Minireview

Lysenin, a unique sphingomyelin-binding protein

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Abstract Sphingomyelin plays complex structural and signaling functions in the plasma membrane. Of special interest is that hydrolysis of sphingomyelin to ceramide can modulate dynamics of membrane rafts, which serve as signaling platforms for various receptors. This review is focused on a recently discovered sphingomyelin-binding protein, lysenin, which can be used as a unique probe to trace distribution and turnover of sphingomyelin in cellular membranes. We analyze the primary and secondary structures of lysenin with respect to its interaction with the plasma membrane. The specificity of lysenin binding to sphingomyelin, revealed by both biochemical and cytochemical approaches, is discussed.

Key words: Lysenin; Sphingomyelin; Lysenin–membrane interaction; Lipid storage disease

1. Introduction

For a long time lipids have been considered only as structural building elements of the plasma membrane. Recently, however, several membrane lipids have been recognized as precursors of bioactive molecules generated in cells after stimulation of a number of cell surface receptors and functioning as secondary messengers.

To follow individual plasma membrane lipids in vivo different peptide and proteinaous probes have been established. These probes interact specifically with target lipids and thus can serve as tools for monitoring the level of selected lipids at activation of signaling pathways in cells. Until now the probes for phosphatidylethanolamine [1], phosphatidylcholine [2], phosphatidylserine [3,4], various phosphatidylinositols [5,6] and cholesterol [7,8] have been developed. Recently, a new unique protein, lysenin, which selectively recognizes sphingomyelin was isolated and cloned [9,10].

Sphingomyelin, a phosphosphingolipid, is an important structural and functional constituent of cellular membranes. Up to 50% of the total sphingomyelin amount in cells is located in the plasma membrane, being concentrated in the outer leaflet of the membrane [11,12]. Together with glycosphingolipids and cholesterol, sphingomyelin is compartmentalized in specialized microdomains of the plasma membrane, known as lipid rafts and caveolae [13,14]. Rafts are separated from glycerophospholipid-rich environment in the plane of the plasma membrane due to tight packing of saturated fatty acyl chains of sphingolipids. The spaces between long acyl chains of sphingolipids are filled by cholesterol. The sphingolipid/cholesterol-enriched rafts acquire the liquid-ordered phase that displays low elasticity but allows diffusion of distinct set of proteins in and out of these membrane structures [15]. Because many of raft constituents, including sphingomyelin, are involved in cell signaling, the domains are likely to serve as signaling platforms, particularly for immunoreceptors. A critical role for sphingomyelin in signal transduction is manifested through the so-called sphingomyelin cycle [16], in which sphingomyelinases are activated by a number of extracellular agents such as 1α,25-dihydroxyvitamin D3, tumor necrosis factor α, γ-interferon, interleukin-1 and arachidonate [17–19]. Membrane sphingomyelin is hydrolyzed by activated sphingomyelinases, which results in production of ceramide serving, in turn, as a second messenger [16,20]. On the other hand, conversion of sphingomyelin into ceramide leads to changes in the physico-chemical properties of the plasma membrane having possible physiological implications. To this end, a tendency of ceramide to self-aggregate is thought to facilitate coalescence of microdomains, a prerequisite phenomenon for signal generation by raft-interacting receptors [21,22]. Accordingly, it was found that activation of acid sphingomyelinase in the plasma membrane and ceramide generation were essential for clustering of CD95 and CD40 receptors and triggering downstream apoptotic signals [23,24]. These ceramide functions may be determined rather by changes in topology of its formation in the plasma membrane (outer or inner leaflet) rather than ceramide action as a second messenger [21]. In a line with this supposition was finding that generation of ceramide in either the outer or the inner leaflet of liposomes induced endocytosis or blebbing of the membrane, respectively [25]. Aside from the plasma membrane pool of sphingomyelin, its mitochondrial pool was recently implicated in a cell death promotion [26]. Therefore, identification of sphingomyelin pools and their membrane localization are very important for understanding the putative specialized sphingomyelin signaling pathways [21,27].

