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Abstract

Phosphatidylserine (PS), a phospholipid with a negatively charged head group, is an important constituent of eukaryotic membranes. Rather than being a passive component of cellular membranes, PS plays an important role in a number of signaling pathways. Signaling is mediated by proteins that are recruited and/or activated by PS in one of two ways: via domains that stereospecifically recognize the head group, or by electrostatic interactions with membranes that are rich in PS and therefore display negative surface charge. Such interactions are key to both intracellular and extracellular signaling cascades. PS, exposed extracellularly, is instrumental in triggering blood clotting and also serves as an “eat me” signal for the clearance of apoptotic cells. Inside the cell, a number of pathways depend on PS; these include kinases, small GTPases and fusogenic proteins. This review will discuss the generation and distribution of PS, current methods of phospholipid visualization within live cells, as well as the current understanding of the role of PS in both extracellular and intracellular signaling events.

Keywords

Phosphatidylserine • Phosphatidylserine detection • Membrane signaling • Protein-lipid binding • Lipid dynamics

10.1 Phosphatidylserine Biosynthesis, Degradation and Cellular Distribution

Phosphatidylserine (PS) is a glycerophospholipid present in cellular membranes of all eukaryotic cells. Like other phospholipids, PS has a polar

phosphate on *sn*-3 of the glycerol backbone; a serine attached to the phosphate gives PS its distinctive head-group, which has a net negative charge under physiological conditions (Fig. 10.1a). This is in contrast to the most abundant lipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE), which have a zwitterionic head group and therefore bear no net charge. When PS is present in large amounts, the net negativity of its head group can confer significant electrostatic charge to membranes, which can have important implications on the recruitment of soluble cations

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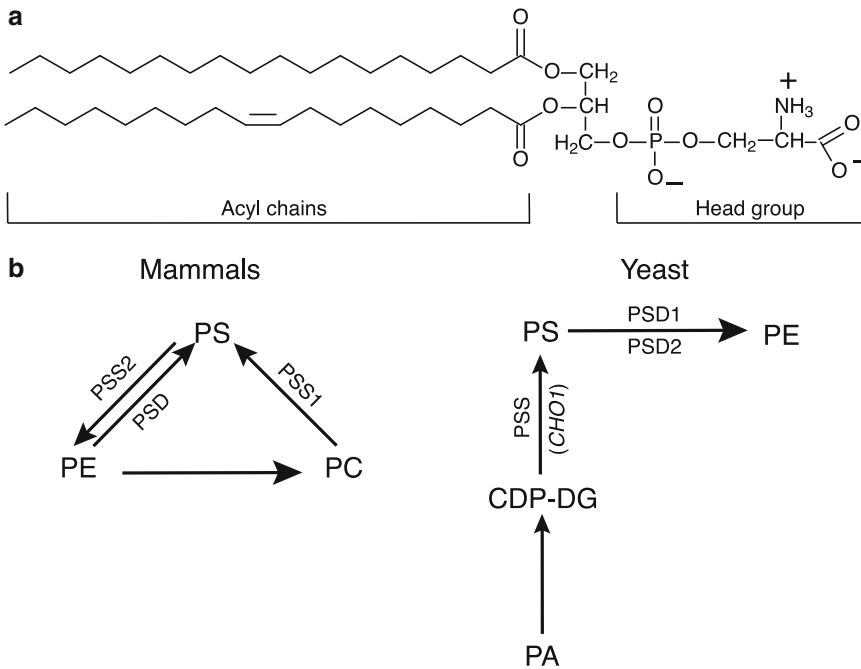


Fig. 10.1 Phosphatidylserine and its production and degradation pathways. (a) Diagram of the structure of PS, with one saturated and one unsaturated fatty acyl chains. Note that at physiological pH the head group bears one net negative charge. (b) Diagram showing pathways of production of PS in both mammalian and yeast cells. In mammalian cells PS can be produced from either

phosphatidylcholine (PC) or phosphatidylethanolamine (PE) by PS synthase (PSS) 1 or 2, respectively, while in yeast phosphatidic acid (PA) is the only precursor that can be converted to PS, via CDP-diacylglycerol (CDP-DG). In both cases, PS is converted to PE by PS decarboxylases (PSDs)

and proteins; such recruitment, in turn, can have important signaling implications, as discussed below.

Both the *sn*-1 and *sn*-2 positions of PS, as with the majority of glycerophospholipids, have fatty acyl chains attached. A large variety of acyl chains are known to exist on PS, with the *sn*-1 position tending to have saturated chains of 16 carbons or longer, and the *sn*-2 position generally having unsaturated chains of 18 carbons or longer [84]. Interestingly, however, PS tends to display the greatest acyl chain composition diversity between tissues, with brain PS being especially enriched in long, poly-unsaturated fatty acyl chains, especially docosahexaenoic acid (22:6n-3); this has been speculated to have implications in neuronal development and function [60, 75, 161].

