The Distribution and Function of Phosphatidylserine in Cellular Membranes

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Key Words

lipid asymmetry, flippase, scramblase, surface charge, C2 domain; apoptosis

Abstract

Phosphatidylserine (PS) is the most abundant negatively charged phospholipid in eukaryotic membranes. PS directs the binding of proteins that bear C2 or gamma-carboxyglutamic domains and contributes to the electrostatic association of polycationic ligands with cellular membranes. Rather than being evenly distributed, PS is found preferentially in the inner leaflet of the plasma membrane and in endocytic membranes. The loss of PS asymmetry is an early indicator of apoptosis and serves as a signal to initiate blood clotting. This review discusses the determinants and functional implications of the subcellular distribution and membrane topology of PS.

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INTRODUCTION

Lipid bilayers form the core structure of the membranes that envelop eukaryotic cells and subdivide them into compartments with distinct structural and functional identities. Lipids not only confer unique dielectric and permeability properties to the bilayer but also dictate the partitioning and folding of intrinsic proteins. Glycerophospholipids account for approximately 70% of the total lipid content of mammalian cells; the other 30% consists of cholesterol, sphingomyelin, and glycosphingolipids. Among the phospholipids, phosphatidylcholine (PC) is the most prevalent and accounts for 40%–50% of the total (90). Phosphatidylethanolamine (PE), which ranges from 20% to 45% of the total phospholipids, depending on the tissue, is the next most abundant (56, 57, 90). Phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidic acid (PA) are present in lesser amounts.

The lipid composition of the membranes of different organelles can vary widely: Cardiolipin is a major constituent of mitochondria but is virtually absent from other organelles (91), whereas *bis*(monoacylglycero)phosphate, more commonly known as lyso*bis*phosphatidic acid, is largely confined to late endosomes and lysosomes (38, 53). Even the content of conventional phospholipids varies among organelles (**Table 1**). In some instances, sharp differences in lipid composition exist also between the subdomains of a single organelle (e.g., the internal vesicles versus the lining membrane of multivesicular bodies). The distinct lipid composition confers characteristic thickness, permeability, and fluidity properties onto the membranes of individual organelles and subdomains (91). Because lipids and their metabolites are effective second messengers, the ability to transduce signals also varies markedly between organelles. In addition, lipid composition can differ drastically between the two monolayers that constitute a defined membrane bilayer, e.g., the cytosolic versus extracellular leaflets of the plasmalemma (**Figure 1**). The resulting asymmetry can induce membrane curvature and can impose a transmembrane electrostatic potential difference.

On a molar basis, PS is a relatively minor constituent of most biological membranes. However, the low abundance of PS is outweighed by its physiological importance, which is attributable to its unique physical and biochemical properties. PS is key to the recruitment and activation of numerous enzymes and structural components, and it signals important events such as the clearance of apoptotic cells and the internalization of viruses by host cells (12). Similar to other lipids, and perhaps more so, PS is distributed unevenly in the cell. In view of its varied and critical functional roles,

PC: phosphatidylcholine

PE: phosphatidylethanolamine

PS: phosphatidylserine

Table 1 Phospholipid content and cholesterol/PS molar ratio of different organelles

Membrane	PC	PE	PI	PS	Chol/PS	References
	(%)[a]					
Endoplasmic reticulum	55	30	15	3–5	3	46
Golgi complex	50	15	10	5	4	46
Early endosome	47	23	8	8.5	–	33
Late endosome	48–49	18.5–20	4–7	2.5–3.9	–	33, 34
Mitochondria	40–46	30–35	5–10	1	10	17, 46, 104
Plasma membrane	42	25	3	12	8	46

[a]Each phospholipid species is expressed as a percentage of total phospholipids.
Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; Chol, cholesterol.

the task of defining the distribution of PS in cells acquires an important dimension. The objective of this review is to briefly summarize our knowledge of the subcellular distribution of PS and its determinants, while recognizing that the field is still in its infancy.

PHOSPHATIDYLSERINE: AN OVERVIEW

Structure, Biosynthesis, and Degradation

Phosphatidylserine species were first identified in whole brain lipid extracts by Folch and coworkers in the 1940s (21–23). Similar to other phospholipids, PS contains two acyl chains at the *sn*-1 and *sn*-2 positions of the glycerol moiety, with the polar headgroup attached to position *sn*-3. Although the acyl chains in PS vary among cell types and organelles, saturated fatty acids of 16 or more carbons are generally attached to the *sn*-1 position, whereas unsaturated fatty acids are generally found at the *sn*-2 position. Although the presence of an unsaturated acyl chain is not conducive to partitioning in sphingolipid- and cholesterol-enriched microdomains, there are reports that PS is enriched in putative rafts in the plasma membrane (63). This may reflect a higher degree of saturation of the plasmalemmal PS.

The most distinctive feature of PS, however, is the linkage of serine at position *sn*-3. Unlike choline or ethanolamine, which are cationic, serine is neutral. Thus, whereas PC and PE are zwitterionic, PS is anionic. When present in substantive amounts, PS endows membranes with a sizable electrostatic potential that concentrates soluble cations in the electrical double layer, in proportion to their charge. Polycations and proteins with cationic clusters bind avidly to electronegative surfaces, a major mode of protein recruitment to membranes that is often overlooked.

In yeast, PS is synthesized by the conjugation of serine to CDP-diacylglycerol (**Figure 2**), a pathway reminiscent of that described in prokaryotes (90). The phosphatidylserine synthase (PSS) that catalyzes this reaction is encoded by *CHO1*, and the deletion of this gene precludes the formation of PS (44, 59). However precariously, *CHO1*-deficient yeast survive provided the growth medium is supplemented with ethanolamine (59). In mammalian cells, two separate pathways can accomplish PS biosynthesis. Rather than using CDP-diacylglycerol, mammalian cells produce PS by exchanging the headgroup of PC or PE for serine. PSS1 mediates the release of choline from PC, whereas PSS2 releases ethanolamine from PE, with the concomitant insertion of serine and generation of PS in both instances (90). These pathways are at least partially redundant, which was demonstrated by the development of PSS-deficient cells (40, 41, 95) and by gene knockout experiments (4, 6).

