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Lysenin: A new tool for investigating membrane lipid organization

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Abstract

Sphingomyelin is a major sphingolipid species in animal cells and is a major lipid constituent of plasma membranes. Recent reports have established important roles for sphingomyelin and its metabolites as second messengers in signal transduction events during development and differentiation. Sphingomyelin is also a major component of sphingolipid, cholesterol-rich plasma membrane microdomains, known as 'lipid rafts'. However, little is known about the organization of sphingomyelin in biological membranes. Lysenin is a recently discovered sphingomyelin-specific toxin. In The present review, we summarize the current characterization of this protein and describe our recent attempt to elucidate the organization of sphingomyelin in cellular membranes using lysenin as a unique tool.

Key words: lipid rafts, lysenin, membrane domain, pore-forming toxin, sphingomyelin.

Introduction

The biological membrane of eukaryotic cells contains more lipid species than are needed to form a simple bilayer. This has long raised interest in the question of what purpose underlies this diversity. Certain lipids could serve to organize membranes into discrete, specific domains with different properties. Sphingomyelin is a major sphingolipid species in animal cells and is a major lipid constituent of the plasma membranes, being concentrated in the outer leaflet of the membranes (Barenholz & Thompson, 1999; Gatt, 1999; Ramstedt & Slotte, 2002). Sphingomyelin has attracted attention as a reservoir of ceramide in sphingomyelinase-dependent signal transduction (Kolesnick & Hannun, 1999). Sphingomyelin is also a major component of sphingolipid, cholesterol-rich plasma membrane microdomains called 'lipid rafts' (Brown, 1998; Rietveld & Simons, 1998; Ostermeyer *et al.*, 1999). Lipid rafts are believed to play an important role in a variety of cellular functions, such as signaling, adhesion, motility and membrane trafficking (Brown & London, 1998; Simons & Toomre, 2000). However, little is known about the functional or structural organization of sphingomyelin-containing membranes, mainly because of a lack of appropriate probes. Several proteins that interact with sphingomyelin have been reported (Bernheimer & Avigad, 1976; Lange *et al.*, 1997; Valcarcel *et al.*, 2001; Tomita *et al.*, 2004; Zitzer *et al.*, 2000). Among them, lysenin is known to be highly specific to sphingomyelin. We characterized lysenin as a unique protein tool to study the membrane organization of sphingomyelin. The results indicate that lysenin is not only a lipid-specific protein, but also a lipid organization-specific toxin.

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Lysenin and lysenin-related proteins specifically recognize sphingomyelin

The coelomic fluid of the earthworm *Eisenia foetida* exhibits antibacterial, hemolytic, cytotoxic and hemagglutinating activities (Vaillier *et al.*, 1985; Roch *et al.*, 1989, 1991). Lysenin was isolated from the coelomic fluid of *E. foetida* as a 41 kDa protein that induced contraction of strips of rat isolated aorta (Sekizawa *et al.*, 1996, 1997). Subsequently, it was shown that lysenin induced hemolysis and was cytotoxic towards vertebrate spermatozoa as well as cultured mammalian cells (Yamaji *et al.*, 1998; Kobayashi *et al.*, 2000). Lysenin is unique in that it specifically recognizes sphingomyelin (Yamaji *et al.*, 1998). In Fig. 1, the binding specificity of maltose-binding protein (MBP)-tagged lysenin was examined

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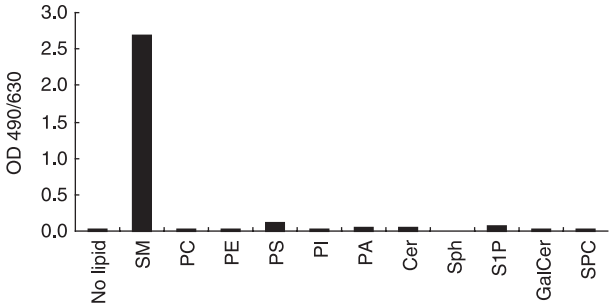

Figure 1. Lysenin specifically recognizes sphingomyelin. Binding of maltose-binding protein (MBP)-tagged lysenin to various lipids was determined by ELISA. Lipids (500 pmol) immobilized on wells of a microtiter plate were probed with MBP-lysenin (1 μg/mL), followed by anti-MBP polyclonal antibodies, biotinylated anti-rabbit IgG and peroxidase-conjugated streptavidin. SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; Cer, ceramide; Sph, sphingosine; S1P, sphingosine 1-phosphate; GalCer, galactosylceramide; SPC, sphingosylphosphorylcholine.