Compartmentalization of sphingomyelin in plasma membrane rafts plays an essential role in pathogenesis of Creutzfeldt–Jakob disease [28]. As prion, human immunodeficiency virus (HIV)-1 and Alzheimer proteins possess a common sphingolipid-binding motif, membrane rafts are likely to be involved in development of corresponding diseases [29]. Re-

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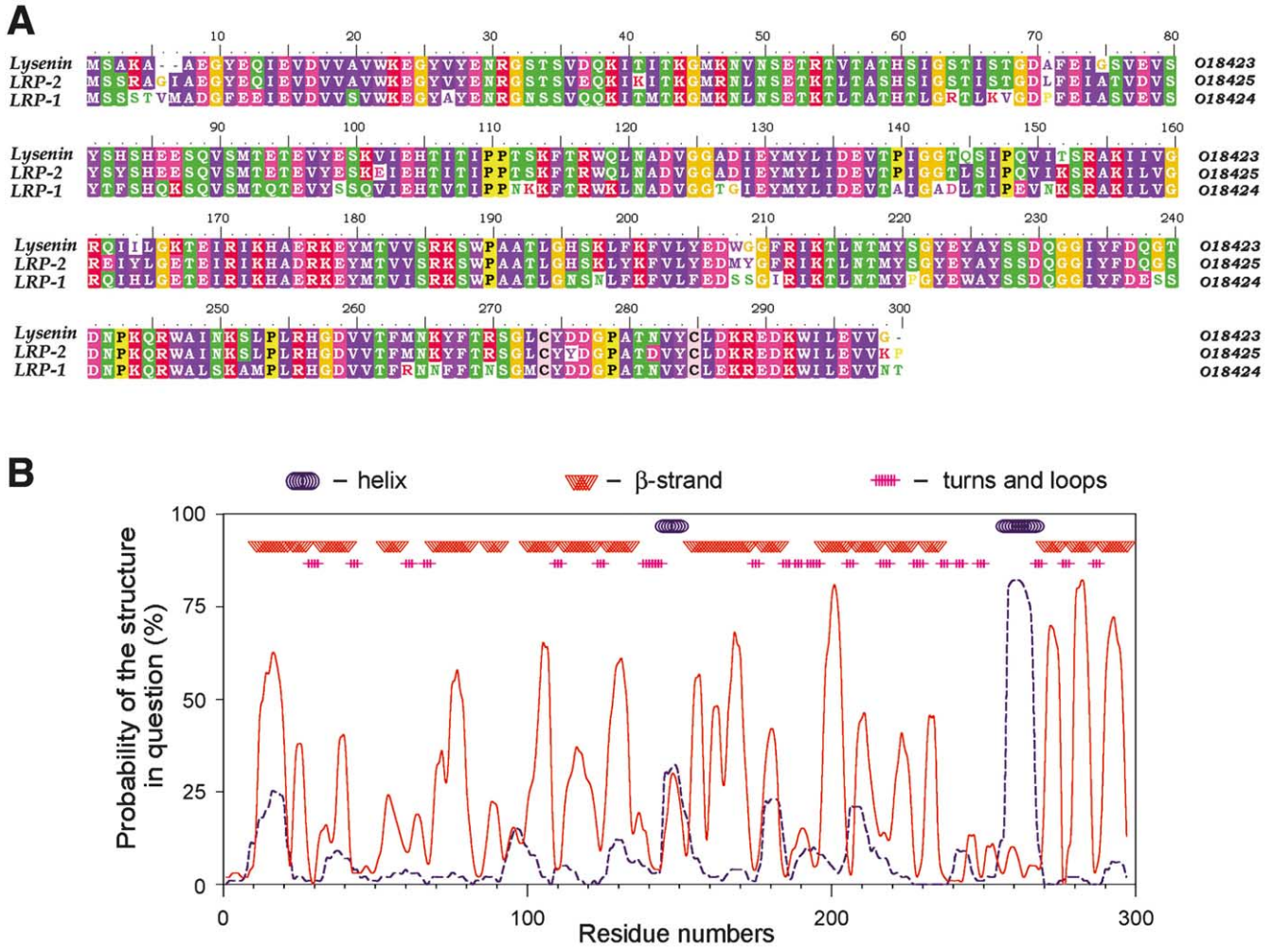