In mammalian cells, PS synthesis can occur via two pathways, using either PC or PE as a precursor (Fig. 10.1b). The enzyme PS synthase

1 (PSS1) exchanges the choline on PC for a serine, while the enzyme PSS2 can exchange the ethanolamine on PE for serine. These two synthesis pathways are at least partially redundant, as mice without either PSS1 or PSS2 are viable, though double-knockout of both synthases is lethal [8, 16], indicating that PS is an essential phospholipid in mammals. In yeast cells, a single PSS enzyme exists, encoded by *CHO1*, which converts phosphatidic acid (PA) to PS via cytidine diphosphate-diacylglycerol (CDP-DAG) (Fig. 10.1b). In contrast to mammals, yeast mutants without PSS expression – and thus no PS – are able to survive, albeit rather precariously, in medium supplemented with ethanolamine [112].

Degradation of PS occurs mainly by decarboxylation of the PS head group to produce PE, a reaction mediated by the enzyme PS decarboxylase (PSD). There is one PSD in mammals, encoded by the *Psd* gene, and two in yeast, *psd1p* and

Table 10.1 Phospholipid content of organellar membranes

Membrane	PC	PE	PI	PS	References
Endoplasmic reticulum	55 ^a	30	15	3–5	[84, 160]
Golgi complex	50	15	10	5	
Early endosome	47	23	8	8.5	
Late endosome	48	18.5–20	4–7	2.5–3.9	
Mitochondria	40–45	30–35	5–10	1	
Plasma membrane	42	25	3	12	

^aNumbers indicate percentage of total lipid present in mammalian cells

psd2p, encoded by the *PSD1* and *PSD2* genes, respectively (Fig. 10.1b) [84, 161]. Mammalian PSD and yeast Psd1p are found on the outer leaflet of the inner mitochondrial membrane [161], leading to production of a unique pool of PE from PS within the mitochondria. This mitochondrial production of PE is unable to be fully complemented by the CDP-ethanolamine pathway, as *Psd*^{-/-} mice do not survive beyond embryonic day 9 [145]. Additionally, in mice missing three of four PSS alleles, where the catalytic levels of PSS are reduced to ~10 % of wild type mice and PS levels are consequently reduced, PE levels are concomitantly reduced [8]. Thus, PS is an important precursor for the production of mitochondrial PE, which is in turn important for the functioning of mitochondria. Finally, though a minor contributor to overall degradation, some phospholipases are additionally capable of degrading PS with some of the resulting products, especially lyso-PS, having important signaling effects [7, 65]; these will be discussed later in greater detail.

The intracellular (organellar) distribution of PS is not homogeneous, with the majority of the PS of the cell being on the plasma membrane (PM) (Table 10.1). The endoplasmic reticulum (ER) is the main site for lipid synthesis, and in mammals both PSS1 and PSS2 are localized and active in specialized areas of the ER, which are known as mitochondria-associated membranes due to their close relationship and tendency to be isolated with mitochondria [163]. Despite being produced at the ER, PS is not particularly abundant in this compartment (Table 10.1), suggesting that selective transport or removal of PS must occur. One means of removal of PS from the ER is by transport to the mitochondria where, as

mentioned above, PS can be converted to PE. Transport from the ER to mitochondria appears to occur at the mitochondria-associated membranes. At this specialized junction PS delivery occurs by direct inter-membrane movement, possibly assisted by soluble or membrane-bound carrier proteins; vesicular-mediated transport is not involved [114, 140, 160, 162]. The other main method of PS removal from the ER is via anterograde vesicular transport to the Golgi apparatus, likely occurring via bulk flow, although specific transport mechanisms cannot be ruled out [84, 160]. How high PS levels are maintained at the PM without being returned to the Golgi apparatus via retrograde traffic, is not entirely clear.

Another important aspect of PS is its tendency to be asymmetrically distributed between leaflets of membranes. This is especially evident at the plasmalemma, where virtually all the PS is on the inner (cytoplasmic facing) leaflet in healthy cells, with none detectable on the outer leaflet. This asymmetry of PS at the surface membrane substantially increases the mole percentage of PS in the inner leaflet to 25–30 % of the total lipid. By contrast, the transmembrane distribution of PS in other organelles remains poorly defined; according to some reports the ER has little, if any, PS remaining on the outer (cytoplasmic-facing) leaflet, with the majority of detectable PS being on the inner (lumen-facing) monolayer [22, 42, 61, 74].

The distribution of PS across membrane leaflets is maintained by various flippases, floppases and scramblases. Flippase is a general name for lipid translocases that move lipid toward the cytoplasmic-facing leaflet of a bilayer. ATP-dependent flippases with selectivity for PS are known to function at the PM; these translocases belong to the family of P₄-ATPases [34, 135, 154]. Five

different P₄-type ATPases have been identified in yeast (DRS2, DNF1, DNF2, DNF3 and NEO1), and over a dozen have been deduced by sequence homology in humans; a number of these have been shown experimentally to have PS flippase activity [34, 51, 119, 134]. A number of the yeast proteins are located on intracellular membranes and their deletion has implications in membrane traffic [31, 66]. This implies that PS asymmetry occurs, and is important for, the function of intracellular organelles.

