Phosphatidylethanolamine critically supports internalization of cell-penetrating protein C inhibitor

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Although their contribution remains unclear, lipids may facilitate noncanonical routes of protein internalization into cells such as those used by cell-penetrating proteins. We show that protein C inhibitor (PCI), a serine protease inhibitor (serpin), rapidly transverses the plasma membrane, which persists at low temperatures and enables its nuclear targeting in vitro and in vivo. Cell membrane translocation of PCI necessarily requires phosphatidylethanolamine (PE). In parallel, PCI acts as a lipid transferase for PE. The internalized serpin promotes phagocytosis of bacteria, thus suggesting a function in host defense. Membrane insertion of PCI depends on the conical shape of PE and is associated with the formation of restricted aqueous compartments within the membrane. Gain- and loss-of-function mutations indicate that the transmembrane passage of PCI requires a branched cavity between its helices H and D, which, according to docking studies, precisely accommodates PE. Our findings show that its specific shape enables cell surface PE to drive plasma membrane translocation of cell-penetrating PCI.

Introduction

Although endocytosis represents the prevailing route for the internalization of proteins into cells (D’Hondt et al., 2000; Itoh et al., 2001; Conner and Schmid, 2003), additional pathways have been suggested that only partially use or might even bypass the endocytic machinery. These pathways apparently facilitate the internalization of polybasic peptides such as magainin-2 and buforin (Matsuzaki et al., 1995, 1998; Takeshima et al., 2003), the HIV-1–based Tat protein, and the third helix of antennapedia from Drosophila melanogaster (penetratin), which mediates the transmigration of antennapedia through the plasma membrane and nuclear pore complex (Derossi et al., 1994; Joliot and Prochiantz, 2004). Although the mechanisms supporting cellular incorporation might vary among the different types of cell-penetrating peptides, membrane lipids could participate in several of the proposed pathways (Mäe and Langel, 2006). Lipids are indeed capable of directly supporting the membrane passage of proteins as shown for the release of (apo)cytochrome c from mitochondria (Diekert et al., 2001; Kuwana et al., 2002).

Distinctive properties of the lipid architecture of the cell membrane include lateral microdomains enriched in cholesterol and (glyco)sphingolipids, which constitute a platform for signal transmission and the internalization of microorganisms (rafts and caveolae; Simons and Vaz, 2004; Jacobson et al., 2007). Another key feature of the plasma membrane is represented by the transverse asymmetry of lipids. Under specific biological conditions, this asymmetry is attenuated, as exemplified by the surface exposure of phosphatidylserine (PS), which provides a recognition signal for the phagocytosis of apoptotic cells (Gardai et al., 2006) and establishes a catalytic surface for proteases implicated in blood coagulation (Bevers et al., 1999). Under the same conditions, phosphatidylcholine (PC) is exposed to the inner leaflet, where it exerts a proapoptotic activity (Robert et al., 2000). Since lipids are essential for cellular structure and function, thus representing a limited resource, specific lipid microdomains might serve as entry points for cell-penetrating peptides. In support of this view, recent work has shown that the insertion of a transmembrane helix of the cell-penetrating peptide penetratin into bacterial membranes is mediated by a conical cavity lined by PE (Diekert et al., 2001).

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Abbreviations used in this paper: CF, carboxyfluorescein; GUV, giant unilamellar vesicles; HCII, heparin cofactor II; LUV, large unilamellar vesicles; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PCI, protein C inhibitor; PE, phosphatidylethanolamine; Pr3+, praseodymium; PS, phosphatidylserine; rPCI, recombinant PCI; SUV, small unilamellar vesicles; uPCI, urinary PCI.

The online version of this paper contains supplemental material.
conditions, the exposure of phosphatidylethanolamine (PE) is enhanced (Emoto et al., 1997; Smirnov et al., 1999). However, in contrast to the well-known functions of PS exposure, the functional meaning of PE externalization is largely unknown.

We found that the cellular internalization of the serpin protein C inhibitor (PCI) is crucially supported by plasma membrane PE, which enables its rapid targeting to the nucleus both in vitro and in vivo. Our findings position PCI as an eminent candidate for the nuclear supply of cargo. On the basis of the crystal structure of PCI, a hydrophobic cavity has been characterized as the binding site for PE, which is recognized by specific lipids of conical morphology. Our findings indicate cell surface PE as a mediator for the cell membrane translocation of proteins and suggest that this requires the ability of the lipid to foster formation of transient nonbilayer domains within the membrane.

**Results**

**PCI acts as selective transferase for PE**

The lipid structure of the plasma membrane can be dynamically regulated by the selective insertion of extracellular lipids via specialized proteins such as scavenger receptor class B type I, which transfers cholesterol and phospholipids into cells (Acton et al., 1996; Urban et al., 2000), CD14, a transporter for phosphatidylinositol (Wang and Munford, 1999), and the fatty acid carrier CD36 (Koonen et al., 2005). These also include unknown proteins mediating the cellular import of PE (Engelmann et al., 1998). To identify the latter proteins, we separated the supernatants from activated blood platelets by gel filtration. Then the phospholipid transfer activities of the fractions were measured by determining the exchange of fluorescent-labeled phospholipids between donor and acceptor vesicles. Among the fractions analyzed, fraction 23 caused the strongest stimulation of the introversicular transfer of fluorescent-labeled PE (Fig. 1 A).

Because activated platelets secrete the PE-binding serpin PCI (Nishioka et al., 1998; Prendes et al., 1999), we analyzed whether fraction 23 contained this protein. PCI could indeed be detected in the active fraction (Fig. 1 A). The serpin was additionally recovered in the total supernatant (Fig. 1 A). To evaluate whether PCI was principally capable of mediating the PE transfer through aqueous media, the serpin was added to suspensions of labeled donor vesicles and unlabeled acceptor vesicles.
Isolated PCI was found to transfer fluorescent-labeled PE to the acceptor vesicles within short time periods (Fig. 1 B), which documents that the serpin supports the intermembrane exchange of PE. Moreover, PCI markedly augmented the incorporation of extracellular fluorescent-labeled PE into platelets (Fig. 2 C) and several other cells (human monocytes, neutrophils, and umbilical vein endothelial cells; not depicted). However, PCI failed to enhance the incorporation of fluorescent-labeled phosphatidylcholine (PC), sphingomyelin (Fig. 1 C, SM), and PS (not depicted). The enhanced PE uptake by the activated platelets was almost completely abolished by the anti-PCI antibody (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200707165/DC1).

