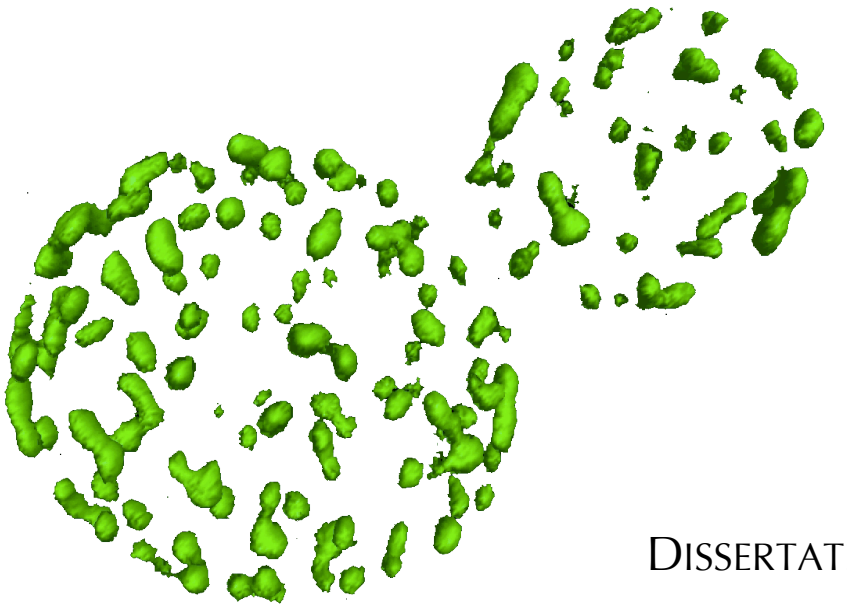
Plasma membrane compartmentation

in *Saccharomyces cerevisiae*



DISSERTATION

ZUR ERLANGUNG DES
DOKTORGRADES DER NATURWISSENSCHAFTEN (DR. RER. NAT.)
DER NATURWISSENSCHAFTLICHEN FAKULTÄT III
– BIOLOGIE UND VORKLINISCHE MEDIZIN –

UNIVERSITÄT REGENSBURG

vorgelegt von

Guido Großmann

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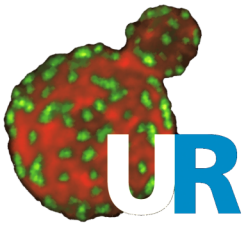

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im Juli 2008

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Die Arbeit wurde angeleitet von Prof. Dr. Widmar Tanner.

Prüfungskommission:

Prof. Dr. Thomas Dresselhaus (Vorsitzender)
Prof. Dr. Widmar Tanner
Prof. Dr. Wolfgang Seufert
Prof. Dr. Gernot Längst
Externe Begutachtung durch:
Prof. Dr. Gerrit van Meer (Universiteit Utrecht, NL)

Für Sabrina

Contents

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Watching the world through lenses can enormously broaden one's horizon. And it doesn't matter whether one is gazing at galaxies or entering the microscopical universe of cells - the fascination is always to "explore strange new worlds, to seek out new life, to boldly go where no man has gone before."[1]

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[1] From the title sequence of „Star Trek".

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Regensburg, im Juli 2008

List of publications

P1 **Grossmann G**, Opekarová M, Nováková L, Stolz J and Tanner W (2006) Lipid raft-based membrane compartmentation of a plant transport protein expressed in *Saccharomyces cerevisiae*. *Eukaryot Cell* **5**(6), 945-53

P2 **Grossmann G**[*], Opekarová M[*], Malínsky J[*], Weig-Meckl I and Tanner W (2007) Membrane potential governs lateral segregation of plasma membrane proteins and lipids in yeast. *EMBO J* **26**(1), 1-8

P3 Lauwers E, **Grossmann G** and André B (2007) Evidence for coupled biogenesis of yeast Gap1 permease and sphingolipids: essential role in transport activity and normal control by ubiquitination. *Mol Biol Cell* **18**(8), 3068-80

P4 **Grossmann G**, Malínsky J, Loibl M, Stahlschmidt W, Weig-Meckl I, Frommer WB, Opekarová M and Tanner W (2008) Plasma membrane microdomains regulate turnover of transport proteins in yeast. *Journal of Cell Biology* DOI: 10.1083/jcb.200806035

[*] These authors contributed equally to the work.

1 Introduction

1.1. Bordering life – the plasma membrane of cells

At the moment when for the first time self-replicating molecules were enclosed by a thin hydrophobic film the foundation for all cellular life on Earth was laid. Since then, a barrier of 30 Å confines life from inanimate matter. Over billions of years a complex plasma membrane has evolved which protects the interior by restricting import and export of water-soluble compounds and thereby enables the living cell to maintain a stable chemical environment for biological processes. Special components of the plasma membrane specifically facilitate the import of essential nutrients, while others allow waste products to leave the cell. As the plasma membrane is the contact zone to the surroundings, its task is also to perceive environmental changes or communication signals from other cells. In eukaryotes, also the cell interior is compartmentalized by membranes that delimit free diffusion of metabolic intermediates, and thus ensure the efficiency of organelle-specific biochemical reactions.

As every biological membrane, the plasma membrane is composed of two molecular classes of equal weight and importance: proteins and amphipathic lipids. While lipids are responsible for the property of membrane impermeability, the selective and controlled pervasion by water-soluble compounds is mainly accredited to the protein fraction.