PSS: phosphatidylserine synthase

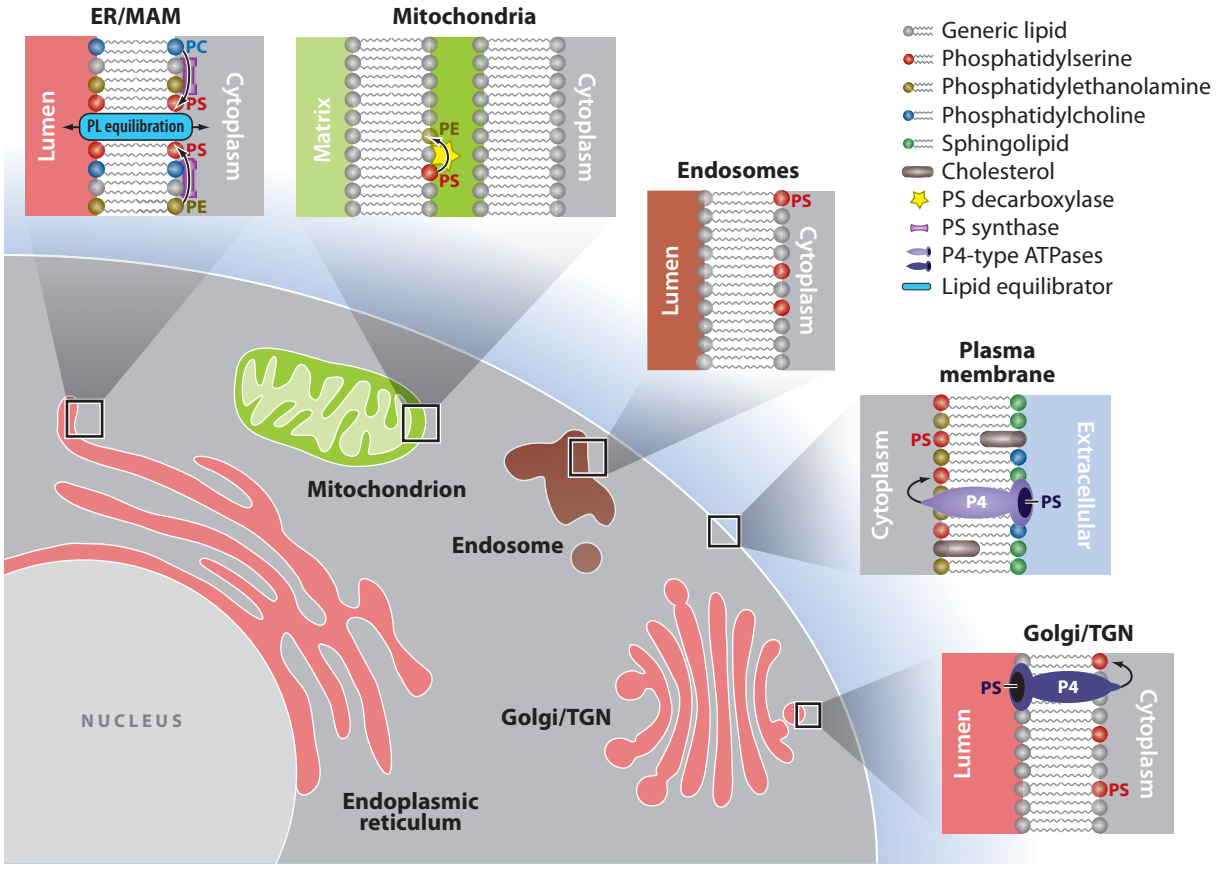

Figure 1

Phosphatidylserine topology, synthesis, and transport across bilayers in mammalian membranes. The topology of phosphatidylserine (PS; *red headgroup*) is illustrated where known. PS is produced in the mitochondrial-associated membranes (MAMs) of the endoplasmic reticulum (ER) from phosphatidylethanolamine (PE; *light brown headgroup*) or phosphatidylcholine (PC; *blue headgroup*) by PS synthases. PS is decarboxylated to PE by PS decarboxylase on the outer leaflet of the mitochondrial inner membrane. Phospholipids (PLs) are translocated to the inner (luminal) leaflet of the ER by a nonspecific equilibrator mechanism, similar to a scramblase. P4-type ATPases in the late Golgi complex/*trans*-Golgi network (TGN) and plasma membrane actively transport PS to the cytoplasmic leaflet to create or maintain PS asymmetry. The topology of cholesterol (*dark brown oblong*) and sphingolipids (*dark green headgroup*) at the plasma membrane is also shown. Unspecified lipids are represented by a gray headgroup.

Mice that lack either PSS1 or PSS2 are viable and fertile (although male *Pss2*$^{-/-}$ mice have reduced fertility; see Reference 6). In contrast, the elimination of both genes is lethal (4).

PS degradation occurs primarily by decarboxylation, yielding PE. Yeast have two PS decarboxylases (PSDs), encoded by *PSD1* and *PSD2* genes. The products of these genes, Psd1p and Psd2p, are localized in mitochondrial and in the Golgi complex/vacuole membranes, respectively (85–87). The single mammalian PSD, like Psd1p, is found in the outer aspect of the inner mitochondrial membrane. PS can also be hydrolyzed by phospholipases that are located in the plasmalemma. Mammalian PS-specific phospholipase A1 (which also acts on 1-acyl-2-lyso-PS) hydrolyzes the *sn*-1 acyl chain of PS, which generates -lyso-2-acyl-PS. The latter compound can signal platelet degranulation, mast cell activation, and T-cell growth suppression (3, 32). Phospholipase A2 hydrolyzes the *sn*-2