Floppases function to transport lipids away from the cytoplasm to the luminal-facing leaflet of organelles, or the topological equivalent of the PM, the extracellular-facing leaflet. Most belong to the ATP-binding cassette (ABC) superfamily but to date, only limited evidence exists for the ability of these proteins to move PS across membranes [34]. Scramblases, by contrast, are ATP-independent, bidirectional and function to randomize lipids across membranes [128]. Scramblases are important in the exposure of PS on the cell surface during apoptosis, discussed in more detail later.

The abundance of PS in the PM, together with its asymmetric distribution between leaflets and its negative charge, have important electrostatic implications. Soluble cations, polycations and proteins with cationic clusters can all be concentrated in the immediate vicinity of membranes, where they can influence biochemical and signaling events. Thus, the unique subcellular and transmembrane distribution of PS confers onto this lipid a special signaling connotation.

The importance of PS in cellular signaling and survival is highlighted by examination of cells lacking PS. Yeast cells lacking the PSS gene (*cho1Δ*) survive, but grow very poorly and have elevated phosphatidylinositol levels [9], possibly a compensatory attempt to increase levels of alternative negatively charged lipids. *Cho1Δ* cells do have normal fluid phase uptake, α -factor maturation, CPY trafficking and septin ring maturation [41, 109], but polarization of the yeast PM and recruitment of CDC42 are greatly inhibited, preventing proper formation of buds and mating projections [41]. The role of PS is even more prominent in higher organisms. In contrast to yeast,

mammalian cells completely lacking both PSS genes are not viable [8], and cell lines that have greatly reduced PSS functionality are only viable when PS is added exogenously [129]. Although mice with only one of three PSS alleles remaining are viable (despite some tissues only having 10 % of wild type PS levels [8]), PS is clearly an important lipid for proper survival and growth and participates in many signaling cascades, as discussed below.

10.2 Detection of Phosphatidylserine

In order to determine the involvement of PS in signaling cascades, it is first necessary to determine the presence and localization of PS. Traditional methods of PS detection were biochemical in nature, such as the covalent reaction of its head group with amino-reactive chemicals such as 2,4,6-trinitrobenzenesulfonate, followed by extraction and thin layer chromatography or mass spectrometry. Such methods were successfully used to determine that PS resides exclusively on the cytoplasmic leaflet of the PM in healthy mammalian cells [92, 132], as well as to establish the sidedness of PS in a limited number of organelles [143, 158]. However, the usefulness of such probes is limited as they are generally slow to react, also label PE, and their use can compromise cellular viability [23].

Fluorescent analogues have also been successfully used to study the properties of PS within the cellular environment. Fluorescent PS analogues generally have a fluorescent moiety attached to a shortened *sn*-2 acyl chain, where it is presumed to interfere minimally with the unique head group. The most commonly used fluorophore has been 7-nitro-2-1,3-benzoxadiazol-4-yl (NBD). Indeed, NBD-PS has been useful in measuring the activity of proteins involved in PS flipping [35, 93, 121]. However, NBD has a strong dipole moment which forces the attached acyl chain to loop towards the aqueous interface and distorts the PS-like molecule [29, 30, 104], reducing its hydrophobicity and allowing it to be removed easily from the membrane [74, 93]. More recently, a second

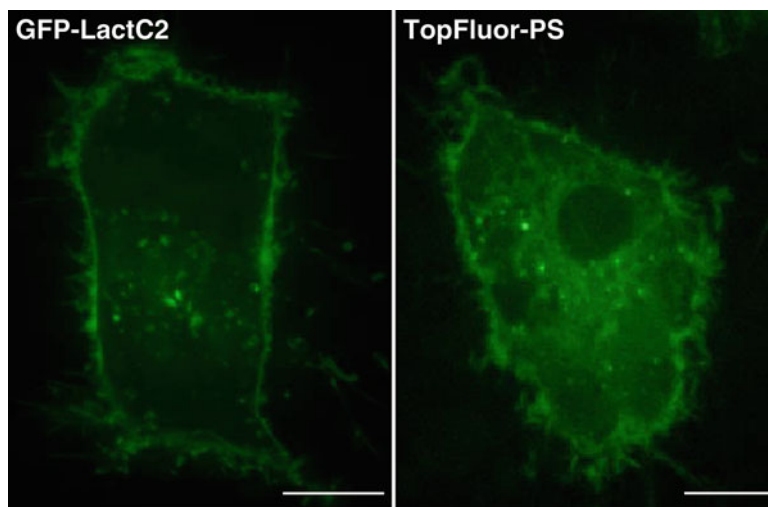


Fig. 10.2 Phosphatidylserine detection in cellular membranes of live cells. Two examples of PS detection in live HeLa cells. Cells were either transfected with a genetically encoded GFP-LactC2 (*left*), or were loaded with TopFluor-PS analogue (*right*). Both methods of PS

