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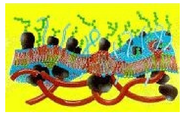
A Large-scale Investigation Of The Cellular Plasma Membrane Nanostructure With Native, Genetically Modified, Membrane Proteins As Molecular Reporters



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ABSTRACT

We are using genetic engineering to permanently annotate single native membrane proteins in live mouse embryo fibroblasts with short biotin ligase acceptor peptide sequences that are specifically biotinylated with the bacterial enzyme biotin ligase. Thus annotated native membrane proteins will be used as molecular reporters of the plasma membrane nanostructures. This will create a maximally versatile cell based system which will enable 1) in vivo studies of native membrane proteins at physiological expression levels with any biotin specific probe, 2) efficient scale-up of current studies from typically only a few membrane proteins to hundreds of membrane proteins all with a single probe type, and 3) efficient implementation of all current microscopy techniques. We anticipate that this project will reveal unprecedented detail about the organization and dynamics of native membrane proteins and of the plasma membrane. This project will also, as an added benefit, help identify and characterize novel membrane proteins.

INTRODUCTION

The hypothesized existence of spatially enriched signaling platforms, also known as lipid rafts, in the cellular plasma membrane has generated much interest[1]. Yet, despite a decade long search, that started with the original definition of lipid rafts as the *in vivo* equivalent of the low buoyancy detergent resistant membrane (DRM) fractions, the existence of lipid rafts in intact plasma membranes has not been proven. Unfortunately, putative lipid rafts, in contrast to caveolae and clathrin-coated pits, are apparently featureless by transmission electron microscopy. As a result, their identification require either lipid or protein markers, all of which to date have been defined by the DRM composition. It is now thought that lipid rafts in cells are very small (≤ 50 nanometer diameter), very short lived (≤ 30 ms) or both.

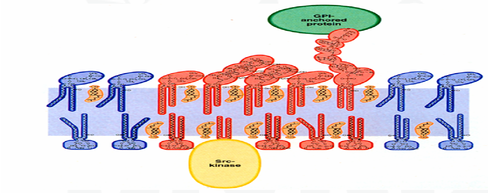

FIGURE 1: Classical schematic of hypothesized lipid rafts as a plasma membrane nanostructure enriched in cholesterol, GPI-anchored proteins and signaling molecules[2].

Our specific aim is to further investigate the cellular plasma membrane nanostructure, including the possible existence of morphology featureless nano-domains such as lipid rafts. In stark contrast to earlier work, we will not limit our studies to only a few protein markers that have been defined by the DRM composition. Rather, we will perform a large-scale random search for protein markers of morphology featureless plasma membrane nanostructures. We hypothesize that by gaining a better understanding of possible nanostructures in the plasma membrane, it will eventually be possible to design new and better drugs with increased disease specificity but decreased toxicity.

APPROACH

We expect that if plasma membrane nanostructures exist, there must also exist membrane proteins that preferentially reside within these nanostructures. To find such protein markers, we are genetically annotating random native membrane proteins in live mouse embryo fibroblasts (MEFs) by use of the Central Dogma (CD) tagging approach[3]. In this approach, custom guest exons containing mammalian splice acceptor and donor signals are inserted at random into genomic DNA with a retroviral delivery system. During gene splicing and protein translation, only modifications that are inserted in introns in the correct reading frame are detectable at the protein level. In addition, beacuse successful insertions can only occur within introns, these annotations are expected to minimally interfere with the native protein expression level and function.

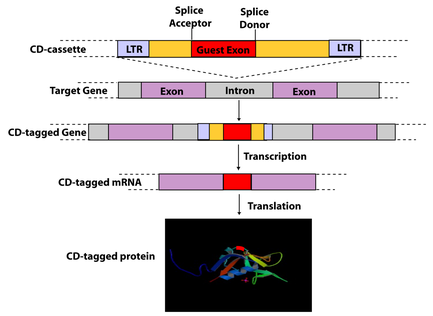

FIGURE 2: Principle of CD tagging. Upon insertion into an intron, the transcribed guest exon is spliced into mRNA by virtue of included splice acceptor and splice donor signals and subsequently translated resulting in a CD-tagged protein.

