when driven into apoptosis. This observation suggests that the scramblase is intact in Scott cells, and that the defect is in the Ca^{2+}-ionophore activation mechanism. Moreover, it implies that the scramblase activation pathway in apoptosis does not depend on the product of the Scott gene and to that extent, it represents a distinct mechanism that leads to PS exposure. It has been observed that the defect in Ca^{2+}-induced lipid scrambling is preserved in resealed erythrocyte ghosts from Scott patients [56]. This result suggests that the putative regulatory protein affected by Scott syndrome is likely to reside in the plasma membrane.

5.4. Two scramblase activation pathways in platelets

The existence of (at least) two distinct pathways that lead to lipid scrambling, one which is strictly Ca^{2+}-dependent and one that is independent of intracellular Ca^{2+} (the 'apoptotic pathway') may explain the partial defect in the development of procoagulant activity in Scott platelets treated with collagen and thrombin (see Fig. 3). If this physiological stimulus switches on both pathways in platelets, the block to the Ca^{2+}-dependent branch in Scott cells might result in only a partial reduction in the rate and/or extent of PS exposure. Recent data suggest this may well be the case. Various components of the apoptotic pathway, including caspases and regulatory proteins from the Bcl-2 family, are present in platelets. Moreover, several characteristics of apoptotic cells, including loss of mitochondrial inner membrane potential ($\Delta\psi_m$)

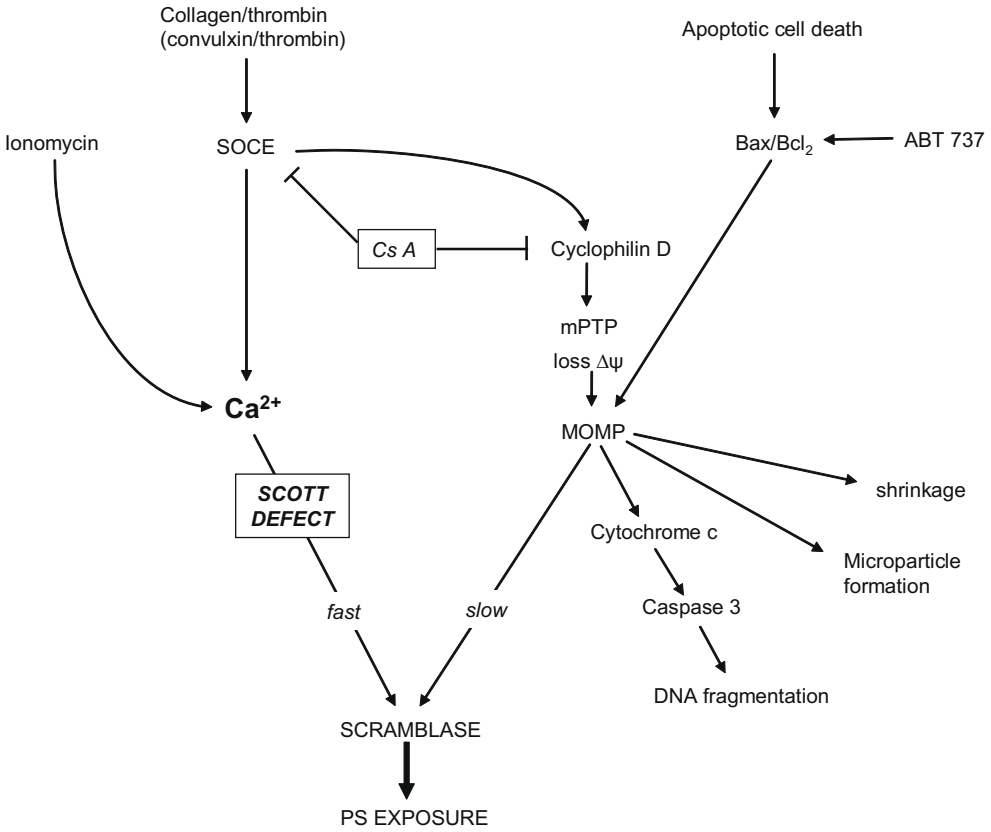

Fig. 3. Distinct pathways leading to PS exposure in platelets. In cells from the hematopoietic lineage, scramblase can be activated by a persistent elevation of $[Ca^{2+}]_{in}$ for example by means of a Ca^{2+} ionophore. Dual agonist stimulation of the collagen and thrombin receptor evokes a sustained high $[Ca^{2+}]_{in,}$ mediated by store operated Ca^{2+} entry (SOCE). PS exposure by this pathway, in both cases, is blocked by the Scott mutation. Alternatively, lipid scrambling and PS exposure can result from activation of the Bax/Bcl_2 pathway of apoptosis, independent of elevated intracellular Ca^{2+}. Stimulation with collagen and thrombin may compromise the mitochondrion, causing formation of the cyclosporine A (CsA) sensitive permeability pore (mPTP) and collapse of the mitochondrial inner membrane potential ($\Delta\psi$) with subsequent by permeabilisation of the mitochondrial outer membrane (MOMP), leading to PS exposure. Stimulation in this fashion can thus lead to PS exposure through both the 'mitochondrial' and 'Scott, pathways.

