Characterization of endocytic recycling and sorting of glycosylphosphatidyl inositol-anchored proteins in fibroblastic cell lines.

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Abstract:

The majority of glycosylphosphatidylinositol (GPI)-anchored proteins enter mammalian cells via the clathrin-, and dynamin-independent GPI-enriched early endosomal compartment/ Clathrin-independent carrier endocytic (GEEC/ CLIC) pathway. Using artificially lipid-anchored proteins, we have examined how the 'anchor' structure of these proteins affects their endocytic trafficking after initial internalization from the plasma membrane. In the first part of this thesis, I show that soluble proteins, anchored to cell-inserted saturated and unsaturated phosphatidylethanolamine (PE)-polyethylene glycol (PEGs), distribute within the cell identical to the GPI-anchored folate receptor α, all of which colocalize significantly with markers of the ERC, but not with markers of the late endosomes/lysosomes. Soluble proteins, tightly bound to saturated PE-PEG anchors in CHO cells recycled back to the plasma membrane with a half life ($t_{1/2}$) of 25-30 minutes, similarly to the folate receptor α. By contrast, proteins bound to unsaturated PE-PEG anchors recycled back to the plasma membrane with a $t_{1/2}$ of 7-9 minutes, similarly to the transferrin receptor (TfR). These results support a potential role for membrane rafts in differential endocytic recycling of GPI-anchored proteins from transmembrane proteins (such as TfR) in these cells.

Earlier reports indicated that GPI-anchored proteins are targeted to late endosomes in BHK cells, in contrast to CHO cells, and suggested that this phenomenon is due to differential association with membrane rafts in each cell line (Fivaz et al., 2002; Sabharanjak et al., 2002; Kalia et al., 2006). In the second part of this thesis, I studied the possible role of membrane rafts in this differential endocytic sorting using our artificially lipid-anchored proteins in BHK cells. Our results showed that, in BHK cells, endocytosed proteins artificially tethered to either saturated or unsaturated PE-PEG 'anchors' are again distributed within the cell essentially identical to the GPI-anchored folate receptor, and differently from internalized transferrin receptor. These preliminary findings indicate that, contrary to previous suggestions, the endocytic trafficking of GPI-anchored proteins in BHK cells is not dependent on their potential to associate with membrane rafts.

Résumé:

La majorité des protéines liées au glycosylphosphatidylinositol (GPI) entrent dans les cellules mammifères par le biais du compartiment de l'endosome précoce par l'intermédiaire d'une voie indépendante des clathrines et dynamines enrichie en GPI et du transporteur endocytosomique indépendant des clathrines (GEEC/ CLIC). En utilisant des protéines attachées artificiellement à des lipides, nous avons étudié la manière avec laquelle ces « ancres lipidiques » influencent le trafic endocytosomique des protéines suite à l'internalisation initiale à partir de la membrane plasmique. Dans la première partie de cette thèse, je démontre que les protéines solubles, lorsque attachées à du phosphatidylethanolamine (PE)-polyethyleneglycol (PEGs) directement injecté dans les cellules de manière saturante ou non-saturante, suit une distribution intracellulaire identique à celle du récepteur du folate α lié au GPI, c'est-à-dire qu'ils colocalisent presque parfaitement avec des marqueurs de la compartiment de recyclage d'endocytose (CRE), mais non avec ceux de l'endosome tardif/lysosomes. Les protéines solubles liées solidement aux ancres de PE/PEG saturés dans les cellules CHO se recyclent vers la membrane plasmique avec une demi-vie ($t_{1/2}$) de 25-30 minutes, comme c'est le cas pour le récepteur folate α. Par contre, la $t_{1/2}$ des protéines liées au PE/PEG non-saturé n'est que de 7-9 minutes, tel qu'observé pour le récepteur de la transferrine (TfR). Ces résultats suggèrent un rôle potentiel pour les 'radeaux' membranaires dans le recyclage différentiel des protéines liées au GPI à partir de protéines transmembranaires (comme TfR) dans ces cellules.

Des publications antérieures ont indiqué que, contrairement à ce qui a été observé dans les cellules CHO, les protéines liées au GPI sont ciblées vers l'endosome tardif dans les cellules BHK et suggère donc que ce phénomène est dû à leur association différentielle aux « radeaux » membranaires dans chacune des lignées cellulaires (Fivaz et al., 2002; Sabharanjak et al., 2002; Kalia et al., 2006). Dans la seconde partie de cette thèse, j'ai étudié le rôle potentiel des 'radeaux' membranaires dans le classement endocytosomique des protéines dans les cellules BHK en utilisant notre système artificiel de fixation de lipides aux protéines. Les résultats démontrent que les protéines endocytosées et liées artificiellement PE-PEG saturé ou insaturé sont distribuées de manière quasi-identique au récepteur du folate lié au GPI et de manière différente du récepteur de la transferrine. Ces découvertes préliminaires indiquent que, contrairement à ce qui a été suggéré précédemment, le trafic endocytosomique des protéines liées au GPI dans les cellules BHK n'est pas dépendant du potentiel d'association avec les « radeaux » membranaires.

Preface:

This thesis is based on a manuscript in preparation, on which I am a first author.

Chapter2:

Mohammad Refaei, Rania Leventis, and John R. Silvius. (2010-to be submitted). **Assessment of the Roles of Ordered-lipid Microdomains in Intracellular Sorting of Endocytosed Glycosylphosphatidylinositol-anchored Proteins in Mammalian Fibroblasts.**

Authors' contributions: Dr. J. R. Silvius has supervised this project, prepared PEG-PE Lipids for all of my experiments, provided advice on experimental protocols, acquired micrographs on the confocal microscope for the colocalization part, and contributed to the writing and preparation of the manuscript. Rania Leventis has helped with fluorescent labelling of DHFR, majority of colocalization experiments in CHO and BHK cell lines, prepared stably transfected BHK-21 cells with construct of the human Folate Receptor, and maintained tissue culture work for all cell lines used in this project (CHO-FRa-Tb1, BHK-21, and BHK21-R32). I performed the majority of experiments in this project, including the purification and fluorescent labelling of DHFR protein, recycling experiments in CHO, and the colocalization experiment in CHO and BHK cells.

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Abbreviations:

AMF	Autocrine motility factor
ATP	Adenosine triphosphate
BHK	Baby Hamster kidney cells
CCP	Clathrin coated pit
CCV	Clathrin coated vesicle
CHO	Chinese Hamster Ovary cells
CIE	Clathrin-independent endocytosis
CI-MPR	Cation-independent mannose-6-phosphate receptor
CLIC	Clathrin-independent carriers
CME	Clathrin mediated endocytosis
CTxB	Cholera toxin subunit B
D-AKAP2	Dual-specificity A-Kinase anchoring Protein 2
DHFR	Dihydrofolate reductase
di 12:0	didodecyl-PE-PEG
di 16:0	dipalmitoleoyl-PE-PEG
di 18:1	dioleoyl-PE-PEG
DNP	Dinitrophenol
DRM	detergent-resistant membrane
EE	Early endosome
EEA	Early Endosomal antigen
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EM	Electron microscopy
ER	endoplasmic reticulum
ERC	Endocytic recycling compartment
ESCRT	Endosomal sorting complex required for transport
EtNP	ethanolaminephosphate
Fra	Folate receptor a
FRAP	Fluorescent recovery after photobleaching
GEEC	GPI-anchored proteins enriched early endosomes
GFP	Green fluorescent protein
GLcN	Glucosamine
GLcNac	beta-N-acetylgalactosamine
GPI	glycosylphosphatidylinositol
GPI-GnT	GPI-N-acetylglucosamine transferase
GPI-MT	GPI-mannosyltrasferase
HIV-1	Human Immunodeficiency virus-1
IL-2R-alpha	Interluekin-2 receptor alpha subunit
IL-2R-beta	Interluekin-2 receptor beta subunit

KSHV	Kaposi's sarcoma-associated herpes virus
LE	Late endosome
MHC I	Major Histocompatibility Complex I
MTX	Methotrexate
NBD	N-(7-nitro-2,1,3-benzoxadiazol-4-yl)aminohexanoyl
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PE-PEG	phosphatidylethanolamine-polyethylene glycol
PI	phosphatidylinositol
PI3K	Phosphatidylinositol-3-kinase
PI-PLC	phosphatidylinositol-specific phospholipase C
PM	Plasma membrane
PNH	paroxysmal nocturnal hemoglobinuria
PS	Phosphatidylserine
SE	Sorting endosome
TfR	Transferrin receptor
TGN	Trans-Golgi network
TNP	Trinitrophenol
TTP	Transferrin receptor trafficking protein
UDP	Uracil Diphosphate

Chapter1: General Introduction

1.0 General Introduction

1.1 Eukaryotic Membranes

1.1.1 The Plasma Membrane

The plasma membrane is the boundary of all living cells. It serves as a selective barrier by which the cell is able to import only essential chemicals and nutrients, and to retain newly synthesized nutrients while excreting waste products to the surroundings (Alberts et al., 2002). It is also important for a number of other cellular functions such as ATP production (Brand and Murphy, 1987), cellular signalling (Werlen and Palmer, 2002) and cellular adhesion (Gumbiner, 1996).

The plasma membrane in animal cells comprises phospholipids, sphingolipids, membrane proteins, and sterols (notably cholesterol).The detailed lipid as well as protein composition of the plasma membrane varies from one cell type to another, and from one organism to another, but certain structural trends are observed despite the absolute differences in representation of each component (van Meer, 1989).

***Plasma membrane Lipids*.** The most abundant membrane lipids in yeast and animal cells are phospholipids. They are made up of a polar head group linked to a glycerol backbone with two non-polar alkyl or fatty acyl chains attached to the glycerol 1- and 2-hydroxyl groups, phospholipids are a large group of lipids which can vary in their head groups and the length and the level of saturation of their fatty acid chains (reviewed in (van Meer, 1989). The fact that membrane phospholipids are amphiphilic, with hydrophobic and hydrophilic portions whose cross-section areas are on average equal, causes them to self-assemble into a bilayer when dispersed in an aqueous environment. This provides the lowest thermodynamic free energy state (Singer and Nicolson, 1972).

Sphingolipids, in contrast to glycerophospholipids, have their fatty acids linked via amide bonds to a long-chain sphingoid base (reviewed in (Barenholz and Thompson, 1980; Hakomori, 1986; Stults et al., 1989; van Meer, 1989). A large

proportion of sphingolipid acyl chains are long and saturated species (Sandhoff, 2010).

Cholesterol is the major membrane sterol in animal cells, and can comprise up to 40% of mammalian cell plasma membrane lipid on a molar basis (van Meer, 1989; Edidin, 2003). It is important for modulating the fluidity of the plasma membrane at different temperatures and lipid compositions. For example, cholesterol is able to intercalate between highly ordered acyl chains, increasing the lipid fluidity and suppressing formation of the gel phase in model membranes. Conversely, cholesterol tends to increase the order of disordered acyl chains, as in liquid crystalline phases, filling in gaps in bilayer packing and decreasing the overall lipid fluidity (Edidin, 2003). It also helps to regulate the permeability of molecules through the plasma membrane (Rietveld and Simons, 1998; Alberts et al., 2002; Ohvo-Rekila et al., 2002; Silvius, 2003). The distribution of different lipids is asymmetrical between the two leaflets of the PM. Sphingomyelin, glycosphingolipids, and PC are found preferentially in the exoplasmic leaflet. By contrast, PS and PE are concentrated in the cytoplasmic leaflet. The distribution of cholesterol between the two leaflets of the PM remains less clear (Allan and Walklin, 1988; van Meer, 1989; Mukherjee and Maxfield, 2000).

Plasma membrane proteins. The specific functions of biological membranes are determined largely by the distinct protein composition of each type of membrane (Alberts et al., 2002). Membrane proteins typically equal or exceed lipids on a mass basis, and can reach up to a 4:1 mass ratio with respect to phospholipids (Korn, 1969; Singer and Nicolson, 1972; Bretscher and Raff, 1975; Bretscher, 1985; Engelman, 2005). They can associate with the plasma membrane of mammalian cells in a number of ways. Transmembrane proteins can span the membrane with hydrophobic amino acid side chains facing lipid hydrocarbon residues in the hydrophobic membrane core. Such proteins can span the membrane once or multiple times, via alpha-helices or multiple beta-strands that form a beta-barrel structure (Buchanan, 1999). Other proteins (peripheral membrane proteins) associate with membranes via noncovalent interactions with

the membrane bilayer surface or via binding to other membrane proteins. (reviewed in (Cross, 1990; Edidin, 1992; Driscoll and Vuidepot, 1999).

1.1.2 Endosomal Membranes:

The membranes of various intracellular compartments possess distinct lipid and protein compositions. This implies specific sorting and regulated transfer of material between these different compartments (van Meer, 1989). Here, I will focus primarily on endocytic compartments and the trans-Golgi network in animal cells.

***Endosomal membrane lipids*.** Most membrane lipids are synthesized primarily in the ER, except for sphingolipids and PE which are synthesized in the Golgi and in part on the inner mitochondrial membrane respectively (Jelsema and Morre, 1978; Pagano and Sleight, 1985; Reinhart et al., 1987; Vance and Ridgway, 1988). Vesicular transport takes place to distribute these lipids to different destinations. Evaluation of endosomal lipid compositions has been challenging due to the lack of proper techniques to isolate pure endosomal membrane fractions (van Meer, 1989). However, the composition of primary endocytic vesicles and endosomal compartments, as well as the TGN, appears to be similar to that of the PM. These compartments were shown to contain high levels of cholesterol, sphingomyelin and PS as compared to other intracellular organelles (Brotherus and Renkonen, 1977; Dickson et al., 1983; Luzio and Stanley, 1983; Helmy et al., 1986; Urade et al., 1988). For example, studies on rat hepatocytes revealed that the lysosomal membrane has a slightly higher concentration of cholesterol, sphingomyelin and glycosphingolipids than the plasma membrane (Wherrett and Huterer, 1972; Henning and Stoffel, 1973; Brotherus and Renkonen, 1977). Furthermore, lysobisphosphatidic acid is only found in late endosomes and lysosomes (Brotherus and Renkonen, 1977).

The asymmetric transmembrane distribution of endosomal membrane polar lipids and proteins is maintained throughout the endocytic pathway, where the extracytoplasmic leaflet of the PM becomes the luminal leaflet in an endosomal

compartment/vesicle (Pagano and Sleight, 1985; Koval and Pagano, 1989). However, cholesterol can move rapidly between the two leaflets of these membranes (Leventis and Silvius, 2001).

***Endosomal Membrane proteins*.** It is difficult to define endosomal compartments biochemically, because they are dynamic and no one molecule (cargoes, receptors, small GTPases) may reside permanently in a given compartment. However, endosomal compartments and organelles can be identified by a selective concentration of particular protein and lipid markers, which may be found to lesser extents in other compartments. These markers will be discussed in more detail in later sections.

1.1.3 The Fluid Mosaic Model:

Initial attempts to describe how polar lipids, sterols, and proteins are organized in biological membranes resulted in the Fluid Mosaic Model (Glaser et al., 1970; Singer and Nicolson, 1972). This model envisages membrane proteins (integral & peripheral) as diffusing freely in a two- dimensional fluid lipid bilayer (Singer and Nicolson, 1972). Being consistent with thermodynamic considerations and with the amphiphilic nature of membrane lipids and many membrane proteins, this model was widely embraced by the scientific community, and served as the basis for explaining many functional properties of membranes. However, it was soon recognized that not all membrane proteins diffuse freely and that there is a more complex level of organization, within and adjacent to membranes, that restrains their mobility (Jacobson et al., 1995). For example, some integral membrane proteins, such as the transferrin receptor (TfR), were shown to be transiently confined to microdomains of 300-600 nm for at least 30 sec as revealed by the SPT technique (Kusumi et al., 1993; Ghosh and Webb, 1994; Sako and Kusumi, 1994; Ritchie et al., 2005; Kusumi et al., 2010). It was later shown that interactions between the cytoskeleton and membrane proteins' cytoplasmic domains restrict the proteins' movement and enforce a non-random organization of many membrane proteins. This concept was incorporated in the 'membrane-skeleton fence' model which was developed initially in studies on erythrocytes

(Edidin, 1992). Since its early conception, the Fluid Mosaic Model has been modified multiple times in order to account for such new emerging findings (Jacobson et al., 1995).

Even in their original model, Singer and Nicholson (1972) did not fail to acknowledge that "short-range order" could exist in membranes in the form of microdomains of distinct composition. They even mentioned that the thickness of the plasma membrane could vary from one region to another depending on the local lipid and protein composition (Singer and Nicolson, 1972). This anticipated later proposals of membrane raft and other microdomains and their potential functional importance.

1.2 Membrane raft microdomains:

'Membrane raft' microdomains are proposed to exist as segregated, non-random regions of membranes with distinct physical, chemical and functional characteristics based on a local "liquid-ordered" state of the lipids (described more fully below in section on Model systems). A more recent, widely adopted definition for membrane rafts is as follows (Pike, 2006): "Membrane rafts are small (10-200nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions." The term "lipid rafts" was dropped in favour of "membrane rafts" because it is clear that more players than just lipids are involved in the formation of these structures. The concept has been surrounded with controversy ever since evidence for the existence of rafts in biological membrane was first reported (van Meer and Simons, 1982; Thompson and Tillack, 1985; Simons and van Meer, 1988; van Meer and Simons, 1988).

The first proposal that membranes might contain specialized membrane raft microdomains was advanced to explain the differential sorting of sphingolipids and glycosylphosphatidyl-anchored proteins (GPI-anchored proteins) between the

apical and basolateral plasma membrane domains of epithelial cells (Edidin, 2003). The apical epithelial membrane is enriched in glycosphingolipids and GPI-anchored proteins, unlike the basolateral side (Rodriguez Boulan and Sabatini, 1978; Lisanti et al., 1989). Because GPI-APs are anchored in the membrane via a lipid moiety, Samson and Van Meer rationalized that both glycosphingolipids and GPI-anchored proteins are sorted by the same mechanism, by selective transport of domains containing both species, from the Golgi to the apical plasma membrane (van Meer and Simons, 1982; Simons and van Meer, 1988; van Meer and Simons, 1988).

Subsequently, researchers were able to develop a few experimental criteria to help distinguish lipid rafts from the rest of the bilayer membrane. Brown and Rose (1992) showed that GPI-anchored proteins from apical epithelial plasma membrane are resistant to Triton X-100 solubilisation at 4^{o}C. Density-gradient fractionation of the detergent-resistant membrane (DRM) fraction remaining after low-temperature Triton extraction of membranes revealed that GPI-anchored proteins are present in low-density fractions enriched in lipid (especially glycosphingolipids). Cholesterol was also shown to be enriched in these DRMs, and one group found that 100% of GPI-anchored proteins become cold-triton-soluble upon treatment of membranes with saponin, a cholesterol binding compound (Cerneus et al., 1993).