We have chosen to annotate single native membrane proteins in live mouse embryo fibroblasts (MEFs) with short biotin ligase acceptor peptide (BLAP) sequences that are specifically biotinylated with the bacterial enzyme biotin ligase. This system takes advantage of the extremely tight binding interaction of biotin and i.e. streptavidin (sAv). Furthermore, BLAP by itself is only 15 aa and is not naturally present on the cell surface of mammalian cells[4].

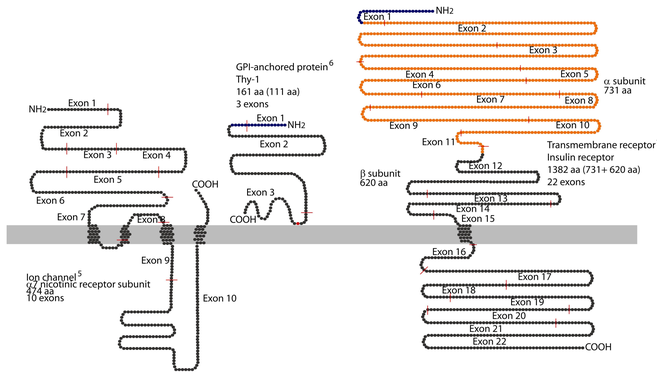

FIGURE 3: Gene structure of a few membrane proteins showing the location of the exon-exon junctions, and hence possible annotation sites, relative to the plasma membrane.

RESULTS AND FUTURE EXPERIMENTS

We have generated a guest exon which inserts the HA epitope and BLAP. This guest exon is 41 aa.

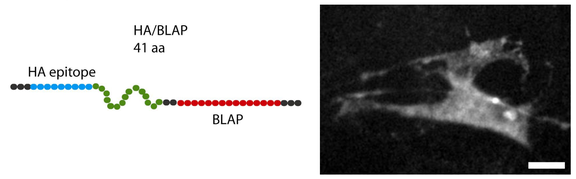

FIGURE 4: BLAP guest exon and an example of BLAP annotated membrane protein in live MEF detected with Alexa 488 anti-HA (Scale bar = 20 μm).

In the first instance, we aim to select between 50-100 single cells each expressing a distinct BLAP annotated membrane protein. Single cells will be grown into cell lines and annotated membrane proteins will be identified by DNA sequencing. We will subsequentally characterize each annotated membrane protein with respect to possible co-existence in featureless plasma membrane nanostructures primarily by single particle tracking (SPT) microscopy with fluorescent sAv conjugated quantum dots (Qdots). This will enable us to simultaneously characterize the annotated membrane proteins at both the expected relevant size (≤ 50 nanometer diameter) and time scales (≤ 30 ms) of lipid rafts.

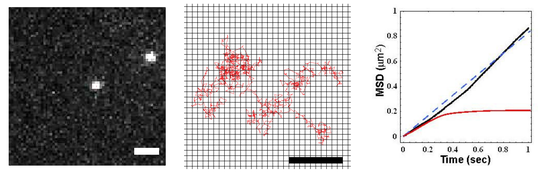

FIGURE 5: In SPT, a time series of the point spread function (PSF) of a single molecule is imaged (left) and the centroid of the single molecule is determined by curve fitting to the theoretical PSF resulting in sub-pixel spatial resolution. Resulting centroids are linked into trajectories (center) from which the mean squared displacement (MSD) is calculated (right). In the case of free random (Brownian diffusion (blue dash), MSD=4Dt where D is the diffusion coefficient. The presence of nanostructures will result in confined diffusion (red solid) in which the MSD reaches steady state at the area of the confining nano-domain. In this example, 5'-nucleotidase (CD73), a GPI-AP, on a live IMR-90 human fibroblast were specifically labeled with biotinylated monovalent Fab antibody fragments and sAv-Qdots and imaged at 189 Hz (left). In this case, the diffusion is Brownian with D = 0.2 μm^2/sec (Scale bars = 1 μm).

SUMMARY

Using this approach, we wil be able to annotate all plasma membrane proteins whose genomic DNA encodes for an exon-exon junction at a site which corresponds to an extracellular protein sequence including, novel membrane proteins, all of which could be important new drug targets. We will use these annotated membrane proteins as molecular reporters of the plasma membrane nanostructure. This will enable a large-scale comparative study of the plasma membrane nanostructure with at least a ten-fold greater number of protein markers than previously and with a single biotin specific probe type. While we plan to primarily use SPT with sAv-Qdots, this system is compatible with any biotin specific probe type or microscopy technique.

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