Protein Effects on Lipid Domain Formation in a Model Membrane

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1. Introduction

Interactions between proteins and the cell membrane are an integral aspect of many biological processes. Lipids are not passive participants in biological membranes and influence the structure and function of membrane proteins. In the lipid raft hypothesis, it is believed that cholesterol- and sphingolipid-enriched membrane microdomains play important roles in cell signaling and viral entry. Those nanoscale transient lipid domains are believed to consist of Sphingomyelin, cholesterol, and membrane proteins in living cells. Because of their transient nature and the size that is smaller than the diffraction limit of conventional optical microscopy, those domains eluded researchers under physiological conditions. Yet, these domains are readily observable in model membranes using optical microscopy and under controlled conditions of two lipid types and cholesterol. As a result, there is an urgent need to elucidate the biophysical properties of lipid domains under different thermodynamics properties.

Cholera toxin (CT), which selectively binds to *ganglioside glycolipids*, is frequently used as a reporter for membrane rafts and as a tool to investigate protein-lipid interactions (1). CT consists of 5 B-subunits and 2 A-subunits. CTB has a pentameric structure with almost perfect 5-fold symmetry that targets the GM_1 gangliosides in the intestinal lumen. The A1 subunit serves as the catalytic subunit that ultimately causes the symptoms of cholera. The A2-subunit serves as the bridging unit between subunits A1 and the 5 B-subunits. The B subunit of cholera toxin (CTB) is responsible for binding the toxin with highest affinity to ganglioside GM_1 (4.61×10^{-12} M), a cell-surface receptor also associated with lipid domains (4). Under the surface lateral pressures in biomembranes, protein binding perturbs lipid packing that results in regional defects and the emergence of lipid phase (4). The altered lipid order in bilayers is usually transmitted from the receptor on the exterior membrane leaflet to the inner leaflet as a mechanism for lipid mediated signaling by multivalent protein binding.

In this UROP project, related primary literature was surveyed concerning the latest state of knowledge of the role of membrane proteins in lipid domain formation and lipid-protein interactions. Our model system was labeled C3H cells with Alexa-CTB in culture. The labeling was optimized using confocal and DIC microscopy as a function of extrinsic GM_1. In order to elucidate the uptake of Alexa-CTB and its intracellular environment, we carried out two-photon fluorescence lifetime imaging microscopy (2P- FLIM). Preliminary results on the translational diffusion of Alexa-CTB were also carried out using fluorescence correlation spectroscopy (FCS) in order to elucidate the conformational state of the toxin and the restriction of its surrounding environment.

2. Research Methodology

Rhodamine green and Alexa-488-Cholera Toxin-B (Alexa-CTB hereafter) were purchased from Invitrogen and used without further purifications. C3H cells were cultured in the recommended cultured media by the supplier and imaged afterwards in Tyrode's buffer.

Differential interference contrast (DIC) and laser-scanning confocal microscopy (Olympus) were used for morphological imaging and Alexa-CTB uptake by C3H cells, respectively. For investigating local environments and conformational changes, we used two-photon (2P) fluorescence lifetime imaging microscopy (FLIM). Subsequent DIC images were recorded after each FLIM image in order to assess any potential photo- induced stress or damage to the labeled cells. Finally, fluorescence correlation spectroscopy (FCS) was also used to examine the diffusion coefficient of cellular Alexa- CTB as a measure of its binding state as well as the degree of restriction imposed by the surrounding environment.

3. Findings and Discussion

3.1. Visualization of Alexa-CTB uptake by C3H Cells in Culture

Monosialo-tetrahexosyl-ganglioside (GM_1) is a member of the ganglio series of gangliosides with important physiological role in neuronal plasticity and repair mechanisms as well as the release of neurotrophins in the brain. GM_1 acts as the site of binding for both cholera toxin, which has 5 B-subunits, each of which has a molecular weight of 11.5 kDa. It is believed that each subunit is labeled with alexa-488 tag that constitutes Alexa-CTB used in these studies. In addition, each subunit binds to the GM_1 ganglioside on the plasma membrane. In order to optimize Alexa-CTB uptake by cultured C3H cells, we varied the concentration and incubation times of GM_1 and Alexa-CTB with cultured C3H cells. The labeling was optimized using confocal and DIC microscopy as a function of extrinsic GM_1. Figure 1 shows two representative images (DIC and confocal) with and without GM_1.

