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Summary

According to the World Health Organization pathogenic bacterium *Pseudomonas aeruginosa* is among the "top priority pathogens" that require immediate attention for development of novel antibacterial strategies. *P. aeruginosa* is an opportunistic pathogen responsible for a high number of hospital-acquired infections. Moreover, it is endowed with versatile resistance mechanisms that make the treatment of infections extremely difficult. Novel antimicrobial drug development thus requires deep understanding of the *P. aeruginosa* defensive mechanisms. Among others, biofilm formation and host cell internalization are powerful defensive mechanisms of *P. aeruginosa*. Both block the access of the immune cells and antibiotics to the individual bacteria. In addition, internalized bacteria are particularly toxic for the host cell as they release their products into the cell interior. Restraining this process will give a considerable advantage in the treatment of bacterial infection. Bacterial internalization is predominantly initiated at the host cell plasma membrane – the semi-permeable barrier between the cytosol and the extracellular environment. Organized on the basis of a lipid bilayer matrix, plasma membrane contains a huge variety of lipids and proteins embedded in the bilayer. Some of them are targeted by the bacteria in order to trigger actin polymerization or directly bend the membrane which will lead to the bacterial engulfment. The local change of content and organization of the lipid bilayer induced by bacteria and bacterial products is an important feature of this process. Plasma membrane glycosphingolipids (GSLs) are important membrane components involved in cell adhesion, signaling and endocytosis. They consist of a hydrophobic fatty acyl backbone embedded in the lipid bilayer and a carbohydrate head group exposed to the extracellular environment. GSLs can be highjacked by viruses, bacteria and bacterial products (such as toxins) that recognize their carbohydrate moieties using special carbohydrate binding proteins (also called "lectins"). Furthermore, lectin-GSL interaction is exploited by pathogens for binding to the membrane surface which results into pathogen internalization by the host cell. Lectin-GSL binding is strong and highly specific. It was demonstrated in artificial membrane systems that such interaction alone is sufficient for membrane tubule nucleation and ligand internalization. This result was successfully reproduced for numerous isolated lectins, viruses and even bacteria. *P. aeruginosa* is not an exception. The efficient binding of this bacterium to the cell membrane strongly relies on the interaction of its surface lectin LecA with the cellular GSL globotriaosylceramide (Gb3). This interaction was reconstituted in artificially prepared giant unilamellar vesicles (GUVs) that contained 5 mol % of Gb3. Bacteria bind to GUVs and form so called "lipid zipper" induced tubular invaginations on GUVs. Deletion of LecA prevents bacterial internalization in both artificial membrane systems and living cells proving the crucial importance of LecA for the *P. aeruginosa* uptake. However, the modification of the membrane content and organization during this process requires further exploration. In this work, we study the local membrane reorganization induced by *P. aeruginosa* and LecA. We explore the changes in membrane order and lateral distribution of membrane lipids induced by LecA and bacteria. In addition, we studied the influence of the molecular structure of Gb3 receptor molecules on *P. aeruginosa* binding and bacteria-induced membrane reorganization.

The association of GSLs with the sphingomyelin and cholesterol enables their incorporation in the highly ordered nanodomains of plasma membrane (referred as “lipid rafts”). Such domains accumulate various membrane components and their assembly and reorganization was proven to be important in the initial steps of cellular signal transduction and endocytosis. Studying such domains in living cells is a demanding task since they are small and highly dynamic. The alternative solution is the reconstitution of such domains in synthetic membrane systems. We employed two types of those – giant unilamellar vesicles (GUVs) and supported lipid bilayers (SLBs). In such systems, lipid domains are stable and microscopic in size which permits studying them using different conventional microscopy techniques. Of note, such microscopic domains only partially resemble the nanodomains in living cell. Nevertheless, such systems enable the elucidation of the role of specific components involved in lipid domain formation and reorganization.