visualization show fluorescence at the plasma membrane and internal (endosomal) membranes. Both probes have been used successfully for the detection and examination of live cell dynamics of PS

fluorescent PS analogue, TopFluor-PS, based on a BODIPY-derivative (1-palmitoyl-2-(dipyrrometheneboron difluoride) undecanoyl-*sn*-glycero-3-phospho-L-serine) has been successfully used to examine PS dynamics in cellular membranes; this analogue is perceived to have fewer drawbacks than NBD-PS [74] (Fig. 10.2).

Specific interactions of proteins with PS can also be used for detection of the phospholipid. There are two main means whereby proteins interact with PS. One of them is charge-based: a number of proteins that are prenylated or lipidated have in addition a stretch of cationic amino acids that can interact electrostatically with PS; however, the interaction is non-selective and such polycationic probes interact also with other anionic phospholipids. A number of Ras and Rho-family GTPases, as well as the tyrosine kinase Src have this property [43, 56, 141]. While these proteins do not interact with PS specifically, they do partition preferentially to PS-enriched membranes, which have a net negative surface charge [56, 141]. While these types of interactions can have important signaling consequences, they are not generally useful for specific detection of PS.

The second type of protein-PS interaction involves protein domains that recognize PS in a

more specific manner, often by a stereospecific PS head group interaction domain. These include the calcium-dependent C2 domain of proteins such as annexin V, protein kinase C (PKC) α and PKC β , the calcium-independent C2 discoidin-like domain of proteins such as lactadherin (also known as MFG-E8), and the γ -carboxyglutamic acid (Gla) domain on prothrombin (Table 10.2). Many of these interactions also have important signaling consequences, which will be discussed below, but some of these domains, or the entire proteins that contain them, can be used as probes for the specific detection of PS in cells. The best known example of this is annexin V, which has been used extensively to stain PS in apoptotic cells [80]. Annexin V works well for detecting PS that has appeared on the exofacial leaflet of the plasmalemma; however, due to its requirement for comparatively high (mM) concentrations of calcium [5], its usefulness for visualization of intracellular PS in live cells is limited. While the approach was reported [27], effective binding required artificial elevation of the cytosolic calcium concentration, which activates signaling cascades, degradative enzymes, etc. Furthermore, binding of annexin V to membranes is not strictly PS-dependent; it is instead sensitive to anionic

Table 10.2 Phosphatidylserine-binding domains

Class	Calcium dependency	Specificity	Examples	References
C2 domain (conventional)	Yes	Electrostatic, limited specificity for PS, but often nonspecific	Annexin V PKC α PKC β	[151] [148]
Discoidin-like C2 domain	No	L-serine stereospecific binding	Coagulation factors V and VIII Lactadherin	[88] [136]
γ -Carboxyglutamic acid (Gla)	Yes	Specific serine head group binding	Prothrombin Gas6	[68]

lipids in general [98, 99]. Thus, while annexin V has been instrumental in furthering the understanding of PS exposure outside cells, its usefulness as an intracellular probe is questionable.

More recently, a new probe based on the discoidin-like C2 domain of the glycoprotein lactadherin has been developed [172]. Unlike conventional C2 domains, the discoidin-like C2 domain of lactadherin binds PS specifically in a calcium-independent manner (Table 10.2) [3, 86]. When the lactadherin C2 domain is linked to green fluorescence protein (GFP), the resulting chimera (GFP-LactC2) is highly specific for PS, has stereospecificity for the phospho-L-serine moiety over phospho-D-serine, has greater avidity for PS than does annexin V and, importantly, binds PS under intracellular physiological conditions [138, 139, 172]. Therefore, this probe provides a new tool to examine PS-mediated signaling events that occur within the cell. When combined with additional probes like TopFluor-PS, GFP-LactC2 can be used with minimal concern about possible effects on lipid scavenging by the probe itself [74]. Although much remains to be discovered, the new probes have been successfully used in a number of recent studies examining PS distribution, dynamics and PS-mediated signaling events in the intracellular environment (Fig. 10.2) [12, 41, 42, 74, 106, 156, 172, 173].

10.3 Phosphatidylserine-Mediated Extracellular Signaling Events

A great deal of attention has been paid to the appearance of PS on the cell surface. This reflects primarily the availability and convenience of

annexin V for exofacial PS detection. A brief overview of what is currently known about extracellular signaling mediated by PS follows.