PSD: phosphatidylserine decarboxylase

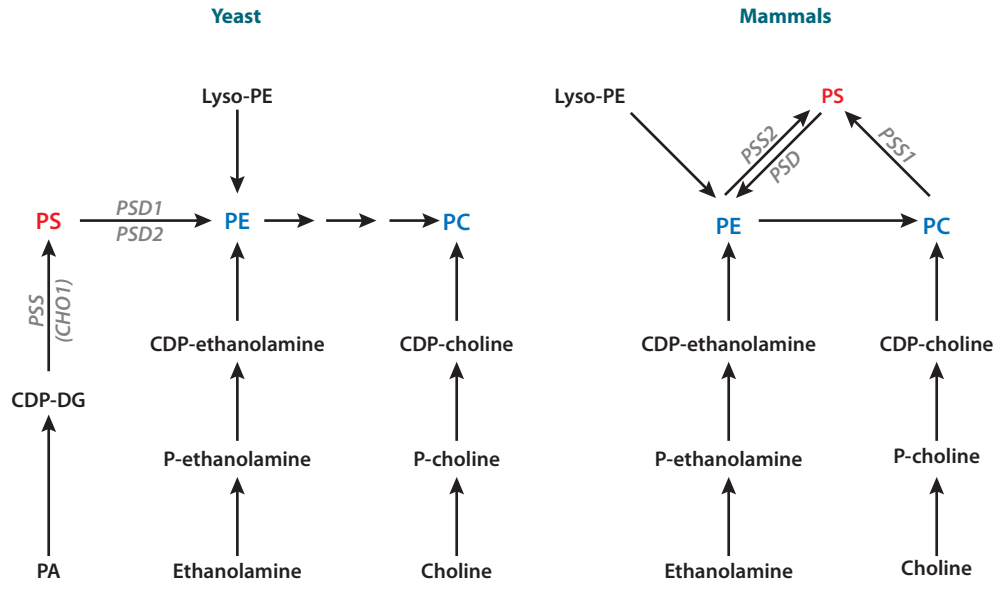

Figure 2

Interrelationship of PS, PE, and PC metabolism in yeast and mammalian cells. In yeast, PS is produced by the conjugation of serine to CDP-diacylglycerol (CDP-DG) by phosphatidylserine synthase (PSS), encoded by *CHO1*. PS is converted to phosphatidylethanolamine (PE) by PS decarboxylase 1 (PSD1) in mitochondria and by PSD2 in Golgi complex/vacuole membranes. In mammalian cells, two PSS enzymes catalyze the synthesis of PS from either PE or phosphatidylcholine (PC). A single mitochondrial PSD decarboxylates PS to form PE. For simplicity, enzymes other than PSS and PSD have been omitted. The synthesis of PE from lyso-PE and CDP-ethanolamine and the synthesis of PC from CDP-choline are similar in yeast and mammalian cells.

acyl of PS to form 1-acyl-2-lyso-PS, which is implicated in mast cell activation and neuronal differentiation (32).

Function

As discussed in more detail below, PS is largely secluded inside the cells and is exposed to the extracellular milieu in exceptional circumstances only. Nevertheless, PS plays important functional roles inside and outside the cells and, ironically, its extracellular functions have been studied more extensively and are better understood. The prototypical example is hemostasis, in which PS is a key signal in the coagulation cascade (7, 99). When activated, blood platelets expose PS on their outer surface; scramblases, the enzymes that promote the translocation of PS to the outer leaflet of the plasmalemma, are discussed in more detail below. The exposure of extracellular PS triggers the attachment and activation of several clotting factors, notably factors V, VIII, X, and prothrombin (48, 80, 108). The recruitment of these proteins to the surface of platelets is mediated by specialized recognition modules, such as discoidin-type C2 or gamma-carboxyglutamic acid domains (**Table 2**) that bind PS with a high degree of selectivity and stereospecificity. The tenase and prothrombinase complexes that promote coagulation become fully active only after binding PS on the surface of activated platelets (7, 108).

Extracellularly exposed PS is also an essential factor in the recognition and clearance of apoptotic cells. When cells undergo programmed cell death, PS appears on their outer surface. Indeed, the extracellular exposure of PS is used by many laboratories as one of the earliest indicators of apoptosis (19, 39, 52). PS externalization is a result of scramblase activation likely coupled with flippase inactivation (5).

Scramblase: one of a group of proteins that causes ATP-independent randomization of phospholipid distribution across membranes, often in response to stimulation

Flippase: an aminophospholipid translocase, which may be a P-type ATPase that causes the specific cytosol-directed transbilayer movement of phospholipids

Table 2 Characteristics of the major phosphatidylserine-binding domains

Class	Ca^{2+} dependency	Specificity	Examples	Function	Structural features	References
C2 domain[a]	Yes	Electrostatic, limited specificity for PS, but often nonspecific	Annexin V	PS masking in placenta to prevent coagulation	Four domains of five α helices coordinate Ca^{2+} and PS	83
			PKCα PKCβ	Protein phosphorylation	Two loops coordinate Ca^{2+} and PS	82, 93
C2 domain, discoidin-like	No	L-serine stereospecific binding	Coagulation factor V	Blood coagulation	β barrel with two loops that associate with PS-containing membrane	46
			Lactadherin	Stabilizes phospholipid lining of milk droplets; integrin bridging during apoptotic cell clearance by macrophages		10, 28, 29, 45, 74
Gamma-carboxyglutamic acid (Gla)	Yes	Specific serine headgroup coordination, nonspecific electrostatic	Prothrombin	Blood coagulation	Hydrophobic patch coordinates PS and Ca^{2+}	34

[a]The two types of C2 domains are unrelated and are so named by coincidence (43).
Abbreviations: PKC, protein kinase C; PS, phosphatidylserine.

However, recent work by Mirnikjoo et al. suggests that PS is also delivered to the surface by lysosomes that fuse with the plasma membrane during apoptosis (54). Unlike necrosis, programmed cell death enables the organism to undergo renewal without the disintegration of cells that would release potentially harmful intracellular enzymes and antigens. The completion of the process, however, requires the rapid and effective clearance of apoptotic bodies by phagocytes. Remarkably, the appearance of PS on the surface of apoptotic cells identifies them as targets for engulfment by phagocytic cells (19, 55, 62). PS is also exposed extracellularly during the course of histamine secretion in mast cells (26, 32). Whether this is a necessary component of the secretory process, or a consequence thereof, remains unclear.

Unlike the sporadic signaling role it plays upon extracellular exposure, intracellular PS likely functions continuously in various capacities. Its negatively charged headgroup, unique structural features, and comparative abundance make PS the preferred target of an assortment of proteins with specialized motifs and domains (43). Some of these are not very selective and interact promiscuously with anionic phospholipids in general. Proteins endowed with a hydrophobic side chain, such as a farnesyl or fatty acyl moiety, in the immediate vicinity of a polycationic stretch display such behavior. Ras and Rho-family GTPases and the tyrosine kinase Src belong to this category (20, 79, 101). Other proteins are slightly more selective towards PS and interact via conventional C2 domains (**Table 2**). Although not entirely PS-specific, C2 domain-bearing proteins such

as synaptotagmin and several protein kinase C isoforms bind PS, often in a calcium-dependent manner, which results in an alteration of their structure and catalytic activity.