Fig. 1. The primary and secondary structure of lysenin. A: The sequence alignment of the lysenin family. The protein names are given on the left, while the entry names of the TrEMBL sequence databases are listed on the right of the alignment. The residues are colored according to the CLUSTAL X protein scheme [56]. In general, if one-letter code of amino acid residue is given on color background it means that it is the same residue as in two other proteins or replacement is strongly similar, otherwise the residue code is given in appropriate color on white background. B: Prediction of the secondary structure elements of the lysenin chain. The solid line denotes the probability of β-strand and the dashed line of helix. Proposed structured segments are marked on the top of the figure.

cently discovered sphingomyelinase activity associated with low density protein implies that plasma membrane sphingomyelin could provide a non-receptor-based gate for the entry of low density lipoprotein (LDL) cholesterol and could also participate in the pathophysiology of atherosclerosis [30]. Finally, Niemann–Pick disease type A caused by deficiency of acid sphingomyelinase extents the list of sphingomyelin-related diseases. Despite sphingomyelin involvement in physiology and pathophysiology of cells, little is known about the topological distribution of sphingomyelin and its dynamics in a variety of cellular processes. The discovery of lysenin, a novel sphingomyelin-specific binding protein will facilitate these studies.

2. Primary and secondary structure of lysenin and lysenin-related proteins

Lysenin was isolated from the coelomic fluid of the earthworm *Eisenia foetida* as a protein that caused contraction of rat vascular smooth muscles [9]. Lysenin polypeptide chain is 297 amino acids long with a calculated molecular mass of 33 440 Da, which is in good agreement with the results of size-exclusion chromatography (33 000 Da) and much less than that measured by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (41 000 Da) [9,10]. This discrepancy could be explained by glycosylation rather than by a high content of acidic residues in lysenin because it only slightly exceeds the average charge value.

Together with two additional proteins presented in coelomic fluid and referred to as lysenin-related proteins, lysenin comprises a family of proteins sharing sequences of high homology which are completely different from known sequences of others proteins available in protein databanks. As shown in Fig. 1A, the lysenin-related proteins have 72% or more of amino acid residues identical to lysenin, about 17% of strongly similar and only about 5% of residues different from lysenin. Different residues are statistically distributed along the entire polypeptide chain except positions 4–7 where different residues are accumulated. In this region lysenin has a two-residue deletion gap as compared to both lysenin-related proteins (see Fig. 1A).

Sometimes lysenin-related protein 2 is called fetidin [31–33]

or hemolysin (gb| > AAB67727.1); according to the TrEMBL protein sequence database (entry > O18425), it is the product of EFL3 gene and is the same protein, which in *E. foetida* cells could be modified by different saccharide components. According to PROSITE motif search [34], the *N*-glycosylation site for lysenin and lysenin-related protein 2 has been predicted at N248 and N250 residues, respectively (Table 1), which is in agreement with experimental observations [31]. For lysenin-related protein 1, two *N*-glycosylation sites at N33 and N151 residues have been predicted. All members of the lysenin family possess a possible *N*-myristoylation site, which could facilitate the anchoring of the proteins in cell membranes.

All three proteins have several potential phosphorylation sites specific for protein kinase C, casein kinase II and tyrosine kinases (Table 1); the lysenin-related protein 1 has one additional site specific for cAMP- and cGMP-dependent protein kinases. The proteins of this family have a heme-ligand signature and possess a weak homology with pyridoxamine 5′-phosphate oxidases (Table 1). Lysenin and lysenin-related protein 1 are homologous with a part of the HypA domain (hydrogenase nickel incorporation protein domain); lysenin has an additional homology with a part of the cellulose-binding domain (CBM-2).