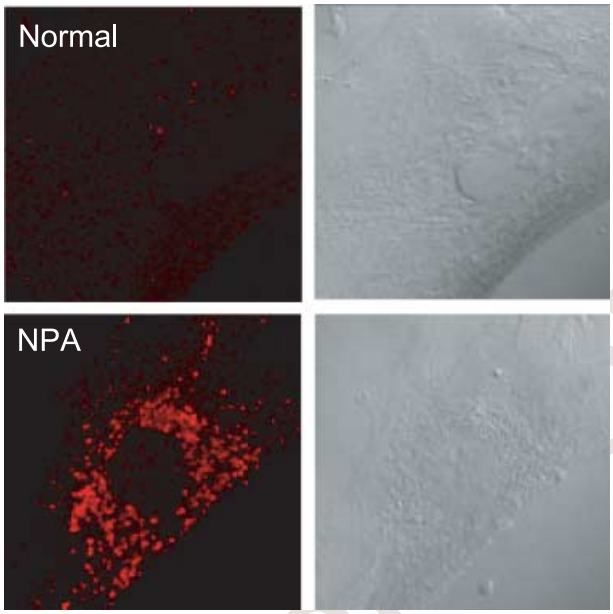

Figure 2. Lysenin recognizes cellular sphingomyelin. Normal and Niemann–Pick type A (NPA) fibroblasts were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min, quenched with 0.1 mol/L NH_4Cl and blocked with 0.2% gelatin in PBS. Cells were then permeabilized by treating them with 50 μg/mL digitonin in PBS for 10 min and were labeled with maltose-binding protein (MBP)-lysenin for 30 min at room temperature, followed by anti-MBP antiserum and Alexa 546-conjugated anti-rabbit IgG. Bar, 20 μm.

by enzyme-linked immunosorbent assay (ELISA; Yamaji *et al.*, 1998; Makino *et al.*, 2003; Yamaji-Hasegawa *et al.*, 2003). It was found that MBP-lysenin bound specifically to sphingomyelin. Other phospholipids, such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and phosphatidic acid, did not bind to MBP-lysenin. In addition, MBP-lysenin did not bind to glycosphingolipids, such as glucosylceramide, galactosylceramide, lactosylceramide or the gangliosides GM1, GM2 and GM3. Intermediates of sphingomyelin synthesis and sphingomyelin metabolites, such as sphingosine, sphingosine-1-phosphate, sphingosylphosphorylcholine and ceramide, also did not bind to MBP-lysenin. The structures of sphingomyelin and phosphatidylcholine share a phosphocholine moiety, whereas both sphingomyelin and glycosphingolipids contain a ceramide backbone. The ELISA results indicated that both the phosphocholine and the ceramide moieties are required for the sphingomyelin to be recognized by lysenin.

The specific binding of lysenin to sphingomyelin makes this protein a unique tool for examining the distribution of cell surface and intracellular sphingomyelin (Yamaji *et al.*, 1998; Nakai *et al.*, 2000). In Fig. 2, normal and Niemann–Pick type A (NPA) fibroblasts were fixed, permeabilized and labeled with lysenin. Niemann–Pick type A cells are characterized by a deficiency in lysosomal acid sphingomyelinase and, hence, intracellular accumulation of sphingomyelin (Kolodny, 2000). Whereas MBP-lysenin labeled small intracellular vesicles in normal cells, NPA cells displayed bright perinuclear MBP-lysenin labeling. However, when fixed and permeabilized NPA cells were treated with sphingomyelinase, MBP-lysenin did not stain the cells (Kiyokawa *et al.*, 2004). Lysenin has also been used to study the biosynthesis and transport of sphingomyelin. Selection of lysenin-resistant variants from Chinese hamster ovary cells yielded cell lines deficient in sphingolipid synthesis (Hanada *et al.*, 1998) or ceramide transport from the endoplasmic reticulum to the Golgi apparatus (Fukasawa *et al.*, 1999; Hanada *et al.*, 2003).