THE DETECTION AND SUBCELLULAR LOCALIZATION OF PHOSPHATIDYLSERINE

Like other lipids, PS is not uniformly distributed in the cell. Because its activity depends critically on location, determining the subcellular distribution of PS is important. Notably, however, although a variety of approaches have been implemented to define the distribution of PS within the cell, our current knowledge is incomplete and imperfect. The techniques applied to this problem and the resulting conclusions are summarized briefly below.

Fractionation

Most of the available information regarding the subcellular distribution of PS was derived from subcellular fractionation studies. The separation of organelles by differential and/or density gradient sedimentation, followed by an analysis of lipid content by chromatographic, isotopic, or spectroscopic methods, has been the procedure of choice. A compilation of the lipid distribution deduced from such studies is presented in **Table 1**, in which only representative references and reviews are cited for the sake of brevity.

Although extremely useful and informative, such studies have limitations. First, the purification of fractions is never perfect, and cross-contamination is widely acknowledged. Second, chemical modification and the redistribution of lipids occur during the often time-consuming fractionation protocols. Third, not all subcellular compartments can be isolated in sufficient quantity and with adequate purity for chemical analysis. Last, global analysis of the lipids disregards asymmetry between monolayers of the membrane, a topic discussed extensively below.

In situ Detection

In principle, the detection of PS in intact cells, in combination with powerful microscopic techniques, should obviate the problems associated with cellular homogenization and fractionation. However, this approach has been hampered by the paucity of suitable probes to detect PS in situ (**Table 3**). 7-Nitro-2-1,3-benzoxadiazol-4-yl-tagged PS (NBD-PS) has been used most extensively, but the results obtained with this analog are perplexing. Specifically, when NBD-PS is added extracellularly, the fluorescence at steady state is weak at the plasma membrane and bright in reticular endomembrane structures, observations that are not easily reconciled with the fractionation studies listed above. Plausible explanations include that the insertion of the NBD moiety distorts the structure (13, 35) and therefore the targeting of the phospholipid and that the lipid may undergo a rapid conversion to other species, especially when structurally altered by the fluorophore.

Rather than labeling the lipid covalently and altering its behavior in the process, researchers have attempted to devise probes to detect endogenous PS. To this end, PS-reactive antibodies have been developed (49, 50), which make immunolocalization possible in fixed cells. Gaining access to the largely intracellular PS, however, requires permeabilization, which is usually effected using detergents that themselves disrupt lipid architecture. A better strategy is therefore to deliver PS-binding probes to the cell interior without disrupting the membrane in the process. Microinjection is a possible approach, but it is technically demanding and limits the number of cells that can be studied. An elegant alternative is the use of molecular biology to genetically encode the PS-binding probes to be expressed within the cells of interest. Although cloning PS-specific antibodies is a formidable task, simpler options exist. Specifically, cells can be transfected with proteins or protein domains that have demonstrated affinity and selectivity for PS. Annexin V has been used extensively to probe for

Table 3 Characteristics of phosphatidylserine probes

Probe	Type	Advantages	Disadvantages	K_d[a]	References
Annexin V	C2 domain protein	Well characterized Commercially available Easily tagged (e.g., GFP, fluorescent dyes) Genetic expression	Binds PE, other phospholipids Requires Ca^{2+} for binding Intracellular PS detection requires Ca^{2+} manipulation	0.5–1 nM	8, 83, 84
Lactadherin	C2 domain protein (discoidin-like)	Ca^{2+}-independent binding Specific Easily tagged (e.g., GFP) Genetic expression	Not readily available Comparatively large	3.3 nM	76
Antibodies	Monoclonal IgG	Specific (although not perfectly) Commercially available	Fixation and permeabilization required to label endomembranes Not amenable to genetic expression	0.6–1 nM	49, 50
TNBS	Reactive with primary amines	Chromatographic quantification Reproducible Cell-impermeant	Unsuitable for in situ detection Alters PS irreversibly	–	25, 78
NBD-PS	PS analog; fluorescent NBD on *sn*-2 6:0 or 12:0 chain (*sn*-1 usually 16:0 or 18:1)	Cells easily loaded Commercially available	NBD distorts acyl chain structure Subject to conversion to other species Fluorescence rapidly bleached	–	51

[a]Dissociation constant for phosphatidylserine binding.

Abbreviations: PE, phosphatidylethanolamine; PS, phosphatidylserine; GFP, green fluorescent protein; NBD-PS, 7-nitro-2-1,3-benzoxadiazol-4-yl-tagged phosphatidylserine; TNBS, trinitrobenzene sulfonate.

extracellular PS and was recently tested also as an intracellular probe, through the generation of a plasmid that encodes a GFP-tagged chimeric construct (11). Of note, the binding of the C2 domain of annexin V to PS requires supraphysiological calcium concentrations, and ionophores or other means are required to artificially elevate the cytosolic concentration of the cation (8, 11, 84). The consequences of these manipulations, together with the fact that annexin V is not entirely selective toward PS, severely limit its usefulness as an intracellular probe.

The discoidin-type C2 domains provide a means to circumvent this problem. Unlike the conventional C2 domain of annexin, discoidin C2 domains such as that of lactadherin, a milk glycoprotein, recognize PS with high affinity and specificity in the nominal absence of calcium and can therefore be used to probe the distribution of the phospholipids in transfected but otherwise unmodified cells (**Figure 3**) (100). One caveat of this approach is that, unless additional targeting determinants are provided, the constructs are expressed in the cytosol and therefore probe only the aspect of the bilayer that faces this compartment. Compartment-specific, luminally targeted constructs can be devised, but assessing their interaction with the membrane poses additional difficulties.

Subcellular Distribution of PS and its Determinants

When the information obtained by all the available methods is combined, a reasonable consensus pattern of the intracellular distribution

of PS begins to emerge. First, it is clear that not all organelles have an identical content of PS because they differ by more than one order of magnitude in some instances (**Table 1**; **Figure 3**). Second, there is general agreement that the plasma membrane is most enriched in PS, followed by early endosomes. At the other extreme of the spectrum are mitochondria, which contain the lowest mole fraction of PS.