Next, resting platelets were incubated with small unilamellar vesicles (SUV) containing 5% of total lipids on a molecular basis (mol%) of PE (reflecting the percentage of PE in human lipoproteins) and, additionally, \([^{14}\text{C}]\text{PE} + [^{3}\text{H}]\text{PC}\). In the absence of PCI, the \([^{14}\text{C}]\text{PE}/[^{3}\text{H}]\text{PC}\) ratio remained unchanged compared with the ratio of the vesicles alone. In contrast, PCI augmented the \([^{14}\text{C}]\text{PE}/[^{3}\text{H}]\text{PC}\) ratio, indicating that the serpin enhances \([^{14}\text{C}]\text{PE}\) transfer into platelets (Fig. 1 D). Hence, PCI promotes phospholipid import at physiological concentrations of extracellular PE. Insertion of PE into the platelet cell membrane could influence the activity of the prothrombinase complex, which is known to be stimulated by cell surface PE (Billy et al., 1995). Platelets were challenged with thrombin, a relatively weak stimulator of the prothrombinase activity, which nevertheless induces a robust secretion of PCI. Thrombin formation by the prothrombinase complex was profoundly enhanced when the PCI-mediated PE transfer into the platelets was operating (Fig. 1 E). In contrast, PCI failed to increase the thrombin generation when the lipid donors were devoid of PE. Control experiments verified that no prothrombinase activity was elicited by the PE vesicles in the absence of the platelets (unpublished data). The anti-PCI antibody abrogated the thrombin formation, which indicates that the secreted PCI is a responsible factor (Fig. 1 E). Additional PCI further promoted the thrombin formation in the presence of the PE-containing donors but not with pure PC vesicles (Fig. 1 E). Our findings characterize PCI as a selective lipid transporter for PE that supports the cellular import of this phospholipid.

**Rapid plasma membrane translocation and nuclear targeting of PCI**

When visualizing the interaction of biotin-PCI with live HL-60 cells, we found that several cells rapidly incorporated the protein. After 10 min, PCI accumulated in the submembraneous region (Fig. 2 A). Heparin cofactor II (HC-II), a serpin with sequence similarity to PCI, was not incorporated (see Fig. 5 C; not depicted). After longer incubation times, PCI was increasingly targeted to the nucleus. We indeed observed accumulation of PCI in the perinuclear space and intranuclear compartments by immunoelectron microscopy (Fig. 2 B). Time-dependent accumulation of PCI was also seen in isolated cytosolic and nuclear fractions (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200707165/DC1). The nuclear targeting of PCI may be facilitated by its H helix, because GFP-linked variants of PCI lacking the basic amino acids of this helix failed to enter the nucleus (unpublished data).

To explore whether the fast cell entry and nuclear targeting of the serpin was also operating in the intact organism, we injected biotin-labeled PCI into the tail vein of mice. The protein was allowed to circulate for 30 min in the vascular system. Thereafter, the total pool of leukocytes was prepared from the murine blood and cells were visualized by confocal microscopy. Thereby substantial containing of PCI with the nuclear marker was noted in the granulocyte fractions (Fig. 2 C), which indicates that the protein had been integrated into the nuclei. We thus conclude that the cell entrance of PCI enables its rapid targeting to intranuclear compartments both in vivo and in vitro.
To quantify the cellular internalization of PCI, neutrophils were first exposed to $^{125}$I-labeled PCI followed by the addition of a ligand dissociation buffer to exhaustively remove any proteins bound to the extracellular surface (Liu et al., 2000). Within the first 2 min of incubation at 37°C, a large proportion of PCI accumulated within the cells (Fig. 3 A). The incorporation then proceeded at a slower rate. The quantity of PCI internalized at 6°C amounted to 2/3 of the uptake at 37°C (Fig. 3 A). In contrast to the PCI internalization, the PE transferase activity of PCI was clearly diminished at low temperatures. Indeed, PCI failed to increase the $^{14}$CPE/$^{3}$HPC ratio at 6°C (Fig. 3 B). To further address the participation of endocytic routes, CHO cells were transfected with the K44A variant of dynamin2 (Altschuler et al., 1998), which prevents the budding of vesicles necessary for internalization of cargo via several major endocytic routes. Intrusion of Alexa 488–PCI into K44A-transfected cells was only slightly lowered compared with the uptake observed in the wild-type cells (Fig. 3 C). Also, transfection with the AP180 variant AP180-C selectively repressing the clathrin-mediated pathway barely reduced PCI incorporation (unpublished data). A major proportion of PCI incorporation thus persists under conditions suppressing the classic endocytic pathways. Inclusion of heparin into the cell suspensions to prevent interaction of PCI with the cell surface (Priglinger et al., 1994) substantially lowered the cell entry of PCI (Fig. 3 D). In contrast, the internalization was largely unaffected by receptor-associated protein, which blocks the function of the low-density lipoprotein receptor–related protein (LRP/α2 macroglobulin receptor), a mediator of the uptake of Tat (Liu et al., 2000). Heparin also abrogated the PCI-mediated cellular import of $^{14}$CPE, as is evident from its ability to prevent the PCI-elicited increase of the $^{14}$CPE/$^{3}$HPC ratio (Fig. 3 E).

**PE essentially supports the internalization of PCI**

To evaluate the contribution of PE for the cell entrance of PCI, the cells were preincubated with duramycin, a cyclic peptide that specifically interacts with this phospholipid (Navarro et al., 1985; Iwamoto et al., 2007). Duramycin dose dependently suppressed incorporation of PCI at 37°C (Fig. 4 A). The peptide also diminished cell entry of PCI at 6°C (Fig. S3 A, available at http://www.jcb.org/cgi/content/full/jcb.200707165/DC1), suggesting that the pathway supporting PCI internalization was still functioning at this temperature. Then, before the addition of duramycin, cell surface proteins were excessively cleaved by trypsin/proteinase K. Even so, the capacity of the PE-binding peptide to abrogate the PCI incorporation was maintained (Fig. S3 B). Although PE is a regular component of the external leaflet of the cell membrane (Zwaal and Schroit, 1997), its cell surface content increases under conditions of PS exposure. PCI can interact...
with both aminophospholipids (Malleier et al., 2007). To specify the interaction of PCI with PE in the presence of PS, we used fluorescence correlation spectroscopy to calculate the diffusion coefficients of PCI bound to lipid vesicles and for free PCI (Fig. 4 B). The ratio of bound/free PCI amounted to 2.9 ± 0.2 in the case of the PE/PC/PS vesicles, whereas it was 1.5 ± 0.1 for the PC/PS vesicles (mean ± SD), indicating that the preferential interaction of PCI with PE persists in the presence of PS. To further evaluate the participation of PE for the internalization of PCI, we added lipid vesicles enriched with high concentrations of PE. This decreased the PCI internalization to an appreciable extent (Fig. 4 C). Cell entry of the serpin remained unchanged, with vesicles containing exclusively PC. In the presence of extracellular PE, heparin was unable to further diminish the protein incorporation (Fig. 4 C). The latter finding establishes that heparin and extracellular PE abrogate the same route of PCI internalization. PCI thus elementarily necessitates PE for its passage through the cell membrane.