Together with two additional proteins in the coelomic fluid, referred to as lysenin-related protein (LRP) 1 (lysenin 2) and LRP-2 (lysenin 3), lysenin comprises a family of proteins sharing sequences of high homology that are completely different from other proteins available in the protein databanks (Sekizawa *et al.*, 1997; Cooper *et al.*, 2002; Shakor *et al.*, 2003). The amino acid sequence of LRP-1 is 76% identical and 88% similar to that of lysenin, whereas the sequence of LRP-2 possesses an 89% identity with and 94% similarity to the lysenin sequence (Fig. 3). The cDNA sequence of LRP-2 is identical to that of fetidin (Lassegues *et al.*, 1997; Cooper *et al.*, 2002; Shakor *et al.*, 2003). It was found that LRP-2 specifically bound sphingomyelin and induced hemolysis in a manner similar to lysenin (Kiyokawa *et al.*, 2004). In contrast, the binding and

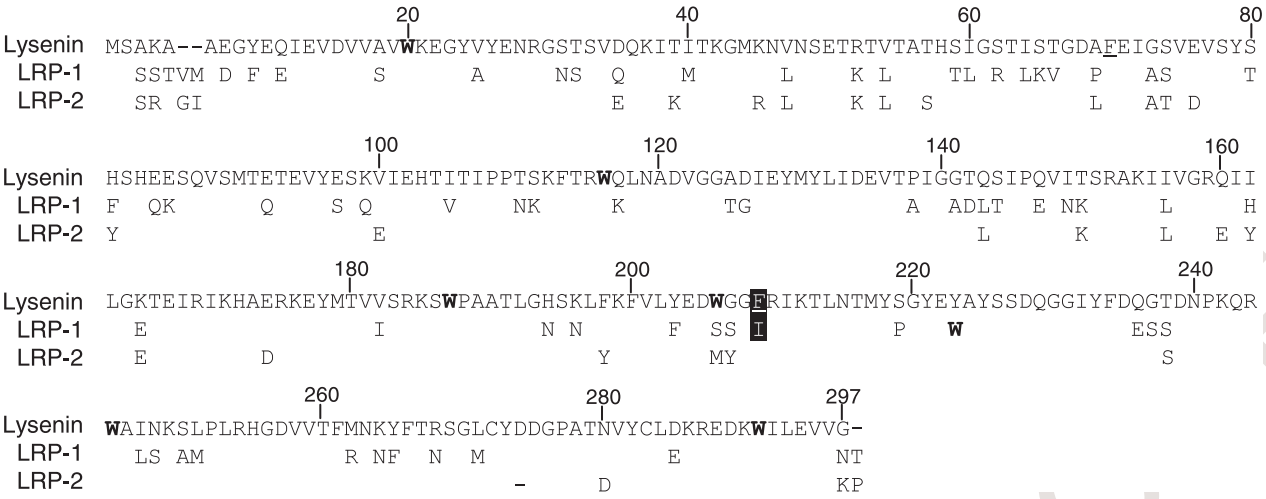

Figure 3. Sequence alignment of the lysenin family. The accession numbers on the GenBank database are D85846 (lysenin), D85847 (lysenin-related protein (LRP) 1) and D85848 (LRP-2). Protein names are given on the left. Sequence numbers for lysenin are shown. The tryptophan residues are indicated in bold. Shading indicates phenylalanine 209 of lysenin, which is substituted for isoleucine 210 in LRP-1.

hemolytic activities of LRP-1 were 10-fold less than those of lysenin and LRP-2. Lysenin and LRP-2 share 30 common sites of aromatic amino acids. Among them, only one position, phenylalanine 210, is substituted for isoleucine in LRP-1. The activity of LRP-1 was markedly increased by introducing a single amino acid substitution of isoleucine 210 to phenylalanine (Kiyokawa *et al.*, 2004), suggesting the importance of aromatic amino acids in the biological activities of lysenin and the LRP. The importance of aromatic amino acids was further demonstrated by a systematic tryptophan to alanine mutation in lysenin (Kiyokawa *et al.*, 2004). Lysenin contains six tryptophan residues at positions 20, 116, 187, 206, 245 and 291. Among the six tryptophan residues in lysenin, five are conserved in LRP-1 and LRP-2. We showed that the conserved tryptophans were required both for the recognition of sphingomyelin and for the hemolytic activity of lysenin, whereas the non-conserved tryptophan was not (Kiyokawa *et al.*, 2004). Our results suggest the importance of the aromatic amino acids of the toxin, most likely due to a direct recognition of sphingomyelin or maintenance of the protein structure. Lysenin and the LRP share putative *N*-glycosylation and *N*-myristoylation sites. Because recombinant MBP-conjugates of lysenin and the LRP expressed in *Escherichia coli* were able to bind to sphingomyelin and induce hemolysis (Kiyokawa *et al.*, 2004), these post-translational modifications do not seem to be essential for the biological activities of these proteins.