Functional evidence that suggests the possible existence of lipid rafts in mammalian plasma membrane has also been reported. For example, a few studies have shown marked association between ligated receptors, such as the epidermal growth factor receptor, phosphatases, and heterotrimeric G-proteins in DRM fractions. It has been proposed that membrane rafts might serve as a docking platform to form these signaling complexes (Sheets et al., 1999; Simons and Toomre, 2000; Pierce, 2002; Werlen and Palmer, 2002). Other studies have shown that cross-linking of GPI-anchored proteins in T-lymphocytes can activate signaling cascades that eventually lead to cellular activation (Stefanova et al., 1991; Werlen and Palmer, 2002; Edidin, 2003). It was suggested that cross-

linking enhances the association of these GPI-anchored proteins with membrane rafts, inducing the formation of signaling platforms (or "signalosomes") within the membrane that eventually activate T-lymphocytes. Many researchers have challenged at least the simple models originally proposed for membrane rafts as signaling platforms, and some have questioned the existence of rafts altogether (Munro, 2003; Shaw, 2006).

1.2.1 Biochemical Studies of membrane rafts composition

Results from quantitative high performance thin-layer chromatography of DRMs isolated from epithelial cell plasma membranes revealed a lipid composition of 34:36:32 (molar proportions) of glycerophospholipids:sphingolipids:cholesterol (Brown and Rose, 1992). It was estimated that the DRM fraction contained 26%, 96% and 26% respectively of the total glycerophospholipids, sphingomyelin, and cholesterol found in the plasma membrane as a whole (Brown and Rose, 1992; Edidin, 2003). The acyl chains of sphingolipids and glycerophospholipids found in DRMs are longer and more saturated in general than the average for glycerophospholipids present in the plasma membrane. This property is crucial for optimal acyl chain packing and formation of lipid rafts (Hansen et al., 2001; Massey, 2001; Edidin, 2003).

Cholesterol is another important component of membrane rafts. Without cholesterol, saturated phospholipids and sphingolipids would form segregated gel phases within the membrane which would be physiologically non-functional. As noted above, cholesterol is able to intercalate into the bilayer and loosen the tight packing of the acyl chains (Miao et al., 2002; Edidin, 2003; Simons and Vaz, 2004). This results in a relatively ordered but dynamic organization which is thought to characterize membrane rafts.

Some classes of membrane proteins, such as GPI-anchored proteins, are preferentially found in membrane rafts, while many transmembrane proteins are excluded (Fridriksson et al., 1999; Chatterjee and Mayor, 2001). Folate receptor α (Sabharanjak and Mayor, 2004), alkaline phosphatase (Brown and Rose, 1992),

FcεR1 receptor (Sheets et al., 1999), src-family kinases (Stefanova et al., 1991) the T-cell antigen receptor (TCR) (Werlen and Palmer, 2002) and the B-cell antigen receptor (Pierce, 2002) have all been shown to co-isolate with DRMs (Edidin, 2003; Simons and Vaz, 2004).

1.2.2 Model Systems:

Intensive investigations of the biochemical and biophysical characteristics of lipid bilayers have been undertaken to clarify the possible physical origins of membrane rafts and their potential implications in various cellular functions. Studies in model membranes initially suggested the existence of two main types of membrane lipid phases: an ordered crystalline solid (or gel s_o) phase and a disordered liquid (l_d) phase. The former has been observed in bilayer vesicles made exclusively of sphingolipids or saturated glycerophospholipids. Bilayer vesicles made of a mixture of unsaturated PC and saturated PC or sphingomyelin exhibits segregated solid (s_o) and fluid (l_d) phases (Simons and Vaz, 2004; Silvius, 2005). However, more recent studies done on membrane models that included cholesterol, making them more similar to biological membranes, revealed the existence of a new intermediate phase, namely the liquid ordered (l_o) or raft-like phase (Ipsen et al., 1987; Ipsen et al., 1989). Cholesterol imposes conformational, but not translational ordering on the phospholipids/proteins in this phase, which in turn allows the membrane rafts to diffuse freely in the plane of the membrane and gives enough motional freedom for proteins to carry out their physiological function (Simons and Vaz, 2004).

1.2.3 Properties and Potential Physiological Importance.

Membrane rafts' main proposed role is to segregate membrane components within cell membranes (the plasma membrane as well as membranes of some intracellular organelles) (Pike, 2006). The generally accepted estimate of their size can be between 10-200 nm, they may exist for times range from microseconds to seconds and at any moment there may be as many as 10^5-10^6 such units within the plasma membrane of a given cell (Pralle et al., 2000; Simons and Vaz, 2004; Pike,

2006). These relatively small microdomains have not been observed to coalesce spontaneously in membranes of living unperturbed cells to form macroscopically visible domains, possibly because of thermodynamic restrictions. However, upon oligomerization or cross-linking of some of their components, lipid rafts tend to cluster into larger domains that are visible under a light microscope (Simons and Vaz, 2004). The potential existence of 'raft' domains in intracellular membranes has been investigated less extensively than for the plasma membrane. However, evidence has been reported for the presence of such domains in endosomal and trans-Golgi membranes (Pike, 2006).

Membrane rafts have been implicated in a number of cellular functions, including membrane trafficking, signal transduction, and cell polarization (Edidin, 2003). For example, the glutamate and EGF receptors were inactivated by cholesterol depletion from the plasma membrane or by reconstitution into membranes lacking cholesterol. These proteins were shown to take on two different conformations; the activated one is only observed when they associate with membrane rafts (Simons and Vaz, 2004). Similarly, heterotrimeric G proteins and S-acylated tyrosine kinases of the src family were shown to require association with membrane rafts in order to function correctly in their physiological signaling cascades (Stefanova et al., 1991; Simons and Toomre, 2000; Werlen and Palmer, 2002; Edidin, 2003). As mentioned earlier, membrane raft microdomains have been proposed to sort sphingolipids and GPI-anchored proteins from the Golgi to the apical surface of epithelial cells (Rodriguez Boulan and Sabatini, 1978; Lisanti et al., 1989). Similarly, it has been suggested that the GPI-anchor, and hence association with membrane rafts, sorts GPI-anchored proteins like the folate receptor α into a distinct endocytic pathway as discussed later (Sabharanjak and Mayor, 2004). From a pathophysiological perspective, some studies have reported that influenza viruses require clustering of two glycoproteins (hemagglutinin and neuraminidase) in lipid rafts in order to form their envelopes (Simons and Vaz, 2004). There are some studies that suggest that HIV-1 has evolved such that it depends on the host cell membrane rafts to support its propagation during multiple stages of its replication cycle (Campbell et al., 2001).

1.2.4 Open Questions.

Despite interesting reports like those noted above, the detailed nature and even the existence of membrane rafts remain issues of considerable controversy. Much of our early picture of membrane rafts was based on studies done on model membranes. There are significant differences between such raft-like model systems and biological membranes (Edidin, 2003). One such difference is the constant influx of materials into and out of biological membranes through processes such as membrane trafficking. Moreover, it has been virtually impossible to reproduce the role of subcortical cytoskeleton in maintaining an inhomogeneous lateral distribution of membrane proteins and potentially membrane lipids (Silvius, 2006). It is still not clear how membrane rafts are coupled across the two leaflets of the cell plasma membrane; unlike model membranes with typically identical compositions in both leaflets, physiological membranes exhibit marked asymmetry in the lipid composition of the two leaflets (Jacobson et al., 1995; Edidin, 2003).

Defining lipid rafts by biochemical means has produced additional uncertainties. Hansen and colleagues (2001), for example, found no effect of cholesterol-binding agents on the insolubility of GPI-anchored proteins in Triton X-100 at $4^{o}C$, while Cerneus and colleagues (1993) reported that GPI-anchored proteins become almost completely soluble after treating the cells with the cholesterol-binding compound saponin (Rothberg et al., 1990; Cerneus et al., 1993; Hansen et al., 2001). There may be additional factors besides cholesterol involved in the formation and clustering of lipid rafts in live cells, which may complicate efforts to link biochemical and functional data to elucidate the properties of 'rafts'.

The cytoskeleton is another factor that regulates the heterogeneity of the plasma membrane. It is well established that the subcortical cytoskeleton interacts with membrane proteins to carry out processes such as cellular fission, formation of focal adhesions, and formation of filopodia/lamellipodia/pseudopodia (Condeelis, 1993; Burridge and Chrzanowska-Wodnicka, 1996; Doherty and McMahon, 2008). More generally, association of membrane proteins with the cytoskeleton

can restrict their free diffusion and thereby induce lateral heterogeneity in the distribution of proteins in the plasma membrane. There is substantial evidence and even more speculation, that subcortical actin microfilaments may regulate the clustering and dynamics of GPI-anchored proteins (Doherty and McMahon, 2009; Park and Cox, 2009). However, it is not understood how this phenomenon might relate to the formation of liquid-ordered 'raft' domains. One group has shown that caveolin-1 positive microdomains (a subset of 'raft' domains) are regulated directly by actin (Rothberg et al., 1992). Deckert and colleagues have shown that cytochalasin H blocked clustering and internalization of CD95 (a GPI-anchored protein) and that clusters of CD95 colocalized with polymerized actin in Jurkat T-lymphocytes (Deckert et al., 1996)

Cell treatments involving cholesterol and/or sphingolipid depletion may lead to perturbation of the cytoskeletal organization (Deckert et al., 1996; Sun et al., 2007; Doherty and McMahon, 2008), which can lead to confusing interpretation of studies in which cellular sterol/sphingolipid composition is manipulated in an effort to manipulate 'membrane rafts'. Thus while there is substantial evidence that the subcortical cytoskeleton can modulate the organization of the plasma membrane, it has been very challenging to relate interactions between the cytoskeleton and the plasma membrane to the formation and behaviour of liquid-ordered microdomains (Mukherjee and Maxfield, 2004; Simons and Vaz, 2004; Doherty and McMahon, 2008).

1.3 Glycosylphosphatidylinositol-anchored proteins:

In the work described in this thesis, we focused on studying the intracellular routing of endocytosed GPI-anchored proteins, with folate receptor α as a representative of this group. This class of membrane proteins plays a critical role in a number of cellular functions including signal transduction, cellular uptake of toxins/prions, and antigen recognition. Studying the kinetics and routes of GPI-anchored proteins endocytosis has generated key findings that have helped to better understand the pathology caused by loss of function of this class of proteins (Ikezawa, 2002; Kinoshita et al., 2008; Lakhan et al., 2009).

1.3.1 Properties.

Glycolipid anchored membrane proteins were discovered in the 1970s, when Ikezawa and colleagues reported that phosphatidylinositol-specific phospholipase C (PI-PLC) cleaves and releases alkaline phosphatase from rat kidney cells (Ikezawa et al., 1976). GPI-anchorage is a unique posttranslational modification that links the C-terminus of a protein (with a specific signal sequence) to the GPI-anchor. The GPI-anchor can be also found unattached to protein on the plasma membrane, and has been shown to be conserved between species (Englund, 1993; Fankhauser et al., 1993; Baumann et al., 2000). GPI-anchored proteins are exclusively found on the extracytoplasmic leaflet of the plasma membrane of mammalian cells and are present in all eukaryotes, but not in eubacteria (Cross, 1990; Englund, 1993; Ikezawa, 2002). Most mammalian GPI-anchored proteins on nucleated cells have two saturated acyl chain moieties, supporting suggestions that GPI-APs may cluster preferentially in lipid-raft microdomains, and which may dictate their functional and endocytic behaviours (Redman et al., 1994; Varma and Mayor, 1998; Chatterjee and Mayor, 2001; Edidin, 2003; Simons and Vaz, 2004; Maeda et al., 2007).

1.3.2 Biosynthesis & remodelling.

The cellular generation of GPI-anchored proteins involves a convergence of biosynthetic pathways for the protein and lipid (GPI) moieties (Englund, 1993; Kinoshita and Inoue, 2000; Ikezawa, 2002). A precursor polypeptide with an appropriate N-terminal signal peptide for import into the endoplasmic reticulum, and a C-terminal sequence for addition of a GPI-anchor, are translocated cotranslationally into the ER, and the N-terminal leader peptide is then removed by a peptidase. All proteins that ultimately become attached to a GPI moiety exhibit common features in their consensus sequences for GPI-anchor addition (Eisenhaber et al., 1998; Ikezawa, 2002). One such feature is a preference for serine, alanine, and alanine/glycine, respectively, as the ω, ω+1, and ω+2 residues near the attachment site of the GPI anchor (Udenfriend and Kodukula, 1995). After import into the ER, the protein is coupled to a GPI-anchor through a

transamidation reaction in which the peptide bond between residues ω and (ω+1) of the GPI-anchor addition sequence becomes replaced by an amide bond between residue ω and an ethanolamine residue of the GPI-anchor (Kinoshita and Inoue, 2000; Ikezawa, 2002; Kinoshita et al., 2008).

Biosynthesis of the GPI-anchor in mammalian cells consists of 10-11 reaction steps (see Figure1 for summary) which involve over 20 genes (Kinoshita et al., 2008; Fujita and Kinoshita, 2009). The first step of GPI-anchor synthesis is the generation of GlcNAc-PI from UDP-GlcNAc and phosphatidylinositol by the action of GPI-N-acetylglucosaminyltransferase (GPI-GnT). The second step entails removal of the acetyl group from the GlcNAc residue by N-acetylglucosaminylphosphatidylinositol de-N-acetylase, following which the inositol ring is acylated at position 2 to generate GlcN-Acyl-PI (step 3). The preceding three steps occur on the cytoplasmic side of the ER, following which a flippase transfers the GlcN-Acyl-PI intermediate to the luminal side (Step 4). At the inner face of the ER membrane three mannose groups are added sequentially through the coordinated action of GPI-α1-4/2/6 mannosyltransferases (GPI-MT-I/II/III respectively) using dolichol phosphate mannose as the mannose donor in each step (steps 5, 7 & 8). Step 6 comprises a non-essential addition of an ethanolaminephosphate (EtNP) group to the first mannose by the PIG-N-encoded enzyme in mammalian cells (Hong et al., 1999). Finally, additional ethanolamine-phosphoryl groups are added to the second and third mannose residues by the PIG-F and PIG-O gene products, which are described as EtNP transferases. Either of the two last intermediates can be conjugated to a protein bearing an appropriate GPI-attachment signal peptide (Kinoshita and Inoue, 2000). After the protein-GPI-anchor coupling step, the inositol-linked acyl group is removed in the ER, and the nascent GPI-anchored protein is trafficked to the Golgi where it may undergo further biochemical remodelling and associate with lipid rafts (Kinoshita and Inoue, 2000; Edidin, 2003; Simons and Vaz, 2004).

Among the further modifications of the GPI-anchor that can occur in the Golgi are sugar/EtNP addition to the core mannose residues, fatty acid re-addition to the

inositol ring, and exchange of the original fatty acid residues of the phosphatidyl-inositol residues with acyl or alkyl groups (Kinoshita et al., 2008). N-actylhexosamine, for example, is added in the Golgi to the first core mannose in all rat brain Thy-1 and in a fraction of human erythrocyte CD95 and bovine liver CD73 (Homans et al., 1988; Taguchi et al., 1994; Rudd et al., 1997). Most nascent GPI-anchored proteins enter the Golgi with one unsaturated fatty acid at the *sn*-2 position of the PI moiety. The enzyme encoded by the PGAP3 gene removes this unsaturated chain to produce a lyso-GPI-anchored protein intermediate which is then re-acylated with a saturated chain (typically stearoyl) by the PGAP2 gene product. Bearing two saturated fatty acyl (or alkyl) chains, mature GPI-anchored proteins are more likely to associate with membrane raft microdomains, and DRMs that include GPI-anchored proteins can be isolated from the Golgi (Simons and Vaz, 2004; Kinoshita et al., 2008; Fujita and Kinoshita, 2009).

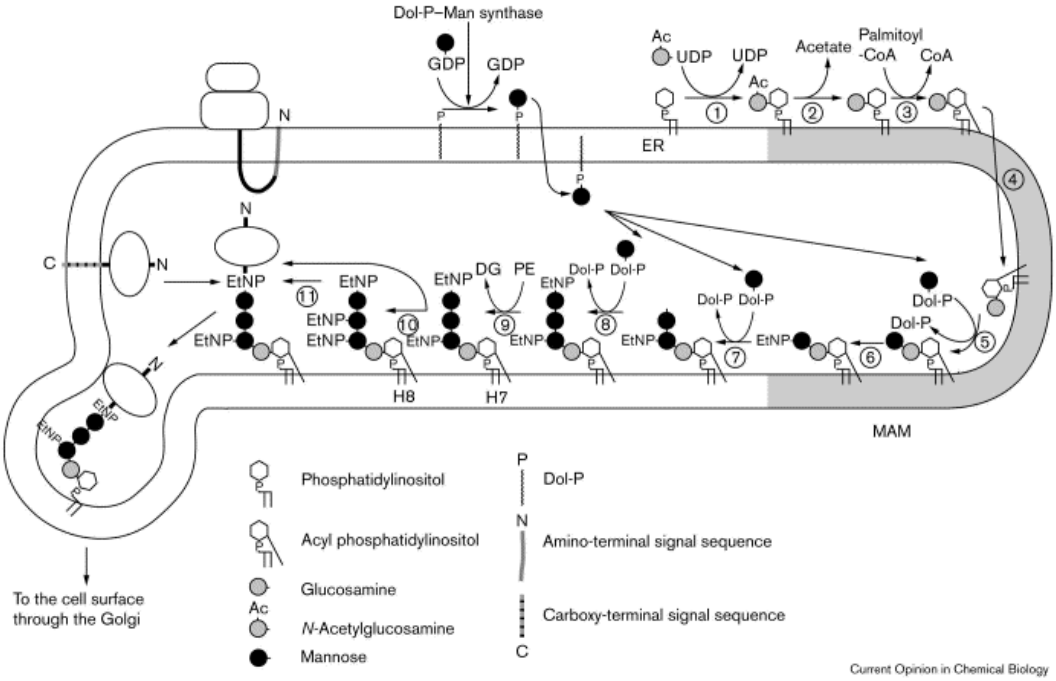

Figure 1: Biosynthesis of GPI-Anchored Proteins in mammalian cells. Reproduced with permission (Kinoshita and Inoue, 2000).

