RESEARCH HIGHLIGHT

Unraveling the mechanism of cholesterol-mediated regulation of receptor dimerization in plasma membranes *in vivo*

Jung Y. Huang[1], Chien Y. Lin[1], Leu Wei Lo[2]

[1]*The T.K.B. Research Center for Photonics, Chiao Tung University, Hsinchu 300, Taiwan*
[2]*Institute of Biomedical Engineering and Nanomedicine, National Health Research Institutes, Zhunan, Miaoli 350, Taiwan*

Correspondence: Jung Y. Huang
E-mail: jungy.huang@gmail.com
Received: March 01, 2015
Published online: August 13, 2015

Cholesterol can restructure lipid membranes and thereby alters the signal transduction of receptor proteins in living cells. The process that membrane cholesterol modulates the interaction of receptor proteins remains unclear. To improve our understanding on this important question, we performed single-molecule optical tracking of epidermal growth factor receptors (EGFRs) in two cancerous cell lines (HeLa and A431) and one normal endothelial cell line (MCF12A) and developed a theoretical model to unravel the correlated diffusion processes of liganded receptor proteins prior to the formation of dimers. We discovered that unliganded EGFRs typically reside in non-raft regions of the plasma membrane and translocate from the non-raft lipid domain to the cholesterol-enriched domain upon ligand binding. We further manipulated the total amount and spatial distribution of membrane cholesterol with methyl-β-cyclodextrin and nystatin and disclosed that the stability of EGFR dimers is relevant to the amount of membrane cholesterol. Among the three cell lines under study, the EGFR dimers in the normal cells are more sensitive to changes in the local concentration of cholesterol than that in the cancer cells. Our result can serve as the foundation to shed light on the way in which protein-protein interactions are regulated in plasma membranes *in vivo*.

Keywords: Receptor signaling; lipid rafts; membrane proteins; single-molecule tracking

To cite this article: Jung Y. Huang, *et al*. Unraveling the mechanism of cholesterol-mediated regulation of receptor dimerization in plasma membranes *in vivo*. Receptor Clin Invest 2015; 2: e684. doi: 10.14800/rci.684.

Living cells must execute a variety of signaling processes to thrive in a fluctuating environment. The first step in the signaling process occurs on the plasma membrane of a living cell at various types of receptors. Receptor signaling dysregulation is responsible for the pathogenesis of several diseases. During the estimation of a signal that varies spatially and temporally, an optimization problem is encountered in cells and this problem entails balancing two opposing objectives. These are the need to locally assemble sensors to reduce estimation noise and the need to spread them to reduce spatial error [1]. An information-theoretic approach serves as a solution to this problem and entails the organization of distributed and mobile receptors into active mobile clusters [1]. Thermodynamics further constrains the

ability of signaling networks to estimate the concentration of an external signal [2]. Receptors can undergo multiple transitions between unbound nonsignaling and bound signaling states by consuming energy to approach this thermodynamic limit. However, the real situation in living eukaryotic cells is more complex because they are highly heterogeneous and stochastically dynamic. Lipid nanodomains, rich in saturated lipids and cholesterol [3, 4], can form spontaneously. It remains unknown whether cells have evolved to make such an approach probable in appropriate situations. Researchers have increasingly determined that lipid raft domains can facilitate signaling receptors to form a dimer in living eukaryotic cells [5]. The process by which this cholesterol modulates the interaction between receptor
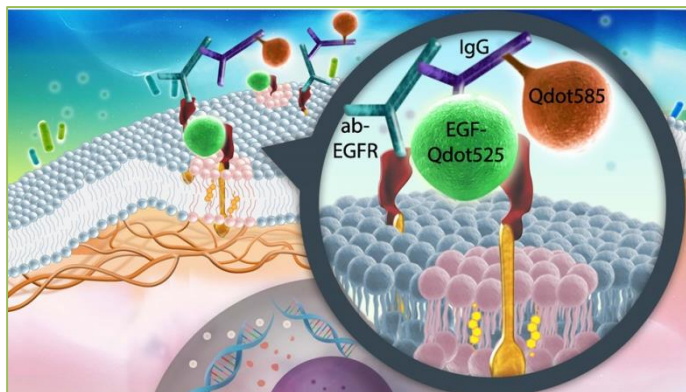
Figure 1. Schematic diagram showing epidermal growth factor receptor (EGFR) dimers in the heterogeneous environment of the plasma membrane. The following labeling scheme is used in this study: Ab-Qdot585 denotes a Qdot585 conjugated to an EGFR antibody; EGF-Qdot525 is a fluorescent epidermal growth factor (EGF) synthesized by conjugating EGF with Qdot525.