PCI is expressed in several body cavities (Geiger, 2007) and hence macrophages poised to clear invading microorganisms from these compartments are continuously exposed to the serpin. Resting macrophages exhibit increased aminophospholipid exposure, which is enhanced during phagocytosis (Marguet et al., 1999). We found that the rapid internalization of PCI by THP-1 macrophages (unpublished data) was associated with an increased phagocytosis of Escherichia coli (Fig. 4 D). The effect was preserved when the phagocytes were first allowed to incorporate PCI and then exposed to the bacteria. In contrast, preincubation of the bacteria with PCI before the addition of THP-1 cells reduced phagocytosis. PCI stimulated phagocytosis of E. coli in cells in which the incorporation of the serpin was restricted to extranuclear regions of the cells, suggesting that the nuclear localization of PCI is not required for its ability to increase phagocytosis. Quantification by flow cytometry confirmed that PCI fosters the cell entry of bacteria (Fig. 4 E). Stimulation of phagocytosis was almost identical when PCI was removed with bacteria and macrophages, respectively. (F) Stimulation of phagocytosis requires PCI–PE interaction. THP-1 macrophages were preincubated with or without uPCI in the absence or presence of 0.5 μM duramycin. The cells were washed once and FITC-labeled E. coli was added. The intracellular localization of the bacteria was visualized by confocal microscopy. The images are representative of three independent experiments.
after preincubation, whereas preincubation of PCI with bacteria suppressed their uptake (Fig. 4 E). This shows that stimulation of phagocytosis by PCI requires a direct interaction of the serpin with the cell membrane. Pretreatment of the macrophages with PCI in the presence of duramycin, which also inhibited PCI internalization into THP-1 cells (not depicted), decreased cell entry of *E. coli* (Fig. 4 F). In contrast, basal uptake of bacteria (absence of PCI) was unaffected by duramycin (Fig. 4 F). Hence, the specific membrane partitioning of PCI facilitates bacterial phagocytosis.

**Membrane insertion of PCI is facilitated by cone-type lipids**

Because several of the translocating peptides were previously shown to generate transmembrane pores (magainin-2 and mastoparan X; Matsuzaki et al., 1995; Schwarz and Arbuzova, 1995), we investigated whether PCI elicited pore formation. As a positive control, the peptide mastoparan X rapidly evoked nucleation of pores in large unilamellar vesicles (LUV; Fig. 5 A), as was evident from the leakage of carboxyfluorescein (CF). In contrast, PCI did not elicit the formation of leakage sites for CF (Fig. 5 A), indicating its protrusion into the vesicle interior. Conversely, the integrity of the protein is fully preserved in pure PC-LUV. Control cleavage (absence of vesicles) reveals complete PCI degradation (all cleavage products <15 kD). All protease experiments were repeated at least once.
PC, the peak pattern was unchanged by prolonged incubation with the cation. However, the addition of PCI markedly reduced the height and broadened the peak representing the inner leaflet phospholipids when long chain PE was present (Fig. 5 B). This demonstrates a direct interaction of Pr$^{3+}$ with the inner monolayer phospholipids. As a negative control, no changes in the inner monolayer peak were seen without PCI. PCI thus generates transient and subtle changes in the bilayer structure that result in the formation of an aqueous pathway within the membrane.

To evaluate whether PCI itself is inserted into the membrane, we added trypsin into the vesicle core. Whereas the serpin remained largely uncleaved by the intravesicular protease in pure PC vesicles, it was clearly degraded when the membrane contained long chain (unsaturated) PE (Fig. 5 C). In contrast to PCI, the integrity of HC-II was fully conserved in the presence of the intravesicular trypsin, irrespective of the lipid composition of the vesicles (Fig. 5 C). In a complementary procedure, PCI was first incubated with the lipid vesicles and subsequently treated with extravesicular trypsin. In the presence of PE, part of the PCI remained refractory toward cleavage by the extravesicular trypsin (Fig. 5 D). In contrast, PCI was completely degraded in suspensions of the pure PC and PC/PS vesicles. The ability of PCI to penetrate into the bilayer thus crucially requires PE.

Partitioning of PCI into the lipid bilayer could be facilitated by a specific binding to PE (e.g., via interactions with the ethanolamine head group) and/or changes in the physicochemical properties of the membrane. In the latter case, nonbilayer domains, which are particularly favored by the conical shape of PE, could be involved (Israelachvili, 1980; de Kruijff, 1997). When the LUV were prepared from a short chain species of PE with a cylindrical morphology (di-14:0-PE), the protein was entirely degraded by extravesicular trypsin, similar to the findings obtained with the pure PC vesicles (Fig. 5 D). However, in LUV consisting of a lipid with a conical configuration but devoid of the ethanolamine head group DAG, PCI was partially resistant toward cleavage by the protease (Fig. 5 D). This suggests that the conical shape of long chain PE is essential for the membrane penetration of PCI. To further substantiate the extent of membrane insertion of the serpin, we exploited the ability of proteinase K to excessively degrade the serpin (Fig. 5 E). When the protease was integrated into the aqueous core of vesicles containing long chain PE, PCI was cleaved into several high molecular mass degradation products (Fig. 5 E), confirming that PCI protrudes into the vesicle core. In LUV supplemented with DAG, very similar fragmentation products were apparent. Conversely, after inclusion of the protease into vesicles only consisting of PC, the integrity of the protein was fully conserved (Fig. 4 E). This confirms that the serpin failed to reach the vesicle core in the presence of PC alone. Overall, our findings suggest that the conical shape of long chain PE is essential for the membrane penetration of PCI, which adopts a location that completely spans the bilayer.

**PCI translocation requires key components of the hydrophobic pocket allowing selectivity of lipid interaction**

Molecular docking studies based on the crystallographic structure of PCI (Huntington et al., 2003) were undertaken to characterize the structural components of PCI enabling its distinctive interaction with phospholipids. In view of the flexibility of the entire PE molecule, we chose a rigid fragment of the saturated hydrocarbon chain (C11 fragment) for the initial docking analysis because saturated fatty acyl chains are esterified to the C1 atom of the glycerol backbone of (diacyl)-PE under biological conditions (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200707165/DC1). Docking of the C11 fragment onto the PCI structure revealed 276 clusters with energies (kJ/mol) better than $-15$ among a total of 25,242,070 calculations. The two best clusters were nearly identical in position and energy (both better than $-28$ kJ/mol) and were significantly separated from the other clusters, with the next best hit at $-24.3$ kJ/mol. The best-docked C11 fragment fit into a capped hydrophobic pocket along helix D (D’ channel; Fig. 6 A). The orientation of the saturated chain was thus fixed. Consequently, the possible positions of the unsaturated chain, canonically esterified to the C2 atom of PE, were spatially limited to the same region. Of the top 10 clusters, two provided a portion of the possible binding site for the unsaturated hydrocarbon chain (9th and 10th best energies). It was found to be best accommodated by a second hydrophobic channel running along helix H (H’ channel), in close proximity to the PCI-binding site for heparin (Figs. 6 A and S4, helix H). Moreover, in view of the locations revealed for the acyl chains, the position of the head group of PE was also suggested. The entire PE molecule was built on the two hydrocarbon chains placed by the docking studies and subjected to energy minimization. In the final model (Fig. 6 A), the ethanolamine group is found to be located in close proximity to Leu78, whereby the formation of an H bond between the N atom of the ethanolamine group and the main chain oxygen of Leu78 could be enabled. Space limitations within this region principally impair the interaction of PCI with phospholipids containing larger head groups than PE such as PC.

