The Organisation of Rafts and its relation to the Endocytosis of the Cell Membrane

by

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DECLARATION

I hereby declare that this thesis is composed independently by me at the Raman Research Institute, Bangalore, under the supervision of Dr. Madan Rao. The subject matter presented in this thesis has not previously formed the basis of the award of any degree, diploma, associateship, fellowship or any other similar title in any other University.

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CERTIFICATE

This is to certify that the thesis entitled **The Organisation of Rafts and its relation to the Endocytosis of the Cell Membrane** submitted by Sarasij R C for the award of the degree of DOCTOR OF PHILOSOPHY of Jawarharlal Nehru University is his original work. This has not been published or submitted to any other University for any other degree or diploma.

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Madan, a very shy man, will be embarrassed to read this page. But I have to write it down, to be true to myself.

Having little acumen for abstract concepts of theoretical physics, I could not acquire my supervisor's formidable skills in manipulating cabalistic symbols on the blackboard! But there is one thing, I know, that has rubbed off on me — his "love of the game of knowledge", to borrow Stephen Jay Gould's words.

Voyaging with Madan, from the purgatory of physics to the wonderland of biology, and sharing in his child-like zeal for grasping every little quirk in the life of a cell, is an experience that has permeated the deepest folds and crevices of my subconscious self — the depths from which I draw joy and sustenance whenever I need to.

Rajat and Jitu ushered me in to the kingdom of the cell and I owe more to them than they will ever know. Soon I made friends with a veritable gang of cellular superstars — Pranav, Samit, Shefali, Sriram, Aadish, and — oh yes, Arjun! It is the unabashed delight in being educated by great friends, and not the outcome of the process in the form of published results, that lends such a magical charm to interdisciplinary research!

Sanat, Raghu and Yashodhan hammered the physics of liquid crystals into the head of a dunce. Without their help, much of the discussion of physical concepts in this book would have been little more than a garbled mumble.

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Roopa, Gayathri, Gautam, Kripa, Sudipto and Shiva have been keenly interested not only in my work but also in my future plans. To every one of them, and to the most beloved absent-minded professor, N Kumar, I am everlastingly grateful.

And before I leave, one more time — Thank you, Madan. From you I learnt to put people first.

Sarasij R C.
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SYNOPSIS

In this thesis we have studied the organisation of a class of proteins anchored to the exoplasmic (outer) leaflet of the cell membrane by means of a lipid chain that is preferentially associated with "rafts". Rafts are hypothesized lipid-based platforms on the cell membrane, never before observed, wherein proteins tethered to the membrane by the lipid anchor, glycosylphosphatidylinositol (GPI), are sequestered and subsequently internalised in the absence of any coat protein such as clathrin.

Our thesis is that the deformation of the plasma membrane that enables rafts to be endocytosed without the help of any coat protein is engendered by a special physical property of the membrane — its ability to transmit a chiral interaction over the entire raft through an ordered field of vectors decorating the membrane. Based on.this premise, we have proposed a novel way for a patch of a lipid bilayer membrane to form a bud.

BACKGROUND

GPI is a conserved glycolipid structure found across diverse phyla. GPI-anchored proteins serve diverse purposes — some are receptors, some transduce signals across the plasma membrane of mammalian cells, others are cell adhesion proteins, while still others play an important role in maintaining the distinction of the apical from the basolateral domain of epithelial cells. Many of these functions demand a high concentration of the protein and the raft has been implicated as a platform for the sorting and aggregation of the protein.

An important feature of the GPI anchor is its ability to confer specific intracellular trafficking routes to GPI- anchored proteins, distinct from the routes followed by trans-membrane anchored proteins. The raft has also been postulated as a determinant in this special pathway of internalisation of GPI-anchored proteins.

The usual definition of rafts is based on the existence of patches on the plasma membrane resisting the action of detergents on living cells. These patches are rich in cholesterol, glycosphingolipids and GPI-anchored proteins. Unfortunately, these patches do not indicate a ***priori*** organisation of rafts; they reflect an organisation induced on treatment with detergents, as has been recently reported in artificial lipid bilayers. We need a non-invasive probe of the organisation of rafts on living cells.

The phenomenon called FRET (fluorescence resonance energy transfer) furnished us with the desired probe.

If the emission spectrum of one molecule (the donor) overlaps with the absorption spectrum of another (the acceptor) then there is a probability of an excited donor transferring its energy non-radiatively to a neighbouring acceptor. The probability is vanishingly small if the separation between the fluorophores is more than two fold greater than the Forster radius — a length scale, characteristic of the pair of fluorophores, determined by (a) the degree of overlap of the emission spectrum of the donor with the absorption spectrum of

the acceptor, and (b) the relative orientation of the transition dipole moments of the two fluorophores. The Forster radius is typically smaller than 100 Å; therefore FRET or the extent of non-radiative transfer of electronic excitation is a probe of the nanoscale organisation of fluorescent molecules. So if we label the protein of our interest with a fluorescent dye then FRET can be used as a "spectroscopic ruler" to measure the proximity of the protein molecules in a raft.