How is the inhomogeneous distribution of PS generated? The enzymes that synthesize PS are located in the ER, where they concentrate in mitochondrial-associated membranes known as MAMs (69, 81). These are highly specialized contact zones that mediate communication and functional interactions between mitochondria and the ER. As such, one would anticipate the ER and possibly the mitochondria to be richly endowed with PS, but this is clearly not the case. There is evidence that some of the PS generated at the MAMs is transported directly to the mitochondria where it is rapidly decarboxylated by PSD to form PE (89, 94), which may account for the low amount of PS seen in this organelle. The newly synthesized PS that initially remains in the ER presumably transits to the endoplasmic reticulum to Golgi intermediate compartment (ERGIC) and Golgi cisternae via the secretory pathway. Upon reaching the *trans*-Golgi network (TGN), PS can potentially undergo two fates: proceed along the secretory pathway to the plasma membrane or be diverted to the prelysosomal endocytic compartment. Although the PS content of late endosomes and prelysosomes is a subject of debate, the early endosomal compartment (which includes the recycling endosomes) and particularly the surface membrane are enriched in PS compared to other organelles, which include the ER where it is synthesized. Where and how the concentrative step occurs is unknown.

Because its generation appears to be restricted to the ER, mechanisms that do not involve biosynthesis must be invoked to account for PS accumulation in the plasma membrane and endosomes. Two general models can be contemplated: (*a*) PS may be preferentially concentrated in secretory vesicles that deliver lipids to the plasma membrane. Such a concentrative event could take place at the TGN or in recycling endosomes, inasmuch as at least a fraction of the TGN-derived vesicles traverse the recycling endosomal compartment en route to the plasmalemma; and (*b*) PS enrichment may result from the selective abstraction of lipids other than PS at the plasma membrane or in the recycling compartment. For instance, the formation and internalization of lipid subdomains that exclude PS would promote its preferential retention in the membrane.

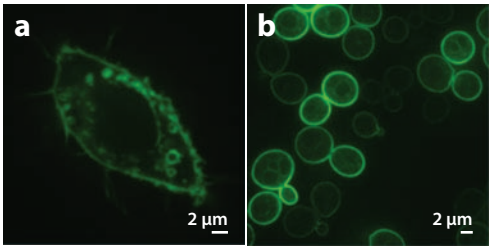

Figure 3

In vivo detection of cytoplasmic leaflet PS using a GFP-tagged discoidin-type C2 domain. (*a*) RAW 264.7 macrophage that expresses a GFP-tagged lactadherin-C2 domain. PS is on the plasma membrane and on endomembranes identified as components of the endocytic pathway (88). Image courtesy of Dr. T. Yeung. (*b*) Wild-type *S. cerevisiae* that express GFP-tagged lactadherin-C2 domain. PS is visible mainly on the plasma membrane. Image courtesy of Dr. G. Fairn. Both images are representative confocal slices.

We have preliminary evidence suggesting that PS concentrates in secretory vesicles: In yeast that harbor mutant alleles of *SEC1* or *SEC6*, in which secretory carriers generated from the Golgi complex are unable to fuse with the surface membrane, vesicles densely labeled by the C2 domain of lactadherin accumulate (G. Fairn and S. Grinstein, unpublished observations). This is accompanied by the diminution of plasmalemmal staining, which indicates the partial depletion of PS. These observations are consistent with the secretory enrichment model discussed initially. However, they appear superficially inconsistent with the recent report by Klemm et al. (37), in which light secretory vesicles isolated from similar yeast mutants showed no accumulation of PS. Their findings and ours could be reconciled by considering that two distinct types of secretory vesicles emerge from

MAM: mitochondrial-associated membrane

ERGIC: endoplasmic reticulum to Golgi intermediate compartment

TGN: *trans*-Golgi network

the Golgi complex: the light kind studied by Klemm and colleagues, and a heavy type that is coated by clathrin and was not studied by these authors. PS enrichment may occur exclusively in clathrin-coated vesicles. Indeed, as we speculate below, the enrichment may be coupled to the clathrin-assembly process.

Although consistent with the enrichment of PS in secretory vesicles, our observations do not rule out the occurrence of an abstraction mechanism. Unlike the light secretory vesicles studied by Klemm et al., which follow a direct path from the Golgi complex to the membrane, heavy secretory vesicles intersect the recycling compartment. PS accumulation may result in this compartment from the sorting of PS-enriched membranes for delivery to the surface, away from other PS-depleted regions that are targeted elsewhere, either retrogradely to the Golgi complex or to deeper endosomal compartments.

Clearly, much remains to be learned about how PS is selectively targeted to certain membranes while being excluded from others. Although the mechanism remains far from clear, we suspect that lipid asymmetry between monolayers of individual organelles plays a role in the process. A description of such asymmetry, the underlying mechanisms, and the rationale for our hypothesis that it plays a role in organellar redistribution are the subjects of the following sections.

TRANSBILAYER ASYMMETRY OF PS

Lipid distribution differs not only among organelles but frequently also between the two leaflets of the membrane. Although several lipid species are distributed differentially in the two monolayers of several biological membranes, we restrict our discussion to the transmembrane disposition of PS. Several methods have been used to quantify the exposure of PS on a given surface by measuring the extent of labeling with impermeant reagents (78), the fraction susceptible to degradation by lipases (110), or the accessibility to probes such as annexin V or lactadherin (39, 75–77). Even bioassays that measure the ability of PS to trigger clotting have been employed on occasion to assess PS sidedness (16). Of note, all these procedures hinge on the physical separation of the exposed and contralateral (latent) sides of the membrane and therefore entail the analysis of sealed structures that preserve a permeability barrier. The fraction of contralateral PS is then calculated by subtracting the exposed fraction from the total PS.

Transbilayer asymmetry was demonstrated initially at the plasma membrane. Human erythrocytes, which are plentiful and have a single membrane, were the first system to be analyzed in detail. Labeling with the impermeant amino-group reagent trinitrobenzene sulfonate and subsequent thin-layer chromatographic analysis showed that PS is virtually inaccessible to the probe in intact cells (25). PS was labeled only when the membrane was disrupted, which implies that PS is confined to the cytosolic monolayer. Accordingly, inside-out erythrocyte membranes—but not intact red cells—initiated clotting, a PS-stimulated cascade (109). These and other studies estimated that >96% of the PS resides in the inner leaflet of the erythrocyte membrane (102). Subsequent reports established that a similar extreme asymmetry in the distribution of PS exists across the surface membrane of platelets (71) and in a variety of nucleated cell types (31, 92). NBD-tagged analogs have been used to verify the distribution of PS and to monitor the development of the asymmetry. When added to the external surface of intact cells, NBD-PS is rapidly translocated to the inner leaflet, a process that can be monitored using impermeant fluorescence quenchers or a back-washing procedure that selectively removes exofacial NBD-PS (42, 51).