Lysenin is a pore-forming toxin

The lysenin polypeptide chain is 297 amino acids long with a calculated molecular weight of 33 440, which is in good agreement with the results of size-exclusion chromatography (Sekizawa *et al.*, 1996). The apparent molecular weight of lysenin was determined to be 41 000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). In the presence of sphingomyelin, lysenin assembled into SDS-resistant oligomers with a molecular weight greater than 250 000 according to SDS-PAGE (Fig. 4; Yamaji-Hasegawa *et al.*, 2003). The oligomerization was accompanied by pore formation in target membranes. The diameter of the pore induced by lysenin was approximately 3 nm, as shown by the osmotic protection of hemolysis (Yamaji-Hasegawa *et al.*, 2003). Negative-staining electron microscopy revealed that lysenin produced honeycomb-like hexagonal structures on sphingomyelin-containing membranes (Fig. 5). The diameter of the hexagonal unit was 10–12 nm and pore-like structures, 3–5 nm in diameter, were found inside the hexagonal unit. The tryptophan fluorescence of lysenin increased and the wavelength of maximum emission underwent a blue shift in the presence of sphingomyelin-containing liposomes. This result suggests the migration of the tryptophan residues of lysenin to a less polar environment during oligomerization. Indeed, differential scanning calorimetry revealed that lysenin interacted with the hydrophobic tails of sphingomyelin in the membrane (Yamaji-Hasegawa *et al.*, 2003).

When lysenin was incubated with various sphingomyelins of different fatty acid composition at 37°C, oligomerization was observed irrespective of the fatty acid species. In contrast, lysenin oligomerized at 4°C only when it was incubated with sphingomyelin of unsaturated fatty acids. Lysenin oligomerized at 4°C when cholesterol was added to the sphingomyelin that contained saturated fatty acids (Yamaji-Hasegawa

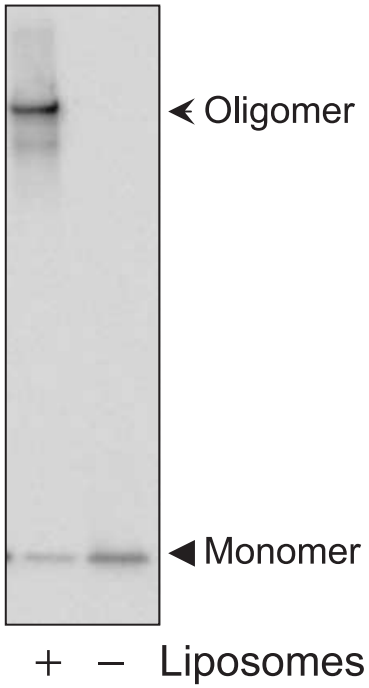

Figure 4. Lysenin forms sodium dodecyl sulfate-resistant oligomers in the presence of sphingomyelin. Lysenin (0.2 μg) was incubated with or without egg sphingomyelin/cholesterol (3 : 2) liposomes (20 nmol lipids) for 30 min at room temperature. The monomer and oligomer of lysenin were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (6–10% gel) followed by western blotting using anti-lysenin antiserum.