The uptake of water-soluble nutrients is an essential task because only small hydrophobic molecules can cross the plasma membrane by passive diffusion. For this purpose, numerous channels and transport proteins exist that can have very

narrow or wide range substrate specificities. They are divided into ion channels, transporters and adenosine-5′-triphosphate (ATP)-powered pumps. Ion channels e.g. for K^+, Na^+ or Ca^{2+} can be seen as selective pores that help to equilibrate a concentration gradient of their specific ion. In many cases such channels are gated, i.e. they open and close upon certain stimuli. Transporters can be sub-classified into uniporters, symporters and antiporters. Uniporters specifically transport molecules down their concentration gradient. Symporters and antiporters both use the concentration gradient of a second substrate (in most cases protons or Na^+/K^+) to actively translocate their main substrate against its concentration gradient in either the same (sym-) or the opposite (anti-) direction. Thereby they enable cells to accumulate compounds several thousand fold in the cytoplasm or in the lumina of organelles.

Among the first actively transporting proteins described in plants, was the hexose uptake protein HUP1 of *Chlorella kessleri* (Komor and Tanner, 1971; Komor et al., 1973; Sauer and Tanner, 1989). HUP1 and all other active sym- or antiporters obtain the necessary energy from a concentration gradient. In case of HUP1, a proton gradient is established by an ATP-powered pump. To transfer ions or small molecules against their electrochemical gradient these pumps couple the translocation with the energy-releasing hydrolysis of ATP phosphoanhydride bonds. Pumps are divided into plasma membrane located P-type pumps, vacuolar or lysosomal V-type proton pumps, and bacterial, plastidial or mitochondrial F-type proton pumps. P-type pumps export protons in plants and fungi and Na^+/K^+ in animal cells, while V-type proton pumps acidify the lumen of the degrading organelle. Related to pumps are ATP-binding cassette (ABC-) transporters that translocate small soluble molecules, lipophilic compounds and peptides.

Concentration gradients of ions result in an electrical potential across the plasma membrane ($\Delta\psi$), which is indispensable for cellular life. The proton gradient across membranes of bacteria, plants, fungi as well as of mitochondria and chloroplasts combines $\Delta\psi$ and ΔpH to form a proton motive force. In addition to membrane transport, the membrane potential is also directly involved in other membrane-associated processes such as binding of charged proteins or other molecules as well

as insertion and orientation of integral membrane proteins (McLaughlin, 1989). Action potentials, a specialized form of electrochemical gradients across the plasma membrane of neurons, are used as signals that bridge long distances in fractions of a second.

Ionophores, as valinomycin for K^+ or FCCP[1] for protons, are amphipathic compounds that are highly toxic for cells. These, so called uncouplers, penetrate the lipid bilayer and depolarize the membrane by equilibrating the ion gradient. The voltage difference across the plasma membrane of all organisms is such an essential driving force for nutrient uptake that a break down of the potential can be taken as an infallible sign for cell death.

Many membrane proteins, however, do not act as nutrient transporters but are involved in cell signaling. They can perceive environmental changes or communication signals between cells. Due to abundant receptor proteins, sensors or signaling channels the cellular processes can react on various stimuli, for instance, to adjust the expression of genes during embryonic development. On the other hand, by secreting messenger molecules or enzymes, the cell is able to communicate with neighboring cells or to adapt the environment to its requirements.

When studying the manifold functions of the plasma membrane, its proteins have always been the focus of interest while the importance of lipids, as the immediate lipid environment for all membrane bound enzymes, was often underrated. Similarly to cytoplasmic proteins, which are in tight interaction with and dependent on water, transmembrane proteins depend on lipids as their solvent and also react to changes in lipid composition with an altered conformation and partially or completely abolished function. Numerous transport proteins, including the yeast arginine/H+ symporter Can1 that also has been used here as a marker for membrane compartmentation, were shown to depend on certain lipids for correct targeting to the plasma membrane and proper function (Opekarová et al., 2002; Opekarová and Tanner, 2003). Hence, the understanding of the membrane composition on a

[1] FCCP, Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone

molecular level is of great interest when addressing the architecture of the plasma membrane and the organization of its constituents.

1.2. Structure and components of biological membranes

1.2.1 General architecture

The main structural component of all biological membranes is a flexible lipid bilayer of approximately 30 Å that is formed mainly by amphipathic lipids (Lee, 2003; Andersen and Koeppe, 2007). This bilayer organization was first recognized by Gorter and Grendel in the early twenties of the last century, when they realized that the determined surface area of extracted lipids from erythrocyte membranes spread on water was exactly twice the calculated surface area of the same number of intact erythrocytes (Gorter and Grendel, 1925).

The "fluid mosaic model", developed by Singer and Nicolson in the seventies, described the membrane bilayer further as a two-dimensional fluid, in which lipids and integrated proteins diffuse without restrictions. They stated that proteins are embedded discontinuously (as a mosaic) throughout the plane of the bilayer and that lipids and proteins are distributed asymmetrically across the membrane (Singer and Nicolson, 1972). Over thirty years later, the common view of membranes changed to "more mosaic than fluid" (Engelman, 2005). In textbook drawings, the fluid mosaic model is usually illustrated by monomeric proteins that swim distant from each other in a sea of lipids, which is homogenous in composition and thickness. The recent knowledge changes this image to a much more crowded and compartmentalized view of a biological membrane. For example, measurements of rhodopsin density in the rod outer-segment disc membranes revealed a tight packing of 30,000-55,000 molecules per μm^2 (Fotiadis et al., 2003). Hence, in the current view only few rings of lipid molecules form a tight shell around individual or oligomeric proteins (Jacobson et al., 2007).