The secondary structure of lysenin has been predicted by us using several recent methods available on the NPS@ server (http://pbil.ibcp.fr) [35], by ALB algorithm [36] and by the PHD method based on the neuronal network [37]. The average results of secondary structure prediction with the probability of the structure in question are presented on Fig. 1B. More than half of the residues of the lysenin chain (53%) tend to form β structures and 23% form turns and loops, while helices are formed only by 6% of residues composing two helical segments. The first one (shorter and with low probability) covers I145–T150 residues, while the second helix (with high probability) is located close to the C-terminus (G256–T267) of the lysenin chain. Each of the three proteins has 150–160 of exposed residues and this is more than the expected number (126) of exposed residues for a protein of the same molecular mass, so they could form ellipsoidal globules.

All these proteins have two cysteine residues: C272, C283 (C274, C285 in lysine-related proteins) with different potential ability of forming a disulfide bond. The first residue C272 (C274) has a high probability (over 0.80) of forming a disulfide bond while the second residue C283 (C285) has almost the same probability of staying in the SH form. This may suggest a tendency of these proteins to form dimers.

Lysenin has been classified as a membrane transport protein by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) (http://www.chem.qmul.ac.uk/iubmb/mtp/). Recommended on the mentioned website, WHAT software [38] based on analyses of hydropathy and amphipathicity predicted in lysenin a transmembrane helix located between residues V148 and G164. In the case of both lysenin-related proteins, the predicted transmembrane helices were located between residues Y131 and I147. This shift in the location of the transmembrane helix is unexpected, since the difference between the sequences of lysenin-related proteins 1 and 2 in region Y131–I147 seems to be essential and more distinct than the difference between these sequences in lysenin-related protein 2 and lysenin. On the contrary, the sequence of the transmembrane helix suggested for lysenin only, V148–G164, looks similar in all three proteins (for details see Fig. 1A). Moreover, the subroutine of the WHAT program designed for protein secondary structure prediction did not reveal any helical segments in any region of lysenin molecule, but indicated two helical segments in the lysenin-related protein 2 chain, both shifted to its C-terminus. The discrepancy between results of analysis of hydropathy and amphipathicity and helical segments predicted by all the methods indicates that lysenin and lysenin-related proteins have no helices capable of forming a transmembrane domain. Taking into account a strong lytic activity of lysenin, a possibility of an involvement of other lysenin segments in the penetration of the plasma membrane remains open.

3. Lysenin specifically binds to sphingomyelin

There is a substantial body of evidence that lysenin binds specifically to sphingomyelin in the plasma membrane of various cells and may therefore be used as a probe to study distribution and function of sphingomyelin in cellular membranes. Aside from sphingomyelin, lysenin isolated from *E. foetida* does not bind to any other sphingolipid nor to ceramide, sphingosine, sphingosine-1-phosphate, sphingosylphosphorylcholine or galactosylceramide, as shown by immunosorbent assay, thin layer chromatography immunostaining and liposome lysis assay. These studies indicated also that lysenin recognizes a precise molecular structure of sphingo-

Table 1
PROSITE and PFAM motifs and sites for lysenin family proteins

Motifs and sites	Residue numbers[a]		
	Lysenin	LRP-1	LRP-2
N-Glycosylation site	248	33; 151	250
N-Myristoylation site	61; 140	142	6; 63; 142
Protein kinase C phosphorylation site	2; 110; 150; 183	65; 185; 262	2; 112; 185
Casein kinase II phosphorylation site	32; 65; 82; 89; 219	93; 127; 146	34; 67; 84; 91; 221
Tyrosine kinase phosphorylation site	171	173	173
cAMP- and cGMP-dependent protein kinase phosphorylation site	–	113	–
Peroxidases proximal heme-ligand signature	50ETRTVTATHSI60	52ETKTLTATHTL62	52ETKTLTASHSI62
Homology with (hydrogenase nickel incorporation protein) HypA domain	63–78	69–80	–
Homology with cellulose-binding domain	12–86	–	–

[a]According to multiple sequence alignment lysenin has deletion at positions 6, 7 and 300 as compared to two other members of the lysenin family.

