Article Addendum

EGF induces rapid reorganization of plasma membrane microdomains

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Abbreviations: A488, alexa fluor 488; CTB, cholera toxin B-subunit; FLIM, fluorescence lifetime imaging microscopy; FRET, Förster resonance energy transfer

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The plasma membrane of mammalian cells is composed of a great variety of different lipids which are laterally organized into lipid domains. The segregation of lipids into domains has been studied in great detail in vesicles but domain formation of lipids in the plasma membrane of live cells is still unclear. We have previously used fluorescence lifetime imaging microscopy to study the colocalization of the receptor for EGF with the ganglioside GM1 and the GPI-anchored green fluorescent protein. Here we have used this technology to study the effect of EGF on the organization of GM1 in the plasma membrane. Our data show that stimulation of the cell with EGF induces rapidly a strong increase in colocalization of GM1 molecules, suggesting the formation of large lipid domains. These results support the notion that activation of EGFR signaling may result in the formation of signaling platforms.

The plasma membrane functions as a barrier separating the cells interior from the extracellular environment. In addition to its barrier function the plasma membrane also functions as a recruitment site for signaling processes, for instance by the binding of inositol lipids to PH-domain containing signaling molecules as the serine/threonine kinase Akt.¹,² The membrane is composed of a large variety of different lipids of which some have a clear structural function (phospholipids), while others can act as first and second messenger for signaling processes, preventing or enhancing favorable conformations for signaling. This view is supported by the ability of EGFR to directly bind the ganglioside GM1, which caused a 93% reduction of cellular ganglioside levels.⁶ These cells display a highly reduced EGF binding although EGFR levels were unaffected. Also EGF-induced proliferation and migration were reduced, which correlated with reduced EGFR phosphorylation and Rho/Rac1 activation. How gangliosides exert their influence on the EGFR is far from clear. It has been suggested that the glycan head-groups can differentially affect the conformation of the ectodomain, preventing or enhancing favorable conformations for signaling. This view is supported by the ability of EGFR to directly bind the ganglioside gangliosides, depending on the glycosylation of the receptor.⁷

Although the biochemical analysis of lipid rafts in vitro has yielded a large amount of information, application of this approach to the cellular situation suffers from a number of limitations that frustrate reliable interpretations. Detergent extraction has the risk of artificial clustering of membrane components, gives no information on the spatial distribution of domains, and does not discriminate between different domains in a heterogeneous population. Moreover, the sensitivity of proteins for extraction varies with the type of detergent used (TX-100, Brij-98 or 96, Tween-20, octylglucoside), suggesting that the discrimination between Lₒ and L_D is more complex.⁵,⁸

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To study the orientation of the EGFR in the plasma membrane we have previously set up a microscopical approach.\(^9\) The putative submicron scale of membrane domains cannot directly be visualized in the light microscope, because the optical resolution limit, determined by the wavelength of light, does not allow discrimination of separate objects smaller than ~200 nm. The colocalization of the EGFR and components of lipid rafts was therefore investigated by Förster resonance energy transfer (FRET).\(^{10}\) FRET is a process in which energy is transferred from an excited fluorescent donor molecule to a fluorescent acceptor probe. It can be used to detect the 5–10 nm proximity of molecules. The extent to which FRET takes place is measured by means of fluorescence lifetime imaging microscopy (FLIM); the occurrence of FRET results in a reduction of the donor’s fluorescence lifetime. 

An important aspect in this approach concerns the conjugation of the fluorescent probes to the molecules of interest, i.e., EGFR and GM1. For the labeling of GM1 we have used the B-subunit of cholera toxin (CTB) directly conjugated to an Alexa Fluor probe. As a marker for the EGFR we have used nanobodies which are the single chain epitope-binding subunits from the heavy chain only antibodies from \textit{Llama glama}.\(^11\) Besides their small size (~15 kDa), nanobodies can be selected using phage display for their high affinity binding to the ectodomain of the EGFR.\(^11\) Likewise, we generated a number of anti-EGFR nanobodies that neither compete for EGF-binding nor activate the EGFR, and which binding characteristics are not affected by conjugation of the fluorescent probe.\(^9\) Our results demonstrated the presence of GM1 in the proximity of the EGFR. However, another typical lipid raft resident molecule, a GPI-anchored green fluorescent protein did not colocalize in the resting cell with the EGFR. Interestingly, the colocalization of GM1 with GPI-GFP appeared to be cholesterol-dependent while the colocalization of the EGFR with GM1 was independent of cholesterol. These results suggest the presence of different classes of lipid raft in the resting cell. 

Stimulation of EGF signaling did not affect the colocalization of EGFR with GM1. By contrast, EGFR activation induced the colocalization of the EGFR with GPI-GFP, suggesting the coalescence of different lipid rafts upon receptor activation. To further analyze the effect of EGF on the organization of lipid rafts we analyzed the nanoscale colocalization of GM1 during EGF signaling. FRET efficiency between differentially labeled CTB molecules was analyzed in time by measuring the fluorescence lifetime of the donor probe (Fig. 1). In the resting cell no significant colocalization was observed between CTB-A488 (donor) and CTB-A594 (acceptor). Already after 1 minute of EGFR activation a significant increase in FRET was observed between the CTB molecules demonstrating an increase in proximity of GM1 lipids. Such data suggest that EGF induces the formation of larger lipid rafts, which may lead to alterations in the inner leaflet of the plasma membrane as well. Evidence for such transbilayer effects have been demonstrated in vitro using asymmetric planar bilayers.\(^12\) Considering the more effective activation of the PH-containing serine/threonine kinase Akt within lipid microdomains,\(^1,2\) the EGF-induced changes in the organization of the plasma membrane may result in the formation of signaling platforms, which represent highly efficient signaling sites in the plasma membrane.

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