To explore the role of the hydrophobic pocket for the PE-dependent membrane insertion of the serpin, PCI residues potentially implicated in the lipid binding were mutated. L78 was substituted by Trp to prevent the proposed interaction of the protein with the ethanolamine head group. Consequently, the variant remained completely undegraded in the presence of the intravesicular proteinase K, oppositely to the native protein (Fig. 6 B). Substitution of V374, a component of the H’ channel suggested to mediate the binding of the sn-2 fatty acyl chain of PE, by Trp also conferred resistance toward cleavage by the intravesicular protease (Fig. 6 B). Both amino acid exchanges thus suppress the ability of PCI to partition into the vesicle core. In contrast, mutations of residues R35 and T341, both being unrelated to the proposed lipid binding site, did not perturb the PCI penetration into the vesicle center (unpublished data). A qualitatively different digestion pattern was noted when three Ala were inserted at position 376 (Fig. 6 B). This insertion was implemented to restitute the loop between the last two $\beta$ strands specifically truncated in PCI while attenuating its hydrophilic properties. Part of the PCI species was accessible by the intravesicular enzyme, as was evident by the appearance of digestion products (Fig. 6 B), arguing for its protrusion into the vesicle core. To further detail the extent of membrane penetration, the
nuclei were stained with TO-PRO-3 (red).

The mutant was exposed to extravesicular proteinase K. Thereby, the mutant remained almost entirely refractory toward proteolytic attack (Fig. 6 C). In contrast, under the same conditions, the complete loss of the substrate and limited cleavage into a single high molecular mass degradation product was noted for the native protein (Fig. 6 C). The differential digestion pattern for the wild-type PCI in the presence of the intra- versus extravesicular protease (Fig. 6, B and C) excluded a passage of the enzyme across the bilayer. The findings thus suggest that the 376AAA insertion strongly facilitates the membrane translocation of PCI. This conclusion was enforced by experiments with intact cells. After a 10-min incubation period, the labeled 376AAA variant was found to be rapidly internalized by HL-60 cells (Fig. 6 D). In particular, during the short exposure, a noticeable proportion of the mutant was incorporated into the nucleus (Fig. 6 D), in contrast to what had been observed with the native serpin (Fig. 2 A). Overall, the findings establish that the branched hydrophobic cavity in between helices H and D is of basic importance for the membrane insertion and transmigration of PCI.

Discussion

We demonstrate that the internalization of a new cell-penetrating protein, the serpin PCI, is essentially supported by the plasma membrane lipid PE. Lipids are known to contribute to the interaction and insertion of proteins into intracellular and bacterial membranes (van Klompenburg et al., 1998; Kuwana et al., 2002). However, their contribution to the protein insertion into the plasma membrane and consequently the protein internalization into cells is less well documented. Our findings now reveal that cell surface–exposed PE is a major mediator of the protein translocation across the plasma membrane. The essential contribution of PE is corroborated by the ability of the PE-binding peptide duramycin to abrogate protein uptake, the failure of the serpin to cross the cell membrane in the presence of extracellular PE, and the selective PE requirement for membrane penetration of PCI. Interaction of PCI with PE is preceded by docking of the serpin to negatively charged surface molecules such as glycosaminoglycans. This suggests that two basic steps, initial cell surface docking and subsequent interactions with PE, mediate the membrane insertion and cell entry of the protein. Importantly, we show that the distinct conical shape of PE (Israelachvili et al., 1980; de Kruijff, 1997) represents a major driving force for the membrane translocation of PCI.

PE is the eminent plasma membrane lipid with a cone-type morphology because the concentrations of similarly shaped lipids (DAG and phosphatidic acid) are considerably lower. Mechanistically, DAG also efficiently supported the membrane penetration of PCI, whereas the protein intrusion was prevented by cylindrical PE species. This suggests that the conical shape of PE is of greater relevance for the interaction with PCI than specific interactions with the ethanolamine head group. Despite the bilayer-spanning location of PCI, the protein was not entirely transferred to the vesicle core, suggesting that its incorporation into the cell interior requires additional membrane components. Moreover, although transmembrane pores were not implicated in the PCI translocation, which is consistent with the inability of the conical lipids to support the pore formation (Matsuzaki et al., 1998), the serpin was found to elicit subtle perturbations of the membrane organization, as indicated by the leakage of ions from the lipid vesicles. This indicates that the PCI–PE interaction induces the formation of aqueous compartments within the membrane.
Lipids of cone-type morphology are principally capable of generating areas of negative curvature in membranes (Farsad and De Camilli, 2003). This can facilitate the formation of intramembrane nonbilayer domains (inverted micelles), which might assist in the formation of aqueous compartments within the membrane. At present, it is unclear how the PCI–PE interaction mediated by the docking of the lipid to a distinct hydrophobic groove in PCI in the molecular system (see last paragraph of Discussion) relates to the alterations in physicochemical properties by the cone-shaped lipids that allow PCI transfer across the membrane. Hence, more in-depth analyses will be required in future studies to clarify whether these two observations are indeed interrelated. Overall, our results indicate that the membrane passage of PCI is crucially driven by lipids of a distinct morphology that generate an aqueous translocation route for the protein via the generation of nonbilayer domains.

The lipid-dependent internalization of cell-penetrating PCI principally agrees with a model theoretically proposed for the internalization of the translocating peptides (Joliot and Prochiantz, 2004). However, a contribution of PE and its peculiar shape for the cellular uptake of proteins has never been revealed. Cone-shaped lipids facilitate membrane fusion and budding processes (McMahon and Gallop, 2005), which are required for the formation of phagosomes. Consequently, the enhancement of bacterial phagocytosis induced by the PCI–PE interaction as observed here could be enabled by the stimulation of phagosome maturation. The ability of PCI to foster removal of pathogens suggests its participation in host defense and thus overlaps with the functions of other cell-penetrating proteins implicated in innate immunity. The internalized PCI was found to be rapidly directed to the nucleus, both in vitro and in the intact organism. Our findings position PCI as a promising candidate for the rapid delivery of cargo to the nucleus, including viruses, nucleotides of differing length and structures, and proteins/peptides. Being a component of the human proteome, potential applications of cargo-loaded PCI in humans are not immunogenic. PCI might be exploited in particular for the targeting of cargo into cells with increased surface exposure of PE (Vance, 2003), including capacitated sperm (Gadella and Harrison, 2000) and others.