The study of lipid asymmetry in internal membranes has proven far more complicated. To our knowledge, no in situ determinations of the transmembrane distribution of PS in organelles have been reported, owing to the paucity of probes and the difficulties inherent in targeting and quantifying their association

with defined membranes in intact cells. Instead, research in this area has relied on a combination of subcellular fractionation and the type of PS exposure determinations described above. It is important to note that, however gentle, the homogenization procedures required for fractionation can disrupt the permeability barrier of the organellar membrane, which allows access to otherwise latent PS and leads to an overestimation of the exposed (cytosolic leaflet) fraction. In extreme cases, homogenization can alter the sidedness of the resulting membranes, particularly when large membrane sheets fragment and reseal with an inverted orientation. Therefore, results obtained with fractionated cells must be interpreted with caution.

Because they can be isolated intact and in fairly large numbers, chromaffin granules have provided reliable determinations of the transmembrane distribution of PS. Using spin-labeled probes, Zachowski et al. estimated that approximately 85% of PS is found on the cytosolic face of these organelles (103). The ER, which is also abundant, has similarly been studied by several groups. In this case, however, the sealed structures required for sidedness determinations ostensibly form by the fragmentation of an intricate network of tubules. Whether the resulting vesicles retain their original orientation and seal homogeneously is a matter of conjecture. Nevertheless, the available results consistently show a marked asymmetry in the distribution of PS. Both in the ER and in its muscle cell counterpart—the sarcoplasmic reticulum—PS is predominantly found in the internal (luminal) monolayer (9, 88). The enrichment varies in different reports, with the luminal fraction ranging from approximately 70% of the total to over 90% (9, 30, 88). The scarcity of PS in the cytosolic face of the ER is consistent with the failure of annexin V and of the C2 domain of lactadherin to bind to this membrane (11, 100). Remarkably little is known about the lipid composition of the individual leaflets of other organelles.

How is lipid asymmetry established? Three types of lipid transporters may dictate the transbilayer distribution of phospholipids: flippases, floppases, and scramblases.

Flippases

Floppase: a protein that directs the transbilayer translocation of phospholipids away from the cytosol; may be an ABC protein

Flippases are aminophospholipid translocases that transport specific phospholipids, including PS, from the extracellular or topologically equivalent luminal leaflet of an organelle to the cytosolic side. Flippase activity was found to require ATP (72) and therefore suspected to involve a phospholipid-translocating ATPase. However, the paucity of the enzyme, together with the lack of specific pharmacological antagonists, hampered the molecular identification of the responsible enzyme for over a decade. Eventually, painstaking biochemical analysis of chromaffin granules and parallel studies of yeast genetics converged to demonstrate that flippase activity is, in all likelihood, mediated by P4 subtype P-type ATPases similar to those engaged in ion transport.

S. cerevisiae have five genes that encode P-type ATPases (*DRS2*, *DNF1*, *DNF2*, *DNF3*, and *NEO1*), and at least 14 human homologs have been identified by bioinformatics (27). Immunofluorescence and cell fractionation studies detected Drs2p and Dnf3p in the late Golgi complex (14, 33), whereas Dnf1p, Dnf2p, and Neo1p are found in the plasma membrane and endomembranes (33, 98). The P-ATPases located in the Golgi complex generate phospholipid asymmetry prior to the arrival of secretory membranes to the cell surface. This is supported by the observation that NBD-PS is translocated across isolated TGN membranes or post-Golgi secretory vesicles in an ATP-dependent manner and that this activity requires Drs2p and Dnf3p (2, 58). Unlike wild-type yeast, strains that lack Drs2p display PS on their outer surface (15), and a further increase in PS exposure was noted when Dnf1p and Dnf2p were also eliminated (65). These observations suggest that PS is flipped across internal membranes as well as the surface membrane and that these activities cooperate to minimize PS exposure to the extracellular milieu. Caution must be exercised

in interpreting these deletion studies, however, because the observed effects may be secondary to the disruption of vesicular trafficking.

Floppases

Floppases transport lipids in the opposite direction, i.e., from the cytosolic to the extracellular or luminal leaflet. This activity has been attributed to members of the ATP-binding cassette (ABC) transporter superfamily. ABC transporters are a large and rather diverse collection of transmembrane proteins that participate in the ATP-dependent export of amphipathic compounds, notably xenobiotic drugs, from the cell (64). Some members of the family, which include ABCA1, ABCB1, ABCB4, and ABCC1, seem to specialize in the translocation of lipids across membranes. Unlike the drug-exporting members of the family, which are rather promiscuous in their selection of ligands, ABCA1, ABCB1, ABCB4, and ABCC1 are more selective. Neither ABCB1, ABCB4, nor ABCC1 can transport significant amounts of PS. In contrast, ABCA1 has been linked to PS transport, even though the export of cholesterol appears to be its main function (61). Whether the transport of these two lipids is somehow linked is still unclear.

Scramblases

Unlike flippases and floppases, which transport lipids unidirectionally in an ATP-dependent manner, scramblases (recently reviewed in Reference 70) are bidirectional and function to randomize or at least reduce the asymmetry of phospholipids in membranes in an ATP-independent manner (36). In the ER, scramblases act constitutively and translocate lipids synthesized on the cytosolic side to the internal leaflet. In this manner, newly formed PS can reach the lumen of the secretory compartment.

A second type of scramblase is quiescent in cells at rest but becomes activated when cytosolic calcium is elevated. These enzymes are located on the plasma membrane and are at least partly responsible for exofacial PS exposure when platelets, mast cells, and phagocytes are activated, and also during apoptosis (19). A protein identified with this activity, termed PLSCR1, has been cloned and characterized (105). Because the deletion of the *Plscr1* gene in mice did not measurably affect lipid transport, however, the role of this protein as a scramblase has been questioned (106). The existence of redundant genes could account for the apparent discrepancy, although other identified members of the PLSCR family reside in mitochondria and in the nucleus (106). Putative scramblase genes have also been identified in *Drosophila* (e.g., *Scramb1* and *Scramb2*), and in *C. elegans* (*SCRM-1*), although functional studies have yielded confounding results (1, 97).