On the other hand, the lipid composition of the membrane is far from being homogenous, suggesting that the membrane structure is also characterized by lateral heterogeneity. Thousands of lipid species compose a typical eukaryotic plasma membrane (Dowhan, 1997) and even few of them already promote phase separation in synthetic membranes (Dietrich et al., 2001).

1.2.2 Lipid components of biological membranes

The complex diversity of membrane lipids can be divided into phospho-, sphingo- and glycolipids as well as sterols. Phospholipids or phosphoglycerides are the most abundant and the major structural lipids in eukaryotic membranes. They are derivatives of glycerol 3-phosphate, of which the two hydroxyl groups are typically esterified to two fatty acyl chains, resulting in phosphatic acid (PA), the smallest phospholipid and precursor of many others. In PA the polar head group consists only of a phosphate. Miscellaneous modifications of this phosphate group with alcohols further classify the phospholipids into phosphatidyl-choline (PC), phosphatidyl-ethanolamine (PE), phosphatidyl-serine (PS) or phosphatidyl-inositol (PI).

In most eukaryotic membranes PC represents more than 50% of phospholipids. Usually, PC contains one *cis*-unsaturated fatty acyl chain (Figure 1.1), which results in high fluidity at room temperature within the bilayer. Such a bilayer is formed even spontaneously when purified PC is exposed to aqueous media. As the molecular shape of PC is rather cylindrical, this lipid forms absolutely plane bilayers. In contrast, the geometry of PE is conical due to its smaller head group (Cullis and de Kruijff, 1979). Thus, PE induces curvature if added to a bilayer formed by PC (Marsh, 2007). The structurally related plasmalogens represent further variations of phospholipids. In these ether lipids, the fatty acid at the first carbon position is substituted by an ether-linked alkene.

The second important class is represented by sphingolipids, where a long-chain fatty acid is attached in an amide linkage to the amino group of sphingosine, an amino alcohol with a 18-hydrocarbon chain (Figure 1.1). Similar to phospholipids, sphingolipids contain a phosphate based polar head. In sphingomyelin (SM), for

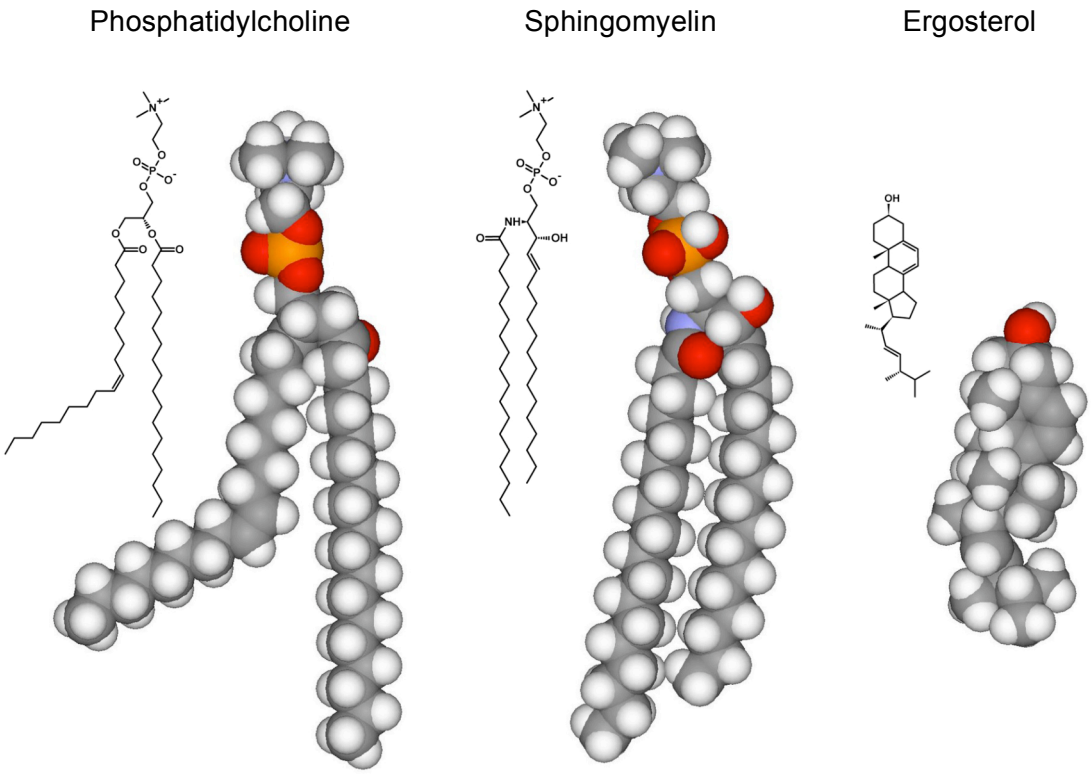

Figure 1.1 Structural formulas and calotte models of phosphatidylcholine, sphingomyelin and ergosterol (the most abundant sterol in fungi).

instance, phosphocholine is attached to the terminal hydroxyl group of sphingosine. If this hydroxyl group is not coupled to a phosphate-linked alcohol but to mono-, di- or oligosaccharides, glycolipids are formed. Glucosylcerebroside, the simplest glycosphingolipid, contains a single glucose attached to sphingosine.