PCI is shown to selectively extract PE from lipid particles, consecutively inserting the lipid into the plasma membrane of various cells. PCI thus belongs to a new class of proteins promoting the cellular uptake of selective lipids, including those specific for phosphatidylinositol (Wang and Munford, 1999) and sphingomyelin (Stoeckelhuber et al., 2000). The latter proteins do not share structural homologies with PCI, a situation known from the intracellular lipid transfer proteins (Wirtz, 1997). During the transfer, the phospholipid would have to be largely protected from the contact with water, as revealed for scavenger receptor class B type I, the prototype mediator of the cellular phospholipid import (Urban et al., 2000). Based on the velocity, extent, and temperature dependence of the PE import, we assume that a principally similar process allows PCI to ferry PE into the cells. Only a limited change in the conformation of the protein–lipid complex might be required to switch between the extra- and intramembrane modes of the PCI–PE interaction. Indeed, the protein is most likely integrated into aqueous compartments during the membrane translocation. Nonetheless, at low temperatures, the lipid transferase activity of PCI is suppressed, whereas the PE-dependent PCI internalization is largely maintained. This indicates that the PE transfer is energy independent, whereas the membrane translocation of PCI exhibits a limited energy requirement, a property common to several cell-penetrating proteins. Whereas PCI facilitates PE enrichment on the cell surface by virtue of its lipid transferase activity, PE accumulation is further assisted by the delayed transbilayer movement of the lipid (Morrot et al., 1989). We observe that PE insertion accelerates the activity of the prothrombinase complex, thereby amplifying the formation of thrombin, a major coordinator of blood coagulation. Once internalized, PCI could potentially also support the intracellular movement of PE, such as between the cell membrane and membranes of intracellular organelles. Overall, our findings characterize PCI as the first transferase specifically fostering the cell import of PE.

Using the crystal structure of PCI as a template, we revealed that the selective interaction of PCI with PE is facilitated by a dual-channelled hydrophobic groove that is generated by the shortened A helix and a truncated loop between strands 3 and 4B. The H′ and D′ channels arising therefrom accommodate the extensions of the fatty acyl chains of the phospholipid with sharp precision. Indeed, because the H′ channel is not capped, it can house hydrocarbon chains of up to 22 C atoms, the longest unsaturated fatty acids esterified to PE in mammals. Moreover, the restricted size of the structure in which the phospholipid headgroup is buried markedly favors interactions with conical phospholipids. Hydrogen bonds between the ethanalamine group and specific amino acids, suggested to permit the folding of bacterial lactose permease (Bogdanov et al., 1999), might additionally ease the interaction with the phospholipid, as exemplified by the functional contribution of Leu78. Together, this structural constellation is supposed to enable the higher affinity of PCI for PE over PC. The docking and energy minimization studies were corroborated by the mutagenesis of single amino acids designed to specifically remodel the hydrophobic cavity. In particular, the insertion of a triple Ala sequence on top of the H′ channel substantially promoted the intrusion and membrane translocation of the serpin. The insertion, which reconstitutes the truncated loop intercalated between strands 3 and 4B, is likely to strengthen the interaction with the fatty acyl chains of the phospholipids by virtue of its specific location and enhanced hydrophobicity. In conclusion, the hydrophobic pocket appears to represent a unique structure for accommodating lipids capable of supporting the membrane passage of proteins.

Materials and methods

Cells

Human platelets were isolated from citrated blood as described previously (Engelmann et al., 1998). For isolation of neutrophils, the buffy coats from human blood anticoagulated with citrate were incubated with microbeads coupled to anti-CD15 antibodies for 15 min at 8°C. The suspensions were applied onto the positive selection column (Miltenyi Biotech) and the neutrophils were eluted with buffer. The purity of the neutrophil suspensions was >95% and the viability of the cells was 98% (trypan blue exclusion). CHO cells were transfected with pcDNA3 wild-type dynamin2 and its dominant-negative K44A mutant (provided by S. Egan, The Hospital for
Sick Children, Toronto, Canada). Cells were cultured in Iscove’s modified Dulbecco’s medium containing 20% FBS (HL-60) or DME supplemented with 10% FCS (CHO).

Preparation of native PCI and PCI variants

For preparation of recombinant PCI, the cDNA of human PCI was amplified from a human liver cDNA library using a forward (5′-GGCGTCAACCAATGC-3′) and reverse (5′-CCGGGATCCATCAGGGGCGGTTACCTGGAAC-3′) primer. Mutagenesis was performed using the QuickChange site-directed mutagenesis kit (Stratagene). The following variants were generated: V374W, T341R, L78W, R35A, and 376AAA.

Phospholipid exchange

For the determination of the intervesicular phospholipid exchange, py-labeled donor vesicles and unlabeled acceptor vesicles were coincubated for different time periods. The suspensions were passed over an anion exchange column (BioRad Laboratories), and the fluorescence was determined in Triton X-100 (Sigma-Aldrich) solubilates of the acceptor vesicles as described previously (Stoeckelhuber et al., 2000). The vesicle preparations and incubations were performed in the presence of 10 mM butylated hydroxytoluene and under argon or nitrogen. The transfer of py-labeled phospholipids into the cells was analyzed as described previously (Urban et al., 2000). Under principally similar conditions, the cells were incubated with lipid donor vesicles containing traces of [14C]PE (1-palmitoyl, 2-linoleoyl species) and [3H]cholesterol (both from GE Healthcare).

Confocal microscopy

Washed HL-60 cells and murine leukocytes (see Incorporation of PCI… above) were incubated with Alexa fluor 488-labeled PCI at 6 or 37°C. The cells were fixed with 4% paraformaldehyde for 60 min at room temperature, permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min at 4°C, and washed thereafter. Nonspecific binding sites were saturated overnight at 4°C with antibody dilution buffer (Dako) containing 0.2% goat serum. Subsequently, the cells were incubated for 60 min with Cy2-labeled streptavidin (GE Healthcare) and an additional 30 min with TO-PRO-3 (Invitrogen). The cells were transferred onto slides and covered with Vectashield (Vector Laboratories). Slides were analyzed at room temperature with a laser scanning microscope (LSM 510 Meta; Carl Zeiss, Inc.) using a Plan-neofluar 40×, 1.3 oil interference contrast 1056–602 (1083–997) objective lens (Carl Zeiss, Inc.) and 488-nm and 633-nm HeNe2 lasers. Images were obtained with a PFM photomultiplier tube (Hamamatsu Photonics). Image acquisition and processing was performed with LSM 510 image examiner software (Carl Zeiss, Inc.).