TRANSBILAYER ASYMMETRY AND THE SUBCELLULAR DISTRIBUTION OF PS

We have reason to believe that the transbilayer disposition of PS is a key determinant of its unique organellar distribution. As discussed briefly above, PS enrichment or depletion could in principle be caused by localized synthesis or degradation, respectively. Thus, decarboxylation to PE may contribute to the paucity of PS in mitochondria. However, the PS enrichment observed in the surface and endocytic membranes cannot be attributed to excessive synthesis because both PSS isoforms reside in the ER. Alternatively, the enrichment of defined membrane components, such as PS, can be generated by their segregation and directed targeting in the course of membrane trafficking. Proteins and lipids can be selectively delivered to their cellular destination by preferential sequestration into transport vesicles that are guided by vectorial transport mechanisms. In this manner, transferrin receptors are routed through the recycling compartment, avoiding degradation in lysosomes, and glycosphingolipids are preferentially delivered to the apical membrane of epithelial cells. Importantly, the molecules that induce the fission of transport vesicles, their translocation to their destination, and fusion with the target membrane are all exposed to

the cytosolic environment. We therefore assume that the selective targeting of PS to the plasma membrane and endosomes requires it to be exposed to the cytosol. Two mechanisms can be proposed, based on earlier observations and hypotheses. First, because flippases translocate lipids unidirectionally, their continued activity creates a structural imbalance between the donor and acceptor leaflets of the membrane. The bilayer bulges in the direction of the acceptor leaflet, and, if flippase activity is high and/or localized, the resulting curvature could promote vesicular fission (18). The generation at the TGN of PS-enriched secretory vesicles may be formed by active aminophospholipid-translocating flippases such as Drs2p or its mammalian homologs. Second, a more conventional means of selectively targeting PS-enriched vesicles to the plasma membrane would entail the preferential association of PS with proteins involved in membrane fission or fusion. PS may promote the association of coat proteins required for membrane budding or scission or, conversely, coat proteins recruited by protein cargo may attract and stabilize PS in the region where vesicles are generated. The result, in both instances, would be the formation of vesicles with an elevated PS content. The interaction of coat and severing proteins such as clathrin and dynamin with anionic phospholipids in general and with PS in particular has been documented (47, 66, 73), and a genetic linkage has been established between Drs2p and the generation of clathrin-coated vesicles (15, 24). Selective PS delivery to the membrane could also result from its association to vesicular SNARE proteins targeted to the plasmalemma. Accordingly, some syntaxins are associated tightly with PS (68, 96). Yet, this option seems less appealing because it would require PS to be the predominant species in a sizable lipid annulus that surrounds SNAREs.

Regardless of the specific mechanism(s) involved, the exposure of PS on the cytosolic aspect of secretory membranes is likely to participate in their generation and trafficking, which provides an attractive model to account for enrichment of the phospholipid in the surface and early endocytic membranes. In this context, anionic phospholipid flippases may be critical not only to the maintenance of transbilayer PS asymmetry but also to the differential distribution of PS in cellular organelles.

ESTABLISHMENT OF THE SUBCELLULAR DISTRIBUTION OF PS: A SPECULATIVE MODEL

Why is the concentration of PS highest in the plasma membrane and lowest in mitochondria? Why are flippases needed to deliver PS to the cytosolic side of membranes when PS is generated on this side of the membrane in the first place? How the steady state distribution of PS is established in the cell may be best understood by following the journey of the phospholipid in the cell (**Figure 4**).

There is little doubt that, in animal cells, PS is generated by PSS at the ER, specifically at the MAMs. A fraction of the newly formed PS reaches mitochondria, in which much of it is decarboxylated to PE, which accounts for the scarcity of PS in this organelle. How the inner mitochondrial membrane acquires PS—and maintains levels higher than those found in the outer membrane (107)—remains a mystery. The remainder of the PS is redistributed across the ER membrane by a scramblase-like activity. There is, however, a glaring inconsistency between the expected randomizing effect of a scramblase and the experimental finding that PS is largely secluded in the luminal monolayer of the ER (9, 30, 88). If the sidedness determinations are correct, other transport or sequestration processes must be at work.

The PS in the ER seemingly travels to the ERGIC and Golgi compartments, possibly by bulk flow, although specific delivery systems cannot be ruled out. What fraction of the PS is in the lumen of the Golgi complex is, to our knowledge, not yet known. To the extent that cytosolic C2 domain probes failed to attach visibly to the Golgi membranes, we surmise that little PS is present on the cytosolic surface of the cisternae. Regardless of what fraction of the PS reaches the lumen of the TGN, current