10.3.1 Hemostasis

As mentioned above, in healthy, unstimulated cells PS is normally present on the cytoplasmic leaflet of the cell, with virtually none on the exofacial leaflet. Changing this resting distribution of PS has a strong potential for initiating signaling. This was first recognized in the case of platelet activation. When stimulated, platelets expose PS on their surface, thereby triggering the coagulation cascade. The collagen receptor glycoprotein VI on platelets, which recognizes the underlying collagen upon breakage of a blood vessel, is a potent activator of platelets [17, 57, 58, 77, 177]. Platelet activation and PS exposure are also triggered by fibrin-binding receptors such as glycoprotein Ib-V-IX (vWF receptor) and integrin $\alpha_{IIb}\beta_3$ [20, 124]. Engagement of the receptors by their cognate ligands triggers a cascade of tyrosine and serine protein phosphorylation, with resultant activation of phospholipase C γ 2 and subsequent generation of inositol 1,4,5-*tris*phosphate. Inositol 1,4,5-*tris*phosphate is required for the release of calcium from intracellular stores and their depletion, in turn, activates calcium entry from the extracellular medium, amplifying the response [58]. While elevated calcium inhibits PS flippase activity, this is not enough on its own to cause the observed rapid appearance of exofacial PS required for efficient blood clotting to occur [85, 178]. Instead, the main effect of calcium is thought to be the activation of lipid scrambling. Scramblase activity

requires a sustained calcium elevation, and it can be inactivated simply by the removal of calcium [19, 167]. While the underlying molecular entity was elusive for many years, a bona fide calcium-dependent scramblase, TMEM16F, was identified recently and is thought to be involved in the translocation of PS in platelets [85, 149].

Once PS is exposed on the surface of the platelet, it functions as a scaffold for the coagulation cascade to occur in the correct location, which is in the vicinity of the activated platelet. Factor VIII, a co-factor for the factor Xa complex and factor V, a co-factor for the prothrombinase enzyme complex, both have discoidin-like C2 domains that specifically bind exposed PS by interacting with its unique head group [49, 88, 122, 126, 159]. In addition, factors X, VII, IX and II (prothrombin) contain 'Gla' domains, rich in glutamic acid residues, which are γ -carboxylated in a vitamin K-dependent reaction [171]. These domains also interact with PS specifically, but in a calcium-dependent manner [68], and serve to bring these additional factors to the activated platelet. The importance of the scaffolding function of PS in bringing together the required clotting factors is highlighted by patients with Scott syndrome. In these patients, platelets fail to scramble PS in response to activation, at least in some cases due to the lack of a functional TMEM16F scramblase; as a result these patients present with a bleeding disorder [28, 125, 149, 165]. Further, it has been shown that the Xa and prothrombinase enzyme complexes depend indirectly on PS, as their enzymatic activity is completely lost or highly diminished by blocking the PS-binding capability of factor V and VIII with either antibodies, blocking peptides, or by mutations in the PS-binding sites [50, 69, 76, 137].

In addition to PS exposure on the surface of activated platelets, microvesicles formed by the evagination of the plasma membrane are released from platelets; these microvesicles also expose PS [33, 46]. The PS exposed on the surface of the microvesicles can also promote procoagulant activity, as highlighted by the fact that mice lacking serum lactadherin (which binds and promotes the clearance of microvesicles) suffer from excessive coagulation [37].

Finally, exofacial PS on activated platelets can be bound specifically in a calcium-dependent manner by the proteins Gas6 and protein S through their Gla domains [108]. When these proteins interact with their receptors – members of the TAM family (Tyro3, Axl and Mer) of tyrosine kinase receptors – under hemostatic conditions, they appear to stabilize thrombus formation by promoting platelet aggregation [6, 32].

10.3.2 Apoptosis

Cells turn over constantly, undergoing death and replacement. Apoptosis is the programmed death that allows for the removal of cells without the release of potentially harmful intracellular enzymes and antigens, and therefore without causing an inflammatory response. PS exposure on the surface of apoptotic cells is another highly studied area, as it is known to be the signal for the rapid uptake of cell corpses by phagocytes. Exposure of PS is therefore thought of as an 'eat-me' signal [40]. While the signaling cascades and cellular processes that result in a commitment to apoptosis are the subject of intense study and are quite well defined [39, 105], the determinants of PS exposure, other than a requirement for caspase activation, are not well known [94, 107]. In apoptosis, as during platelet activation, the movement of PS to the exofacial leaflet likely involves the activation of scramblases. However, the time-scale for PS scrambling is vastly different, with PS exposure occurring in platelets on the scale of minutes, while PS exposure on apoptotic cells develops over hours [24, 168, 169, 178]. Additionally, while calcium has been implicated in upstream apoptotic signaling [1, 21, 70, 73, 96], whether calcium elevation is an absolute requirement for the scrambling of PS during apoptosis is not clearly defined. While most studies show there is no requirement for influx of extracellular calcium, the possible involvement of intracellular calcium storage sites is less clear [10, 13, 36, 53, 101, 102, 133, 168, 176]. What is evident, however, is that patients with Scott syndrome have normal apoptotic PS exposure despite having no calcium-induced PS exposure on activated platelets or lymphocytes [18, 125, 165, 168]; this