For analysis of phagocytosis of E. coli into THP-1 macrophages, the cells (10⁵/jwell) were seeded in 24-well plates in 500 μl RPMI-1640 supplemented with 10% heat-inactivated FCS, 1% Hepes, 500 U/ml penicillin, and 500 μg/ml streptomycin (GE Healthcare). The cells were incubated for 60 min with Cy2-labeled PCI and 633-nm HeNe2 lasers. Images were obtained with a PFM photomultiplier tube (Hamamatsu Photonics). Image acquisition and processing was performed with LSM 510 image examiner software (Carl Zeiss, Inc.).

Prothrombinase activity

The activity of the prothrombinase complex was determined as described previously (Zieseniss et al., 2001).

Western blots

HL-60 cells were incubated with 100 nM PCI at 37°C, and the cell pellet was resuspended in nuclear extraction buffer 1 (10 mM Tris-HCl, 10 mM KCl, 0.5% IGEPAL CA-630, and protease inhibitor cocktail Complete; Roche), incubated for 30 min on ice, and lysed by sonication. The supernatants obtained from the homogenates were centrifuged, and the resulting nuclear or cytosolic proteins were recovered. After 100 nM PCI or one ml of detergent-containing pellets by detergent extraction buffer 2 (10 mM Tris-HCl, 0.5% NaCl, and Complete), incubated for 30 min on ice, and centrifuged for 30 min. The pellets were resuspended in polyvinylidene fluoride membranes (Millipore), blocked overnight with 5% dry milk, and incubated for 60 min with anti-PCI antibody. After incubation for 45 min with horseradish peroxidase-conjugated anti-rabbit IgG, the proteins were visualized.

Translocation of PCI across pure lipid membranes

LUV were prepared from 10 mg of total lipids per milliliter in a buffer composed of 20 mM Heps and 150 mM NaCl, pH 7.5, and supplemented with 0.5 mg/ml trypsin or 0.1 mg/ml proteinase K in a final volume of 500 μl. The LUV were then incubated with 100 nM PCI for 1 h, washed once with cold PBS, and added to 1 μl of Triton X-100. The samples were visualized with a confocal laser scanning microscope. The images were obtained with a PFM photomultiplier tube (Hamamatsu Photonics). Image acquisition and processing was performed with LSM 510 Meta; Carl Zeiss, Inc.) using a Plan-neofluar 63×, 1.4 oil objective lens (Carl Zeiss, Inc.) and 488-nm Ar2 and 633-nm HeNe2 lasers. Images were obtained with a PFM photomultiplier tube (Hamamatsu Photonics). Image acquisition and processing was performed with LSM 510 image examiner software (Carl Zeiss, Inc.).

Immuno EM

HL-60 cells previously incubated with or without 90 nM of biotin-labeled PCI were fixed with 4% paraformaldehyde/5% glutaraldehyde for 60 min at room temperature. Before embedding in LR white medium resin (Lew), the HL-60 cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer and then rinsed in 0.1 M phosphate buffer. Ultra-thin serial sections of 150 nm were cut and mounted on gold grids. Non-specific binding sites on the sections were blocked with 5% BSA in PBS, and grids were incubated for 120 min at 37°C with 10 nm of gold-labeled goat anti-biotin IgG (Biocell Laboratories, Inc.). The sections were counterstained with 3% uranyl acetate and lead citrate and examined with a transmission electron microscope (JEOL TEM 1200 EXII; JEOL Ltd.).

Incorporation of PCI by murine leukocytes in vivo

4 μg of biotin-labeled recombinant PCI (rPCI) was injected into the tail vein of anesthetized mice. After 30 min, blood was drawn from the peritoneal sinus into 0.38% sodium citrate, immediately diluted by 1:10 with erythrocyte lysis buffer (1.55 mM NaN3, 10 mM KHCO3, and 0.1 mM EDTA, pH 7.4), and centrifuged at 400 g for 20 min. The pellets were washed three times with decreasing volumes (1.5, 0.5, and 0.1 ml) of erythrocyte lysis buffer. The leukocytes obtained were processed for confocal microscopy as described previously.

Phagocytosis assay

THP-1 cells were differentiated with PMA as described in Confocal microscopy. Subsequently, the cells were washed in serum-free medium, mixed with 2 × 10⁶ FITC-labeled E. coli (Wybrant Phagocytosis Assay kit; Invitrogen) in a final volume of 500 μl, and incubated for 2 h at 37°C in the presence or absence of 100 nM PCI, 50 μM cyclohexatin D, or 10% (vol/vol) human serum. In the preincubation experiments, either macrophages or bacteria were separately preincubated with 100 nM PCI in serum-free medium for 60 min at 37°C and washed once before being added to the phagocytosis assay. After 2 h, noninternalized bacteria were rinsed away (after rinsing fluorescence images were taken) and loosely attached E. coli were removed by trypsinization. The phagocytosis were collected by centrifugation, resuspended in ice-cold PBS, and analyzed by flow cytometry (FACScalibur; BD Biosciences).

Quantification of cellular internalization of PCI

uPCI was labeled with [125I] (GE Healthcare) by adding 100 μCi of the isotope to 50 μl of 0.25 M sodium phosphate buffer, pH 7.0, followed by the immediate mixing with 10 μl of 5 mg/ml chloramine T solution. 5 min after addition of 2.3 μg PCI, the reaction was stopped by inclusion of 50 μl of a saturated sodium ascorbate buffer, pH 7.0, and the labeled protein was purified from the unreacted 125I via passage over a Sephadex-G10 column (GE Healthcare). After incubation with the labeled PCI, the cells were washed once with PBS and incubated for 30 min at 4°C with ligand-dissociation buffer (0.5% trypsin, 0.5 M EDTA, and 50 μg/ml protease K in PBS; Liu et al., 2000). The suspensions were centrifuged, the extracellular media were discarded, and the radioactivity of the pellets was determined. Alexa fluorescence intensity was measured in detergent-treated pellets (2% Triton X-100).
were performed in the presence of 2 mM of the protease inhibitor PMSF. Where indicated, the preparation of the LUV was performed before the proteases, and trypsin or proteinase K were added after the incubations with PCI (extravesicular proteases).

**Efflux of CF from LUV**

The lipids were dispersed in a buffer composed of 10 mM Hepes, 100 mM KCl, and 1 mM EDTA, pH 7.4, containing 60 mM CF (mixed isomers; Sigma-Aldrich). The vesicles were sized using 200-nm polycarbonate filters and separated from the extravesicular CF by dialysis. The fluorescence signal F was recorded after addition of mastoparan X and PCI at 37°C. In the case of pore formation, dequenching of the dye upon dilution into the medium will increase the values above the initial value Fo. To determine the fluorescence signal for the maximally releasable amount of CF (F0), the vesicles were solubilized using the detergent C12E8.