The various classes of phospho- and sphingolipids do not only differ in their head groups but also in the number of carbons (commonly 16 or 18) of the attached fatty acids and in the degree of their saturation. These modifications enormously increase the variability. For example, mass spectrometry revealed that in membranes of Madin-Darby canine kidney cells of type II (MDCK II) almost 100 molecule species exist only within the family of PC (Ekroos et al., 2003). Hence, lipid research entered the world of "omics" and lipidome analyses became a subject of broad interest (Han and Gross, 2003; van Meer, 2005; van Meer et al., 2008).

Among the vast diversity of membrane lipid some phospholipids (e.g. PI and various phosphorylated derivatives) are capable to serve as signaling molecules.

Commonly, the head group is cleaved from the hydrophobic portion of the lipid, thus resulting in two messenger molecules. However, the main physiological functions of the majority of membrane lipids might be the fine tuning of the membrane fluidity and the ability to provide individual environments for various membrane proteins.

1.2.3 Membranes are 2D fluids

All plasma membrane constituents are subject to permanent turnover. Parallel events of exo- and endocytosis continuously deliver newly synthesized proteins and lipids to the cell surface or internalize material. As in intracellular membranes, this dynamic exchange mainly happens through fusion and budding of vesicles – a process that depends on the flexibility of a fluid membrane. The balance between sufficient fluidity and necessary rigidity is determined by the molecular interactions of membrane lipids.

While ionic and hydrogen bonds promote interactions of phospho- and sphingolipid head groups with each other, water and proteins, their contribution to the membrane stability is comparably low. The main stabilizing parameter responsible for close packing of the membrane constituents are van der Waals forces between the non-polar fatty acid chains. These forces are relatively weak between individual molecules allowing transient interaction which is essential for the high molecular mobility. The strength of van der Waals forces, and thus the viscosity of the membrane is determined by three parameters: temperature together with the length and degree of *cis*-saturation of fatty acid side chains. A lower viscosity of the membrane can result from higher temperature or shorter fatty acids or a lower degree of their saturation. Sphingolipids, for example, usually have long saturated or single trans-unsaturated tails that result in a molecular geometry of narrow cylinders and tighter packing. To prevent the formation of a gel-like or solid ordered (so) phase in the plasma membrane, such lipids are fluidized by sterols, nonpolar lipids that are in shape and properties a class of their own.

The basic structure of sterols is a four-ring hydrocarbon (Figure 1.1). Only the hydroxyl substituent on one ring is responsible for the amphipathic character of the molecule. Thus, its polar character is too weak to form a bilayer on its own. Sterols can be found abundantly in most eukaryotic plasma membranes, where they function as a fluidity buffer: at low temperatures, when phospholipids tend to order in a solid phase, resulting in a freezing of the membrane, sterols intercalate with their bulky structure and keep the membrane fluid to a certain extend; at rising temperatures their large surface favor van der Waals forces that stabilize the membrane and avoid melting. Thus, the membrane viscosity is well stabilized in living cells and was determined to be in average about 100-times greater than the viscosity of water (Frye and Edidin, 1970; Cone, 1972). As Edidin illustrated in his review: "The commonplace view now is that the average bilayer lipid viscosity is similar to that of olive oil – a more 'exotic' standard is the viscosity of crocodile fat on a warm summer's day" (Edidin, 2003).

Nematodes represent an exception, where sterols are not required as plasma membrane components. In *Turbatrix aceti* and *Caenorhabditis elegans*, sterols were only found to be necessary precursors for steroid hormones during development (Silberkang et al., 1983; Merris et al., 2003), which is a second important function of these lipids.

1.3. Horizontal and vertical lipid sorting

1.3.1 Lipid-lipid interactions promote lateral domain formation

All cellular membranes have a very specialized composition to fulfill their specific tasks. This composition differs by species, tissue and subcellular localization. On the subcellular level, high concentrations of sterols and sphingolipids can be found only within the plasma membrane. This results in higher viscosity and a more rigid and stable membrane as compared to the intracellular ones (Di Paolo and De Camilli, 2006).