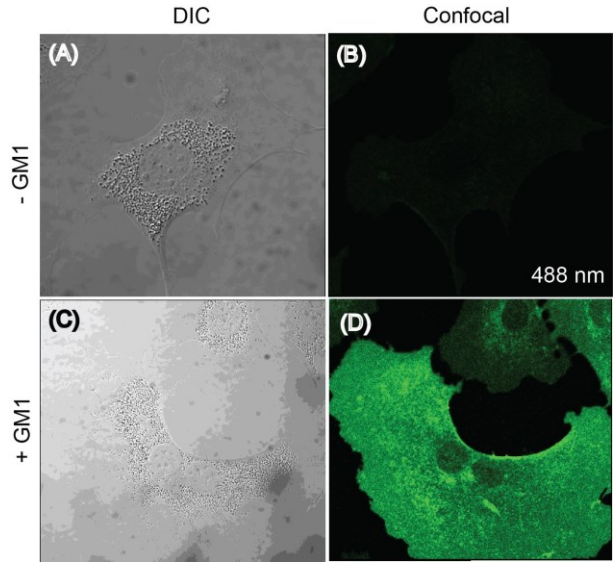

Figure 1: GM_1 facilitates the uptake of Alexa-Cholera Toxin-B by cultured C3H cells. The DIC (A, B) and confocal (B, D) images were recorded using 488 nm. The incubation time (10 minutes) with Alexa-CTB was the same in both the absence (B) or the presence (D) of GM.

In these images, the cells were first labeled with GM_1 by Alexa-CTB, the concentrations and incubation times were optimized for a high level of uptake suited for the confocal imaging. The labeled cells were then washed out three time using Tyrode's buffer prior to imaging. Our results indicate that the cholera toxin crosses the plasma membrane only in the presence of GM_1. In addition, the labeled toxin also crosses the nuclear membrane as well, albiet at a lower efficiency. In some cases, a higher concentration of Alexa-CTB stay attached to the plasma membrane via its binding with the GM_1.

3.2. Probing the Conformation and Surrounding Microenvironment of Alexa-CTB in Cultured C3H Cells

Unlike fluorescence intensity based imaging, fluorescence lifetime is sensitive to both the chemical structure of a given fluorophore and the surrounding enviroment. In order to elucidate the uptake of Alexa-CTB and its intracellular environment, we carried out two- photon fluorescence lifetime imaging microscopy (2P-FLIM). In these measurements, laser pulses (730 nm, 76 MHz, 120 ps in width) were used in a scanning mode for these FLIM studies.

Representative 2P fluorescence intensity (B, E) and FLIM (C, E) images are shown in Figure 2. DIC images were also recorded soon after FLIM in order to assess any potential photostress/photodamage of the living C3H cells. These FLIM imaging conditions introduced no apparent cellular photo-induced stress or damage. Two-photon FLIM of Alexa-488-CTB stained cells indicates biexponential fluorescence decays per pixel with 1.6 ns (56%) and 3.2 ns (44%) with a full-width-half-maximum of ~15%. The average fluorescence lifetime throughout the cells is shown in Figure 2 with a peak around 2.5 ns in the pixel-lifetime histogram (not shown). It is interesting to notice that the average fluorescence lifetime of Alexa-CTB is heterogeneous due to variations of the surrounding microenvironment. It also depends on the physiological state of the cells (Figure 2C and F). The observed fluorescence lifetime of cellular Alexa-CTB is significantly shorter that that of free Alexa-488 (~4.0 ns) in a aqueous solution. As a result, the observed short lifetime observed in Alexa-CTB complex may suggest homo-FRET (fluorescence resonance energy transfer) between Alexa-488 fluorophores on each subunit.