1.3.3 Physiological function of GPI-anchored proteins.

GPI-anchored proteins fulfill diverse functions as adhesion molecules, antigen receptors, enzymes, signal-transduction proteins, complement regulatory proteins and in other capacities (Ikezawa, 2002). Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hematopoietic stem cell disorder arising from a global defect in biosynthesis of the protein-GPI anchor. Erythrocytes from PNH patients are highly susceptible to haemolysis (Rosse, 1989; Kinoshita et al., 1995). Sperm maturation requires decapacitation factors to bind to receptors on the sperm membrane in order to remove inhibitory signals. The GPI-anchored protein PGAP-1 is a receptor for one of these decapacitation factors, and PGAP-1 knock-out mice are found to be sterile (Gibbons et al., 2005; Kinoshita et al., 2008). Mice lacking the GPI-anchored folate receptor α (FR α) have developmental abnormalities, and FRα is overexpressed in cancer cells (Ross et al., 1994; Piedrahita et al., 1999; Sabharanjak and Mayor, 2004). The GPI-anchor plays an important role in the proper sorting of the FRα; replacement of the GPI-anchor with a transmembrane-helical anchor leads to impairment in folate uptake (Ritter et al., 1995). GPI-anchored proteins also play important roles in various pathological processes. Replacement of the GPI-anchor of the cellular prion protein (Pr^{c}) prevents its efficient conversion to the scrapie (Pr^{sc}) form (Taraboulos et al., 1995). GPI-anchored receptors on the cell plasma membrane can act as receptors for toxins (*Diphtheria*, aerolysin, *Clostridium botulinum*, cholera toxin, and others) or prions (Skretting et al., 1999; Rudd et al., 2001; Doherty and McMahon, 2009). These examples illustrate the importance of studying GPI-anchored proteins in more depth, including their endocytosis and trafficking within the cell.

1.4 Endocytosis

Compartmentalization is a key organizational feature of eukaryotic cells. Many cellular processes are regulated by coordinated exchange of proteins and lipids between membrane-bound intracellular compartments. This takes place through a highly dynamic and regulated network of vesicular traffic events (Doherty and

McMahon, 2009). Endocytosis and exocytosis are tightly regulated so that interactions between the cell and its environment can be coordinated.

For this section, I will begin with a general overview of endocytic uptake and intracellular sorting, followed by an overview and classification of the known endocytic pathways. I will finally discuss the known major components and regulators of the machinery of the different pathways, with a focus on the CME and CLIC/GEEC pathways.

1.4.1 Endocytic Uptake:

Endocytic pathways are often classified based on the machinery involved in the internalization process (endocytic uptake). Most fundamentally, they are categorized as either clathrin-dependent (clathrin-mediated endocytosis, or CME) or clathrin-independent (CIE). The uptake of diverse cargos by CME appears to depend on the same fundamental machinery, although reports that different cargos may be concentrated in distinct populations of clathrin-coated vesicles suggest that the process may not be entirely uniform for all cargo species (Puthenveedu and von Zastrow, 2006; Puthenveedu et al., 2007; von Zastrow, 2010). Clathrin-independent endocytosis comprises a wider variety of distinct pathways, as discussed later. CME and CIE contribute roughly equally to the total endocytic flux in fibroblastic cells but may represent different proportions of total endocytic traffic in other cell types (Mayor and Pagano, 2007; Doherty and McMahon, 2009).

It is still not entirely clear why cells require so many endocytic pathways. Endocytosis mediates and/or regulates many cellular functions including nutrient uptake, cell adhesion, pathogen entry, synaptic transmission, migration, signalling, cell polarity, growth and differentiation (Doherty and McMahon, 2009; Kumari et al., 2010). A multiplicity of endocytic pathways may be required to provide sufficient flexibility for differential regulation of the trafficking of the many plasma membrane and extracellular molecules that are involved in these diverse processes. Defects in endocytosis are associated with a number of diseases including muscular dystrophies, neurodegenerative diseases (Alzheimer's,

Huntington's, ataxia and Down syndrome) and rare cases of haemophilia (Cataldo et al., 2000; Garuti et al., 2005; Atwal et al., 2007; Cataldo et al., 2008; Nonis et al., 2008).

1.4.2 Intracellular Endocytic Sorting:

After an endocytic vesicle/tubule buds off the plasma membrane, it undergoes a series of complex and iterative molecular sorting events which target its different components to specific destinations within the cell (Mellman, 1996). Generally, newly formed endocytic vesicles fuse together to form early endosomes (EE) that have varying morphological and compositional characteristics depending on the specific endocytic pathway utilized. Multiple EEs can fuse with one another and with a specialized peripheral endocytic compartment known as the sorting endosome (SE). The SE is a tubular-vesicular structure with luminal pH ~5.9-6, a property that can assist in releasing ligands from their receptors. Nascent sorting endosomes, once their formation is initiated, continue to fuse with EEs for 5-10 minutes, after which time they become more acidic, acquire acid hydrolases and translocate along microtubules towards the perinuclear region of the cell to "mature" into late endosomes (Maxfield and McGraw, 2004). As the SE matures, membrane protein and lipid molecules are removed rapidly and efficiently through repeated pinching off of tubular regions of the SE (Dunn et al., 1989; Mellman, 1996). Because the surface-area-to-volume ratio of the tubules is larger than that of the vesicular portion of the SE, this process gradually removes a large fraction of membrane components from the SE, while concentrating soluble cargo in the vesicular central region of this structure. Molecules remaining in the vesicular region of the SE will be targeted to late endosomes. Therefore, this first step of endocytic sorting is geometry-based.

There are two main routes by which membrane components (and a small amount of luminal contents) return to the PM from the SE: a very rapid direct route, and an indirect slower route via a long-lived compartment known as the endocytic recycling compartment (or ERC) (Yamashiro et al., 1984; Dunn et al., 1989; Mayor et al., 1993; Maxfield and McGraw, 2004). The transferrin receptor (or

TfR), as an example, is recycled back to the plasma membrane directly with a half-life of 2-3 minutes, or via the ERC with a half-life of 9-12 minutes (van der Sluijs et al., 1992; Johnson et al., 1993; Mayor et al., 1993; Presley et al., 1993). The ERC is a collection of tubular organelles with diameters of 60 nm that are associated with microtubules. ERC are found mainly in perinuclear regions of cells, but they can also be found more diffusively distributed throughout the cytoplasm (Hopkins, 1983; Yamashiro et al., 1984; McGraw et al., 1993; Ullrich et al., 1996; Lin et al., 2002). The molecular differences that determine whether a given endocytosed membrane component is recycled by the rapid pathway or more slowly via the ERC are still being elucidated. It has been shown that Rab4, 5 & 11 regulate these two recycling routes and segregate to distinct domains on the sorting endosomes. Rab4 seems to play a role in rapid recycling to the PM, whereas Rab11 (which is also found on the ERC and trans-Golgi network) regulates recycling through the ERC (Urbe et al., 1993; Ullrich et al., 1996; Ren et al., 1998; Sonnichsen et al., 2000). Overexpression of a dominant negative form of Rab11 has been shown to result in enhanced accumulation of TfR in the ERC (Ullrich et al., 1996; Ren et al., 1998). It is still unknown how cargoes are selected for Rab4- or Rab11-positive domains on sorting endosomes, but preferential association with/exclusion from lipid rafts has been hypothesized to play a role (Sonnichsen et al., 2000; Zerial and McBride, 2001; Choudhury et al., 2004; Chen et al., 2008). Besides recycling molecules back to the PM, the ERC also sorts certain molecules to late endosomes (Wilcke et al., 2000; Iversen et al., 2001; Lin et al., 2004; Maxfield and McGraw, 2004). The recycling pathways discussed above are essential for maintaining the proper composition of the PM and various organelles in various mammalian cells (Maxfield and McGraw, 2004).

As already noted, certain endocytosed molecules are sorted for degradation in late endosomes/lysosomes. This can happen in two major ways. First, bulk fluid and soluble ligands (such as reduced folate) released from their receptors accumulate in the vesicular region of sorting endosomes and are delivered to late endosomes through a process of maturation of SEs as described above (Dunn et al., 1989; Dunn and Maxfield, 1992; Maxfield and McGraw, 2004). As mentioned earlier,

this is a geometry-based, bulk phase separation that does not require any specific signal (Dunn and Maxfield, 1992; Mayor et al., 1993). Alternatively, some membrane proteins (e.g. signaling receptors) can be targeted to LE via ubiquitylation of their cytoplasmic domains, which can be recognized by a number of factors. For example, Hepatocyte-growth-factor-Regulated tyrosine kinase Substrate (HRS) links ubiquitylated receptors to flat clathrin lattices in endosomes, an interaction which is thought to retain these receptors in the vesicular portion of the SE and ultimately deliver them to LE (Raiborg et al., 2002; Maxfield and McGraw, 2004). Endosomal Sorting Complex Required for Transport (ESCRT) proteins are another group of factors with important implications in targeting particular membrane proteins to late endosomes (Luzio et al., 2009; Luzio et al., 2009; Pryor and Luzio, 2009). MHC class I molecules in T-lymphocytes have been studied extensively in this regard (Hewitt et al., 2002; Lehner et al., 2005; Duncan et al., 2006). Many viruses down-regulate MHC class I as part of their infection/replication cycle. Kaposi's Sarcoma-associated Herpes Virus (KSHV), expresses a viral integral membrane protein, K3, which is a ubiquitin ligase capable of promoting the polyubiquitination of MHC class I molecules, thereby, promoting their rapid internalization and degradation in lysosomes (Lehner et al., 2005). Clathrin, the clathrin accessory protein epsin and ESCRT-0 and -1, but not ESCRT-2 or -3, have been implicated in subsequent recognition and targeting of the polyubiquitinated MHC class I receptors to late endosomes and lysosomes (Hewitt et al., 2002; Lehner et al., 2005; Bowers et al., 2006; Langelier et al., 2006). It remains unclear, however, how membrane proteins lacking cytoplasmic domains (such as GPI-anchored proteins) can be targeted to LE (Fivaz et al., 2002; Maxfield and McGraw, 2004).

1.4.3 The endocytic pathways:

In the following sections, I describe the characteristics and itineraries of specific known endocytic pathways, with a particular focus on CME and the CLIC/GEEC pathways in mammalian systems.

1.4.3.1 Clathrin-mediated endocytosis (CME):

A large number of transmembrane receptors with a specific cytoplasmic signal sequence or domain are internalized via CME. The recruitment signal can comprise either YXXF, DEXXXLLI, or FXNPXY motifs, which are recognized by different adaptor proteins (Kumari et al., 2010). Transferrin receptor (TfR) is a classical example of a membrane protein endocytosed by CME. The cytoplasmic domain of TfR includes a YTRF motif that mediates its binding to the μ2 subunit of the adaptor protein AP-2 (Collawn et al., 1990). Membrane internalization via CME entails the recruitment of clathrin and accessory proteins to form a coat structure around the vesicle (Schmid and McMahon, 2007), which can be visualized by both electron and total internal reflection fluorescence microscopy (Pearse, 1976; Marsh and McMahon, 1999; Bellve et al., 2006).

Adaptor proteins coordinate the nucleation of clathrin at the plasma membrane and link it to the membrane-protein cargo. Clathrin then starts to polymerize forming clathrin-coated pits (or CCPs). As polymerization continues, the curvature of such pits increases, ultimately forming a more constricted neck which is acted upon by dynamin. Dynamin is a GTPase that forms a helical polymer around the neck of maturing CCPs and mediates the generation of detached clathrin-coated vesicles (CCVs). Subsequently, the clathrin coat is released by two chaperone proteins, auxilin and hsc70, and the vesicles continue along the endocytic pathway as described above (Doherty and McMahon, 2009). There is a wide range of adaptor and accessory proteins that can assist in membrane protein recruitment, in clathrin nucleation at the PM and in CCP and CCV formation.

The sorting of cargoes and their receptors after internalization via CME follow the routes discussed earlier. After shedding their clathrin coats, CME-derived vesicles fuse with one another and with SE, where many receptor-bound ligands are released. Soluble molecules and ubiquitylated free receptors accumulate in the vesicular portion as SE mature into late endosomes and lysosomes where these molecules are degraded. Other membrane bound molecules, including non-

ubiquitylated receptors, can be returned back to the cell surface via either the Rab4- or the Rab11-regulated recycling routes. TfR has been shown to be efficiently recycled (99% per transit through the cell interior) via these recycling itineraries in a number of cell lines including BHK21 and CHO (Presley et al., 1993; Ullrich et al., 1996; Maxfield and McGraw, 2004).

1.4.3.2 Clathrin-independent endocytosis (CIE):

In recent years, researchers have found that upon inhibition of CME, the uptake of a large number of endocytic cargoes was relatively unaffected, an observation that led to the discovery of clathrin-independent endocytic pathways (Doherty and McMahon, 2009; Howes et al., 2010; Kumari et al., 2010). Lacking a well characterized specific protein 'coat', the endocytic structures involved in many of these pathways have been more difficult to visualize under EM, but they are traceable by fluorescent labelling of specific cargo molecules (Duprez et al., 1994; Sabharanjak et al., 2002; Kalia et al., 2006; Nishi and Saigo, 2007). CIE has been divided into apparently distinct pathways which differ in their abilities to internalize specific cargo, their reliance on certain proteins and lipid domains, and their differential sensitivity to drugs and dominant-negative mutants of different monomeric G-proteins (Mayor and Pagano, 2007; Doherty and McMahon, 2009; Howes et al., 2010; Kumari et al., 2010).

1.4.3.2.1 Caveolin1- & Flotillin-dependent pathways.

These forms of CIE utilize identified coating proteins (caveolin or flotillin-1 and -2) to form proteinaceous scaffolds/coats around the forming endocytic vesicles (Nevins and Thurmond, 2006; Lundmark et al., 2008). There are three caveolin species in mammalian cells, which exhibit cell-specific, differential expression and are necessary for the formation of caveolae (Lipardi et al., 1998; Kumari et al., 2010). Numerous signaling proteins have been found to be associated with and regulated by caveolins, but less is known about proteins regulating caveolar biogenesis (Krajewska and Maslowska, 2004; Parton et al., 2006; Bastiani et al., 2009). SV40 virions, cholera toxin B subunit, tetanus toxin, albumin, and

autocrine motility factor (AMF) have all been shown to be endocytosed via a caveolin-dependent pathway, which also relies on the activity of dynamin (Kumari et al., 2010).

Flotillins share significant overall homology with caveolins but seem to form a distinct population of nascent endocytic structures at the plasma membrane. Flotillin-dependent endocytosis is not directly interconnected with that mediated by caveolins (Frick et al., 2007; Kirkham et al., 2008). The former pathway's main cargoes are proteoglycans and unlike caveolin, they do not require dynamin to complete the fission process (Kumari et al., 2010).

Beside the general morphological similarity of the coated structures that they form, caveolins and flotillins both seem to associate preferentially with liquid ordered lipid domains in the Golgi and the plasma membrane (Doherty and McMahon, 2009; Kumari et al., 2010). Cholesterol depletion flattens caveolae and increases mobility of caveolin-1, again suggesting the potential importance of liquid-ordered cholesterol-dependent lipid domains in the formation of functional caveolae (Rothberg et al., 1992). Likewise, low-temperature detergent-resistant membrane fractions (DRMs) from the Golgi and PM are enriched in caveolin-1 oligomers (Pelkmans and Zerial, 2005). The mechanisms by which cargo is selected for internalization via caveolae or flottilin-associated structures are currently not well understood.

1.4.3.2.2 The CLIC/GEEC pathway.

Some endocytic pathways have been shown to require the activities of neither specific identified coating proteins nor dynamin, a conclusion that required extensive, careful experimentation and genetic manipulations to document (Moya et al., 1985; Doxsey et al., 1987; Conner and Schmid, 2003; Kirkham et al., 2005; Kumari et al., 2010). The formation of membrane invaginations without the need for coating proteins has puzzled workers in the field of endocytosis. Some researchers have suggested either that these pathways have a proteinaceous coat that is too short-lived to be captured by current techniques, or that special types of

lipid/protein organization could initiate membrane deformation (Kirkham et al., 2005; Cheng et al., 2006; Zoncu et al., 2007). Binding of Shiga toxin to Gb3 ganglioside on the cell surface, as well as to model membranes, has in fact shown to induce extensive membrane invaginations (Romer et al., 2007).

GPI-anchored proteins have been shown to be endocytosed via a clathrin/ caveolin-1 and dynamin-independent pathway (Sabharanjak et al., 2002; Mayor and Pagano, 2007). The GPI-linked folate receptor α and CD95 have been used as reliable markers of this pathway. GPI-anchored proteins, a few minutes after internalization, were found concentrated in uncoated tubulovesicular clathrin-independent carriers (CLICs) under the plasma membrane. These structures bud from the PM and fuse together to form larger tubular structures that are selectively enriched in GPI-anchored proteins and that are termed GPI-AP enriched early endosomes (GEECs) (Sabharanjak et al., 2002; Kirkham and Parton, 2005). These are distinct from the EEs derived from other pathways, especially those formed via CME (Chadda et al., 2007; Kumari and Mayor, 2008; Bhagatji et al., 2009). As a result, the process that leads to GEEC formation was termed the CLIC/GEEC pathway (Doherty and McMahon, 2009; Kumari et al., 2010).

The basis for selective sorting of GPI-APs into CLICs remains only partly understood at this stage. It has been proposed that this sorting is dependent on the GPI-anchor which favourably associates with membrane rafts, and some groups have shown that perturbation of the normally "nanoclustered" state of GPI-anchored proteins abrogates endocytosis via this pathway (Varma and Mayor, 1998; Kirkham et al., 2005). However, recent studies have demonstrated that not lipid raft association, but rather steric exclusion of GPI-anchored proteins from coated pits selectively targets GPI-anchored proteins to CLICs (Mayor et al., 1998; Chatterjee et al., 2001; Bhagatji et al., 2009). Endocytosis via the CLIC/GEEC pathway is dependent on the maintenance of subcortical actin architecture (Chadda et al., 2007). This, in turn, is dependent on the cycling of Cdc42 (a Rho family GTPase) which activates the actin-regulating WASP protein. Cdc42 dynamics are under tight regulation by ARF1, which when activated

recruits ARHGAP10 (a GAP) and enhances Cdc42 GTP hydrolysis leading to its deactivation (Howes et al., 2010; Kumari et al., 2010). GRAF1 (GTPase Regulator Associated with Focal Adhesion kinase-1) is another Rho-GAP-related regulator of Cdc42 in HeLa cells (Lundmark et al., 2008; Doherty and Lundmark, 2009). Beside its Rho-GAP domain, GRAF1 also contains BAR and SH3 domains which could aid in promoting membrane curvature and enable interactions with dynamin. The latter might explain why dynamin is found on GEECs only post-internalization (Lundmark et al., 2008).