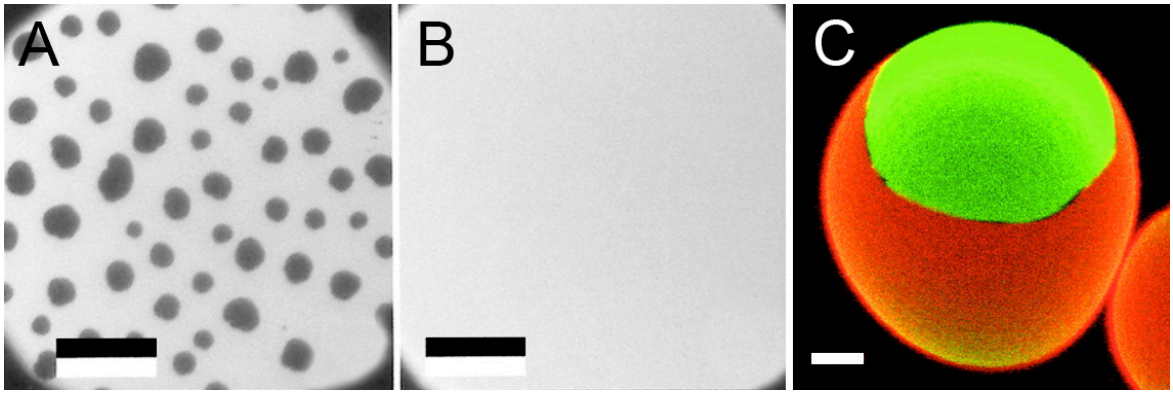

Figure 1.2 Microdomain formation due to phase separation in artificial membranes. (A) In a supported monolayer of PC:chol:SM 2:1:1 two phases emerge, while in a monolayer of PC:chol 2:1 no domains are formed (B). Scale bars, 10 µm. Images reproduced from Dietrich et al., 2001. (C) Three dimensional projections of phase separation in giant unilamellar vesicles (GUVs). Scale bar, 5 µm. Image reproduced from Kahya et al., 2003.

Due to the great lipid heterogeneity it can be assumed that the viscosity of membranes is locally different. In the seventies of the last century van Deenen and coworkers already elucidated lipid-lipid interactions in pioneering studies on artificial membranes. By calorimetric measurements, they found a high affinity of cholesterol for SM, higher than for PC, which again is higher than for PE (van Dijck et al., 1976; Demel et al., 1977). Later, phase separation and microdomain formation was detected in model membranes consisting of equimolar amounts of SM, cholesterol and 1-stearoyl-2-docosahexaenoyl-*sn*-glycerophosphoethanol-amine (SDPE) (Shaikh et al., 2001). Using head group-labeled fluorescent phospholipid analogs, these lipid domains being several microns in diameter could be visualized by two-photon fluorescence microscopy in supported lipid monolayers and in giant unilamellar vesicles (GUVs), where derivatives of PC were used instead of SDPE (Figure 1.2; Dietrich et al., 2001; Kahya et al., 2003). However, phase separation in these model systems depends on the presence of both SM and cholesterol plus any other phospholipid. Lipid mixtures of sole PC and cholesterol appeared to be homogenous (Dietrich et al., 2001). The preferred interaction of cholesterol and SM is suggested to be stabilized by a hydrogen bond between the 3 β-hydroxyl group of cholesterol and the amide group of acylamidosphingosin (ceramide) (Brown, 1998; Veiga et al., 2001). This can result in a shielding of the unpolar sterols by sphingolipids as described by the so-called umbrella model (Ikonen, 2008).

Based on these demonstrations of phase separation in artificial membranes, it is conceivable that in biological membranes liquid-disordered (l_d) phases exist next to liquid-ordered (l_o) phases, where both of them may represent specific individual solvents for different embedded proteins (Simons and Vaz, 2004).

1.3.2 Biological membranes are asymmetric

Studies on model membranes, however, cannot reflect the enormous complexity of biological membranes in lipids and proteins. In addition, such artificial systems lack an electrochemical potential across the bilayer, and usually consist of two symmetric leaflets. In contrast, biological membrane bilayers are asymmetrical in their lipid composition (Bretscher, 1972b; Verkleij et al., 1973). SM and glycosphingolipids are exclusively found in the outer exoplasmic leaflet of the bilayer and also PC is preferentially located here. In contrast, PS, PE, PI and PA reside in the inner cytoplasmic leaflet (Figure 1.3; Zachowski, 1993).

Such a vertical compartmentation has physiological importance since this asymmetry of charged lipids directly contributes to the formation and maintenance of the membrane potential (Latorre and Hall, 1976). Furthermore it is well known that lipids on both sides fulfill specialized functions. As indicated above, PE locates to the inner leaflet, mediating a convex curvature. This feature of PE is used by cells for budding, fission and membrane fusion (Marsh, 2007). It was reported that a locally and temporally regulated translocation of PE is essential for cell cycle progression (Emoto and Umeda, 2000). Moreover, an involvement of PE in cell polarity has been shown (Iwamoto et al., 2004). It was also described that the cytoskeletal component spectrin interacts with PS (Manno et al., 2002). Upon a global collapse of asymmetry, e.g. in apoptotic cells, PS is recognized by phagocytes (Fadok et al., 1992).

The question arises, how this asymmetry is established and maintained in biological membranes. Due to large polar head groups, a spontaneous trans-bilayer movement is very slow in protein-free model membranes (Kornberg and McConnell, 1971; Bai and Pagano, 1997). However, the situation changes when proteins come