Figure 2. Schematic diagram illustrating diffusive motion of single EGFR in the plasma membrane of a live cell.

proteins is still unclear.

We have conducted a series of studies aiming to answer these questions. First, we combined the generalized Langevin equation with the Cahn-Hilliard equation. The resulting model enabled us to integrate a hierarchical structure of actin corrals, protein-induced lipid ordering domains, and dynamic diffusion of receptor proteins into a unified framework [6]. We tagged epidermal growth factor receptors (EGFR/ErbB1) in two cancerous cell lines (HeLa and A431) and one normal endothelial cell line (MCF12A) with anti-EGFR antibody-quantum dot complex (Ab-Qdot585), as illustrated in Fig. 1. Fluorescent EGF (EGF-Qdot525) was used to activate the EGFRs. Using this labeling scheme, we were able to investigate various correlated movements of EGFR species by selecting either a pair of liganded and unliganded EGFR or a pair of liganded EGFR complexes [7].

Although single-molecule optical tracking can be used to probe the microscopic environments and fluctuations faced by receptor proteins in a living cell, this information is embedded within large amounts of data. We developed a data visualization scheme to help researchers identify critical processes from single-molecule trajectories. The visualization scheme starts with the calculation of the local mean-square displacements $\overline{R^2(t)}$ of single-molecule trajectories as illustrated in Fig. 2. $\overline{R^2(t)}$ can be used to quantify the way in which a receptor molecule diffuses in its environment. We also prepared a normalized variance $V(R^2(t)) = \sigma^2_{R(t)^2} / [\overline{R^2(t)}]^2$ with $\sigma_{R^2(t)}$ denoting the variance of $\overline{R^2(t)}$ to reveal information about the nature of
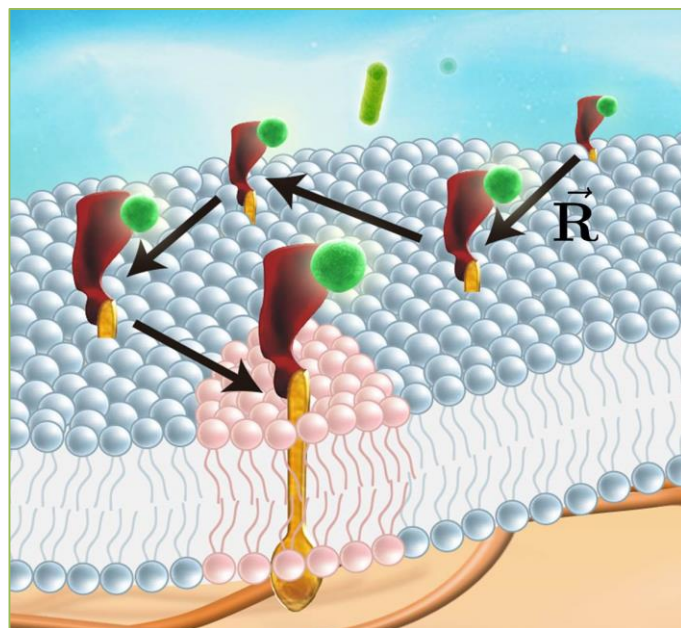
the interaction between a receptor protein and its environment [6]. Fig. 3 presents the histograms of $\overline{R^2(t)}$ and $V(R^2(t))$ data in a 2D contour plot for Ab-Qdot585-EGFR in live A431 cells at rest, single liganded EGF-Qdot525-EGFR, and dual liganded EGF-Qdot525-EGFRs in activated A431 cells, respectively. An attractive feature of this data visualization scheme is that when a molecule repeatedly visits a membrane domain, the characteristic $\overline{R^2(t)}$ and $V(R^2(t))$ of the lipid domain is imposed on the trajectories; this results in the formation of a peak at the corresponding position on the plot.