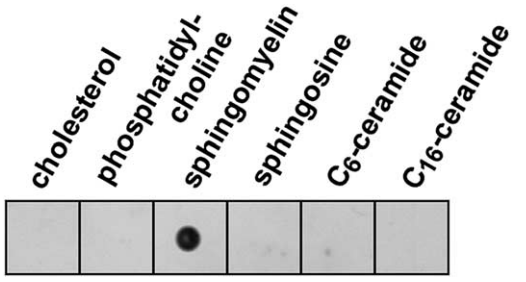

Fig. 2. Lysenin specificity. Lipids (4 pmol) spotted onto nitrocellulose were probed with His-tagged recombinant lysenin (2 μg/ml) followed by rabbit anti-His tag and anti-rabbit peroxidase antibodies.

myelin. Within the sphingomyelin molecule, lysenin required the phosphorylcholine, sphingosine and fatty acid moieties for binding [39]. We established that recombinant His-tagged lysenin recognizes sphingomyelin with the same specificity (Fig. 2). Incorporation of cholesterol into sphingomyelin-based synthetic membrane significantly increased the total amount of lysenin bound to the membrane. Surface plasmon resonance revealed that cholesterol incorporation did not change the kinetic parameters of the lysenin–sphingomyelin interaction, suggesting that cholesterol incorporation may change the topological distribution of sphingomyelin in the membrane, thereby increasing the accessibility of sphingomyelin to lysenin [39].

Owing to the sphingomyelin-specific binding, lysenin was applied as a cytochemical probe to visualize the phospholipid on the surface of oligodendrocyte lineage cells [40]. The immunocytochemical studies showed that during cell development, the content of sphingomyelin increased, although the amount and distribution of cholesterol remained unchanged. This kind of studies may clarify the role of sphingomyelin in oligodendrocyte differentiation, shedding light on the demyelination and remyelination in the brain. Lysenin was also used for immunofluorescent staining of fibroblasts derived from a patient with Niemann–Pick disease type A and permitted to visualize two distinct pools of sphingomyelin in these cells [39]. The disease is accompanied by an up to 50-fold increase of cellular sphingomyelin level caused by deficiency of lysosomal acid sphingomyelinase. Despite this, immunofluorescence staining of sphingomyelin on the surface of Niemann–Pick patient-derived fibroblasts was uniform and resembled the staining pattern of normal fibroblasts. After cell permeabilization, however, lysenin revealed a high level of sphingomyelin in lysosomes of Niemann–Pick fibroblasts, which was absent in normal cells [39]. These observations pointed to lysenin as a potential probe for detection of sphingomyelin in various cellular membranes under both normal and pathological (e.g. lipid storage diseases) conditions. Interaction of lysenin with sphingomyelin was influenced neither by sugar and protein molecules of the cell surface nor by fixation methods (2–4% paraformaldehyde, 10 mM sodium periodate/formaldehyde), but the lysenin/sphingomyelin interaction was abolished when the cells were fixed and permeabilized with alcohol [40].

When applied onto living cells, lysenin exhibits a strong cytotoxic and cytolytic activity directly related to the sphingomyelin content in the plasma membrane of the cells. Sheep erythrocytes, in which the sphingomyelin content is up to 51% of total membrane phospholipids, were much more sensitive to the lytic activity of lysenin than human and rat erythrocytes poorer in sphingomyelin (25% and 13% of total membrane phospholipids, respectively). Preincubation of lysenin with liposomes containing sphingomyelin, but not other phospholipids, inhibited lysenin-induced hemolysis completely and also abolished lysenin immunodetection in erythrocytes. Moreover, treatment of erythrocyte membrane with sphingomyelinase abolished the ability of lysenin to induce hemolysis [39]. By analogy to erythrocytes, the harmful effect of lysenin toward spermatozoa was correlated with sphingomyelin content in these cells, being higher in mouse than in frog spermatozoa [41]. A detailed phylogenetic study performed by Kobayashi et al. [42] indicated that sphingomyelin-containing spermatozoa of vertebrates were damaged by lysenin. On the contrary, spermatozoa of the vast majority of invertebrates were insensitive to lysenin treatment and this correlated with a lack of detectable sphingomyelin level in these cells.

