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Lysophospholipids prevent binding of a cytolytic protein ostreolysin to cholesterol-enriched membrane domains ☆

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ABSTRACT

Ostreolysin, a 15 kDa pore-forming protein from the oyster mushroom (*Pleurotus ostreatus*), binds specifically to cholesterol-enriched membrane domains existing in the liquid-ordered phase, and lyses cells and lipid vesicles made of cholesterol and sphingomyelin. We have monitored binding of sub-lytic concentrations of ostreolysin to membranes of Chinese Hamster Ovary cells and rat somatotrophs, using primary anti-ostreolysin and fluorescence-labeled secondary antibodies detected by confocal microscopy. Depletion of more than 40% membrane cholesterol content by methyl-β-cyclodextrin dramatically decreased ostreolysin binding. Immunostaining showed that ostreolysin is not co-localized with raft-binding proteins, cholera toxin B-subunit or caveolin, suggesting that natural membranes display heterogeneity of cholesterol-enriched raft-like microdomains. Impaired ostreolysin binding was also observed after treating the cells with lysophosphatidylinositol. Effects of lysophosphatidylinositol on binding of ostreolysin to immobilized large sphingomyelin/cholesterol (1/1, mol/mol) unilamellar vesicles were studied by a surface plasmon resonance technique. Injection of ostreolysin during the lysophosphatidylinositol dissociation phase showed an inverse relationship between ostreolysin binding and the quantity of lysophosphatidylinositol in the membranes of lipid vesicles. It was concluded that lysophospholipids prevent binding of ostreolysin to cell and to artificial lipid membranes resembling lipid rafts, by partitioning into the lipid bilayer and altering the properties of cholesterol-rich microdomains.

Abbreviations: CHO, Chinese Hamster Ovary cells; Chol, cholesterol; CT-B, Cholera toxin subunit B; DOPC, dioleoylphosphatidylcholine; DRMs, detergent-resistant membranes; FBS, fetal bovine serum; GPI, glycosylphosphatidylinositol; l_d, liquid-disordered; l_o, liquid-ordered; LP, lysophospholipid; LPC, lysophosphatidylcholine; LPI, lysophosphatidylinositol; LUVs, large unilamellar vesicles; MβCD, methyl-β-cyclodextrin; MLVs, multilamellar vesicles; Oly, ostreolysin; PC, phosphatidylcholine; POPC, palmitoyl-oleoylphosphatidylcholine; RU, resonance unit; S.E., standarad error; SM, sphingomyelin; SPR, surface plasmon resonance; SUVs, small unilamellar vesicles..

☆ *Ethical statement*: The protein used in this study was purified from edible oyster mushrooms (*Pleurotus ostreatus*, strain Plo5), that were taken from the ZIM collection of the Biotechnical Faculty, University of Ljubljana, Slovenia.

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1. Introduction

According to the current model of cell membrane structure, certain membrane lipids, especially sterols, are responsible for the separation of membrane lipid domains into co-existing liquid-disordered (l_d) and liquid-ordered (l_o) domains (McConnell and Vrljic, 2003). The latter are the basis for formation of lipid rafts, cell membrane entities enriched in sphingolipids, cholesterol (Chol), and specific proteins (Pike, 2004). Rafts are involved in several important biological functions, such as exocytosis and endocytosis, signal transduction, pathogen entry, and attachment of various ligands (Edidin, 2003; London, 2002; Simons and Ikonen, 1997). They are believed to be transient, dynamic, and unstable membrane entities, which are stabilized on binding ligand molecules (Hancock, 2006; Kenworthy et al., 2000; Subczynski and Kusumi, 2003), and exist as nanoscale clusters (Hancock, 2006; Sharma et al., 2004) of various sizes (de Almeida et al., 2005).

Membrane domains in the l_o phase are more resistant to solubilization by detergents than the lipids in l_d domains (Lichtenberg et al., 2005) and, hence, lipid rafts are often operationally called detergent-resistant membranes (DRMs) (Simons and Ikonen, 1997). However, recent experimental data indicate that DRMs should not be assumed to describe biological rafts in size, structure, composition or even existence, as detergents themselves can induce the formation of ordered domains (Heerklotz, 2002; Lichtenberg et al., 2005).