Table 10.3 Receptors recognizing phosphatidylserine on apoptotic cells

Phagocytic receptor	Direct PS recognition?	Accessory ligand	References
TIM-1, TIM-4	Yes	–	[79]
BAI1	Yes	–	[115]
Stabilin-2	Yes	–	[117]
Mer	No	Gas6, protein S	[4]
$\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins	No	Lactadherin	[54]
CD36	Oxidized lipid	?	[52]

suggests that a scramblase other than TMEM16F may be involved in exofacial exposure of PS during apoptosis. Possible candidates are the phospholipid scramblases 1–4, or members of the ABC transporter family, though recent evidence suggests that these are not efficient PS scramblases and that phospholipid scramblase proteins are in fact signaling proteins [19, 91, 128].

Once exposed on the surface of apoptotic cells, PS is one of the most potent ‘eat me’ signals, directing phagocytic cells to internalize and degrade the apoptotic cell. There are a number of receptors on the phagocyte membrane that recognize exofacial PS on apoptotic cells (Table 10.3), either by directly interacting with the lipid or through an intermediate protein. Gas6, protein S and lactadherin are proteins identified as PS-binding proteins that can act as bridging molecules that allow receptors to engage and take up apoptotic cells [4, 54, 108]. In addition to their role in hemostasis described above, protein S and Gas6 are important for phagocytic cells to recognize targets and signal through their TAM receptors. This applies not only to macrophages and dendritic cells, but also to Sertoli cells that clear the apoptotic cells formed during spermatogenesis, and to retinal pigment epithelial cells that take up sections of photoreceptor cells that are shed daily [83]. Lactadherin is expressed by macrophages and immature dendritic cells and, when interacting with PS via its C2 domain as described earlier, it can simultaneously associate with $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins on phagocytic cells, thereby stimulating the engulfment of the apoptotic cell [54, 170].

Apoptotic receptors that have been reported to bind directly to PS include stabilin-2, brain specific angiogenesis inhibitor 1 (BAI1) and members of the T cell immunoglobulin mucin (TIM) family

[79, 115, 117]. Stabilin-2 appears to interact with PS specifically, in a calcium-dependent manner [118], while BAI1 interacts with phosphatidylinositol 4-phosphate, PA and the mitochondrial lipid cardiolipin as well as PS [115]. Both TIM-1 and –4 bind PS in a calcium-independent manner, holding the head group of PS in a specific metal ion-dependent pocket [130]. Finally, a portion of the exposed PS appears to be oxidized during the apoptotic process [155], and CD36 has been shown to interact with oxidized lipids and with oxidized PS preferentially over other oxidized lipids, promoting the uptake of apoptotic bodies [52]. How all these receptors capable of PS binding interact and/or cooperate to allow for the recognition and uptake of apoptotic cells is still not clear, but PS is most certainly a signal from apoptotic cells that they need to be engulfed and eliminated [107, 123].

There is evidence to suggest that lyso-PS, a deacylated form of PS having increased aqueous solubility [111], may be involved *in vivo* in cellular signaling, especially in the context of the immune system [45, 90]. Lyso-PS was shown by mass spectrometry to be present in the serum of lipopo-lysaccharide-treated mice and in peritoneal lavages of casein- or zymosan-treated animals [44]. Because there is no known pathway for its *de novo* synthesis, lyso-PS is thought to be produced only by a deacylation reaction catalyzed by phospholipase A (PLA) enzymes. A secreted isoform that is PS-specific (PS-PLA₁), is massively upregulated by various inflammatory stimuli [65, 90] and was shown to be released from activated platelets [131]. When treated with lyso-PS, mast cells undergo enhanced degranulation [95, 142], T-cell growth is inhibited [15] and macrophage uptake of activated or apoptotic neutrophils is

enhanced [44]. Additionally, lyso-PS stimulates fibroblast migration [116] and may therefore play a role in tissue remodeling following injury. Thus, while there are tangible suggestions that lyso-PS may be playing important roles in both immune regulation and wound healing, much work is clearly required to understand the full consequences and significance of this more soluble and potentially more mobile form of PS.

10.4 Phosphatidylserine-Mediated Intracellular Signaling Events

While the detection and visualization of PS in live cells has until recently been difficult, there is still a fair amount known about the involvement and importance of PS in various intracellular signaling events. Recall that PS is heterogeneously and asymmetrically distributed throughout the cell (Table 10.1 and Fig. 10.2). It makes up to 30 % of the lipid on the inner leaflet of the PM; this, combined with its negatively charged head group, confers onto PS a nonpareil ability to direct the recruitment of both proteins containing polycationic stretches, as well as proteins that possess a specific PS-recognition site (Table 10.2).