The molecular mechanism of membrane damage caused by lysenin is not elucidated, although it does not follow sphingomyelinase action [39]. The presence of lysenin sequence able to form a transmembrane domain is in question, therefore the protein may attach to the membrane surface without penetration of the membrane bilayer. Subsequently, hydrophobic domains of lysenin could cause a local distortion of the lipid bilayer. In addition, an assembly of lysenin into oligomers cannot be precluded, since fetidins and eiseniapore, other *E. foetida* proteins, were shown to oligomerize during interaction with sphingomyelin-containing membranes [33,43,44]. It is possible that accumulation of sphingomyelin in distinct membrane microdomains such as sphingolipid/cholesterol-rich rafts [45,46] can render the microdomains especially susceptible for lysenin binding. Concentrated lysenin–sphingomyelin complexes within microdomains may evoke a local damage of the plasma membrane with subsequent cell lysis. From this point of view, studies of cytolysis induced by lysenin may help to reveal if alterations of lipid rafts are sufficient to induce cell death. There are several ways by which lysenin/sphingomyelin complexes can promote membrane leakage, one of them related to destabilization of the bilayer due to a local altering of membrane curvature. This mode of action was described for pro-apoptotic protein tBid in mitochondrial membrane [47]. One might imagine that after the plasma membrane permeabilization, lysenin gets access to a mitochondrial pool of sphingomyelin and the cytotoxicity of the protein is augmented.

Owing to the cytolytic property of lysenin, the protein was applied for selection of CHO mutant cells with deficiency in sphingolipid biosynthesis that rendered the cells resistant to lysenin [48,49]. On the other hand, the lysenin cytotoxicity limits the application of the protein as a probe for tracing sphingomyelin in living cells. Thus far, lysenin was used for sphingomyelin detection in fixed cells such as oligodendrocytes and fibroblasts of Niemann–Pick patients. This kind of studies can be extended for ultrastructural analysis of sphingomyelin topography in cell membranes, similarly to a procedure employed for visualization of cholesterol molecules [50]. The use of sublethal lysenin doses can help to circumvent the problem of its cytotoxicity. It seems, however, that modification of the protein, which will eliminate its lytic activity while preserving sphingomyelin recognition can provide a lysenin derivative useful for in vivo studies. A preparation of perfringolysin O derivative is a good example of such an approach

[51]. Lysenin devoid of its toxic property can be applied for confocal microscopy analysis of sphingomyelin pools and their dynamics upon triggering of the sphingomyelin cycle. Taking into account an accumulation of sphingomyelin in lipid rafts of the plasma membrane, labeled lysenin can be used to pursue behavior of rafts and these receptors, which are thought to transiently associate with rafts for signal generation. Particularly attractive are novel microscopic approaches developed to reveal native structure of lipid rafts, including fluorescence resonance energy transfer microscopy.

4. Lysenin, a puzzling component of defense mechanisms in earthworm *E. foetida*

Specific interaction of lysenin with sphingomyelin predisposes this protein as a tool for exploring sphingomyelin distribution and turnover under physiological and pathological conditions in mammalian cells. Surprisingly, the physiological function of lysenin in *E. foetida* is less clear. Lysenin is produced in large chlorogocytes and large coelomocytes and secreted into coelomic fluid of the earthworm [52]. Aside from lysenin, the coelomic fluid contains a wide spectrum of other bioactive peptides involved in innate protective pathways [53–55]. Lytic activity of lysenin and other lytic components of the coelomic fluid are believed to be an essential mechanism of defense. However, infectious microbes (bacteria, fungi), accessing the coelomic cavity after mechanical damages of the body wall, do not possess sphingomyelin required for the lytic activity of lysenin. Instead, bacteriostatic properties of fetidins were attributed to its peroxidase activity [33]. The presence of peroxidase signature in lysenin suggests a similar antibacterial activity of the protein.

Taking into account the sphingomyelin-binding ability of lysenin it was suggested that the protein might contribute to protection against predator vertebrates, when secreted through the dorsal pores of the earthworm. Lysenin may also serve as a defense substance for protection of the earthworm sperm, lacking sphingomyelin and discharged under influence of some stimuli, against soil insects, some of which possess sphingomyelin [42].

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