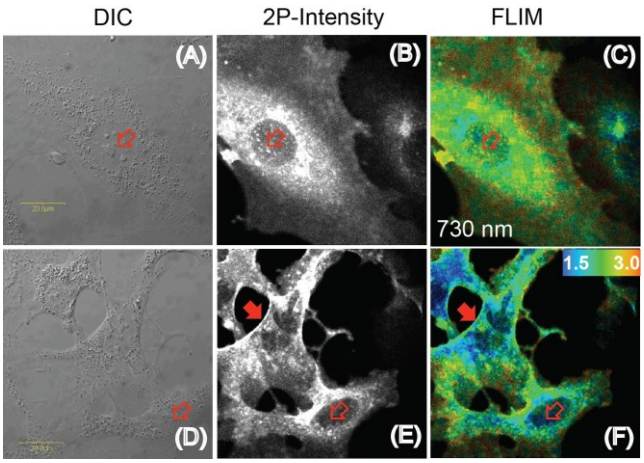

Figure 2: Two-photon FLIM of intracellular Alexa-CTB reveals a heterogeneous environment in cultured C3H cells. The DIC (A, D) and 2P-fluorescence intensity (B, E) images reflect the Alexa-CTB distribution through the C3H cells, which seems comparable to those shown in Figure 1. The 2P-FLIM (C, F), however reflect a hetrogeneous in the local environment of Alexa-CTB inside C3H cells. The solid arrows indicate regions where the Alexa-CTB was localized specifically on the plasma membrane of the cultured cells. The unfilled arrows point to representative nuclei with low concentration of Alexa-CTB. Color code: 1.5 – 3.0 ns for both FLIM images (C and D).

The question now is whether Alexa-488 cleaves from its construct with Cholera Toxin B. To answer this question, we examined the translational diffusion and binding state of Alexa-CTB in culturd C3H cells at room temperature (below).

3.3. Translational Diffusion of Alexa-CTB in Cultured C3H Cells

Preliminary results on the translational diffusion of Alexa-CTB were also carried out using fluorescence correlation spectroscopy (FCS) in order to elucidate the conformational state of the CTB and the restriction of its surrounding environment. Representative autocorrelation decay of Alexa-CTB is shown in Figure 3. In these experiments, FCS was calibrated using rhodamine green in PBS at room temperature with a diffusion time of ~0.07 ms, which corresponds to a diffusion coefficient of ~2.8×10^{-6} cm^2/s.

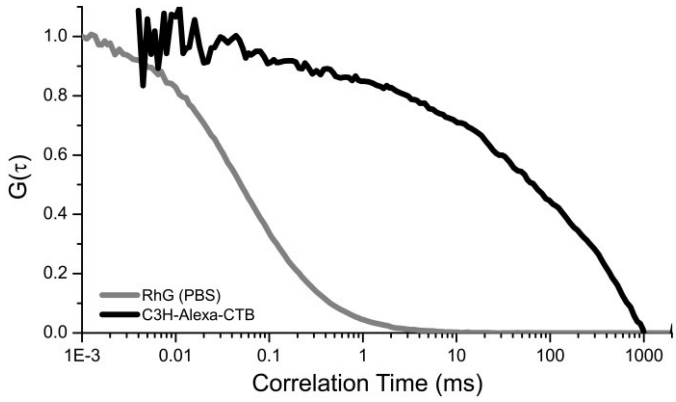

Figure 3: Translational diffusion of internalized Alexa-CTB indicates an intact toxin in a restricted environment. The autocorrelation curve of Alexa-CTB (black curve) indicate that intracellular toxin diffuses at significantly slow diffusion coefficient. Under the same conditions, the FCS was calibrated using free rhodamine green in PBS (gray curve) at room temperature.

In contrast, Alexa-CTB in randomly selected regions of interest diffuses at much slower diffusion time ~100 ms. The corresponding diffusion coefficient of Alexa-CTB in cellular cytosol is ~$1.4x10^{-9}$ cm^2/s, which indicates the absence of free Alexa-488 inside the cells. These result suggest that the Alexa-CTB is internalized intact inside the C3H cells. Importantly, the the diffusion time seems significantly longer than expected for 5x11.5 kDa complex, which suggest a restricted microenvironment surrounding Alexa-CTB inside living cells.

4. Conclusion

In this project, we investigated the uptake of Alexa-488-cholera-toxin B in cultured C3H cells. In addition, the conformational state and local environment of Alexa-CTB were investigated using two-photon FLIM. At the single-molecule level, our preliminary results indicate the significantly slower diffusion coefficient as compared with the predicted one in a pure buffer, assuming 57.5 kDa.

During the course of this project, I surveyed related literature and discussed experimental design with my advisor. I also learned new wet lab skills, cell culture, cell staining, and confocal imaging. Finally, I enjoyed the experience of working with other group members in the lab and during the weekly group meetings.

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