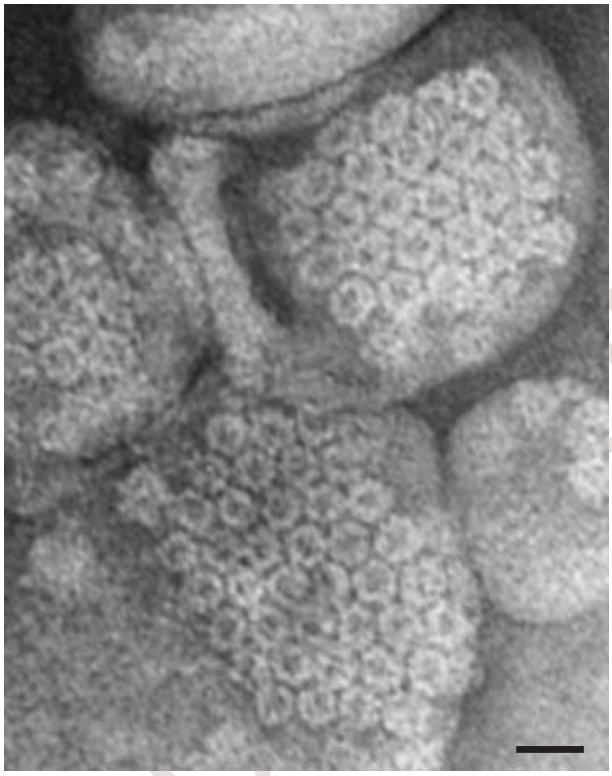

Figure 5. Lysenin produced honeycomb structures in sphingomyelin-containing membranes. Brain sphingomyelin/cholesterol (1 : 1) liposomes (1 mmol/L lipids) were incubated with 400 μg/mL lysenin at 37°C for 30 min. The mixture was fixed with 2.5% glutaraldehyde for 1 h at 37°C, washed with phosphate-buffered saline, stained with 4% aqueous uranyl acetate and observed under a transmission electron microscope. Bar, 20 nm.

et al., 2003). In contrast with oligomerization, the binding of lysenin to sphingomyelin was not significantly affected by the fatty acid composition of sphingomyelins. It is known that cholesterol increases the membrane fluidity of gel-phase lipids. Our results suggest that oligomerization, but not binding, was influenced by the fluidity of sphingomyelin.

Lysenin recognizes the membrane organization of sphingomyelin

Naturally occurring sphingomyelin has a relatively high gel-to-liquid crystalline phase transition temperature (36–40°C). Therefore, at physiological temperatures, sphingomyelin is in a gel or solid phase. Most of the sphingolipids also have high phase transition temperatures and, thus, are in the solid phase under physiological conditions. In contrast, most of the glycerophospholipids are in a liquid crystalline phase. Gel phase lipids, such as sphingomyelin, are well mixed with gel phase, but not with liquid crystalline phase, lipids. Therefore, when sphingomyelin is mixed with liquid crystalline (disorderd) lipids, such as dioleoylphosphatidylcholine (diC18 : 1 PC), sphingomyelin forms domains. In contrast, the presence of solid (ordered) lipids, such as dipalmitoylphosphatidylcholine (diC16 : 0 PC) or glycosphingolipid and galactosylceramide (GalCer), decreases the local density of sphingomyelin because the lipids are miscible with each other (Fig. 6A). Figure 6B shows the lipid distribution of the different binary systems in giant unilamellar vesicles (GUV). Red fluorescence from 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI C18) identifies the sphingomyelin-rich ordered phase, whereas the green fluorescence from 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoyl)–1-hexadecanoyl-*sn*-glycero-3-phosphocholine (BODIPY-C12-PC) localizes in the diC18 : 1 PC-rich fluid phase (Feigenson & Buboltz, 2001; Ishitsuka *et al.*, 2004). In sphingomyelin/diC18 : 1 PC vesicles, two phases were clearly separated, indicating that sphingomyelin forms clusters. In contrast, in GUV of sphingomyelin/diC16 : 0 PC, a uniform fluorescence of DiI C18 was observed. Figure 6C shows the binding of fluorescently labeled lysenin (His-Venus-lysenin) to these GUV (Ishitsuka *et al.*, 2004). Fluorescent lysenin bound sphingomyelin/diC18 : 1 PC liposomes, but not sphingomyelin/