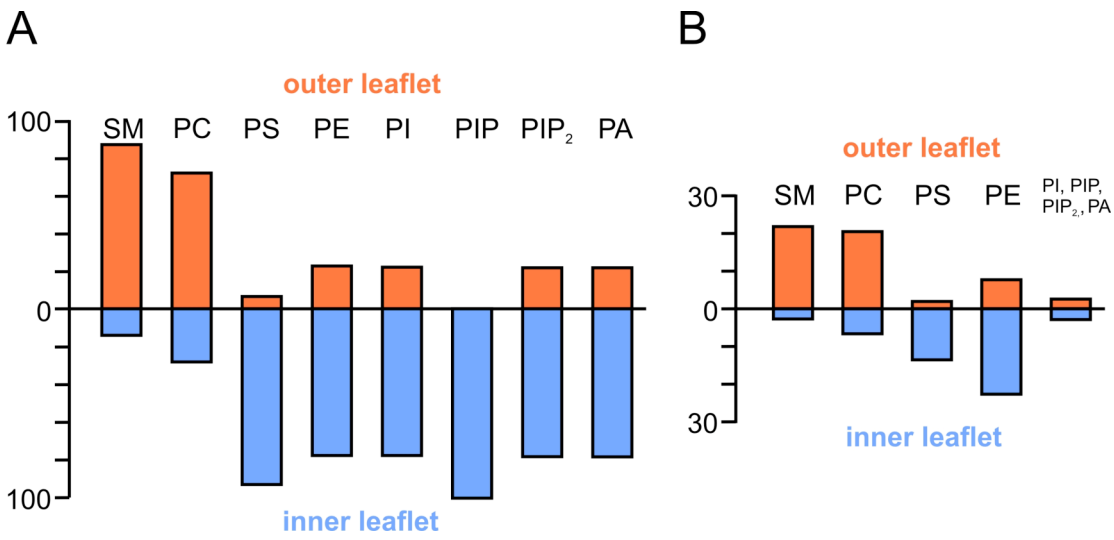

Figure 1.3 Lipid asymmetry in the plasma membrane of human erythrocytes. The distribution among the two leaflets is given as percentage of each lipid (A) and of total lipids, respectively (B). The figure is adapted from Fig. 2 in Zachowski, 1993, which includes results from Bretscher (1972a,b), Gordesky et al. (1972, 1975), Verkleij et al. (1973), van Meer et al. (1981), Bütikofer et al. (1990), Gascard et al. (1991).

into play. Although the synthesis of glycerophospholipids occurs exclusively at the cytosolic membrane leaflet of the endoplasmatic reticulum (ER; Bell et al., 1981), this membrane is almost symmetrical (Herrmann et al., 1990). In the ER, the translocation of glycerophospholipids proceeds rapidly and independently from energy (Herrmann et al., 1990; Buton et al., 1996), as well as from specific translocases. In liposomes translocation of fluorescent labeled phospholipids occurred as soon as any transmembrane peptides or proteins without known translocase activity were reconstituted into the vesicles (Kol et al., 2001). Interestingly, this translocation was inhibited by the presence of cholesterol (Kol et al., 2003). This is in accordance with the fact that symmetric ER membranes are very poor in sterols, while these lipids accumulate in the asymmetric plasma membrane (Ikeda et al., 2006).

As a consequence, the membrane asymmetry found in the plasma membrane has to develop in maturing membranes on their way from the ER to the final destination. For this purpose specialized translocases exist. Translocation towards the cytoplasmic leaflet (FLIP) is mediated by flipases, whereas translocation towards the exoplasmic leaflet (FLOP) is mediated by flopases.

Several P-type ATPases are known to exhibit flipase activity. This protein family of aminophospholipid translocases preferentially facilitates the translocation of PS and PE from outside to inside. In *S. cerevisiae*, the redundant P-type ATPases Dnf1, Dnf2, Dnf3 and Drs2, as well as the essential Neo1, fulfill this function in the Golgi apparatus, plasma membrane and endosome (Siegmund et al., 1998; Hua et al., 2002; Hua and Graham, 2003; Saito et al., 2004; Wicky et al., 2004). On the other hand, FLOP is performed by ABC transporters. In yeast these are Pdr5, Ste6 and Yor1, which translocate glycerophospholipids with low specificity (Ruetz et al., 1997; Decottignies et al., 1998; Pomorski et al., 2003).

For SM and glycosphingolipids the situation is not very different. Although their biosynthesis takes place at the cytosolic side of the Golgi membrane (Jeckel et al., 1992), they are further processed in the lumen (Nomura et al., 1998; Huitema et al., 2004), and later exclusively found in the exoplasmic leaflet of the plasma membrane. In yeast, the sphingolipid precursor sphingoid base (long chain base, LCB) is therefore translocated in an ATP-dependent manner by Rsb1 from the cytosolic to the extracytosolic side (Kihara and Igarashi, 2002). Interestingly, the expression of Rsb1 is regulated according to membrane asymmetry. If the asymmetry of glycerolipids is artificially changed, *RSB1* expression is increased. On the other hand, if RSB1 is overexpressed, flip of PE and PC is stimulated while flop of both of them is repressed. These findings suggest some crosstalk between sphingolipids and glycerolipids to maintain the functional lipid asymmetry of the plasma membrane (Kihara and Igarashi, 2004).

Although a method for asymmetric bilayer formation in model membranes is already known since the seventies of the last century (Montal and Mueller, 1972), only recent technological advances allowed to study unsupported asymmetric bilayers in terms of phase separation and domain formation (Collins and Keller, 2008). The authors showed that leaflets, composed of ternary lipid mixtures, can influence the ability of their counterpart to phase-separate. A lipid mixture that normally bears lateral homogeneity can be induced to form lipid domains by the second leaflet that is composed to support phase separation. Conversely, a change in

lipid composition of the first leaflet can also inhibit domain formation in the second one.