From the study of the three cell lines, we were surprised to find that unliganded EGFRs typically reside in non-raft regions and EGF-binding causes EGFR to translocate from the non-raft domain to the cholesterol-enriched domain.[7] This suggests that this ligand-induced translocation may be a common behavior. The total amount of cholesterol in the plasma membrane can be reduced using methyl-β-cyclodextrin (MβCD). We can also redistribute membrane cholesterol with nystatin while keeping the total amount of membrane cholesterol constant. Applying the two drugs to perturb the cells, we disclosed that the amount of membrane cholesterol can affect the stability of EGFR dimers in the plasma membranes. The EGFR dimers in the normal cells are more sensitive to changes in the local concentration of cholesterol than that in the cancer cells. Using our energetic model [6], we attributed the cholesterol-mediated interaction between receptors to from the protein-induced lipid ordering effect. Through the receptor-lipid interactions, a receptor
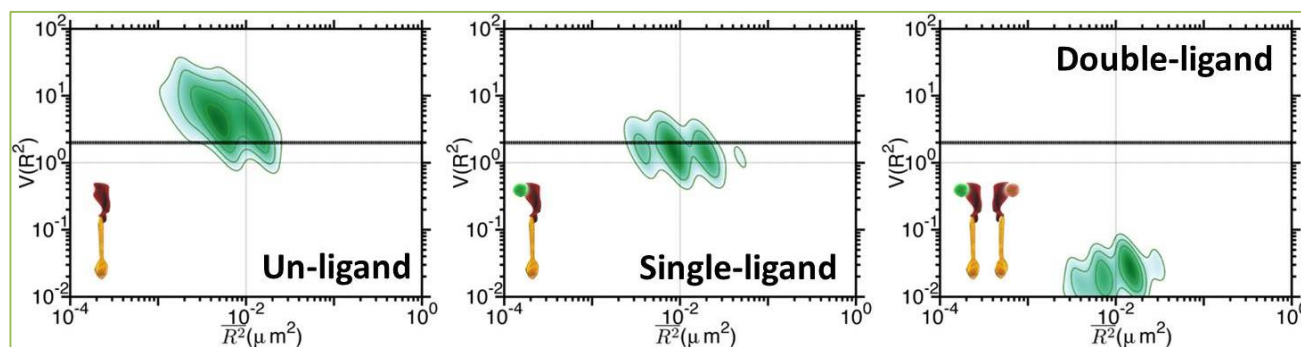
Figure 3. The histograms of $\overline{R^2(t)}$ and $V(R^2(t))$ are presented as 2D contour plots for (a) single-molecule Ab-Qdot585-EGFR in live A431 cells at rest, (b) singly liganded EGF-Qdot525-EGFR in activated A431 cells, and (c) liganded EGF-Qdot525-EGFR moving relative to a nearby EGF-Qdot525-EGFR companion.

protein can induce order in nearby raft lipids. The protein and ordered lipids can then be viewed as a dressed protein. The degree of induced order depends on the receptor-lipid interaction and the local amount of cholesterol.

The amount of membrane cholesterol is known to have an effect on ligand-induced activation of receptors [8-10]. Recent biochemical study has also revealed that the functions of SNARE proteins in the trans-Golgi network change with cholesterol concentration [11]. Live cells may evolve to use cholesterol concentration to regulate protein-protein interactions for survival.

Receptor dimerization is a common mechanism for signal transduction. The cholesterol-mediated protein-protein interactions discovered in our study may play a larger role in cells. Our method successfully captures dynamic interactions between receptors at the single-molecule level and the result can serve as the foundation to shed light on the way in which protein-protein interactions are regulated in plasma membranes *in vivo*.

Competing interests

The authors have declared that no competing interests exist.

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