Most of what we know about the CLIC/GEEC pathway has been discovered in the past decade. GEECs are acidic tubular-shaped primary endosomal compartments that are devoid of any markers of the CME pathway, such as Rab4, Rab5 or early endosomal antigen (EEA). Ten minutes after internalization, these structures start to acquire Rab5 and EEA1 before they undergo homotypic fusion with other GEECs, and heterotypic fusion with early sorting endosomes. These fusion events have been shown to depend on Rab5 GTPase activity and phosphatidylinositol-3'-kinase (PI3K) (Kalia et al., 2006). From this point onwards, cargoes of the CLIC/GEEC pathway may undergo the same geometrically based sorting in SE as do cargoes internalized via CME. Alternatively, there have been reports that some cargoes of the CLIC/GEEC pathway may be transported directly from the GEECs, bypassing the SE, to the ERC, late endosomes, or the trans-Golgi network. However, the mechanism and regulation of such trafficking are still unclear (Chatterjee and Mayor, 2001; Doherty and Lundmark, 2009; Doherty and McMahon, 2009). It has been shown that GPI-anchored proteins recycle back to the plasma membrane through Rab-11 positive ERC in CHO cells. This seems to happen at a slower rate ($t_{1/2}$ ~ 30 min) than that for the TfR ($t_{1/2}$~8 min). The ultimate fate of GPI-anchored proteins internalized by the CLIC/GEEC pathway may however be cell-type dependent. Fivaz and colleagues (2002) reported that GPI-anchored CD95 is targeted to the ERC in CHO cells, but to the late endosomes in BHK cells. It has been suggested that association with membrane rafts may influence the differential sorting of GPI-anchored proteins in different fibroblastic cells (Fivaz et al., 2002).

A number of questions remain to be answered concerning the endocytic trafficking of GPI-anchored proteins. How, for example, can GPI-anchored proteins be targeted to late endosomes? How can they be sorted to the Golgi directly from the ERC? If they are destined for recycling to the PM, how are they sorted between the fast, Rab4-depandent and the slow, Rab11-dependent recycling pathways? A few hypotheses have been proposed to answer these questions, most of these centered around a potential role of membrane rafts. I have sought to test some of these hypotheses in this thesis.

The functional importance of the CLIC/GEEC pathway as a whole remains to be fully clarified. Given its constitutive nature, the heterogeneous structure of its transport intermediates, and its slow kinetics, the CLIC/GEEC pathway might help to maintain the homeostasis of the PM (Kumari et al., 2010). A large number of molecules/receptors are endocytosed via this pathway, such as fluid phase markers, CTxB, VacA toxin, and the majority of GPI-anchored proteins (Kumari et al., 2010). The slow kinetics of the CLIC/GEEC pathway allow the folate receptor enough time to release the bound folate in the SEs, from which folate can be transported to the cytoplasm to be used in different metabolic reactions (Mayor and Riezman, 2004; Sabharanjak and Mayor, 2004; Kalia et al., 2006). The CLIC/GEEC pathway may also serve as an alternative endocytic pathway leading to a different functional outcome for membrane proteins that can also be internalized by CME. The fate of epidermal growth factor receptor (EGFR), for example, depends on the endocytic pathway it follows. At low EGF concentrations, EGFR is endocytosed via CME and is recycled back to the cell surface. However, at high concentrations of EGF (leading to activation of the receptor), EGFR is endocytosed via clathrin-independent pathways whereby the receptor is ubiquitinated and degraded (Sigismund et al., 2008).

There appear to be other clathrin/caveolin-independent pathways, which bear some resemblance to the CLIC/GEEC pathway but may involve different intermediate carriers and requirements for GTPases or dynamins, as discussed in the next two sections.

1.4.3.2.3 RhoA-regulated pathway.

A clathrin/caveolin1-independent pathway seemingly distinct from the CLIC/GEEC pathway, and more specific for the Interleukin-2 receptor β subunit (IL-2Rβ) in lymphocytes has been characterized (Lamaze et al., 2001). This pathway is regulated by the activity of the small G proteins rhoA and rac1, and the kinases PAK1 and PAK2 (Grassart et al., 2008). Unlike the CLIC/GEEC pathway, this is a dynamin-dependent pathway which generates 50-100 nm vesicular intermediates. It has been shown to be responsible for the internalization of γ_c cytokine receptor and FcεRI receptor in lymphocytes (Sauvonnet et al., 2005; Fattakhova et al., 2006). The prevalence of this pathway is limited to lymphocytes, and it is not clear if it plays a major role in other cell types (Doherty and McMahon, 2009).

1.4.3.2.4 Arf6-regulated pathway.

Another pathway that is seemingly similar to the CLIC/GEEC pathway, yet appears to be at least subtly different is a clathrin/caveolin1/dynamin-independent, Arf6-dependent pathway (Naslavsky et al., 2003). It has been shown that MHCI, carboxypeptidase E, and Tac (Interleukin-2 receptor α subunit (IL-2Rα)) are internalized into Arf6-positive tubular endosomal invaginations, where Arf6 is found associated with transport intermediates throughout this pathway (Donaldson et al., 2009; Kumari et al., 2010). Arf6 has been mainly implicated in recycling of membrane/cargoes/receptors back to the plasma membrane, as has been shown for the herpes simplex protein vp22 (Balasubramanian et al., 2007; Nishi and Saigo, 2007). HeLa cells in which GRAF1 has been perturbed showed altered internalization of fluid phase markers but not of MHC-I (Lundmark et al., 2008). GRAF1, as mentioned earlier, is a Rho-GAP that is involved in the regulation of Cdc42, the main small GTPase implicated in regulation of many clathrin-independent pathways, including the Arf6-regulated pathway. Post-internalization, early tubular intermediates in the Arf6-regulated pathway have been shown to divide up into two populations, one of which is Arf6-positive and devoid of CME markers, while the other becomes associated with the Rab5-

/EEA1-positive SE. Interestingly, however, these two populations merge later in late endosomes and deliver their cargoes for degradation. Rab7 is required to complete this process (Naslavsky et al., 2003).

1.5 Project overview

The formation and functional importance of ordered membrane raft microdomains have received a great deal of interest from workers in the fields of membrane trafficking and membrane signaling in recent years. Segregation of 'raft-like' liquid-ordered lipid domains from liquid-disordered lipid domains has been extensively characterized in lipid model membranes (Simons and Vaz, 2004). A variety of functional evidence has been accumulated to suggest that similar segregation of liquid-ordered domains may occur and play important functional roles, in membranes of mammalian cells. Still, it has proven technically challenging to assess whether such domains are indeed present in mammalian cell membranes under 'native' conditions and to link them conclusively to membrane function (Edidin, 2003).

My project sought to test the currently favoured hypothesis that the distinctive intracellular endocytic sorting of GPI-anchored proteins is a consequence of their association with liquid-ordered membrane microdomains (Chatterjee and Mayor, 2001; Chatterjee et al., 2001; Sabharanjak et al., 2002). Replacing the GPI-anchor of the GPI-anchored folate receptor α with a "non-raft-associating" transmembrane helix was shown to abolish this specific trafficking in CHO cells. This finding has been taken as evidence to support the 'raft' hypothesis of GPI-anchored protein trafficking noted above. It is also possible, however, that GPI-anchored proteins are sorted in a distinctive manner because of specific receptors that recognize distinctive *structural* features of these proteins. To date, it has not been possible to determine which of these hypotheses is correct, and this is what my project sought to elucidate. As a novel means to approach this issue, Dr. Silvius' laboratory has designed artificially lipid-anchored protein that can be incorporated into living cells where their behaviour can be compared to that of endogenous GPI-anchored proteins (Wang et al., 2005; Bhagatji et al., 2009).

1.5.1 Artificially lipid-anchored proteins:

1.5.1.1 Structural components.

The artificially lipid-anchored protein species utilized in this thesis comprise lipid-linker-ligand and protein moieties. The former moiety is a phosphatidylethanolamine linked to a ligand through a polyethyleneglycol-1500 linker (Figure2). The ligand at the end of the PEG chain is bound with high affinity by a specific protein. The fatty acyl chains of the PE can vary in length and/or saturation. Dipalmitoyl-PE-PEG- (di16:0-), dioleoyl-PE-PEG (di18:2-), and didodecyl-PE-PEG (di12:0-) 'anchors' and their diether counterparts were synthesized and employed for this project. As expected from model system studies, our laboratory showed that proteins anchored to di16:0-, but not di18:2- or di12:0-lipid-PEG moieties partitioned substantially into DRM fractions (and hence into membrane raft microdomains) isolated from Jurkat or fibroblast cells (Wang et al., 2005). The major advantage of using artificially lipid-anchored proteins is the ability to assess the role(s) that membrane raft association plays in the behaviour of GPI-anchored protein analogues without using methods that cause pleiotropic disturbances to the cells. We used two types of ligand/protein combinations to form artificially lipid-anchored proteins: methotrexate (MTX) which can be bound by dihydrofolate reductase (DHFR), and trinitrophenyl (TNP-) residues, which can be bound by anti-DNP antibodies. These proteins were labelled with fluorescent groups, which enabled us to monitor their endocytic itineraries (once assembled with appropriate lipid 'anchors') inside live cells by fluorescence microscopy.

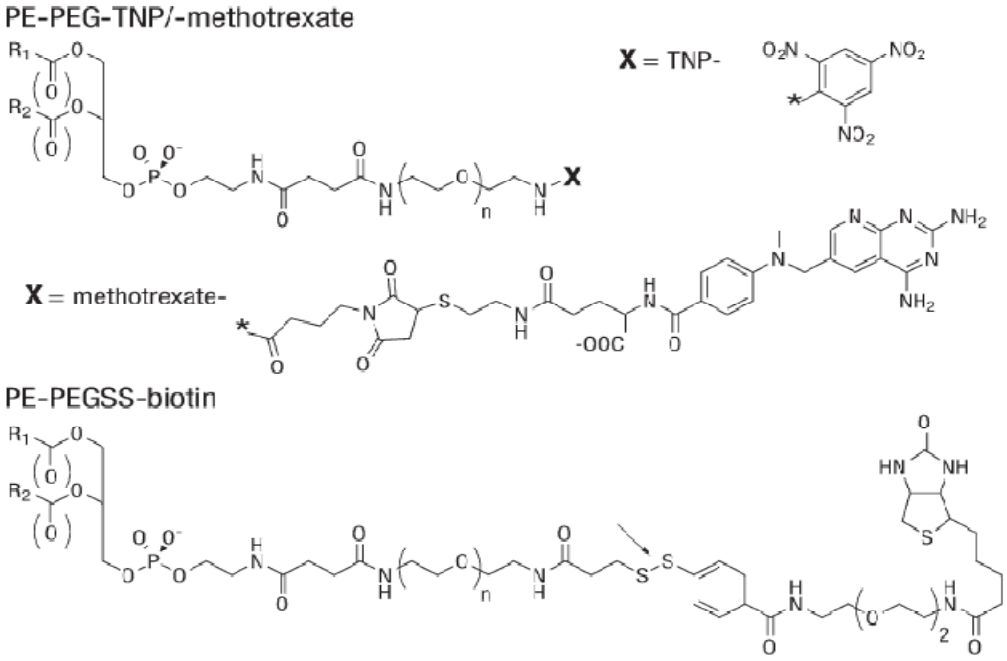

Figure 2: Structural Components of the Lipid Moiety of the Artificially Lipid-Anchored Proteins. Reproduced with permission (Bhagatji et al., 2009).

PE-PEGs, when dispersed in aqueous solution, can form micelles and integrate spontaneously into model lipid bilayers as well as the plasma membrane of live mammalian cells. They were shown to remain stably anchored to the membrane thereafter over long periods of times (hours or longer) (Silvius and Zuckermann, 1993; Johnsson et al., 2003; Palmer et al., 2003; Wang et al., 2005; Bhagatji et al., 2009). After the PE-PEG-ligand conjugate is incorporated into cell membranes, the protein is allowed to bind tightly to its ligand at the cell surface, producing a lipid-anchored protein whose overall structural features resemble these of GPI-anchored proteins but that lack specific structural features found in these proteins that might be recognized in a specific manner by possible 'GPI-anchored protein receptors'. The steps of incorporation of artificially lipid-anchored proteins into cell surface membranes are summarized in Figure3.

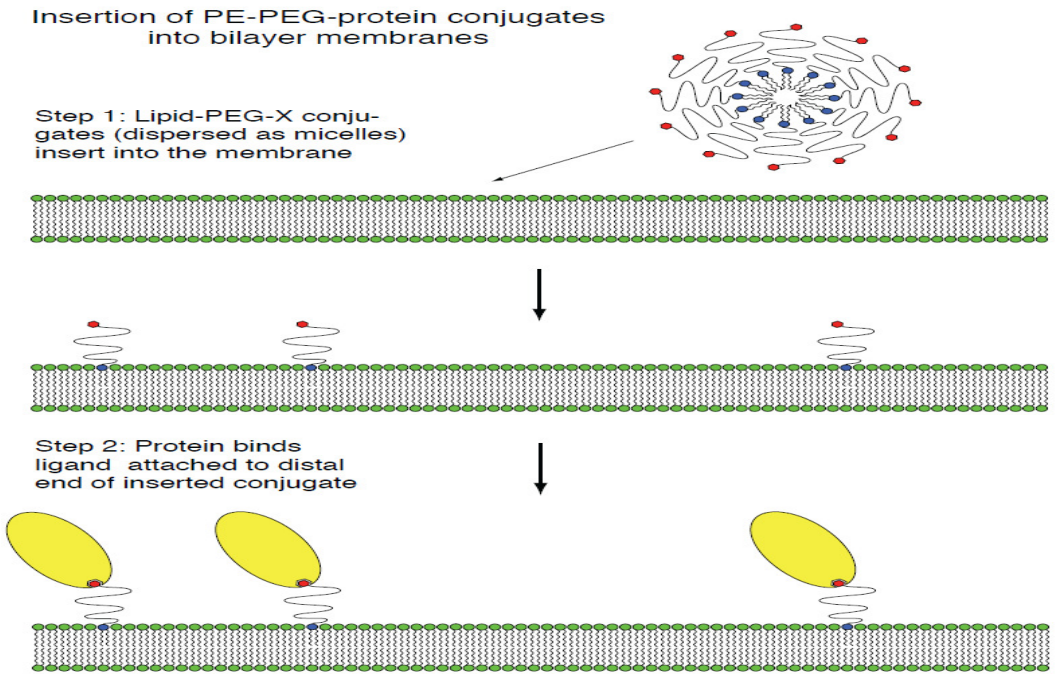

Figure 3: Incorporation of Artificially Lipid-Anchored Proteins into the Plasma Membrane of Live Cells.

1.5.1.2 Previous applications.

Our artificially lipid-anchored protein systems were first employed in order to study the relevance of membrane-raft association of GPI-anchored proteins to the activation of Jurkat T-lymphocytes induced by clustering of such proteins at the plasma membrane (Wang et al., 2005). We have also employed this system more recently in order to determine the role of membrane rafts in sorting GPI-anchored proteins into the CLIC/GEEC endocytic pathway from the plasma membrane of CHO cells (Bhagatji et al., 2009).

A similar GPI-anchored protein artificially system has been developed by another group (Paulick et al., 2007). This group used oligo-PEG's with a few mannose residues at the end and attached it chemically to the green fluorescent protein (GFP). These modified GPI-GFPs were incorporated into supported lipid bilayers as well as in live cells (CHO and HeLa cells) with the intention of studying the effect of remodelling the different chemical constituent groups of the GPI- anchor (e.g. removing a mannose residue). The results from these studies suggested that such analogues behave like actual GPI-anchored proteins in CHO and HeLa cells

where they were shown to be internalized and recycled back to the plasma membrane via similar endocytic routings (Paulick et al., 2007). An interesting finding was that the loss of a monosaccharide unit from the GPI-anchor decreased the rate of lateral diffusion of these analogues significantly, which may suggest the existence of transient interactions between the GPI-anchored protein and the underlying lipid bilayer surface (Paulick et al., 2007).

1.5.2 Using artificially lipid-anchored proteins to study the role of membrane rafts in endocytosis in Fibroblastic cells.

As noted above, artificially lipid-anchored proteins can be readily incorporated into the plasma and endosomal membranes of living mammalian cells (Wang et al., 2005; Bhagatji et al., 2009), and can be constructed to mimic the *physical* characteristics of native GPI-anchored proteins without carrying their specific *structural* features. This allows us to determine whether or not membrane rafts, or biospecific recognition of the GPI-anchor is crucial for a given biological function. My work has focused on the endocytic trafficking of the GPI-anchored folate receptor α, and of artificially lipid-anchored proteins in CHO and BHK cells in which the endocytic trafficking of GPI-anchored proteins has been most extensively characterized (Fivaz et al., 2002; Wang et al., 2005; Kalia et al., 2006; Mayor and Pagano, 2007; Howes et al., 2010)..

Recently, our group has used artificially lipid-anchored proteins, incorporated into live CHO cells as a tool to show that the initial sorting of GPI-anchored proteins into a distinctive endocytic pathway (the CLIC/GEEC pathway) at the plasma membrane requires neither association with ordered-lipid microdomains nor biospecific recognition of the GPI-anchor, but depends instead on steric interactions between such lipid-anchored proteins and other macromolecules at the 'crowded' membrane surface (Bhagatji et al., 2009).

In my project I have compared the trafficking of GPI-anchored proteins and artificially lipid-anchored proteins subsequent to their uptake from the plasma membrane, with the specific aim of testing previous proposals that partitioning of GPI-anchored proteins (and other membrane-components) into ordered lipid

microdomains underlies both their slow kinetics of recycling (compared to recycling transmembrane proteins) to the plasma membrane of CHO cells (Mayor et al., 1998; Chatterjee et al., 2001; Fivaz et al., 2002).

1.5.2.1 Role in Endocytic recycling in CHO-FRa-Tb1.

Measurements of the rates of recycling from the ERC to the PM of GPI-anchored proteins (Folate Receptor α & decay accelerating factor), TfR, and C6-NBD-sphingomyelin (a bulk membrane lipid marker) in CHO cells revealed differential sorting in the ERC. TfR and C6-NBD-sphingomyelin return to the PM with a $t_{1/2}$ of about 10 minutes, whereas the GPI-anchored proteins return with a $t_{1/2}$ of 30 minutes (Mayor et al., 1998). When cholesterol or sphingolipid levels are depleted, the recycling rate of such GPI-anchored proteins is increased three fold to match that of TfR (Mayor et al., 1998; Chatterjee et al., 2001; Sabharanjak et al., 2002; Chadda et al., 2007). Since the majority of the GPI-protein anchors possess long saturated fatty acyl/alkyl chains, such studies suggested a role for lipid rafts in regulating this endocytic retention of GPI-anchored proteins in the ERC (Mayor and Riezman, 2004).