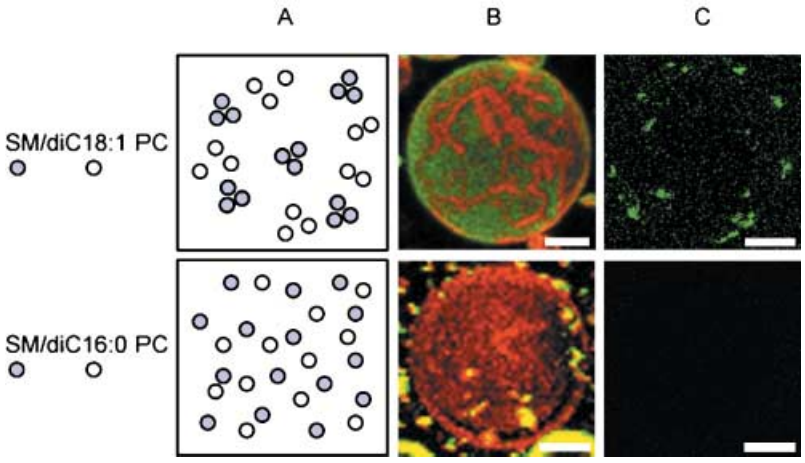

Figure 6. The local density of sphingomyelin (SM) influenced the binding of lysenin to SM in lipid bilayers. (A) Model of SM distribution on the membranes. When SM is mixed with dioleoylphosphatidylcholine (diC18 : 1 PC), the local density of SM is high. The presence of dipalmitoylphosphatidylcholine (diC16 : 0 PC) decreases the local density of SM. (B) Giant unilamellar vesicles composed of palmitoylsphingomyelin (C16 : 0 SM)/diC18 : 1 PC (molar ratio 7 : 3) containing 7 mol % dipalmitoylphosphatidylglycerol (diC16 : 0 PG) and 3 mol % dilauroylphosphatidylglycerol (diC12 : 0 PG), and C16 : 0 sphingomyelin/diC16 : 0 PC (molar ratio 1 : 1) containing 10 mol % diC16 : 0 PG were labeled with 0.1% DiI C18 (red) and 0.1% BODIPY-C12-PC (green). Color-merged images are shown. Bar, 2 μm. (C) Giant unilamellar vesicles composed of C16 : 0 SM/diC18 : 1 PC (3 : 7) or C16 : 0 SM/diC16 : 0 PC (3 : 7) were incubated with histidine and Venus-tagged lysenin (His-Venus-lysenin). The His-Venus-lysenin fluorescence images were obtained with a confocal microscope. Bar, 5 μm.

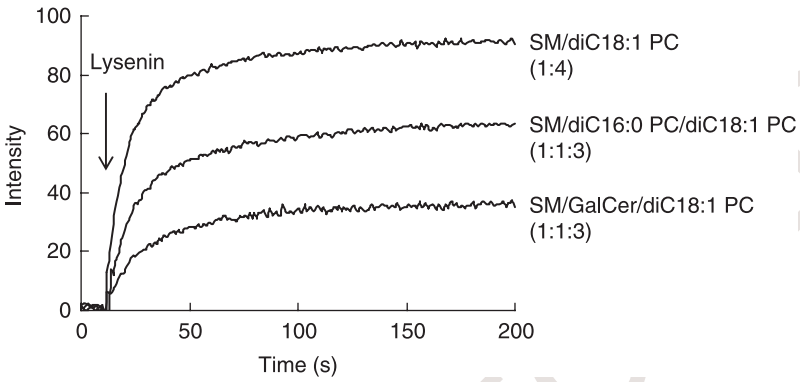

Figure 7. Energy transfer between pyrene-labeled sphingomyelin (SM) and lysenin reveals organization-dependent interaction of SM with lysenin. Fluorescence of pyrene-SM was measured continuously after the addition of 0.06 μmol/L (final concentration) lysenin to 1.25 μmol/L large unilamellar vesicles of egg SM/dioleoylphosphatidylcholine (diC18 : 1 PC; 1 : 4), egg SM/dipalmitoylphosphatidylcholine (diC16 : 0 PC)/diC18 : 1 PC (1 : 1 : 3) and egg SM/galactosylceramide (GalCer)/diC18 : 1 PC (1 : 1 : 3) in the presence of 1 mol % pyrene-SM. The excitation and emission wavelengths were 280 and 420 nm, respectively; temperature, 25°C.