and shedding of membrane microparticles, as well as PS exposure, occur in platelets in response to physiological stimuli [60,61]. Cyclosporin A, which binds to cyclophilin D, a component of the mitochondrial permeability transition pore (mPTP), and prevents loss of $\Delta\psi_m$, strongly inhibits PS exposure in convulxin/thrombin stimulated platelets. These agonists also fail to induce PS exposure in cyclophilin D deficient mice [62]. Although these data suggest that the mPTP contributes to the platelet procoagulant response, recent data showed that PS externalisation can occur independent from $\Delta\psi_m$ loss, and vice versa [63,64]. Ca^{2+}-ionophore induced PS exposure occurs normal in cyclophilin $D^{-/-}$ platelets and is not inhibited by cyclosporin A [62].

Recently, Schoenwaelder et al. obtained evidence for two distinct pathways of PS exposure in platelets [61]. As indicated above, platelets express many well known apoptotic regulators including members of the Bcl-2 protein family. PS exposure in platelets is induced by the apoptosis-inducing BH3 mimetic compound ABT737, although in comparison to physiological agonists, the rate of PS exposure is slow. The proapototic proteins Bak and Bax appear to play a role in the pathway(s) leading to PS exposure, since this response is completely absent in platelets from Bak/$Bax^{-/-}$ knock out mice, although convulxin/thrombin-induced PS exposure is still present. PS exposure in platelets induced by ABT-737 is caspase dependent, but is unaffected by inhibitors of platelet activation (prostaglandin E1 and theophiline) or extracellular Ca^{2+}-chelating agents [61]. Moreover, chelation of extracellular Ca^{2+} blocked activation-induced, but not apoptosis-induced PS exposure, once more suggesting that PS exposure induction by apoptosis is distinct from induction by Ca^{2+}. Nevertheless, these pathways may cooperate in platelets. BH3 mimetics such as gossypol and methoxy-antimycin, that interfere in the interaction between the pro-apoptotic Bak/Bax and the anti-apoptotic Bcl-2, potentiate the platelet procoagulant response [65].

5.5. Mixed messages

The existence of two pathways leading to scramblase activation and PS exposure in the same cells may be an anomaly confined largely to the hematopoietic system. It is reasonable to suppose that the apoptotic pathway is evolutionarily older, since apoptosis and PS exposure appear as a cell fate in organisms such as *C. elegans* which diverged well before the invention of the coagulation pathway so familiar in vertebrates. The apoptotic pathway and PS exposure are also characteristic of ancient cell types such as epithelial cells, and there is evidence that these cells lack the Ca^{2+}-dependent pathway [45]. Why then is there a second pathway in hematopoietic cells? It is perhaps too early to draw any clear conclusions, but two possibilities might be considered. One, of course, is that the Ca^{2+}-dependent pathway may have been required in order to produce a system in which PS exposure could occur with the great rapidity and reliability required for the blood coagulation system. Another possibility is that the system evolved to support the PS-dependent removal of cells such as erythrocytes in which mitochondria have been lost, but elevation of cytoplasmic Ca^{2+} results in the similar membrane changes, including PS exposure [9]. Indeed, erythrocyte cell death seems to be independent of the normal caspase-dependent apoptotic pathway [66], making red cells the mirror image of epithelial cells where only the apoptotic activation pathway is operative.

6. Concluding remarks

Lipid scrambling with surface exposure of PS is an important process in several physiological and patho-physiological contexts. Although much is known about the characteristics of the scrambling activity, the protein responsible for it remains unknown, and its identification is the single most important problem in the effort to understand the mechanism and regulation of the scramblase. The problem of scramblase activation is also crucial, and there has been progress toward identifying the basis of a known defect in scramblase activation, the Scott mutation. One largely unexploited approach which deserves attention is the use of modern proteomic tools to identify the protein encoded by the gene inactivated by the Scott mutation. In the end, identification of the responsible proteins will undoubtedly provide ways to manage pathophysiological consequences of PS exposure in the clinic.

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