1.4. Plasma membrane compartmentation – *in vivo veritas*

1.4.1 From shells to rafts – isolation of microdomains

From bacteria to mammalian cells, increasing in vivo evidence suggests that the ability of the plasma membrane to form subcompartments by the generation of microdomains is a wide-spread feature throughout all organisms. The most prominent type of membrane microdomains are lipid rafts, which are enriched in sterols, sphingo- and glycolipids. While in mammalian cells the existence of lipid rafts is already discussed for more than two decades (Simons and van Meer, 1988; Brown and London, 1998; Lagerholm et al., 2005), the topic is still young in the fields of plants (Mongrand et al., 2004; Borner et al., 2005; Martin et al., 2005) and prokaryotes (Matsumoto et al., 2006). The development of the lipid raft hypothesis was originally based on the putative existence of lipid domains in the Golgi membranes as an explanation for sphingolipid sorting in epithelial cells, where these lipids are accumulated especially in the apical plasma membrane (Simons and van Meer, 1988). Later, such domains were postulated in the plasma membrane (Parton, 1994) and in endosomes (Sharma et al., 2003).

For years, the proposed domains were mainly characterized by in vitro approaches like analysis of artificial membranes and detergent extraction assays. The tighter packing of membranes rich in sterols and sphingolipids or other lipids with saturated fatty acids provides a relatively higher resistance towards mild detergents. Especially the insolubility in 1% TritonX-100 at 4°C (Brown and London, 1998) was extensively used to find out whether membrane proteins are part of detergent resistant membranes (DRMs), which were considered to be the biochemical equivalent to lipid rafts (Simons and Ikonen, 1997; Brown and London, 1998; Rietveld and Simons, 1998). Thereby, it was found that GPI-anchored proteins localize

preferably to rafts (Schroeder et al., 1994). These microdomains have subsequently been linked to a wide range of processes including signaling (Simons and Toomre, 2000), virus entry and exit (Nguyen and Hildreth, 2000; Marsh and Helenius, 2006), and caveolae-mediated membrane turnover. Caveolae are structures of a certain type of receptor-triggered endocytosis. These flask-shaped invaginations of the plasma membrane are enriched in raft lipids (Dupree et al., 1993). Their main protein constituent caveolin is believed to polymerize by a clustering of lipid rafts (Rothberg et al., 1992; Anderson, 1998; Parton and Simons, 2007). To show that caveolae-triggered endocytosis depends on these microdomains, cells were treated with β-cyclodextrin (β-CD), which is thought to perturb lipid rafts by depleting sterols from membranes. Indeed, the caveolae-mediated uptake of fatty acids into adipocytes was inhibited after sterol depletion (Pohl et al., 2004). However, such a treatment, where large amounts of lipids are extracted from membranes of living cells, has to be taken with caution as controls are missing whether other membrane linked processes are still properly working (Munro, 2003).

In polarized cells like the fission yeast Schizosaccharomyces pombe or mating factor treated, shmoo forming bakers yeast, the growth tips are thought to contain super-rafts as here a high accumulation of certain proteins and of sterols was observed (Bagnat and Simons, 2002; Wachtler et al., 2003). However, this accumulation might be due to preferential secretion towards the polarized tip, endocytic cycling, and slow lateral diffusion (Valdez-Taubas and Pelham, 2003).

Phase separation of lipids could be a sufficient explanation for domain formation on the level of lipids. Though, it remains unclear how proteins are selectively sorted into these domains and how integral proteins affect the phase behavior. One possibility are preferential interactions of certain molecules that directly result in lateral inhomogeneity. Independently of the situation in model membranes, protein-lipid interactions could result in a phase behavior that substantially differs from that of pure lipid mixtures. Specific protein-lipid interactions that can be resolved in crystal structures of membrane proteins have been reported previously (McAuley et al., 1999; Valiyaveetil et al., 2002; Lee, 2003; Hunte and Richers, 2008). It is known for the transporter HUP1 that after purification from

plasma membranes the protein still binds two or three molecules of PC, PE and ergosterol molecules per protein molecule (Robl et al., 2000). This observation is consistent with the lipid shell model that proposes an encasement of transmembrane proteins with a specific lipid shell already during the protein biosynthesis. This shell corresponds to the future membrane domain and may confer the detergent resistance of its protein (Anderson and Jacobson, 2002).

But besides specific attraction, also specific exclusion can result in domain formation. In theory, a protein could stabilize sterol-free areas by avoiding sterols and thereby promote the concentration of sterols in the remaining membrane regions (Epand, 2008).

As another possibility, membrane domains are preformed only by lipids and proteins successively incorporate into these micro-environments regarding their particular requirements. Independent of specific interaction, this sorting could be based on membrane thickness and length of transmembrane domains (Bretscher and Munro, 1993). Indeed, membranes are usually thicker at sites in l_o-phase as compared to sites in l_d-phase. A theoretical membrane of C18:0-SpM and cholesterol (l_o) would be 4.6 nm thick, while di-C18:1-PC reaches only 3.5 nm (Simons and Vaz, 2004). Such differences in height can be verified and visualized in planar model membranes by atomic force microscopy (Chiantia et al., 2006). Due to extra long ceramide molecules in yeast this effect can be increased. Here, a specific elongase activity results in a chain length of 26 carbons instead of normally 22 to 24 carbons (Eisenkolb et al., 2002).