Interpretation of the results just noted is complicated by the pleiotropic effects of the approaches employed in these studies (e.g. perturbation of actin organization or of membrane cholesterol/sphingolipid levels). As an alternative method to test the hypothesis that 'rafts' are important for differential sorting of GPI-anchored proteins in the ERC, I have examined the recycling of artificially lipid-anchored proteins in unperturbed cell using quantitative fluorescence-microscopic methods to compare the kinetics of endocytic recycling of proteins with artificially 'raft-loving' vs. 'raft-avoiding' anchors and of endogenous GPI-anchored proteins in CHO-FRα-Tb1 cells.

1.5.2.2 Role in Endocytic sorting in BHK-21 & CHO-FRa-Tb1.

A recent study has reported differential sorting of GPI-anchored proteins between different fibroblastic cell lines: BHK and CHO cells. They showed that such GPI-anchored proteins are also endocytosed through the CLIC/GEEC pathway in BHK

cells. This study employed the aerolysin toxin: a bacterial toxin that enters animal cells through binding to GPI-APs as a monomer. After binding, the toxin is activated which leads to circular polymerization and the formation of an amphiphillic heptameric complex. Fivaz and colleagues (2002) generated a mutant form of this toxin that cannot be activated and hence remain bound to a monomer GPI-anchored protein. Their study revealed that GPI-anchored proteins, whether bound by monomeric or oligomerized toxins, are endocytosed to the late endosomes in BHK cells. This was not observed for monomer-toxin-bound GPI-APs in CHO FRa-Tb1. Instead, GPI-anchored proteins were targeted to late endosomes only after toxin-induced oligomerization in CHO cells. It is reported in the literature that such oligomerization enhances GPI-anchored proteins association with liquid-ordered domains (Mayor and Riezman, 2004; Sabharanjak and Mayor, 2004). Moreover, the same group were able to find aerolysin-bound GPI-APs (monomer & oligomers) in DRMs isolated from BHK late endosomal fractions. This reported phenomenon was independent of the nature of the GPI-anchored protein, since the same study involved a different protein; namely CD95.

Despite these interesting findings, this study has been criticized at a number of occasions. It is not yet clear whether oligomers have longer residence times in lipid rafts than their monomeric counterparts. Sabharanjak and Mayor (2004) suggested that depletion of cholesterol or sphingolipid might be a necessary condition to implicate membrane rafts in this differential endocytic sorting. Additionally, it is likely that the aerolysin toxin, or at least its heptameric complex, is recognized by a specific protein inside the cell which, in turn, targets it to late endosomes/lysosomes for degradation. For these reasons, the use of our artificial lipid-anchored protein system can be advantageous. I employed confocal fluorescence microscopic methods to follow the intracellular endocytic itineraries of “raft-phillic” and “raft-phobic” artificial lipid-anchored proteins in these two different fibroblastic cell lines: BHK-21 and CHO-FRα-Tb1, and compared it to markers of the CLIC/GEEC pathway and CME (FRα & TfR) as well as markers of different endocytic compartments such as Rab7, Rab11, and Lysotracker.

Chapter2: Assessment of the Roles of Ordered-lipid Microdomains in Intracellular Sorting of Endocytosed Glycosylphosphatidylinositol-anchored Proteins in Mammalian Fibroblasts.

Mohammad Refaei, Rania Leventis, and John R. Silvius

1.0 Abstract

We have used artificially lipid-anchored proteins, incorporated into living mammalian cells and comprising soluble proteins bound to phosphatidylethanolamine-polyethyleneglycol- (PE-PEG-) anchors, to evaluate previously proposed roles for ordered lipid domains ('rafts') in the intracellular endocytic trafficking of glycosylphosphatidylinositol- (GPI-) anchored proteins in CHO and BHK cells. In CHO cells, endocytosed PE-PEG-protein conjugates with saturated or unsaturated anchors colocalized strongly in the central region of the cell with simultaneously internalized folate receptors and with GFP-rab11, a marker of the endosomal recycling compartment. However, internalized conjugates with long-chain saturated anchors recycled to the plasma membrane at a slow rate comparable to that measured for the folate receptor, while conjugates with short-chain or unsaturated anchors recycled at a faster rate also observed for the transferrin receptor. These findings support the proposal (Mayor et al., *EMBO J.* 17 (1998), 4628-4638) that the slow recycling of GPI-proteins in CHO cells rests on their association with ordered lipid domains. In BHK cells internalized PE-PEG-protein conjugates with either long-chain saturated or unsaturated anchors colocalized strongly with simultaneously endocytosed folate receptor, a finding that challenges previous suggestions that the sorting of GPI-proteins to late endosomes in these cells rests on their association with lipid rafts.

2.0 Introduction:

Membrane proteins can be internalized by a variety of endocytic pathways, including both clathrin-dependent and clathrin-independent processes (Kirkham and Parton, 2005; Mayor and Pagano, 2007; Sandvig et al., 2008; Donaldson et al., 2009), and may subsequently undergo diverse fates, including recycling to the cell surface from early endosomes or sorting to late endosomes/lysosomes for eventual degradation (Bishop, 2003; Maxfield and McGraw, 2004; Grant and Donaldson, 2009; Jovic et al., 2010). The signals that direct some membrane proteins to particular endocytic pathways have been clearly established; –YXXF-

and dileucine-based cytoplasmic sequence motifs, for example, target various transmembrane proteins for clathrin-mediated endocytosis by binding to subunits of AP-2 (Traub, 2009) and specific patterns of ubiquitination lead to ESCRT-dependent incorporation of endocytosed proteins into luminal vesicles as late endosomes mature into multivesicular bodies (Hicke and Dunn, 2003; Raiborg and Stenmark, 2009). For many other membrane proteins, however, and particularly for proteins internalized by clathrin-independent pathways, the targeting information that determines sorting into particular endocytic routes and compartments remains obscure.

The mechanisms of endocytic trafficking of glycosylphosphatidylinositol-anchored (GPI–) proteins are of particular interest from a conceptual standpoint, as these species lack transmembrane or cytoplasmic domains and therefore cannot interact directly with scaffolding or adaptor proteins found at the cytoplasmic faces of the plasma and endosomal membranes. In CHO cells GPI-proteins are internalized via the clathrin-and dynamin-independent GEEC/CLIC pathway, are delivered to sorting and recycling endosomes where they substantially colocalize with transferrin receptor internalized via clathrin-mediated endocytosis (Fivaz et al., 2002; Sabharanjak et al., 2002; Kirkham et al., 2005; Kalia et al., 2006). Mayor and colleagues (Mayor et al., 1998; Chatterjee et al., 2001) have shown that the transferrin receptor is returned from the latter compartment to the plasma membrane much more rapidly than is the folate receptor. However, these workers also reported that in cells grown under conditions that reduce cellular cholesterol levels, both proteins recycled to the cell surface at a rapid rate comparable to that observed for the transferrin receptor in normal cells. This observation led to the proposal that in normal (cholesterol-replete) cells, association of the folate receptor with ordered-membrane lipid (‘raft’) microdomains reduces the rate of recycling of this protein from the endosomal compartment to the cell surface. Suggestions that ‘raft’ association can influence the endocytic trafficking of GPI-proteins have not been limited to CHO cells alone. Fivaz et al. (2002) reported that in BHK cells, internalized GPI-anchored proteins are not efficiently recycled

to the cell surface but instead are delivered to late endosomes, where they colocalize with rab7 and the late endosome-specific lipid marker lyso-bisphosphatidic acid. These researchers also observed that internalized GPI-proteins were associated with a cold detergent-insoluble membrane fraction and suggested that sorting of these proteins to late endosomes could be determined by association with raft microdomains.

Rigorous assessment of the role of lipid rafts in various cellular functions remains a challenging task, given the limitations of available methods to demonstrate directly the association of membrane components with rafts in living cells and the potential that treatments designed to manipulate rafts *in situ* (e.g., partial depletion of cellular cholesterol) may perturb a variety of membrane properties, not all of which are related to raft formation (Subtil et al., 1999; Liu et al., 2006; Shah et al., 2006). As a novel tool to address this problem we have developed artificially lipid-polyethyleneglycol-anchored protein conjugates that can be incorporated into living cells, where their behaviour can be compared to that of endogenous GPI-anchored proteins, and whose lipid 'anchor' structures can be altered to vary their tendencies to associate with ordered-lipid microdomains (Wang et al., 2005; Bhagatji et al., 2009). We have previously shown that like endogenous GPI-proteins, these species are internalized in CHO cells via the GEEC/CLIP pathway and are excluded from the clathrin-mediated endocytic pathway, but that the basis for this selectivity rests not on the affinities of these lipid-anchored proteins for lipid rafts but rather on their steric exclusion from coated pits (Bhagatji et al., 2009). In this study we have compared the intracellular trafficking in CHO and BHK cells of artificially lipid-anchored proteins and of endocytosed GPI-proteins, to assess the degree to which association of these species with ordered lipid microdomains affects their endocytic routing after initial uptake from the plasma membrane. Our findings indicate that partitioning into ordered lipid microdomains does not determine the endocytic itinerary of these species in either cell type, at least as reflected at the microscopic (slightly sub-micron) level of resolution. However, we also find that in CHO cells the affinity of artificially

lipid-anchored proteins for ordered-lipid domains strongly influences the kinetics of recycling of these proteins to the plasma membrane after internalization, strongly supporting the proposal of Mayor et al. (1998) that association of GPI-proteins and other species with 'lipid rafts' in sorting/reycling endosomes modulates the kinetics of their recycling to the cell surface.

3.0 Materials and Methods:

Materials- Phosphatidylethanolamine-polyethyleneglycol1500 (-PEG1500-) conjugates substituted at the distal end of the –PEG1500- chain with biotinyl (PE-PEG-Bio), trinitrophenyl (PE-PEG-TNP) or methotrexate residues (PE-PEG1500-MTX) were synthesized as described previously (Wang et al., 2005; Bhagatji et al., 2009). A rhodamine-labeled folate analogue was prepared as described previously (Bhagatji et al., 2009). *E. coli* dihydrofolate reductase (DHFR), prepared as described previously (Bhagatji et al., 2009), transferrin and streptavidin (Sigma/Aldrich, St. Louis, MO), and rabbit polyclonal anti-dinitrophenyl (anti-DNP) IgG (Molecular Probes/Invitrogen, Burlington, ON) were labelled with Alexa488- or Alexa555-pentafluorophenyl ester (Molecular Probes) according to the manufacturer's instructions..

Cell labelling- FRαTb-1 cells, a CHO cell line stably expressing the human GPI-linked folate receptor and the transferrin receptor (a generous gift of Dr. Satyajit Mayor, National Centre for Biological Sciences, Bangalore, India), were cultured in folate-free Ham's F12 medium containing 10% dialyzed fetal bovine serum as described previously. BHK cells were cultured in Dulbecco's minimum essential medium (D-MEM) containing 5% serum. BHK cell lines stably expressing the human folate receptor were prepared by transfecting cells with a derivative of the plasmid pcDNA3.1(–) (Invitrogen) incorporating the complete coding sequence of the human folate receptor (prepared by standard PCR methods and ligated between the *Xba*I and *Eco*RI sites of the plasmid) and were selected and maintained in D-MEM containing 10% fetal bovine serum and 0.8 mg/ml G418. Cells were transiently transfected with plasmids encoding rab7- or rab11-GFP

fusion proteins (generously provided by Dr. Marino Zerial, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden) using Fugene6 (Roche Canada, Mississauga, ON) as per the manufacturer's instructions.

PE-PEG/bovine serum albumin (BSA) complexes were prepared and used to incorporate PE-PEG conjugates into cell monolayers, grown on coverslips or in glass-bottomed dishes, as described previously (Pagano et. al., 2000; Bhagatji et al., 2009); cells were incubated with 5-50 mM PE-PEG/BSA complexes for 2 h in serum-free medium at 37^{o}C, then postincubated in serum-containing medium for 1.5 h at 37^{o}C. Cells were incubated for the indicated times at 37^{o}C with fluorescent-labelled proteins and/or folate, then fixed for 15 min at 37^{o}C with 3% paraformaldehyde in phosphate-buffered saline and mounted in 10% Mowiol, 25% glycerol, and 2.5% DABCO in 0.2M Tris pH 8.5 for microscopic imaging.

Quantitation of marker internalization and recycling- To monitor the kinetics of marker uptake, CHO (FRaTb-1) cell monolayers were washed 4 times at 37^{o}C in Hanks' buffered saline solution plus 25 mM HEPES, pH 7.2 (HBSS/HEPES), then incubated for varying times at 37^{o}C in folate-free, serum-supplemented Ham's F12 medium containing either Alexa555-DHFR (20 µg/ml), Alexa555-transferrin (20 µg/ml), Alexa555-antiDNP antibody (20 µg/ml) or rhodaminyl-folate (20 nM). At the end of the incubation period the cells were rapidly washed three times in ice-cold HBSS/HEPES, then incubated at 4^{o}C in the following media to remove surface-bound ligand: ascorbate buffer (160 mM ascorbic acid, 40 mM sodium ascorbate, 1 mM each $CaCl_2$ and $MgCl_2$, pH 4.5, 5 x 5 min) for rhodaminyl-folate and transferrin, 10 mM DNP-lysine in ascorbate buffer (4 x 10 min.) for anti-DNP antibody and 30 µM methotrexate in HBSS/HEPES (4 x 15 min) for DHFR. The cells were then fixed with 3% paraformaldehyde for 30 min at 4^{o}C, mounted as described above and imaged on a Nikon TE300 fluorescence microscope with a 20x (NA 0.45) Plan-Fluor objective. The total fluorescence intensity within the cell contour (determined by phase-contrast imaging),

corrected for background fluorescence, was measured and averaged for 60-100 cells for each time point to determine the time course of marker uptake.

To monitor the kinetics of recycling of internalized fluorescent markers to the cell surface, cells were first incubated at 37^{o}C with the fluorescent species in serum-supplemented, folate-free medium at the concentrations indicated above, for 1 h (for transferrin) or 2 h (for rhodaminyl-folate, DHFR in cells incorporating PE-PEG-methotrexate conjugates or anti-DNP antibody in cells incorporating PE-PEG-TNP conjugates) to reach steady-state labelling. The cells were stripped of surface-bound ligand in the cold as described above, then rewarmed to 37^{o}C to initiate recycling of internalized marker to the cell surface. After varying times at 37^{o}C the cells were rapidly chilled to 4^{o}C and again stripped of surface-exposed fluorescent molecules as described above, then fixed and mounted for microscopic observation. Residual cell-associated fluorescence after varying times of incubation at 37^{o}C was determined by microscopy in the same manner as described above for monitoring marker uptake.

Confocal imaging- Cells were labelled for confocal microscopy as described above and examined either live or fixed, as indicated, on a Zeiss LSM 5 Pascal confocal microscope with a 63x objective lens (NA 1.45) and a pinhole setting of 0.8 μm.

4.0 Results:

As we have described previously (Wang et al., 2005; Bhagatji et al., 2009), phosphatidylethanolamine-polyethyleneglycol- (PE-PEG-) –ligand conjugates can be stably incorporated into the surface membrane of living cells and subsequently bound, with high affinity and specificity, by appropriate soluble proteins to produce artificially lipid-anchored proteins in the plasma membrane outer leaflet. In this study we used three types of lipid-PEG-protein conjugates, based on *E. coli* dihydrofolate reductase (DHFR) bound to PE-PEG-methotrexate, or anti-

dinitrophenyl (antiDNP) antibody bound to PE-PEG-trinitrophenyl (TNP) or streptavidin bound to PE-PEG-biotin anchors. As a representative GPI-anchored protein we examined the previously studied folate receptor labelled with a high-affinity fluorescent ligand, rhodaminyl-folate. The strong binding of DHFR, anti-DNP antibody and streptavidin to PE-PEG-methotrexate, PE-PEG-TNP and PE-PEG-biotin, respectively, and of rhodaminyl-folate to the folate receptor are maintained at the acidic pH values ($\geq$ 4.5) found in endosomal compartments (Stone and Morrison, 1983; Kamen et al., 1988; Green, 1990; Klingenberg and Olsnes, 1996; Chatterjee et al., 2001; Bhagatji et al., 2009). The observed intracellular distributions of these fluorescent-labelled proteins and of rhodaminyl-folate can thus be taken as representative of those of the corresponding lipid-anchored protein conjugates and of the GPI-anchored folate receptor, respectively.

In CHO cells both GPI-proteins like the folate receptor and artificially lipid-anchored proteins undergo internalization via the GEEC/CLIC endocytic pathway (Sabharanjak et al., 2002; Kirkham et al., 2005; Bhagatji et al., 2009) but later reconverge with membrane proteins internalized via clathrin-mediated endocytosis and destined for plasma membrane recycling, such as the transferrin receptor, in a central endosomal structure that corresponds at least partly to the endosomal recycling compartment (Mayor et al., 1998; Chatterjee et al., 2001). In initial experiments, CHO cells pretreated to incorporate PE-PEG-methotrexate or –TNP 'anchors' were incubated with fluorescent DHFR or anti-DNP antibody, rhodamine-labeled folate and/or labelled transferrin for 2 h to label the major intracellular endocytic compartments to steady-state levels, and the distributions of the internalized molecules were examined by confocal microscopy. In order to preserve optimally the structure of intracellular endocytic compartments (some of which are labile at low temperatures (Sabharanjak et al., 2002; Lundmark et al., 2008), after incubation with fluorescent markers cells were fixed at 37^{o}C.

Figure 4: Comparison of steady state intracellular distributions of different endocytic markers (Rab7/11, di16:0-PE-PEG-DHFR, and TfR) to rhodaminyl-folate in CHO cells.

Comparison of steady-state intracellular distributions of different endocytic markers in CHO cells. Washed cell monolayers, pretreated where indicated to incorporate di16:0-PEG-methotrexate or to express rab-GFP constructs, were incubated for 2 h with the indicated fluorescent markers, then fixed and imaged by confocal microscopy. For illustrative purposes the fields shown in panels A-C and J-L include an entire cell; those shown in the other panels include only the brightly fluorescent central region of a cell, to facilitate comparison of the marker distributions in this region. For the merged images in the right-hand panels, the markers indicated in the left-hand and center panels are shown in green and red, respectively. Marker combinations shown are as follows: (A) – (C), Alexa488-DHFR, bound to cell-incorporated di16:0PE-PEG-methotrexate, and rhodaminyl-folate; (D) – (F), rab11-GFP and rhodaminyl-folate; (G) – (H), rab11-GFP and Alexa555-DHFR, bound to cell-incorporated di16:0PE-PEG-methotrexate; (I) – (L), rab7-GFP and rhodaminyl-folate; (M) – (O), Alexa488-transferrin and rhodaminyl-folate. Other experimental details were as described in Materials and Methods. Scale bar = 10 µm.