In order to study the lipid domain reorganization induced by *P. aeruginosa*, we used SLBs that contained 5 mol % of Gb3 receptor molecules. We created lipid bilayers with two coexisting spatially separated phase domains using a three component lipid mixture of DOPC, cholesterol and sphingomyelin. Such lipid bilayer composition mimics the heterogeneity of the plasma membrane on the microscopic scale. Strong hydrogen bonds between SM and cholesterol allow the formation of tightly packed (liquid ordered) domains. As GSLs preferentially associate to lipid rafts, the majority of Gb3 molecules incorporate into the Lo phase domains in model membranes. Gb3 molecules in model systems can be recognized by galactose specific lectins, in particular – LecA. Coupled to bacterium or isolated, LecA binds specifically to Gb3 carbohydrate moieties exposed to the aqueous environment of the lipid bilayer. Wild-type *P. aeruginosa* that expresses LecA sticks efficiently to the lipid bilayer surface. Interestingly, bacteria tend to aggregate and form bacterial clusters on the supported lipid bilayer. Furthermore, bacteria sequester the lipid material from the model membrane. Finally, lectin-Gb3 interaction alters the spatial organization of the Lo domains in the lipid bilayer. Lo domains become smaller and disappear. LecA deletion inhibits the dissolution of the Lo domains as well as the accumulation of the lipid material on the bacterial surface. In addition, PA ΔLecA does not stick efficiently to the membrane surface and does not form bacterial aggregates. Furthermore, in order to decipher the mechanisms of LecA impact on the membrane organization, we explored the SLB interaction with the isolated LecA. Isolated LecA induces the dissolution of the domains and pulls out the membrane material from the original membrane in order to form membrane multilayers. As a result, the membrane is depleted in lipid material and disintegrates. This effect of isolated LecA is not a common feature for Gb3-specific lectins. The reorganization of Gb3-containing membranes was formerly studied in the context of the cellular uptake of the B subunit of Shiga toxin from *S. dysenteriae*. Studies on model membrane systems (giant unilamellar vesicles and supported lipid bilayers) showed a specific preference of StxB to Gb3 incorporated in Lo domains as well as an efficient clustering of the Gb3 molecules induced by the multivalent binding of StxB. Moreover, StxB shrinks Lo domains and induces the formation of novel Lo domains in supported lipid bilayers. Efficient Gb3 clustering by StxB is granted by the number, position and orientation of binding pockets (all 15 of them are pointing in one direction). Unlike StxB, LecA contains only 4 Gb3-specific binding sites and, in addition, the distance between them is higher as in case of StxB. The geometry of LecA binding pockets is not suitable for glycolipid clustering and, as we observed in our experiments, it did not happen. On the contrary, LecA induces the dissolution of the Lo domains, homogenizing the membrane. The preference of LecA to lipid bilayer interfaces enables another interesting phenomenon – membrane

multilayer formation. Indeed, LecA – Gb3 interaction is strong enough to detach the membrane from the solid support and wrap it around the bacterium or form a membrane multilayer with the LecA concentrated in between membrane layers. Phase dissolution precedes membrane multilayers formation. Non-phase separated SLBs, where phase mixing cannot occur, are completely disintegrated within 5 min after LecA application, whereas in phase separated bilayers membrane defects start appearing only after 15 min. Presumably, phase mixing is required for successful membrane multilayer formation. All in all, we demonstrated that LecA is a crucial protein not only for the bacterial uptake but also for the plasma membrane domains dissolution that precedes bacterial internalization.

By studying the dynamics of the reorganization of membrane domains induced by lectins we also explored the role of lectin (StxB and LecA) valence and geometry in this process. We observed not only differences in the lipid domains reorganization dynamics induced by StxB and LecA, but also variations in the binding preferences to the lipid bilayers. Such variations may originate from the lipid composition of the lipid bilayer and the molecular structure of the Gb3 receptor. Both factors define the exposure of the Gb3 carbohydrate moiety to the extracellular environment and lectins may prefer a specific orientation of Gb3 sugars. In our previous experiments, we always used wild type Gb3 that is extracted from brain porcine and contains a natural mixture of Gb3 molecules with different fatty acyl chains. Here, we reconstituted the lectin-Gb3 binding process in giant unilamellar vesicles containing a natural Gb3 mixture or some single Gb3 species (Gb3-FSL-DOPE, Gb3 C-24:0 and Gb3 C-24:1). We also adjusted the lipid composition in order to elucidate the specific requirements for the efficient binding of both lectins. In order to quantitatively compare the binding efficiencies of lectins to membranes containing different Gb3 species, we developed a FIJI-based macro that enabled fast and unbiased computational processing of the data obtained by fluorescence microscopy. We found that StxB prefers Gb3 molecules incorporated in a more ordered environment whereas LecA demonstrates a slight preference for a less ordered lipid environment.

Reconstitution of complex cellular processes in synthetic systems is a powerful tool for the elucidation of the molecular mechanisms and role of specific components. Here, we reconstituted the interaction of the pathogenic bacterium, *P. aeruginosa,* with its cellular receptor globotriaosylceramide (Gb3) in supported lipid bilayers. We identified a bacterial surface protein (lectin LecA) that is responsible for bacterial binding to the cell surface and modulation of the lipid organization of the plasma membrane. We showed that LecA – Gb3 interaction cause ordered domains dissolution in the lipid bilayer and, presumably, this process is required for successful PA internalization into the host cell.

Furthermore, we studied the lipid bilayer reorganization induced by isolated LecA. In addition to domain dissolution, LecA also forms membrane multilayers on the lipid bilayer surface. We compared impact of LecA on the membrane to the impact of another Gb3 specific protein – StxB. Unlike LecA, StxB does not induce ordered domains dissolution but shrinks existing Lo domains and induces the formation of the novel ones. In addition, we explored the role of the Gb3 receptor structure in the LecA and StxB binding to the lipid bilayer. We used Gb3 molecules with the different structure of fatty acyl chains embedded in the membrane of different order. We found that StxB prefers Gb3 molecules embedded in more ordered environment, whereas LecA has a slight preference to the disordered membranes. In perspective, we plan to explore the role of Gb3 species structure in the binding of the complete bacteria – *P. aeruginosa*.