The increasing amount of experimental evidence emphasizes the crucial biological roles of lipid rafts, thus increasing the need for new techniques and approaches to study these membrane microdomains. In particular, fluorescence-labeled cytolytic proteins that interact specifically with molecules enriched in lipid rafts are gaining interest. The best known, and the only commercially available raft-labeling agent, is the G_{M1} ganglioside-binding cholera toxin B subunit (CT-B) (Bacia et al., 2004). Two new candidates that have been proposed as possible selective markers for rafts are the pore-forming cytolysin, lysenin, whose truncated, non-toxic mutant recognizes sphingomyelin-enriched membrane domains specifically (Kiyokawa et al., 2005; Ishitsuka et al., 2005), and a protease-nicked and biotinylated non-toxic derivative of perfringolysin O (θ-toxin), that binds selectively to Chol-rich lipid rafts and DRMs (Ohno-Iwashita et al., 2004; Waheed et al., 2001). These proteins not only recognize the single lipid component, but are also sensitive to its distribution in the membrane and especially to its enrichment in lipid rafts.

Ostreolysin (Oly) is functionally a sterol-dependent cytolysin that binds specifically to SM membranes rich in Chol or other sterols, and to detergent-resistant fractions of cell membranes (Rebolj et al., 2006; Sepčić et al., 2004). This 15 kDa acidic protein is found in large amounts in young fruiting bodies of the edible mushroom *Pleurotus ostreatus* (Berne et al., 2002; Vidic et al., 2005), and belongs to the aegerolysin family of closely similar proteins that can be found in fungi, bacteria and plants (Berne et al., 2005). Cytolytic and hemolytic effects, that are the consequence of pore formation, are features of some aegerolysin-like proteins (Berne et al., 2002; Kumagai et al., 1999; Sakurai et al., 2004; Sepčić et al., 2003, 2004; Tomita et al., 2004), and can be responsible for their toxicity (Sakaguchi et al., 1975; Žužek et al., 2006). Aegerolysins appear to differ in their affinity for membrane acceptors. Pleurotolysin, an Oly isoform from *P. ostreatus*, was suggested to recognize sphingomyelin (SM) (Tomita et al., 2004), while Asp-hemolysin was proposed to bind lysophosphatidylcholine (LPC) specifically (Kudo et al., 2002). Our previous study on Oly binding and pore-forming properties revealed that the protein does not bind to pure SM or to other membrane lipids, but specifically senses membrane domains in which Chol is combined with either SM or fully saturated glycerophospholipids, suggesting its specific interaction with raft-like domains. This is supported by several lines of evidence. First, Oly can be found in isolated DRMs of both SM/Chol (1/1, mol/mol) vesicles and Chinese Hamster Ovary (CHO) cells (Sepčić et al., 2004). Secondly, permeabilization of SM/Chol vesicles by Oly appears only above 30 mol% Chol, the concentration at which this sterol induces the formation of a l_o-phase (de Almeida et al., 2003). It is interesting to note that between 30 and 40 mol% of Chol, the binding of Oly and its permeabilization of SM/Chol vesicles sharply increase from 0% to 22%, and from 0% to 90%, respectively (Rebolj et al., 2006; Sepčić et al., 2004). The interaction of Oly with Chol-enriched domains can be diminished or disrupted by the addition of mono- and di-unsaturated phosphatidylcholine (PC) (Sepčić et al., 2004), or by replacing Chol with other natural sterols or Chol derivatives (Rebolj et al., 2006). Furthermore, its hemolytic activity can be inhibited by the addition of micromolar concentrations of fatty acids and lysophospholipids, especially lysophosphatidylinositol (LPI), sphingosine-1-phosphate and lysophosphatidylcholine (Sepčić et al., 2003). Lysophospholipids (LPs) are single-chain, water-soluble surfactants that are found in small concentrations in lipid membranes, where they are synthesized de novo or are generated by enzymatic cleavage of glycerophospholipids and SM, and are released to regulate and maintain the organismal homeostasis (Gardell et al., 2006; Goetzl and An, 1998). Their signaling activity is mediated by the activation of transmembrane G-protein coupled receptors (Anliker and Chun, 2004). Thus, many LPs, especially lysophosphatidic acid and sphingosine-1-phosphate, affect fundamental cellular functions like proliferation, differentiation, survival, migration, adhesion, invasion and morphogenesis (Ishii et al., 2004). Impairment of their signaling functions is associated with several metabolic and physiological disorders (Gardell et al., 2006).

The aim of this work was to further investigate the interaction of Oly with Chol-rich raft-like membrane domains. Rabbit anti-Oly and fluorescent Alexa fluor 546-labeled secondary goat antibodies were used to monitor the binding of a sub-lytic concentration of Oly to membranes of CHO cells and rat somatotrophs, both under normal conditions and after treating the cells with methyl-β-cyclodextrin (MβCD) or lysophosphatidylinositol. Additionally, the effect of LPI on Chol/SM vesicles in