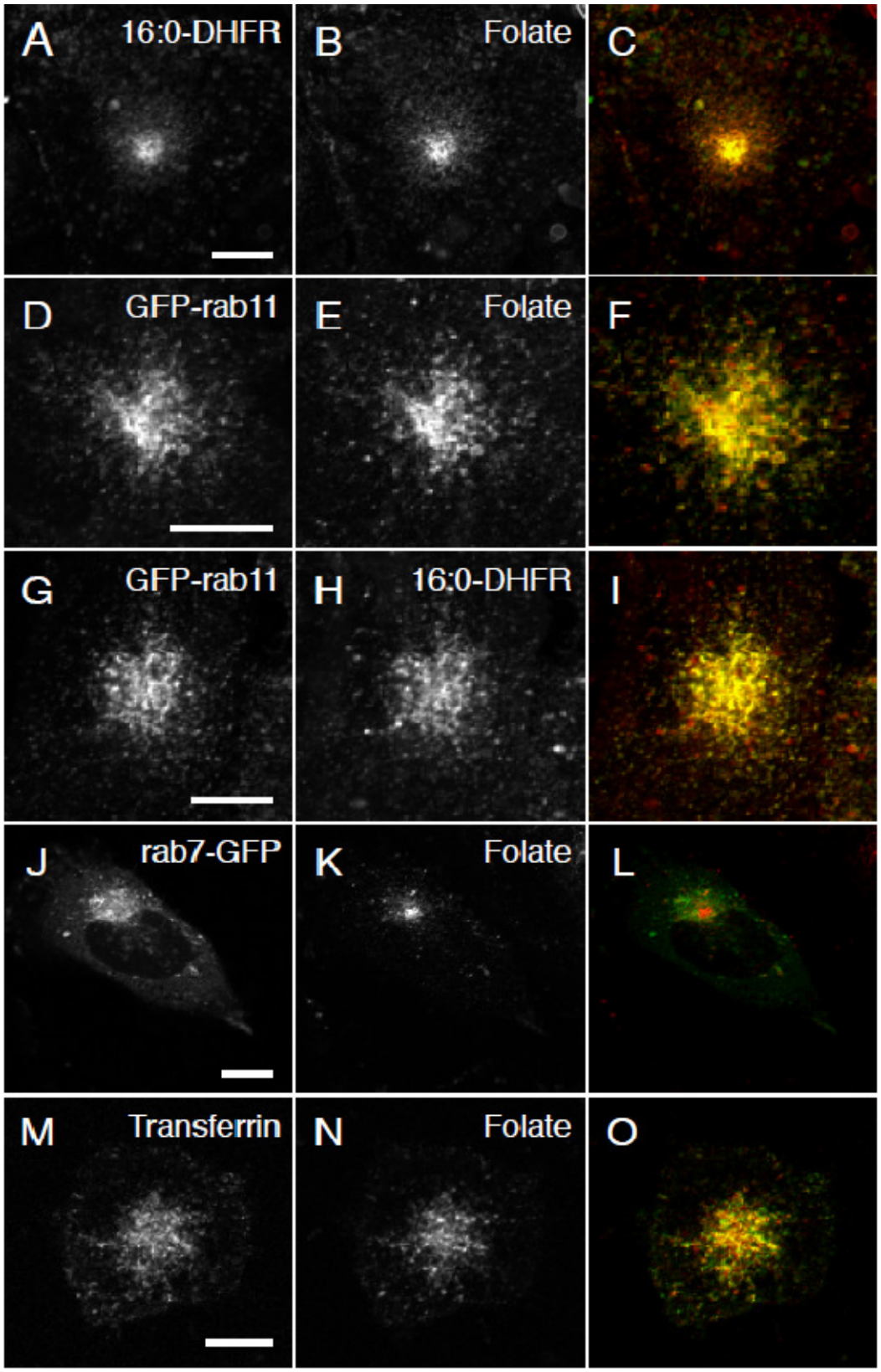
A
16:0-DHFR
B
Folate
C
D
GFP-rab11
E
Folate
F
G
GFP-rab11
H
16:0-DHFR
I
J
rab7-GFP
K
Folate
L
M
Transferrin
N
Folate
O

Figure 4

As shown in Figure 4A-C and Figure 5, CHO cells allowed to internalize a saturated di16:0-PEG-DHFR conjugate along with rhodaminyl-folate for 2 h showed essentially complete colocalization of the two markers in the central region of the cells (Pearson correlation coefficient = 0.95 $\pm$ 0.01). Internalized rhodaminyl-folate and di16:0-PEG-DHFR colocalized strongly in the central region of the cells with GFP-rab11 (Figure 4D-I and Figure 5), a marker for the endosomal recycling compartment (Ullrich et al., 1996; Sonnichsen et al, 2000). Neither marker showed significant colocalization with GFP-rab7, a late endosomal/lysosomal marker (Figure 4J-L), or with LysoTracker Green (not shown), consistent with previous findings that in CHO cells the endocytosed folate receptor is efficiently recycled to the plasma membrane, with at most a small fraction trafficked to late endosomes/lysosomes (Mayor et al., 1998; Fivaz et al, 2002). Transferrin and rhodaminyl-folate also showed substantial colocalization in the central region of the cells (Figure 4M-O and Figure 5), as reported previously (Mayor et al., 1998; Chatterjee et al., 2001; Bhagatji et al., 2009). An unsaturated di18:1c-PEG-DHFR conjugate also showed strong intracellular colocalization with rhodaminyl-folate in CHO cells allowed to internalize both markers for 2 hr (Figure 5 and Figure 6A-C), indicating that the strong colocalization of the endocytosed lipid-PEG-DHFR conjugates with internalized folate receptor was not dependent on the affinity of the lipid-PEG anchor for ordered-lipid domains. To eliminate any possibility that the acyl chains of PE-PEG-DHFR lipid anchors could be remodelled after uptake, we repeated the experiments with PE-PEG-methotrexate anchors bearing two long saturated alkyl chains (di16ether-, Figure 6D-F) or two unsaturated alkyl chains (di18c-ether, Figure 6G-I), again with the same results. Internalized PE-PEG-DHFR conjugates thus colocalize strongly with endocytosed folate receptor in recycling endosomes, independently of the affinity of their PE-PEG ‘anchors’ for ordered lipid domains.

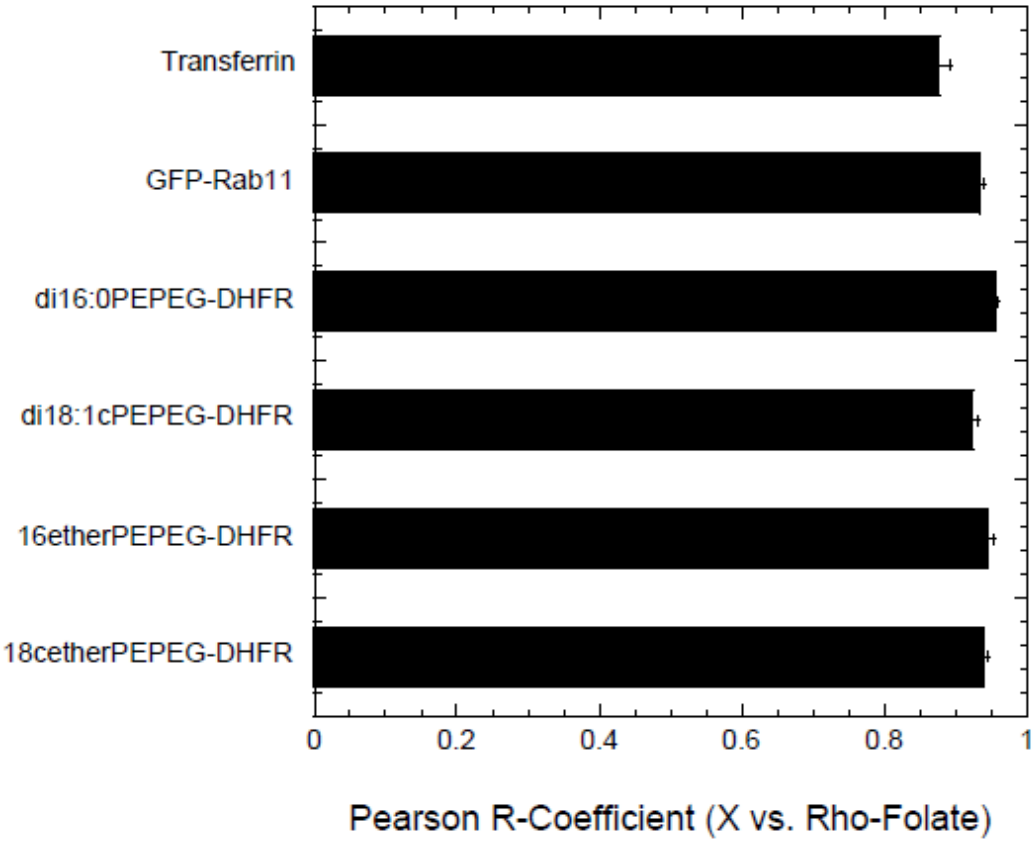

Figure 5: Pearson correlation coefficient (R) for colocalization of the indicated fluorescent markers with rhodaminyl-folate in ERC of CHO cells.
Pearson correlation coefficients (R) for colocalization of the indicated fluorescent markers with rhodaminyl-folate in the central regions of CHO cells allowed to internalize the fluorescent cargos for 2 h at 37°C. Where relevant cells were transfected to express rab11-GFP, or pretreated to incorporate PE-PEG-methotrexate species, as described in Materials and Methods. Values shown represent the average values (± standard error of the mean) determined for 20-25 images.

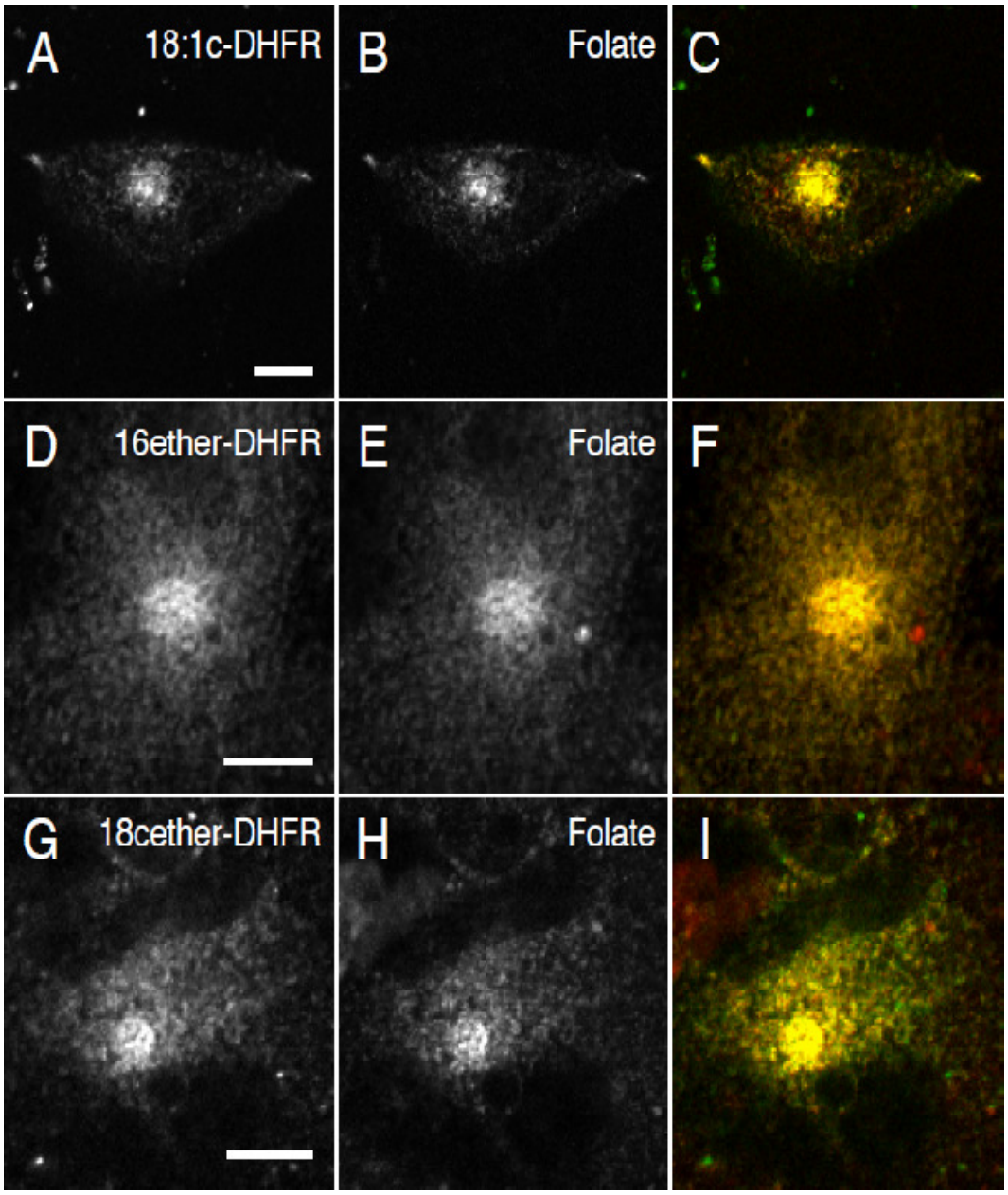

Figure 6: Endocytosed PE-PEG-anchored DHFR conjugate strongly colocalize with internalized rhodaminyl-folate in CHO cells, independent of 'raft-associating' affinity of the PE-PEG anchors.

Endocytosed PE-PEG-anchored DHFR conjugates strongly colocalize with internalized rhodaminyl-folate in CHO cells, independent of the 'raft'-associating affinity of the PE-PEG anchor. Washed cell monolayers, pretreated to incorporate the indicated PE-PEG-methotrexate species, were incubated for 2 h at 37^{o}C with Alexa488-DHFR and rhodaminyl-folate, then fixed and imaged by confocal microscopy. In the right-hand merged images, the markers indicated in the left-hand and center panels are shown in green and red, respectively. Images shown represent cells co-internalizing rhodaminyl-folate and: (A) – (C), di18:1cPE-PEG-anchored DHFR; (D) – (F) dihexadecyl- (di16-ether-) –PE-PEG-anchored DHFR; (G) – (H), di-(cis-9'-octadecenyl)- (di18c-ether-) –PE-PEG-anchored DHFR. Other experimental details were as described in Materials and Methods. Scale bar = 10 µm.

We next compared the rates of endosome-to-cell surface recycling of TfR, folate receptor and PE-PEG-anchored conjugates of DHFR and anti-DNP antibody, using PE-PEG- anchors with different hydrophobic chains and consequently differing affinities for ordered-lipid domains. Cells were loaded to steady-state for 2 h with different fluorescent endocytic markers, stripped of surface-bound marker in the cold and rewarmed to 37^{o}C to initiate recycling of internalized markers to the cell surface. After incubation for varying times at the latter temperature the cells were again chilled, stripped of surface-exposed label and fixed. The average intracellular fluorescence remaining at each time point was then determined by epifluorescence microscopy and quantitative image analysis as discussed in Materials and Methods. As illustrated in Figure 7A, the level of intracellular fluorescence under these conditions decreases exponentially with the time of incubation at 37^{o}C, reaching a plateau determined by the relative rates of recycling and possible subsequent re-internalization of each marker (see Appendix or Mayor et al., 1998). The rate constants for recycling of each internalized marker (k_{rec}) were determined from these time courses as described in the Appendix and are summarized in Figure 7B. Transferrin recycles rapidly to the cell surface, with a calculated k_{rec} value of 0.073 $\pm$ 0.002 min, similar to the value reported previously for this species in CHO cells (Mayor et al., 1993). By contrast, also in agreement with previous findings (Mayor et al., 1998; Chatterjee et al., 2001), the GPI-linked folate receptor shows a much slower rate of recycling (k_{rec} = 0.017 $\pm$ 0.001 min^{-1}). DHFR bound to a PE-PEG-methotrexate anchor with long saturated (di16:0) acyl chains shows a slow rate of recycling comparable to that measured for the folate receptor. In marked contrast, DHFR bound to PE-PEGs with either unsaturated (di18:1c-) or short saturated (di12:0-) acyl chains recycles to the surface at a much faster rate, similar to that measured for the transferrin receptor. To rule out any possibility that the above results could be affected by remodelling of the PE-PEG-methotrexate lipid anchors, we also examined the kinetics of recycling of DHFR bound to analogous dialkyl-PE-PEG-methotrexate anchors. Similar results were obtained; DHFR bound to a long-chain di16ether- anchor recycled with slow kinetics similar to those observed for the

folate receptor, while DHFR bound to unsaturated (di18c-ether–) or short chain saturated (di12ether-) anchors showed a rapid rate of recycling comparable to that determined for the transferrin receptor. In order to ensure that the observed endocytic recycling kinetics of the PE-PEG-anchored DHFR were not dependent on the nature of the protein moiety, we also carried out similar experiments using anti-DNP antibody bound to different PE-PEG-TNP species (Bhagatji et al., 2009). As shown in Figure 7B, anti-DNP antibody bound to a long-chain saturated (di16:0-) PE-PEG-TNP anchor again recycled at a slow rate similar to that observed for the folate receptor, while anti-DNP antibody anchored to an unsaturated (di18:1c-) PE-PEG-TNP showed a rapid rate of recycling. The recycling kinetics of lipid-anchored proteins in the extracytoplasmic leaflet of the sorting endosome thus appear to depend strongly on the hydrophobic chains of the lipid anchor (slow kinetics for anchors with long saturated chains, rapid kinetics for anchors with short or unsaturated chains) but not on the nature of the linkage of the hydrocarbon chains to the lipid glycerol backbone (acyl vs. alkyl) nor on the nature of the passenger protein.

Figure 7: Recycling kinetic curves & rates.