But how do we assess the extent of non-radiative transfer between the dye molecules? There are two means. (A) If the acceptor and the donor belong to different species so that their emission spectra do not overlap then we can excite the donor by shining light of an appropriate frequency on it and from the light emerging from the dyes filter out only the contribution of the acceptor. In this manner we directly isolate the effect of spontaneous emission of the donor from the transfer of excitation to the acceptor. Let us call this method of detecting energy transfer heteroFRET. (B) To a good approximation the light emitted by a fluorophore is dipole radiation, the dipole of the radiation being aligned with the transition dipole moment of the molecule. For a given separation of the acceptor and the donor the probability of FRET is proportional to the electrostatic energy of interaction between the dipole moments of the pair of molecules. As a result, the dipole of the secondary radiation emitted by the acceptor is distributed over a much greater solid angle than that of the radiation spontaneously emitted by the donor. By measuring the depolarisation of the emergent light we can deduce the degree of FRET. We have used both the methods in our effort to study the distribution of GPI-anchored proteins in the raft.

Transmembrane proteins with tyrosine-based internalisation motif in their cytoplasmic domain, for instance, transferrin receptors (TfR) and low-density-lipoprotein receptors (LDLR), are internalised from the plasma membrane into endosomes via a well known mechanism — the clathrin-mediated endocytosis. Endocytosis not involving the clathrin coat, though equally important in most cell types, is a poorly understood process. GPI-anchored proteins are internalised by a clathrin- independent process into endosomes that are very poor in TfR and LDLR.

The first step in the process of internalisation is the invagination of a patch of the cell membrane in the form of a bud. We believe that, at least in the case of endocytosis not assisted by coat proteins, the first step is governed entirely by the mechanical properties of the patch of membrane — the line tension of the patch and the rigidity of the membrane (measured by the elastic modulus of bending deformations). Thus we look for ways in which a patch with sharply defined boundary on a fluid membrane can form a bud. In all that follows we assume that the patch assumes a conformation that minimises its free energy, in other words, the bud is in a *local equilibrium* state.

Budding can be induced by line tension — the membrane deforms in the shape of a bud in order to minimise its interfacial energy proportional to the length of its periphery

(which lies at the neck of the bud).

Budding can be induced by spontaneous curvature — owing to the asymmetrical distribution of lipids in the two leaves of the bilayer the membrane has an innate tendency to deform in the shape of a bud.

Budding can be induced by chirality. If one leaf of the bilayer in the patch is decorated by a vector field, and if the chiral interaction of the vector field be sufficiently strong, then the vectors at every point of the patch arrange themselves in the form of an Archimedes spiral — fanning out from the centre of the patch toward the periphery. The strength of the chiral interaction is strongest at the centre; therefore the patch can further decrease its energy by deforming in the shape of a bud — one centre of chirality being at the neck of the bud and the other at the opposite pole of the bud, the spiral fanning out from one pole and smoothly converging on the other.

In the next section we summarise the main results of the thesis.

RESULTS

A part of the population of GPI-anchored proteins on the cell surface exist in very dense clusters. By shining a pulse of exciting radiation on the protein labeled by a dye and monitoring the depolarisation of the emergent light with the lapse of time, we observe a component of depolarisation much too rapid to be accounted by the rotational diffusion of the protein. This rapid depolarisation is due to FRET. The separation of neighbouring proteins in the cluster is less than a Forster radius (70 Å).

The very dense clusters are very small, not containing more than four proteins. They are so small that upon labeling the protein with an even ratio of acceptors and donors belonging to different species, we fail to detect any heteroFRET signal.

20% to 40% of the GPI-anchored proteins on the cell membrane exist in clusters, the rest are isolated monomers not undergoing any FRET.

Depletion of cholesterol from the plasma membrane leads to the disintegration of a part of the clusters. Depletion of glycosphingolipids has no effect on the aggregation of the protein but facilitates the depletion of cholesterol.

Multiple GPI-anchored proteins can inhabit the same cluster.

The cluster resides in a milieu rich in sphingolipids, thus we have a super-organisation of clusters on the cell. The cluster is labile, freely exchanging its constituent proteins with other clusters in a super-organisation, but proteins in different super-organisations are not free to mix with one another.

We have studied the equilibrium conformations of a lipid domain, decorated by a vector field, surrounded by a fluid and structureless membrane. The lipid domain stands for a super-organisation on the cell membrane, while the vector field represents either one of the axes defining the geometrical orientation of a GPI-anchor, or one of the axes defining the structure of a condensed complex: a nanoscale molecular assembly observed

in artificial membranes formed by cholesterol and sphingolipids. Guided by monte carlo simulations, we have set up variational calculations to study the effect of chirality on the texture and shape of such a lipid domain.

Our most interesting finding has been that sufficiently strong chiral interaction can not only produce buds of small size (50 nm diameter) but can also prevent the aggregation of the super-organisations into large patches on the surface of the cell. The budding induced by chirality therefore preempts the budding induced by a decrease in interfacial tension.

Sufficiently strong chiral interaction likes to arrange the vectors in a raft in the form of a helix wrapping a tube, the tube being capped by a spherical bud. Such flask-like invaginations are indeed observed on the cell, being known as caveolae. Since the composition of the membrane forming caveolae is very similar to that of rafts, we are tempted to propose chirality as a mechanism for the production of caveolae.

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