**Fluorescence correlation spectroscopy**

Giant unilamellar vesicles (GUV) were prepared from 1-palmitoyl, 2-linoleoyl species of PC and PE together with 5 mol% dipalmitoyl-PS. For determination of the translational diffusion coefficient Dt, the 1-palmitoyl, 2-linoleoyl species of PE was built in XtalView (Huntington et al., 1998). The vesicles were sized using 200-nm polycarbonate filters and separated from the extravesicular CF by dialysis. The fluorescence signal F was recorded after addition of mastoparan X and PCI at 37°C. In the case of pore formation, dequenching of the dye upon dilution into the medium will increase the values above the initial value Fo. To determine the fluorescence signal for the maximally releasable amount of CF (F0), the vesicles were solubilized using the detergent C12E8.

**Docking studies**

Based on the crystal structure of cleaved PCI (Huntington et al., 2003), docking studies for PE binding were performed with the program Docking for the membrane interaction of PCI, the fluorescence correlation spectroscopy measurements were performed in the autocorrelation mode with the Confocor 2 system (Carl Zeiss, Inc.) using either the 488-nm line of an Ar-ion Laser or a 633-nm HeNe Laser (at 50–60 μW). The excitation light was reflected by a dichroic mirror (HFT 488/633; Carl Zeiss, Inc.), and focused onto the sample. The emitted fluorescence light was split by a second dichroic mirror (NFT 635; Carl Zeiss, Inc.), and detected in two separate channels. In each case, out of plane fluorescence was reduced by a pinhole of 90-μm diameter. For analysis of the membrane interaction of PCI, the fluorescence correlation spectroscopy focal spot was positioned near the center of the upper membrane of the GUV. For characterizing the diffusion properties of PCI in aqueous solution, the focus was moved to a position 20 μm above the upper membrane. The data were evaluated by Levenberg-Marquardt nonlinear least squares fitting to the appropriate model of the autocorrelation function.

**Pr3+ transfer into vesicles**

LUV of a diameter of 200 nm were prepared by the extrusion method (see Translocation of PCI…) and Pr3+ was added to a final concentration of 5.7 mM. The chemical shifts of the 31P signals from the phospholipids present in the outer and inner leaflet of the LUV were registered. NMR spectra were recorded with a spectrometer (400S; Varian Medical Systems).

**References**

Acton, S., A. Rigotti, K.T. Landschulz, S. Xu, H.H. Hobbs, and M. Krieger. 1996. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. Science. 271:518–520.

Altschuler, Y., S.M. Babas, L.I. Terlecky, K. Tang, S. Hardy, K.E. Mostov, and S.L. Schmid. 1998. Redundant and distinct functions for dynamin-1 and dynamin-2 isoforms. J. Cell Biol. 143:1871–1881.

Bevers, E.M., P. Comfurius, D.W. Dekkers, and R.F. Zwaal. 1999. Prothrombin contributes to the assembly of the factor Va-factor Xa complex at phosphatidylserine-containing phospholipid membranes. J. Biol. Chem. 274:26883–26889.

Bogdanov, M., U. Mueda, and W. Dowhan. 1999. Phospholipid-assisted re-folding of an integral membrane protein. Minimum structural features for phosphatidylethanolamine to act as a molecular chaperone. J. Biol. Chem. 274:12339–12345.

Brunger, A.T., P.D. Adams, G.M. Clore, W.L. Delano, P. Gros, R.W. Grosse-Kunstleve, J.S. Jiang, K. Karasuyama, K. Inoue, and M. Umeda. 1998. The third helix location across the plasma membrane of mammalian cells. Biochem. Biophys. Acta 1439:317–330.

Bilby, D., G.M. Willems, H.C. Henker, and T. Lindhout. 1995. Prothrombin binds to the assembly of the factor Va-factor Xa complex at phosphatidylserine-containing phospholipid membranes. J. Biol. Chem. 270:26883–26889.

Conner, S.D., and S.L. Schmid. 2003. Regulated portals of entry into the cell. Nature. 422:37–44.

de Kruijff, B. 1997. Lipid polymorphism and biomembrane function. Curr. Opin. Chem. Biol. 1:564–569.

Derossi, D., A.H. Jojoli, G. Chassaing, and A. Prochiantz. 1994. The third helix of the Antennapedia homeodomain translocates through biological membranes. J. Biol. Chem. 269:10444–10450.

D’Hondt, K., A. Reese-Peck, and H. Rieemann. 2000. Protein and lipid requirements for endocytosis. Annu. Rev. Genet. 34:255–295.

Diekert, K., A.I. de Kroon, K. Ahting, B. Niggemeyer, W. Neupert, B. de Kruijff, and R. Lill. 2001. Apocytochrome c requires the TOM complex for translocation across the mitochondrial outer membrane. EMBO J. 20:5626–5635.

Emoto, K., N. Toyama-Sorimachi, H. Karasuyama, K. Inoue, and M. Umeda. 1997. Exposure of phosphatidylethanolamine on the surface of apoptotic cells. Exp. Cell Res. 232:430–434.

Engelmann, B., B. Schiap, P. Dobner, M. Stoeckelhuber, A. Cologn, W. Siess, and A. Hermetter. 1998. Platelet agonists enhance the import of phosphatidylethanolamine into human platelets. J. Biol. Chem. 273:27800–27808.

Farsad, K., and P. de Camilli. 2003. Mechanisms of membrane deformation. Curr. Opin. Cell Biol. 15:372–381.

Gadella, B.M., and R.A.P. Harrison. 2000. The capacitating agent bicarbonate induces protein kinase A-dependent changes in phospholipid behavior in the sperm plasma membrane. Development. 127:2407–2420.

Gardai, S.J., D.L. Branton, C.A. Ogden, and P.M. Henson. 2006. Recognition ligands on apoptotic cells: a perspective. J. Leukoc. Biol. 79:986–903.

Geiger, M. 2007. Protein C inhibitor, a serpin with functions in- and outside vascular biology. Thromb. Haemost. 97:343–347.

Huntington, J.A., M. Kjellberg, and J. Stenflo. 2003. Crystal structure of protein C inhibitor provides insights into hormone binding and heparin activation. Structure. 11:205–215.

Israelachvili, J.N., S. Marcelja, and R.G. Horn. 1980. Physical principles of membrane organization. Q. Rev. Biophys. 13:121–200.

Itoh, T., S. Koshita, T. Kigawa, A. Kikuchi, S. Yokoyama, and T. Takenawa. 2001. Role of the ENTH domain in phosphatidylinositol-4,5-bisphosphate binding and endocytosis. Science. 291:1047–1051.

Iwasato, K., T. Hayakawa, M. Murate, A. Makino, K. Ito, T. Fujisawa, and T. Kobayashi. 2007. Curvature-dependent recognition of ethanolamine phospholipids by duramycin and cinacinnamycin. Biochim. Biophys. Acta 93:1608–1619.