diC16 : 0 PC liposomes. The binding of lysenin to the sphingomyelin-containing liposomes was further analyzed by measuring the fluorescence resonance energy transfer between the tryptophan residues of lysenin and the pyrene-labeled sphingomyelin (Fig. 7; Ishitsuka *et al.*, 2004). Efficient energy transfer was observed when lysenin was incubated with sphingomyelin/diC18 : 1 PC liposomes containing pyrene-labeled sphingomyelin. In contrast, energy transfer was much less efficient in sphingomyelin/GalCer/diC18 : 1 PC or sphingomyelin/diC16 : 0 PC/diC18 : 1 PC liposomes. Our results indicate that the binding of lysenin to sphingomyelin is dependent upon the local density of the lipid (i.e. lysenin recognizes sphingomyelin when the lipid forms clusters). An isothermal titration calorimetry study revealed that one lysenin binds five sphingomyelin molecules, suggesting that the binding of lysenin to sphingomyelin is presumably the result of the formation of a sphingomyelin–lysenin complex of specific stoichiometry (Ishitsuka *et al.*, 2004).

Organization of sphingomyelin differs between different cell types and between different membrane domains within the same cell

Little is known about the organization of sphingomyelin in biological membranes. Our results indicate that lysenin is not only a lipid-specific protein, but also a lipid organization-specific toxin. We used lysenin to study the heterogeneous organization of sphingomyelin at the mammalian cell surface (Ishitsuka *et al.*, 2004). Epithelial cells contain two distinct plasma membranes: the apical domains face the external lumen, whereas the basolateral membranes face the underlying cell layer (Hubbard, 1991; Simons *et al.*, 1992). Each plasma membrane domain has a specialized function and contains a different set of lipids and proteins. Of particular interest are the glycosphingolipids, which are highly enriched in the apical domain (Simons & van Meer, 1988). A cultured epithelial cell line (Madin–Darby canine kidney

(MDCK) cells) was highly sensitive to lysenin when the toxin was added from the basolateral side (Ishitsuka *et al.*, 2004). In contrast, MDCK cells showed resistance to apically added lysenin. Because the sphingomyelin content of the apical membrane is sufficient for lysenin recognition, it was speculated that the glycosphingolipid enrichment of the apical membrane resulted in a decrease in the local density of the sphingomyelin and, thus, endowed it with toxin resistance. The inhibitory role of the glycosphingolipids in the binding of lysenin to sphingomyelin in biomembranes was confirmed by comparing a glycosphingolipid-deficient mutant melanoma cell line with its parent cell. GM95 is a mouse melanoma mutant, defective in ceramide glucosyltransferase I (CerGlcTI), which catalyses the first step in glycosphingolipid synthesis (Ichikawa *et al.*, 1994). The GM95 cell line was sensitive to lysenin-induced killing, whereas the parent cell line was resistant to lysenin (Ishitsuka *et al.*, 2004). The involvement of CerGlcTI in lysenin sensitivity was confirmed by the observation that the stable CerGlcTI transfectant of GM95 was resistant to lysenin. Consistent with lysenin sensitivity, lysenin binds the cell surface of GM95 but not of the parent cell line. Detailed characterization revealed that the observed difference between the mutant and parent melanoma cells is due to altered distribution rather than a change in sphingomyelin content in the mutant. These results indicate that lysenin recognizes the heterogeneous organization of sphingomyelin in biomembranes and that the organization of sphingomyelin differs between both different cell types and different membrane domains within the same cell. Our results also support the idea of the existence of small, condensed lipid complexes consisting of just a few lipid molecules in living cells.

Conclusion and perspectives

Several tools have been reported for the investigation of the lipid organization of lipid rafts. Those include cholera toxin (binds ganglioside GM1), perfringolysin O toxin (binds cholesterol-rich membranes; Waheed *et al.*, 2001) and fluorescent poly(ethylene glycol)-derivatized cholesterol (binds cholesterol-rich membranes; Sato *et al.*, 2004). The specific recognition of sphingomyelin by lysenin makes lysenin an additional tool for the investigation of the organization of lipid rafts. Lysenin is unique in that it recognizes domains of sphingomyelin. Apical membranes and melanoma cell membranes are rare examples that are resistant to lysenin and most cells are susceptible to this toxin. This indicates that sphignomyelin forms domains in most cell membranes.

Acknowledgments

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