10.4.1 Phosphatidylserine Charge-Based Interactions

A number of proteins contain lipidation or prenylation sites that drive their association with membranes; when a polycationic motif exists next to a lipidated site, the positive charge will promote the preferential partition and stabilization of the protein on membranes endowed with negative surface charge, e.g., membranes enriched in PS. One such example is the small GTPase K-Ras4B, which is essential for the signal transduction of a number of growth factors. The majority of Ras superfamily member proteins are geranylgeranylated or farnesylated at their C-terminal CAAX box [166], and while many are also palmitoylated or otherwise lipid-modified to allow their attachment to membranes

strictly by hydrophobic means, K-Ras4B is not [55]. Instead, the polycationic stretch present near the C-terminus of K-Ras4B is an essential second signal (in addition to its farnesylation) for the PM association of K-Ras4B [56]. This highly charged polycationic stretch of K-Ras4B does not interact specifically with PS, but this lipid contributes significantly to the overall surface potential of the PM, aiding in the recruitment of Ras4B to the plasmalemma [56, 113, 172, 174].

Src, a tyrosine kinase and the first known oncogene, as well as Rac1, an additional member of the Ras superfamily, are both important for a number of signaling events [2, 25, 64]. Both Src and Rac1 are proteins whose membrane targeting requires a polycationic stretch in addition to lipid modifications [100, 141]. In fact, the distribution of Src within the cell parallels very closely that of PS (as determined by GFP-LactC2), being on the PM and endosomal system [172]. This is due to the fact that not only can the existence of a polycationic stretch of amino acids direct the association of proteins with the PM, but also other anionic membranes, depending on their charge. Thus, the polycationic stretch next to the farnesylation site of K-Ras4B, which has a net charge of +8, locates almost exclusively at the PM, but if the net charge of this stretch is varied, the resulting mutants are directed additionally to other membranes; constructs of intermediate charge (e.g., +5) localize to endosomal membranes [172, 174]. The same behavior was noted for Src, which has a polycationic stretch next to its myristoylated residue at the N-terminus. The cationic motif of Src has a net charge of +5, and the kinase was found to associate not only with the PM but also extensively with PS-enriched endosomal membranes [172, 173]. Further evidence that PS is important for the charge-based distribution of Src was obtained with phagocytosis. When certain pathogens cause depletion of PS from phagosomes, Src is also lost [173]. Rac1, which has a polybasic region with a net charge of +6, associates more strongly with the PM but is rapidly removed from a sealing phagosome, despite the persistence of PS [174], possibly through additional posttranslational modifications [110]. Overall,

we can conclude that the pattern of PS distribution among intracellular membranes (Table 10.1) plays an important role in determining their ability to interact with important signaling proteins, particularly those with cationic characteristics.

In some instances, cationic motifs on proteins are not sufficient to direct proteins to a membrane, but do influence their targeting. This is the case for other signaling proteins, such as with RhoB, TC10 and CDC42 [81, 100]. In such instances cationic motifs likely play a complementary role. Evidence to this effect comes from recent studies in yeast, where polarized PS is required for the recruitment of the signaling and polarity-regulating molecule CDC42 to the forming bud neck [41]. Without PS present, CDC42 remains mainly Golgi-associated and buds are very inefficiently formed, leading to poor growth [41].

Overall, then, there is an increasing body of evidence that the intracellular asymmetry of PS, and the resultant distribution of negative charge, is responsible for recruiting – through charge-based interactions – soluble or amphiphilic molecules to their proper location within the cell, where they serve crucial roles in signal transduction and propagation.

10.4.2 Phosphatidylserine-Specific Interactions: Head Group Recognition

In addition to the general charge-based interactions outlined above, there are a number of proteins that have domains that will stereospecifically interact with the PS head group (Table 10.2). Such interactions are often important for the localized initiation and propagation of various signaling cascades within the cell. Classical PKCs (α , β I, β II and γ) contain a conventional C2 domain, a calcium-dependent cysteine-rich region that recognizes PS and is responsible, in coordination with the C1 domain that binds to DAG, for activating and localizing the kinase to the PM of suitably stimulated cells [127, 152, 153]. The binding of PS is relatively specific, as the binding pocket of the C2 domain coordinates the binding of

calcium and prefers L-PS [38, 164]. Without this calcium-dependent binding to PS, the classical PKC isoforms are not activated. By contrast, the novel PKC isoforms have a modified C2 domain that does not bind calcium and the atypical PKC isoforms lack a C2 domain entirely, making them independent of PS binding [164].