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Jacobson, K., O.G. Mouritsen, and R.G.W. Anderson. 2007. Lipid rafts: at a crossroad between cell biology and physics. Nat. Cell Biol. 9:7–14.

Joliot, A., and A. Prochiantz. 2004. Transduction peptides: from technology to physiology. Nat. Cell Biol. 6:189–196.

Koonen, D.P.Y., J.F.C. Glatz, A. Bonen, and J.J.F.P. Luiken. 2005. Long-chain fatty acid uptake and FAT/CID36 translocation in heart and skeletal muscle. Biochim. Biophys. Acta. 1736:163–180.

Kuwana, T., M.R. Mackey, G. Perkins, M.H. Ellisman, M. Latterich, R. Schneider, D.R. Green, and D.D. Newmeyer. 2002. Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. Cell. 111:331–342.

Liu, Y., M. Jones, C.M. Hingtgen, G. Bu, N. Laribee, R.E. Tanzi, R.D. Moir, A. Nath, and J.J. He. 2000. Uptake of HIV-1 tat protein mediated by low-density lipoprotein receptor-related protein disrupts the neuronal metabolic balance of the receptor ligands. Nat. Med. 6:1380–1387.

Mäe M., and U. Langel. 2006. Cell-penetrating peptides as vectors for peptide, protein and oligonucleotide delivery. Curr. Opin. Pharmacol. 6:509–514.

Malleier, J., O. Oskolkova, V. Bochkov, I. Jerabek, B. Sokolikova, T. Perkmann, J. Breuss, B.R. Binder, and M. Geiger. 2007. Regulation of protein C inhibitor (PCI) activity by specific oxidized and negatively charged phospholipids. Blood. 109:4769–4776.

Marguet, D., M.-F. Luciani, A. Moynault, P. Williamson, and G. Chimini. 1999. Engulfment of apoptotic cells involves the redistribution of membrane phosphatidylserine on phagocyte and prey. Nat. Cell Biol. 1:454–456.

Matsuzaki, K., O. Murase, N. Fuji, and K. Miyajima. 1995. Translocation of a channel-forming antimicrobial peptide magainin 2, across lipid bilayers by forming a pore. Biochemistry. 34:6521–6526.

Matsuzaki, K., K. Sugishita, N. Ishibe, S. Nakata, K. Miyajima, and R.M. Epand. 1998. Relationship of membrane curvature to the formation of pores by magainin 2. Biochemistry. 37:11856–11865.

McMahon, H.T., and J.L. Gallop. 2005. Membrane curvature and mechanisms of dynamic cell membrane remodeling. Nature. 438:590–596.

Morrot, G., P. Herve, A. Zachowski, P. Fellmann, and P.F. Devaux. 1989. Aminophospholipid translocase of human erythrocytes: phospholipid substrate specificity and effect of cholesterol. Biochemistry. 28:3456–3462.

Navarro, J., J. Chabot, K. Sherrill, R. Anea, S.A. Zahrer, and E. Racker. 1985. Interaction of duramycin with artificial and natural membranes. Biochemistry. 24:4645–4650.

Nishioka, J., M. Ning, T. Hayashi, and K. Suzuki. 1998. Protein C inhibitor secreted from activated platelets efficiently inhibits activated protein C on phosphatidylethanolamine of platelet membrane and microvesicles. J. Biol. Chem. 273:11281–11287.

Prendes, M.J., E. Bielek, M. Zechmeister-Machhart, E. Vanyek-Zavadil, V.A. Carroll, J. Breuss, B.R. Binder, and M. Geiger. 1999. Synthesis and ultrastructural localization of protein C inhibitor in human platelets and megakaryocytes. Blood. 94:1300–1312.

Priglinger, U., M. Geiger, E. Bielek, E. Vanyek, and B.R. Binder. 1994. Binding of urinary protein C inhibitor to cultured human epithelial kidney tumor cells (TCL-598). The role of glycosaminoglycans present on the luminal cell surface. J. Biol. Chem. 269:14705–14710.

Schwarz, G., and A. Arbusova. 1995. Pore kinetics reflected in the dequenching of a lipid vesicle entrapped fluorescent dye. Biochim. Biophys. Acta. 1239:51–57.

Sillerud, L.O., and R.E. Barnett. 1982. Lack of transbilayer coupling in phase transition of phosphatidylcholine vesicles. Biochemistry. 21:1756–1760.

Simons, K., and W.L. Vaz. 2004. Model systems: lipid rafts, and cell membranes. Annu. Rev. Biophys. Biomol. Struct. 33:269–295.

Smirnov, M.D., D.A. Ford, C.T. Esmon, and N.L. Esmon. 1999. The effect of membrane composition on the hemostatic balance. Biochemistry. 38:3591–3598.

Stoeckelhuber, M., P. Dobner, P. Baumgartner, J. Ehlert, E. Brandt, R. Mentele, D. Adam, and B. Engelmann. 2000. Stimulation of cellular sphingomyelin import by the chemokine connective tissue-activating peptide III. J. Biol. Chem. 275:37365–37372.

Takeshima, K., A. Chikushi, K.K. Lee, S. Yonehara, and K. Matsuzaki. 2003. Translocation of analogues of the antimicrobial peptides magainin and buforin across human cell membranes. J. Biol. Chem. 278:1310–1315.

Urban, S., S. Zieseniss, M. Werder, H. Hauser, R. Budzinski, and B. Engelmann. 2000. Scavenger receptor BI transfers major lipoprotein-associated phospholipids into the cells. J. Biol. Chem. 275:33409–33415.

Vance, J.E. 2003. Molecular and cell biology of phosphatidylserine and phosphatidylethanolamine metabolism. Prog. Nucleic Acid Res. Mol. Biol. 75:69–111.

van Klompenburg, W., M. Paetzel, J.M. de Jong, R.E. Dalbey, R.A. Demel, G. von Heijne, and B. de Kruijff. 1998. Phosphatidylethanolamine mediates insertion of the catalytic domain of leader peptidase in membranes. FEBS Lett. 431:75–79.

Wang, P.Y., and R.S. Munford. 1999. CD14-dependent internalization and metabolism of extracellular phosphatidylinositol by monocytes. J. Biol. Chem. 274:23235–23241.

Wirtz, K.W. 1997. Phospholipid transfer proteins revisited. Biochem. J. 324:353–360.

Zieseniss, S., S. Zahrer, I. Muller, A. Hermetter, and B. Engelmann. 2001. Modified phosphatidylethanolamine as the active component of oxidized low density lipoprotein promoting platelet prothrombinase activity. J. Biol. Chem. 276:19828–19835.

Zwaal, R.F., and A.J. Schroit. 1997. Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. Blood. 89:1121–1132.