Kinetics of recycling of internalized endocytic markers in CHO cells (A) Representative time courses of the recycling-induced decrease in intracellular transferrin, rhodaminyl-folate and di16:0PE-PEG-anchored DHFR. (B) Measured rate constants for recycling of the indicated markers to the cell surface. Data shown represent the mean (± SEM) of values determined in at least three independent experiments in all cases. Other experimental details were as described in Materials and Methods

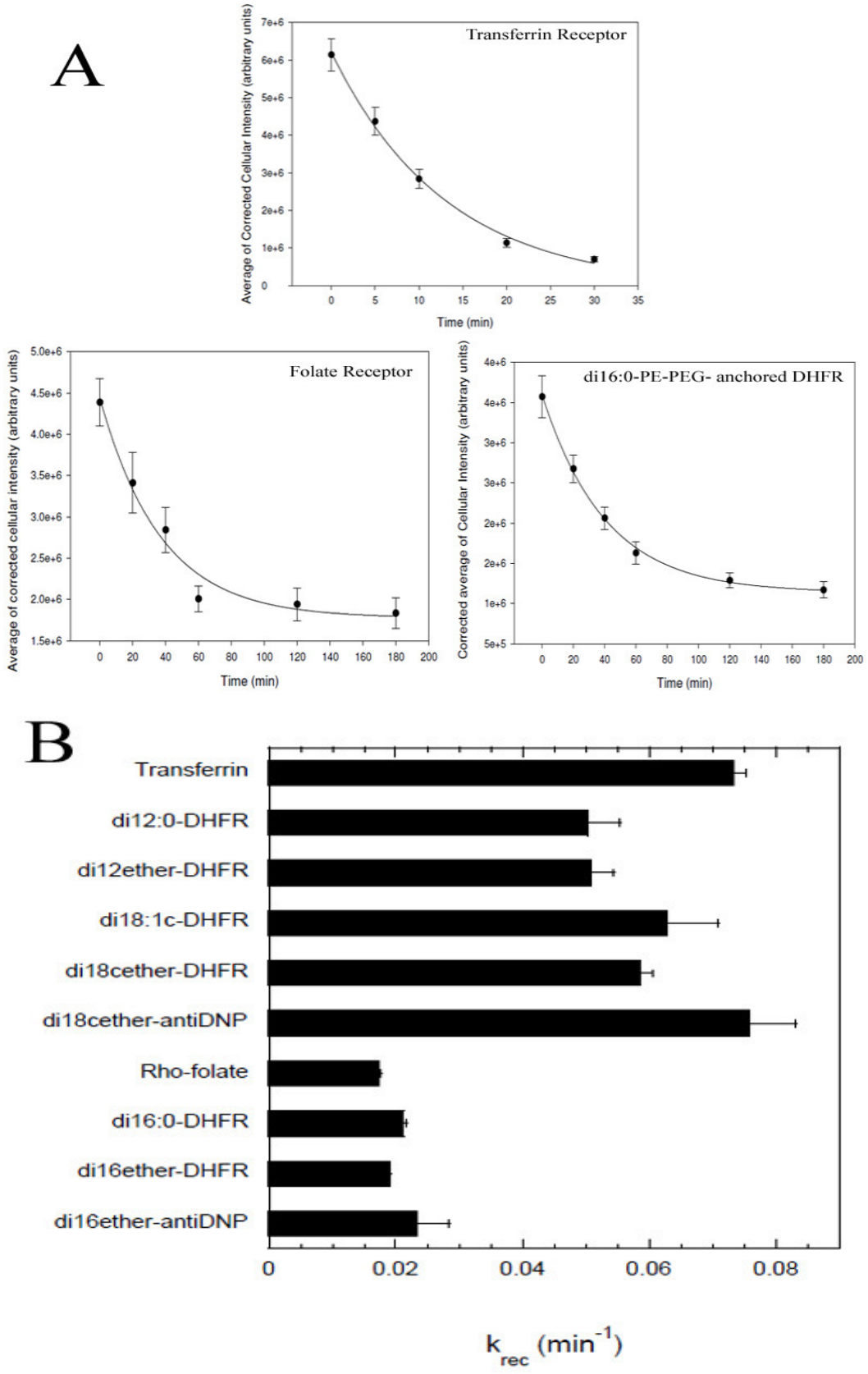
A
Transferrin Receptor
Average of Corrected Cellular Intensity (arbitrary units)
Time (min)
Folate Receptor
Average of corrected cellular intensity (arbitrary units)
Time (min)
di16:0-PE-PEG- anchored DHFR
Corrected average of Cellular Intensity (arbitrary units)
Time (min)
B
Transferrin
di12:0-DHFR
di12ether-DHFR
di18:1c-DHFR
di18cether-DHFR
di18cether-antiDNP
Rho-folate
di16:0-DHFR
di16ether-DHFR
di16ether-antiDNP
0
0.02
0.04
0.06
0.08
k rec (min-1)

Figure 7 (A&B)

Endocytic Trafficking of PE-PEG-anchored Proteins in BHK Cells - Fivaz et al. (2002) have previously observed that endocytosed PI-proteins labelled with a monomeric derivative of the GPI-binding toxin aerolysin are largely trafficked to late endosomes in BHK cells, and they suggested that this behaviour reflects preferential trafficking of raft-associated molecules to late endosomes rather than to recycling endosomes in this cell line.

To assess this possibility, we examined the intracellular distributions of endocytosed DHFR, bound to either saturated or unsaturated PE-PEG-methotrexate 'anchors,' in BHK cells that were allowed to internalize the conjugates (together with other fluorescent endocytic markers as appropriate) for 2 h or 4 h. As illustrated in Figure 8A-C, rhodaminyl-folate and Alexa488-labeled transferrin endocytosed by BHK cells show only a modest degree of intracellular colocalization, in contrast to the behaviour observed in CHO cells. As shown in Figure 8D-I, after either 2 or 4 h of uptake, DHFR bound to a saturated (di16:0-) PE-PEG anchor showed only a moderate degree of colocalization with internalized transferrin but a very high degree of colocalization with rhodaminyl-folate. Similar behaviour was observed for DHFR bound to a saturated (di16ether-) dialkyl PE (Figure 9A-C). Interestingly, DHFR bound to an unsaturated dialkyl (di18:1ether-) PE-PEG-methotrexate anchor also showed a high degree of colocalization with internalized rhodaminyl-folate (Figure 9D-F) but only moderate colocalization with transferrin (not shown). To confirm that the behaviour of our artificially lipid-anchored proteins was not dependent on the nature of the passenger protein, we also compared the intracellular distribution of Alexa488-labeled streptavidin bound to a di16:0-PE-PEG-biotin anchor with that of rhodaminyl-folate in BHK cells allowed to internalize the two species for 2 or 4 h. The intracellular distributions of the two species were very similar after either period of incubation, as illustrated in Figure 9G-I.

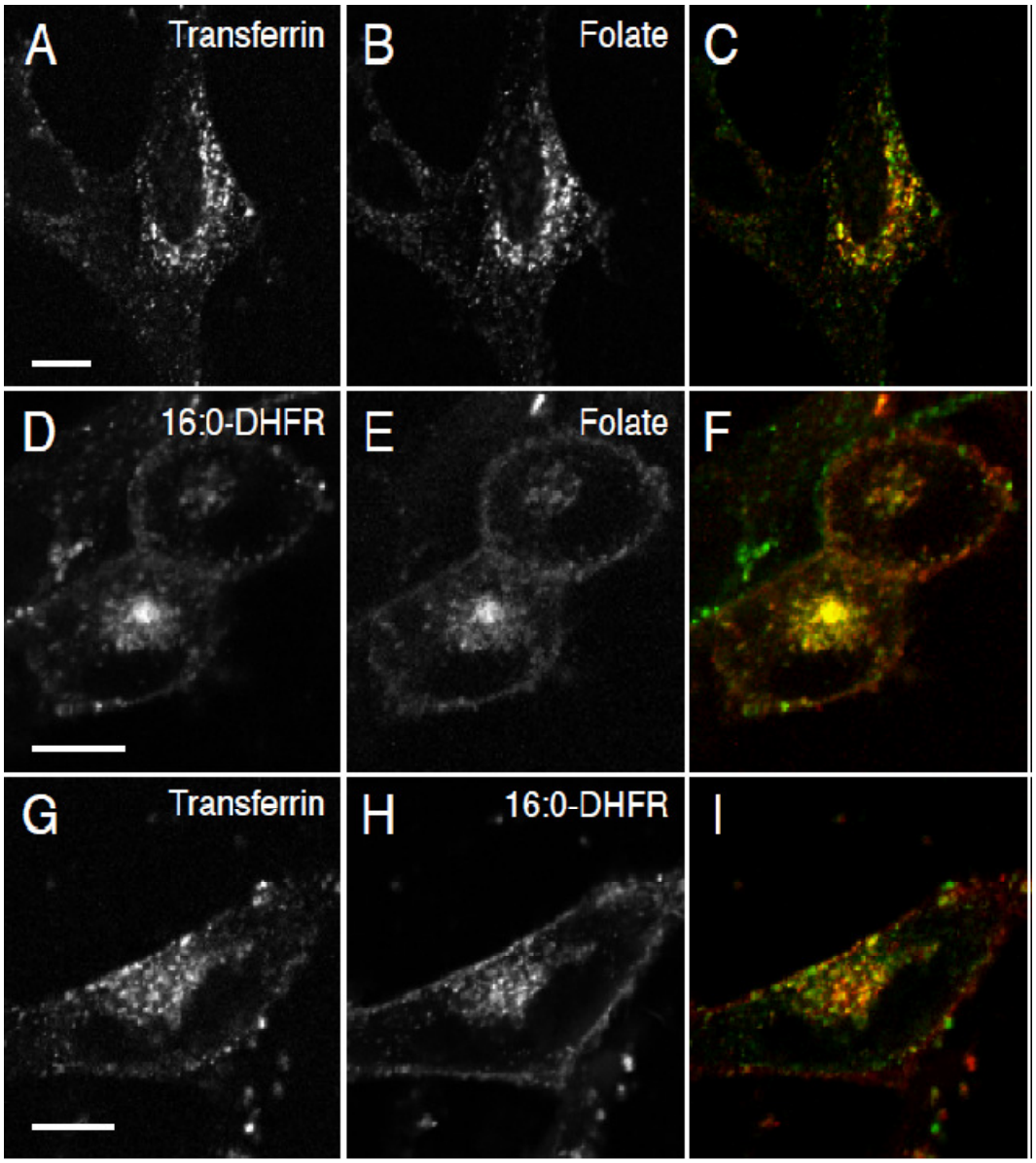

Figure 8: Steady-state intracellular distribution of TfR with either Rho-folate or di16:0 DHFR in BHK cells.

Intracellular distributions of endocytic markers in BHK cells allowed to internalize the markers for 4 h at 37°C. Panels (A) – (C) – Cells coincubated with Alexa488-transferrin and rhodaminyl-folate. Panels (D) – (E) – Cells incorporating di16:0PE-PEG-methotrexate were coincubated with Alexa488-DHFR (to generate di16:0PE-PEG-DHFR) and rhodaminyl-folate. Panels (G) – (I)- Cells incorporating di16:0PE-PEG-methotrexate were coincubated with Alexa488-transferrin and Alexa555-DHFR. Other experimental details were as described in Materials and Methods. Scale bar = 10 µm. For the merged images shown at the right, the images shown in the left-hand and center panels were tinted green and red, respectively.

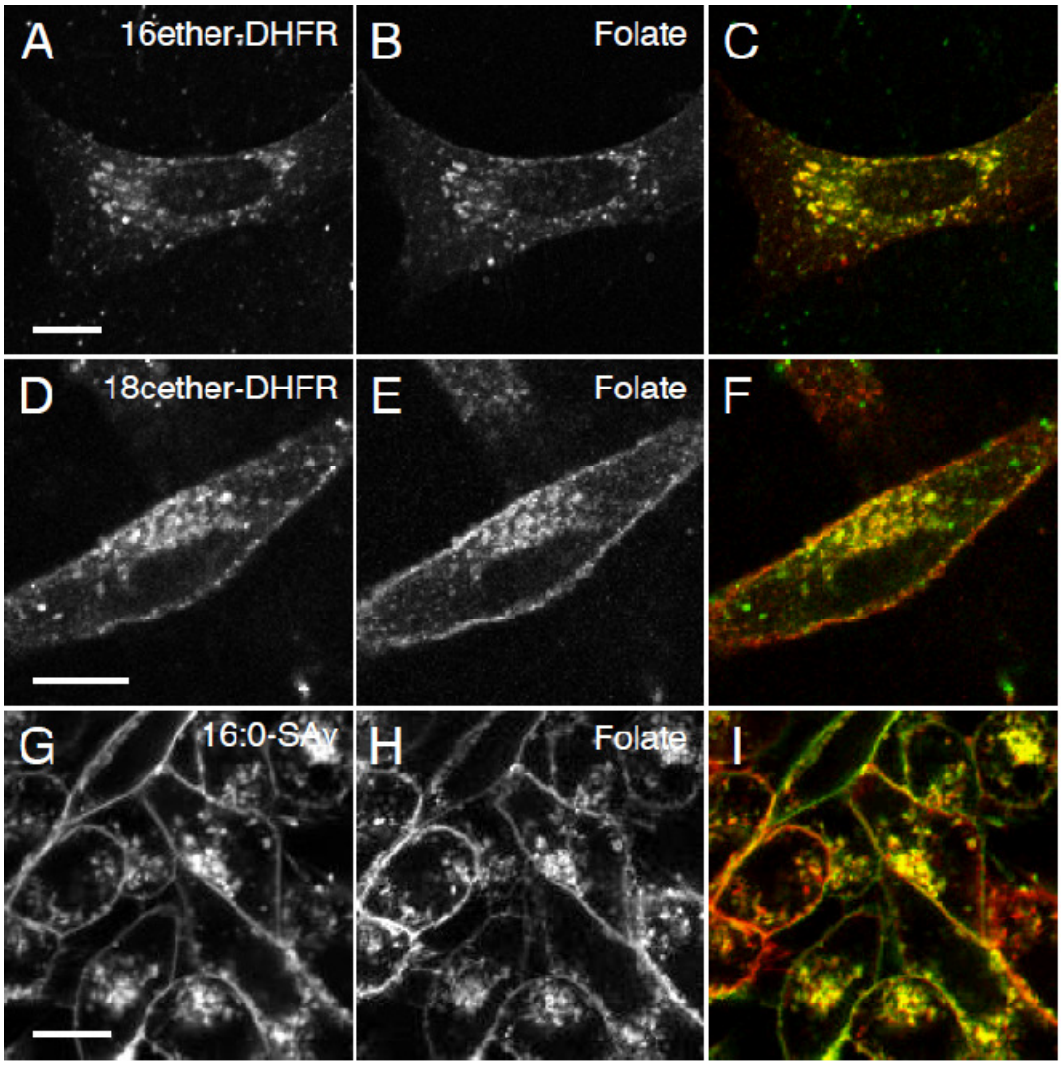

Figure 9: Comparison of the steady state intracellular distribution of different PE-PEG-anchored DHFR/Streptavidin conjugates to rhodaminyl-folate in BHK cells.

Comparison of the steady-state intracellular distributions of different PE-PEG-anchored Alexa488-DHFR or –streptavidin conjugates to that of internalized rhodaminyl-folate in BHK cells allowed to endocytose both markers for 4 h at 37°C. In the merged images shown at the right, the images shown in the left-hand and center panels are tinted green and red, respectively. Panels (A) – (C) – DHFR anchored to dihexadecyl- (16ether-) –PEPEG-methotrexate. Panels (D) – (E) –DHFR anchored to di-(cis-9'-octadecenyl-) (18cether-) – PEPEG-methotrexate. Green-only spots at the tapered ends of the cell arose from spots of material on the cell surface, as revealed by examining successive closely spaced confocal sections. Panels (G) – (I)- Streptavidin anchored to di16:0PE-PEG-biotin. Due to variations in the relative intensities of Alexa488-streptavidin vs. rhodaminyl-folate staining in different cells, not all cells appear yellow in the merged image. Other experimental details were as described in Materials and Methods. Scale bar = 10 µm

5.0 Discussion:

Our present findings indicate that artificially lipid-anchored proteins with long-chain saturated PE-PEG anchors traffic through the endosomal system in a manner very similar to a representative GPI-anchored protein, folate receptor α, in both CHO and BHK cells. As observed previously (Mayor et al., 19989; Fivaz et al., 2002) the steady-state distributions of GPI-proteins within the two cell types are very different; in CHO cells a large fraction of total internalized folate receptor is found in large and well-defined perinuclear recycling endosomes, while in BHK cells internalized GPI-proteins (as well as transferrin) are largely dispersed in smaller structures. Nonetheless, the intracellular distributions of PE-PEG-DHFR species closely mirror that of folate receptor in both cell types, independently of the affinity of the PE-PEG- lipid anchor for ordered lipid domains. Our findings in CHO cells may appear divergent from those of Mukherjee et al. (1999), who observed that in these cells fluorescent lipid probes with long saturated chains are transferred preferentially from sorting endosomes to late endosomes, while short-chain or unsaturated probes are transferred mainly to recycling endosomes. However, as noted by these authors, the fact that GPI-proteins, whose lipid anchors typically bear long saturated hydrocarbon chains (McConville and Ferguson, 1993; Benting et al., 1999), are efficiently transferred to recycling endosomes, not to late endosomes in CHO cells indicates that sorting of membrane components to late endosomes is not determined purely by affinity for ordered-lipid domains. Endocytic sorting of simple lipid probes is thus not predictive for sorting of lipid-anchored proteins, even when the two types of molecules bear similar hydrocarbon chains. We have reported a similar conclusion for endocytic sorting of lipid markers vs. lipid-anchored proteins at the cell surface (Bhagatji et al., 2009).

While the distribution of endocytosed lipid-anchored proteins within CHO or BHK cells, at least at the level of light-microscopic resolution, is not substantially influenced by their affinities for ordered lipid domains, we find that this property

strikingly affects the kinetics of recycling of these proteins in CHO cells from early endosomes to the cell surface. In these cells internalized PE-PEG-anchored DHFR or anti-DNP antibodies with long saturated acyl chains recycle slowly to the plasma membrane, at a rate comparable to that observed for the folate receptor. By contrast, internalized DHFR or anti-DNP antibodies bound to short-chain or unsaturated PE-PEG anchors recycle roughly three times more rapidly, at a rate comparable to that measured for the transferrin receptor, under the same conditions. Mayor and colleagues (Mayor et al., 1998; Chatterjee et al., 2001) have previously observed this difference between the recycling rates of the transferrin and folate receptors in CHO cells and shown that when cellular cholesterol or sphingolipid levels are reduced, both proteins recycle at the rapid rate observed for the transferrin receptor in normal cells. From these results they proposed that the prolonged retention of folate receptor (and other GPI-proteins) in recycling endosomes arises from the ability of these proteins to associate with ordered lipid domains, in contrast to the transferrin receptor which associates preferentially with 'non-raft' lipid domains. Our present results, obtained using cells with unperturbed lipid compositions, provide strong support for this proposal.