Synaptotagmins, a family of proteins whose members also mostly contain C2 domain-like regions that bind to PS, are important for calcium-mediated vesicle fusion, particularly for the fast, calcium-dependent release of neurotransmitters [26, 89, 147, 164]. The C2 domains of synaptotagmins tend to be fairly promiscuous, binding most acidic phospholipids [150]. However, as discussed earlier, PS is the most abundant acidic phospholipid at the PM, where neurotransmitter release occurs. During neurotransmitter secretion, when synaptotagmin binds calcium, in addition to increasing the affinity of synaptotagmin for acidic phospholipids 100–10,000 times, a hydrophobic loop of the protein is released which penetrates the membrane, stabilizing the protein on the lipid bilayer [11, 150]. Numerous *in vitro* studies document the importance of PS in synaptotagmin function during vesicle fusion, including the observation that the phospholipid needs to be present in both the plasmalemma as well as the incoming vesicles [146]. Moreover, recent *in vivo* studies have also shown that PS influences the opening and dilation of fusion pores during calcium-triggered vesicle fusion [175].

PS-specific binding is also important in the function of A-, B- and C-Raf kinases, important regulators of many signal transduction pathways. Raf kinases are generally downstream from the Ras GTPases, and transmit information to activate mitogen-activated protein kinase (MAPK) signaling [157]. When inactive, Raf kinases exist in the cytosol in a closed conformation, with their regulatory domain bound to, and inhibiting, the kinase domain. The protein 14-3-3 is additionally bound to the inactive Raf, stabilizing its inactive conformation. Activation of Raf occurs upon recruitment to the membrane by Ras, but a number of lipids have been found to interact with Raf and potentially play a role in its stimulation

[59, 72, 157]. The Raf proteins have a conserved N-terminal cysteine-rich domain that has some structural similarity to PKC-type C2 domains; this C2-like domain is responsible for PS binding to Raf in a calcium-independent manner [47, 48, 103]. In addition, there is a separate C-terminal domain that binds PA and a region also near the N-terminal that is involved in phosphoinositide binding [47, 59, 72]. While there is still much uncertainty about the functional role of all these lipid interactions [59, 157], the importance of PS binding for the recruitment of Raf to membranes, the release of 14-3-3, and the activation of the kinase has been demonstrated [47, 48, 97]. Thus, PS plays important roles in multiple steps of the cascades initiated by receptor tyrosine kinases that signal through Ras and Raf through to the MAPK system.

Sphingosine kinase (SK) 1 and SK2 are enzymes that convert sphingosine to sphingosine 1-phosphate, an important extracellular messenger involved in stimulating endothelial differentiation, migration and mitogenesis [62], as well as being implicated in intracellular signaling cascades leading to cytoskeletal changes, motility, release of intracellular calcium and protection from apoptosis [63, 82, 144]. SK is a cytosolic protein that can be phosphorylated and recruited to the PM upon stimulation of PKC [71, 120]. Current evidence suggests that SK displays a binding region that is specific for PS, and the binding affinity of SK for PS increases upon phosphorylation; this interaction with PS is essential for its proper recruitment and full enzymatic activity both *in vitro* and *in vivo* [144].

Finally, some signaling proteins with pleckstrin homology (PH) domains have been shown to have the binding of their cognate phosphoinositide enhanced in the presence of PS. For example Grp1, a guanine nucleotide-exchange factor (GEF) for Arf family GTPases, has greatly enhanced binding to phosphatidylinositol 3,4,5-trisphosphate-containing vesicles when PS is also present [78]. Similarly, the PH domain of Akt has been found to have basic residues nearby that are involved in binding of PS, which is

required for its full activation [67]. Additionally, phosphoinositide-dependent kinase-1 (PDK1) is a serine/threonine kinase that functions upon binding to phosphatidylinositol-3,4,5-trisphosphate and phosphatidylinositol-3,4-bisphosphate to regulate a subgroup of 3-phosphoinositide-responsive protein kinase family members including Akt, p70 ribosomal S6 kinase, serum- and glucocorticoid-induced protein kinase, and atypical PKC [14]. PDK1 is partially bound to the PM in the absence of 3'-phosphoinositides and recent studies have shown that there is a site – separate from the canonical phosphoinositide-binding site – that specifically binds PS, is responsible for recruiting PDK1 to the PM and is important for its signaling function [87].

10.5 Conclusions

PS is an essential glycerophospholipid present in all mammalian cells with an asymmetric distribution throughout the cell. It is becoming clear from the research highlighted in this chapter that rather than being an inert component of biological membranes, PS, with its unique head group and combination of abundance and subcellular distribution, plays an important role in a number of signaling pathways. As detailed, signaling via PS is mediated by the interaction of proteins with PS in one of two ways: via domains that stereospecifically recognize the head group, or by electrostatic interactions with membranes that are rich in PS and therefore display negative surface charge, such as the PM. As outlined, such interactions are key to both intracellular and extracellular signaling cascades. Overall, then, there are a number of signaling proteins that depend upon the unique head group and cellular distribution of PS for their proper localization and activation. In closing, it is worth emphasizing that while much of the current understanding of PS involvement in signaling has been derived from *in vitro* studies, future studies will in all likelihood uncover additional signaling roles for PS in the physiological context, now that suitable tools to analyze in live cells are emerging.

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