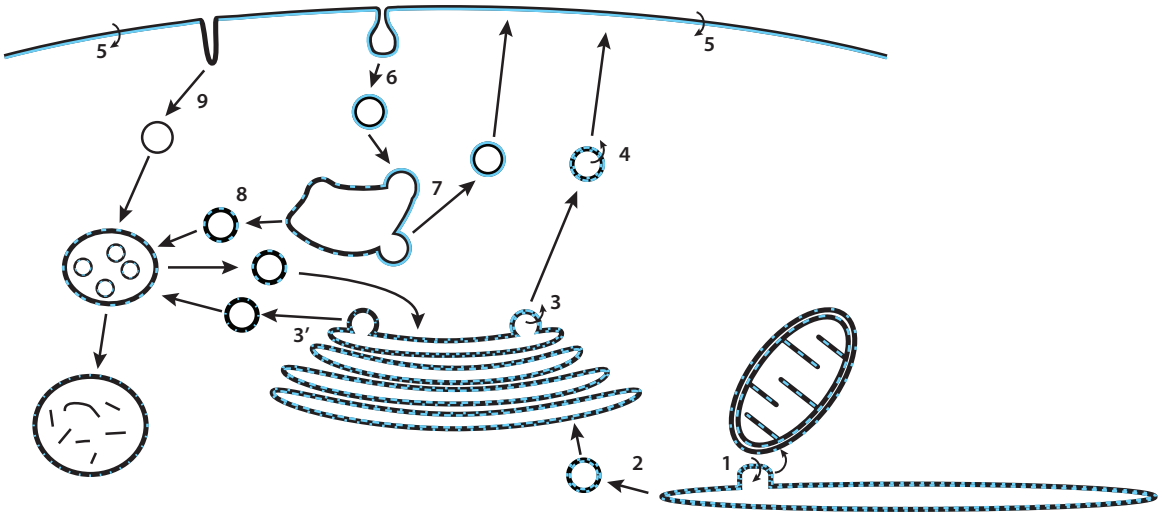

Figure 4

Speculative model of PS traffic and enrichment mechanisms. (Step 1) PS (*solid or dashed blue lines*) is synthesized on the cytoplasmic leaflet of the ER/MAM. Some PS is transported to the mitochondrion where it is rapidly decarboxylated to PE at the inner membrane; PS is also spontaneously translocated to the luminal leaflet of the ER. Current evidence indicates that PS is enriched in the luminal leaflet. (Step 2) PS moves to the ERGIC/Golgi complex via vesicular trafficking. (Step 3) In the TGN, PS is enriched in budding secretory vesicles where it may help recruit proteins necessary for clathrin coat formation; PS may be excluded from vesicles directed to the LE/lysosome (3′). Flippases in the TGN and secretory vesicles translocate PS to the cytoplasmic face. (Step 4) PS continues to be flipped in secretory vesicles en route to the plasmalemma. (Step 5) Plasma membrane flippases maintain PS almost exclusively on the cytosolic leaflet. (Step 6) PS is delivered to sorting endosomes by endocytosis. (Step 7) At the early/recycling endosome, PS-containing/enriched vesicles are recycled to the plasma membrane via vesicular traffic. (Step 8) PS-depleted vesicles generated by sorting endosomes go to late endosomes and/or recycle to the TGN. (Step 9) PS may be excluded from some endocytic events at the plasma membrane. Relative quantities of PS in membrane leaflets are indicated by solid or dashed blue lines. Small curved arrows indicate translocation of PS across the bilayer. Abbreviations: ER, endoplasmic reticulum; ERGIC, endoplasmic reticulum to Golgi intermediate compartment; MAM, mitochondrial-associated membrane; PS, phosphatidylserine; TGN, *trans*-Golgi network.

evidence suggests that flippases can then transport at least a fraction to the cytosolic leaflet. As described earlier, we speculate that the flippase activity contributes to vesicular budding by inducing curvature and/or by promoting the recruitment of coat proteins. This event may be critical in the establishment of the subcellular pattern of PS. Because the TGN is an important traffic sorting station, PS-enriched vesicles may be delivered preferentially to the plasma membrane, whereas other, PS-depleted domains of the TGN membrane are routed retrogradely or to the prelysosomal compartment (**Figure 4**).

The preceding scenario could account for at least part of the accumulation of PS in the membrane, where the asymmetry would be maintained by plasmalemmal flippases (Step 5 in **Figure 4**). Further enrichment may occur at the plasma membrane itself by selective abstraction of other lipids (Step 9 in **Figure 4**). This could be accomplished by the endocytosis of vesicles from membrane domains that exclude PS. However, much of the surface PS must also be continuously internalized because the entire membrane turns over rapidly. This notion is consistent with the observation that PS is also present and rather abundant in early endosomes.

What becomes of the early endosomal PS? Early endosomes are a critical sorting station in cellular traffic. In animal cells, membrane components that are internalized can return to the plasmalemma via rapid and slow recycling pathways or be destined for degradation in late endosomes and lysosomes. In addition, a fraction of the early endosomal material is

delivered retrogradely to the TGN and can thereby reach the rest of the secretory pathway. If, as found in fractionation studies, PS is comparatively depleted in the late endosomes and lysosomes of mammalian cells and in the yeast vacuole, a mechanism must exist for the removal of PS from these compartments, which continuously receive input from earlier endosomal compartments. Two possibilities exist. First, PS may be degraded by lipases, such as those found in the lumen of lysosomes. For this to account for selective PS depletion, however, requires two conditions: (*a*) PS must be preferentially hydrolyzed by the lipases, and (*b*) it must be delivered to the internal vesicles of multivesicular bodies. The latter requirement stems from the fact that hydrolysis of PS in the membrane lining the endosome or lysosome would surely produce organellar leakiness and lysis. The second, and in our minds more attractive, option is that PS-enriched domains of the early endosomal membrane may be preferentially sorted for recycling back to the surface membrane, whereas PS-depleted domains are directed retrogradely and/or to late endosomes and lysosomes (Steps 7 and 8, respectively, in **Figure 4**). The molecular machinery that would accomplish this segregation is presumably similar to that described for the TGN: a combination of flippases and PS-interacting coat proteins. In this regard, the Neo1p flippase is found in the endocytic pathway and is essential for yeast survival (33, 67). Moreover, clathrin, sorting nexins, and other coat-forming and fission-inducing proteins are present and active in early endosomes. Sorting and recycling endosomes may be the main sites where PS enrichment occurs and may potentially surpass the TGN in importance.

Admittedly, the model presented above is a combination of facts and (wild) assumptions, and the latter must be tested experimentally before they can be validated or discarded in favor of more compelling ideas.

SUMMARY POINTS

1. PS is not uniformly distributed in the membranes of eukaryotic cells.
2. PS is produced at sites where the endoplasmic reticulum approaches mitochondria and moves to other membranes via vesicular trafficking.
3. PS is most enriched in the inner leaflet of the plasma membrane.
4. The transbilayer asymmetry of PS in the *trans*-Golgi network (TGN), secretory vesicles, and plasma membrane is likely generated and maintained by flippases.
5. The exposure of PS on the outer leaflet of the plasma membrane signals the onset of apoptosis and triggers phagocytosis and blood clotting.
6. The enrichment of PS at the plasma membrane and in endosomes is likely generated by the segregation of PS-rich and PS-poor vesicles at sorting stations such as the TGN and recycling endosome.

FUTURE ISSUES

1. New approaches must be developed to analyze the sidedness of PS in organellar membranes in situ.
2. The mechanism(s) whereby PS is enriched in some membranes and depleted in others remains to be resolved.

3. The functional implications of the differential PS content of various organelles are unclear and should be addressed.
4. Better probes must be developed to monitor intraorganellar PS.

DISCLOSURE STATEMENT

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Errata

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