The mechanism by which association with ordered lipid domains retards the recycling of GPI-proteins from the early endosomal compartment remains to be clearly elucidated. However, a plausible (if not unique) explanation has been proposed by Chen et al. (2008) based on our current knowledge of the mechanisms of fast vs. slow recycling of materials from early endosomal components to the cell surface. Rab4 mediates rapid return of a large fraction of internalized transferrin receptor (and of bulk lipid markers) directly to the plasma membrane from sorting endosomes, at least in part by recruiting microtubule plus-end-directed motor proteins (kinesins) such as KIF3 (Imamura et al., 2003). Rab11 by contrast mediates slower transport of membraneous cargo from recycling endosomes to the plasma membrane. Rab4-dependent rapid recycling of internalized membrane components is sensitive to elevation of membrane

cholesterol, while Rab11-dependent recycling is not (Choudhury et al., 2004; Chen et al., 2008). The properties of rab4 (specifically, its extractability from membranes by RabGDI, an essential aspect of its physiological functioning) can moreover be modulated by manipulating cholesterol levels in isolated membranes *in vitro* (Choudhury et al., 2004), suggesting that Rab4 and/or its associated effector and regulatory proteins are directly sensitive to the local membrane cholesterol content. It is thus plausible that rab4 cannot associate with or function productively within cholesterol-enriched liquid-ordered domains in early endosomal compartments, and that membrane components associating with these domains therefore cannot undergo rapid rab4-mediated recycling to the cell surface.

Our observations of the distributions of endocytosed folate receptor and artificially lipid-anchored proteins in BHK cells reveal significant differences compared to CHO cells, as Fivaz et al. (2002) have reported previously. In BHK cells these markers colocalize only to a modest extent with internalized transferrin, which in turn shows at best a very modest tendency to accumulate in organized perinuclear structures in these cells. The former result is consistent with the finding of Fivaz et al. (2002) that in BHK cells, in contrast to CHO cells, a substantial proportion of internalized GPI-proteins are sorted to late endosomes. Strikingly, we find that artificially lipid-anchored proteins when endocytosed by BHK cells show steady-state intracellular distributions that are essentially identical to those of internalized folate receptors, independently of the nature or valency of the protein component of the artificially lipid-protein conjugates (DHFR, which can bind a single PE-PEG-methotrexate anchor, and streptavidin, which in principle can bind up to four PE-PEG-biotinyl anchors), or the affinity of their lipid anchor for ordered-lipid domains. This finding is not consistent with the suggestion of Fivaz et al. (2002) that sorting of GPI-proteins in BHK cells is determined by the affinity of their GPI-anchor moieties for ordered lipid domains.

Our findings using artificially lipid-anchored proteins thus confirm one previously proposed role for ordered lipid domains in protein sorting within the endosomal system (modulating the kinetics of recycling of raft-associating vs. raft-excluded proteins within the early endosomal compartment to the cell surface in CHO cells) but argue against a second previously hypothesized role for such domains in endocytic trafficking (routing GPI-proteins from sorting endosomes to late endosomes in BHK cells). Significantly, the former example constitutes the first case in which clear evidence has been obtained by this approach to support the concept that lipid rafts contribute to the differential trafficking of membrane proteins (specifically, GPI-proteins) in mammalian cells. In a previous study of the endocytosis of artificially lipid-anchored proteins (Bhagatji et al., 2009), we found that the preferential internalization of GPI-proteins from the plasma membrane via the GEEC/CLIC pathway rests not on the affinity of the GPI-anchor for ordered lipid domains but rather on the steric bulk of the anchored protein, which likely causes these species to be excluded from coated pits (Bretscher et al., 1980) and hence from internalization via clathrin-mediated endocytosis. These and our present findings illustrate the potential usefulness of artificially lipid-anchored proteins as tools to assess the potential role of ordered-lipid domains in (and more generally, to elucidate the physical origins of) diverse aspects of the trafficking and other biological properties of GPI-anchored proteins.

6.0 Acknowledgments:

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Chapter3: General Discussion and References

1.0 General Discussion:

Role of membrane rafts in endocytic sorting of GPI-anchored proteins in different fibroblast cell lines. In the CHO cell line, we show that soluble proteins bound to saturated and unsaturated PE-PEG anchors have essentially identical intracellular distribution as compared to the GPI-anchored folate receptor α, and that all of these markers colocalize significantly with markers of the ERC (Rab11 & TfR), but not with markers of the late endosomes/lysosomes (Rab7 & Lysotracker). In agreement with earlier reports, preferential association with membrane rafts does not play a role in targeting GPI-anchored proteins to the ERC in CHO cells. This behaviour is clearly independent of factors that recognize the GPI-anchor, since the PE-PEG anchor does not carry any structural resemblance to the normal GPI-anchor. We employed three different soluble protein/ligand combinations (DHFR/MTX, anti-DNP-antibody/TNP and streptavidin/biotin) to do the colocalization experiments in CHO cells. Clearly, all of these different combinations have identical intracellular distribution to that of GPI-anchored folate receptor, meaning that this phenomenon is not coordinated by 'receptors' that recognize the GPI-anchored protein as a whole unit.

In the BHK cell line, soluble proteins bound to both saturated and unsaturated PE-PEG anchors exhibited identical intracellular distributions to those of GPI-anchored folate receptor. This behaviour provide preliminary indication that sorting of GPI-anchored proteins in BHK cells is independent of preferential association with membrane rafts, in contrast to the model proposed by Fivaz et al. (2002). It is still not clear the destination of GPI-anchored proteins and more colocalization experiments are required with markers of the ERC as well as the late endosomes/lysosomes in BHK cells. So provided that our initial conclusions are correct, what is it then that dictate the fate of GPI-anchored proteins in these two different fibroblastic cell lines?

Fivaz et al. (2000) implicated membrane rafts in this differential hypothesis based on their observation that aerolysin-induced oligomers of GPI-anchored proteins become targeted to late endosomes in CHO cells. This group suggested that this

might increase the GPI-anchored proteins residence time in membrane rafts and, hence, enhance this association leading to sorting to the late endosomes/lysosomes. However, sorting of GPI-anchored proteins takes place in the cell periphery inside sorting endosomes that follow a geometry-based sorting to either the late endosome or ERC. Molecules destined to recycle back to the PM have to enter narrow tubular extensions. Given the large size of the heptameric toxin-GPI-anchored proteins complex, it may be unlikely that this complex can enter these tubules easily, and hence may be destined to late endosomes in CHO cells instead for this reason. Alternatively, this differential sorting might be intrinsic to the CLIC/GEEC pathway in each cell line: meaning that any molecules endocytosed via the CLIC/GEEC pathway in CHO cells will be destined to the ERC where it recycle back to the PM, whereas those endocytosed via the CLIC/GEEC pathway in BHK cells will be destined to the late endosomes where they are degraded. This hypothesis might support a role for the numerous endocytic pathways available to molecules/cargo/receptors at the cell surface, similar to the story of the EGFR under low/high concentration of EGF.

Role of membrane rafts in endocytic recycling of GPI-anchored proteins in CHO cells. The results obtained in this part of the study provide several insights into the mechanisms by which GPI-anchored proteins may recycle at a slower rate than other membrane components. First, preferential association of GPI-anchored proteins with liquid-ordered membrane rafts can markedly reduce their recycling rates. We have shown that artificially lipid-anchored proteins with anchors carrying long, saturated hydrocarbon chains like those found on the majority of naturally occurring GPI-anchored proteins (Maeda et al., 2007), recycle back to the plasma membrane in CHO cells at rates 3-4 fold slower than proteins anchored to lipids carrying short or unsaturated hydrocarbon chains. Second, the endocytic routing of GPI-anchored proteins in CHO cells is clearly not dependent on putative GPI-anchor-binding proteins because our artificially lipid-anchored proteins possess none of the specific structural determinants of GPI-anchored proteins and yet are distributed among endocytic compartments essentially

identically to the GPI-anchored folate receptor. As well, the nature of the soluble protein bound to the PE-PEG is clearly not a key determinant of the endocytic recycling rates of GPI-anchored proteins. We employed two different soluble proteins (DHFR and anti-DNP antibody) attached to PE-PEG anchors, and the rates of endocytic recycling were the same for either protein bound to PE-PEG anchors with a given type of hydrocarbon chain. This suggest that a putative biospecific 'receptor' that recognizes the GPI-anchored proteins as a whole unit is not a key determinant of the slower recycling rates of GPI-anchored proteins. It is rather the structural/physical properties of the hydrocarbon chains in the GPI anchor that determine the endocytic recycling rates at which our artificially lipid-anchored proteins, and GPI-anchored proteins recycle back to the plasma membrane. Our results agree with the conclusion of Mayor et al. (1998) and Chatterjee et al. (2001) that ordered lipid domains ('rafts') play an important role in determining the kinetics of recycling of GPI-anchored proteins from the ERC to the cell surface in CHO cells. Consistent with this conclusion, other groups have shown that cholesterol depletion leads to impairment of folate uptake and prion formation (Chang et al., 1992; Taraboulos et al., 1995). However, the methods employed in previous studies (e.g. depletion of cholesterol or sphingolipids) in order to implicate liquid-ordered membrane rafts have pleiotropic effects on the cell and may potentially interfere with different signalling pathways (Stults et al., 1989; van Meer, 1989; Sheets et al., 1999; Simons and Toomre, 2000; Sun et al., 2007).

As discussed in the introduction, we suggest that the membrane-raft based sorting that takes place in the recycling pathway of CHO cells may be regulated by the two small GTPases; Rab4 and Rab11. Sonnichsen et al. (2000) have reported that Rab4 and Rab11 can colocalize on the ERC in the perinuclear region of A134 cells (a human epithelial carcinoma cell line). It has been reported earlier that Rab11, but not Rab4, associate preferentially with membrane rafts (Choudhury et al., 2004; Balasubramanian et al., 2007; Chen et al., 2008). As such, preferential association of recycling receptors with membrane rafts or, conversely, the exclusion from 'rafts,' may determine their association with distinct regions

marked by either Rab11 or Rab4, and therefore might be destined to either the slower or faster recycling pathway, respectively. This proposal does not exclude a potential role of Rab4 in direct recycling from the sorting endosomes. The above hypothesis still requires more experiments in order to validate (see Future Directions).

The model we propose here may not be universal, as the intracellular distribution and possibly even the mechanisms of sorting of endocytosed GPI-anchored proteins may differ in different cell types. There have been some reports that GPI-anchored proteins may be transported directly from the GEECs to the ERC, late endosomes, or the trans-Golgi network, bypassing the SE in some cell lines. However, the regulation of these routes is still unclear, and may not be incompatible with our findings (Chatterjee and Mayor, 2001; Doherty and Lundmark, 2009; Doherty and McMahon, 2009). Most importantly, our findings provide clear evidence that ordered-lipid domains play an important role in determining the kinetics of endocytic trafficking of GPI-anchored proteins, validating the previous suggestions of Mayor and colleagues (Mayor et al., 1998; Chatterjee et al., 2001) using methods that avoid the complications of prior approaches involving large-scale perturbation of cellular membrane lipid composition. As such, our findings provide some of the most direct evidence to date that 'membrane rafts' can affect the intracellular sorting of membrane components under physiological conditions.

2.0 Future Directions:

Role of clustering of GPI-anchored proteins in endocytic targeting to the late endosomes in CHO cells. Fivaz and colleagues (2002) proposed that clustering of GPI-anchored proteins (as induced by active aerolysin toxin) may enhance their association with membrane rafts and therefore targets them to late endosomes in CHO cells. Some researchers were not at ease with the main conclusion for a few reasons. First, there has not been strong evidence supporting the fact that clustering of GPI-anchored proteins may enhance their association with membrane rafts (Mayor and Riezman, 2004; Sabharanjak and Mayor, 2004).

Second, the large size of these heptameric complexes may restrict their recruitment to the narrow recycling tubules of the sorting endosomes regardless of their association with membrane rafts and hence target them to the late endosomes. Finally, Mayor and Reizman (2004) proposed that depletion of cholesterol or sphingolipid is required to implicate membrane rafts in the targeting of these heptameric complexes of aerolysin and GPI-anchored proteins. Our artificially lipid- anchored proteins might provide insight into the role of clustering of GPI-anchored proteins in enhancing their association with membrane rafts and hence targeting to the late endosomes in CHO cells. This can be possible if we are able to develop a conjugate system that can be induced to cluster after incorporating in live cells. By varying the carbohydrate chains of the PE-PEG 'anchor' from saturated to short or unsaturated chains, we would be able to determine if association with membrane rafts plays a role in their targeting to LE in CHO cells.

Linking membrane rafts to the roles of Rab4 and Rab11 in endocytic recycling.
There has been strong evidence supporting a role for Rab4 and Rab11 in the fast and slow endocytic recycling pathways, respectively, in a number of cell lines (van der Sluijs et al., 1992; Ullrich et al., 1996; Ren et al., 1998; Sheff et al., 1999; Sonnichsen et al., 2000; Wilcke et al., 2000; Engstler et al., 2004). Since these small GTP-binding proteins were shown to associate with the cytoplasmic leaflet of their respective endosomal membranes, it has puzzled researchers how these proteins associate with the specific cargo destined for each of the two recycling pathways. A recent study identified the Dual-specificity A-Kinase Anchoring Protein 2 (D-AKAP2) as an important adaptor protein for Rab4 & Rab11 which link them to the transferrin receptor in HeLa cells (Eggers et al., 2009). Unfortunately, this has been more challenging for peripheral membrane proteins. GPI-anchored proteins are present exclusively in the extracytoplasmic/luminal leaflet of membranes and which lack cytoplasmic domain with which Rab4, Rab11, or any of their adaptor proteins may possibly

interact. A few reports, however, have linked the association of Rab4 and Rab11 with their respective endosomal membranes to their differential association with membrane raft domains (Hao et al., 2002; Choudhury et al., 2004; Maxfield and McGraw, 2004; Rajendran and Simons, 2005; Mayor and Pagano, 2007; Pipalia et al., 2007; Chen et al., 2008; Donaldson et al., 2009). In light of the aforementioned results, one may assume that membrane rafts may play a role in sorting different cargos to either endocytic recycling pathways. For example, it is very likely that because our long saturated di-16:0-PE-PEG anchor associate preferentially with membrane raft domains, with which Rab11 also preferentially associates, that these conjugates are delivered to the ERC and hence recycle at slower rate than the shorter or unsaturated di12:0 or di18:1-PE-PEGs which will segregate out of membrane rafts and hence be in a micro-environment preferred by Rab4. Balasubramanian et al. (2007) have shown marked colocalization between the Cholera toxin subunit B and Rab11 in the ERC in mouse embryonic fibroblasts, as we have shown for the folate receptor and our protein-bound saturated PE-PEG conjugates in CHO cells. Preferential association with membrane raft may not be a 100% efficient means of endocytic sorting. A given GPI-anchored protein may still be recycled back to the plasma membrane via the fast recycling pathway, albeit at smaller proportions as compared to the slower recycling pathway which seems to be the major recycling route for this class of membrane proteins. For this reason, it is essential to investigate the proportions of colocalization between our saturated and unsaturated PE-PEG anchored soluble proteins with each of Rab4 and Rab11, and only then that meaningful insights into the role of membrane raft microdomains in the sorting of recycling components between these two pathways can be drawn.

3.0 References:

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4.0 Appendix.

4.1 Determination of the Rate Constant for Recycling of an Internalized Species from Recycling Endosomes to the Cell Surface.

Following the approach previously described by Mayor (1998), we assume (in agreement with previous findings and with our present confocal-microscopic results) that the principal site of accumulation of endocytosed marker species (fluorescent-labeled transferrin or rhodaminyl-folate bound to their respective receptors, or fluorescent DHFR or anti-DNP antibody bound to a PE-PEG-ligand anchor) is the endosomal recycling compartment, from which molecules can be recycled to the cell surface in a process described by a first-order rate constant k_{rec}. We also assume that surface-associated marker molecules can be endocytosed with a first-order rate constant k_{endo}. The experimental procedure to determine the recycling rate constant is described in the main text. Briefly, cells are first loaded with a fluorescent endocytic marker to steady-state by incubation at 37^{o}C in a marker-containing medium. The loaded cells are rapidly chilled and stripped of surface-bound marker in the cold, then rewarmed to 37^{o}C for a time t, after which they are again chilled, stripped of surface-associated marker and fixed. The average intracellular marker fluorescence F_{int} is subsequently measured (by wide-field fluorescence microscopy) for each time point.

If the marker rapidly dissociates from the membrane upon recycling to the cell surface (as is the case for transferrin), the fluorescence of intracellular marker remaining after allowing recycling to proceed for time t at 37^{o}C is given by the simple expression

$$F_{int}(t) = F_{int}(t=0) \cdot \exp(-k_{rec} t)$$

The initial slope of the time course of F_{int} vs. t is then given by the equation

$$\left.\frac{dF_{int}}{dt}\right|_{t=0} = -k_{rec} \cdot F_{int}(t=0)$$

When the marker remains bound to the membrane after recycling to the cell surface, such that it can subsequently be (partially) re-internalized, the behavior of the system in the above experiment will be governed by the following pair of differential equations:

$$dF_{int}/dt = k_{endo}F_{surf} - k_{rec}F_{int}$$

$$dF_{surf}/dt = k_{rec}F_{int} - k_{endo}F_{surf}$$

where F_{surf} is the average fluorescence per cell of surface-associated marker (a quantity that is not measured, since surface-exposed marker molecules are removed prior to microscopy) and the other parameters are as described above. Solving the above differential equations to describe F_{int} as a function of time (under the conditions that $F_{int} + F_{surf}$ = constant and $F_{surf} = 0$ at $t = 0$), we obtain the result

$$F_{int}(t) = F_{int}(t=0) \cdot \left(\left(\frac{k_{endo}}{k_{endo} + k_{rec}} \right) + \left(\frac{k_{rec}}{k_{endo} + k_{rec}} \right) \cdot \exp\left(-(k_{rec} + k_{endo})t \right) \right)$$

It is apparent that in the latter case the rate constant for the exponential variation of F_{int} with t is not simply equal to k_{rec} but rather depends on both k_{rec} and k_{endo}. However, differentiation of the above expression gives the result

$$\left.\frac{dF_{int}}{dt}\right|_{t=0} = -k_{rec} \cdot F_{int}(t=0)$$

which is identical to the result obtained for recycling of an internalized marker that rapidly dissociates from the membrane once it reaches the cell surface. For all marker species examined in our recycling experiments, we therefore determined

k_{rec} from the initial slope of the time course of F_{int} *vs.* the time (t) allowed for recycling at 37°C:

$$k_{rec} = -\frac{\left(dF_{int}/dt\right)\big|_{t=0}}{F_{int}\left(t=0\right)}$$

In practice the initial slope was determined by fitting the time course of F_{int} vs. t to an exponential equation of the form $F_{int}(t) = A + B \cdot \exp(-kt)$, thereby 'extracting' the information content of all the data points; the initial slope was then calculated from the best-fit estimates of the parameters B and k as $(dF_{int}/dt)\big|_{t=0} = -kB$.

This analysis thus overcomes complications arising from the potential of some markers to undergo additional rounds of endocytosis after recycling to the surface.