In spite of an initial high acceptance, the lipid raft theory was always under debate. In particular, it was argued that this theory may be based on technical artifacts (Heerklotz, 2002; Munro, 2003). In addition, the general tendency to identify the originally distinct concepts of lipid rafts, DRMs and liquid-ordered lipid phases also gave rise to further criticism (Lichtenberg et al., 2005). At the Keystone symposium on lipid rafts and cell function, the lack of an accurate definition finally led to an agreement on what lipid rafts actually are: “Membrane rafts are small (10-200 nm), 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" (Pike, 2006).

1.4.2 Seeing is believing – visualization of domains *in vivo*

Despite its drawbacks, the theory of lipid rafts definitely drew the attention to the previously neglected lipid field. Hence, microscopists were motivated to take a closer look at plasma membranes of living cells, albeit in the first experiments to detect microdomains *in vivo*, lipids still played a minor part. Kusumi and colleagues followed the lateral movement of single particles microscopically and found that the cortical cytoskeleton meshwork forms a fence that inhibits the proteins in free diffusion (Sako and Kusumi, 1995; Dietrich et al., 2002; Murase et al., 2004).

Direct visualization of membrane microdomains in living cells by fluorescence microscopy was first achieved in the budding yeast *Saccharomyces cerevisiae*, where relatively large supramolecular membrane protein clusters could be studied (Young et al., 2002; Malínská et al., 2003). In *Bacillus subtilis*, the ATP synthase AtpA and succinate dehydrogenase SdhA were also shown to localize within discrete but mobile membrane domains (Figure 1.4A; Johnson et al., 2004). In addition, the *B. subtilis* ATPase MinD accumulates within a spiral-like compartment that is

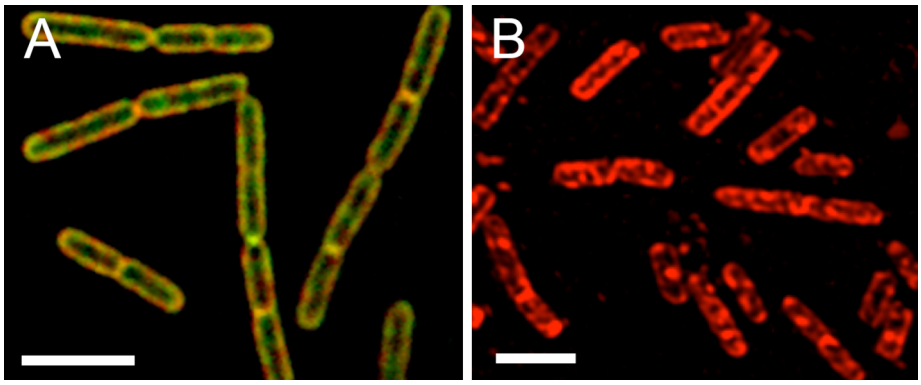

Figure 1.4 Compartmentation of plasma membrane proteins in bacteria and plants. (A) Localization of AtpA-CFP and SdhA-YFP in the plasma membrane of *Bacillus subtilis*. Scale bar, 2 µm. Courtesy of Peter J. Lewis, University of Newcastle, Callaghan, Australia. (B) Visualization of lipid spirals in *B. subtilis* by staining with FM4-64. Scale bar, 2 µm. Courtesy of Imrich Barák, Slovak Academy of Sciences, Bratislava, Slovakia.

suggested to be enriched in anionic phospholipids (Figure 1.4B; Barák et al., 2008).

Among the few examples documenting membrane domains in plant cells, the GFP-tagged version of the K^+ channel KAT1 was shown to be distributed in a stable punctuate pattern in the plasma membrane of *Nicotiana tabacum* leaf epidermis cells (Sutter et al., 2006). Intriguingly, KAT1 forms radial stripes in the plasma membrane of guard cells in *Vicia faba* (Figure 1.5; Homann et al., 2007). However, in animal cells, visualization of micro-domains was most difficult to be ac-complished. While in the other organ-isms the domain size and the distance between each other has been determined to be 200 - 500 nm, the size of mammalian microdomains was estimated to be around 50 nm (Lagerholm et al., 2005). For that reason, the limited resolution of confocal microscopes made visualization difficult.

Figure 1.5 Maximum projection of a guard cell of *Vicia faba* expressing KAT1::GFP (green), autofluorescence is shown in red. Scale bar: 10 µm. Courtesy of Ulrike Homann and Tobias Meckel, University of Technology Darmstadt, Germany.

1.4.3 Functional importance of domain formation

The spatial separation of various biological processes within the membrane implicates a necessity for proper enzymatic activity, regulation or signaling. However, despite extensive studies, the functional importance of this compartmentation is still not well understood.

The model elucidating a functional importance of plasma membrane microdomains is mainly concerned with a role in signaling (Simons and Toomre, 2000). It is suggested that lipid rafts help to sequester receptor proteins into specialized lipid environments. Thereby, platforms for protein clustering and interaction are built,

where local kinases or phosphatases can efficiently modify their substrates, resulting in downstream signaling. Due to the amount of uncertainties concerning size, lifetime or composition of lipid domains, it is still quite intricate to approach this model experimentally.

Recently, convincing evidence was provided that nanoclusters of the small GTPase Ras are involved in regulation of the signaling *via* the mitogen activated protein kinase (MAPK) pathways (Tian et al., 2007). The cytosolic Ras protein, an oncogene product, had previously been shown to be recruited to microdomains at the plasma membrane when activated by GTP (Murakoshi et al., 2004). This finding suggested the formation of activated Ras-signaling complexes, which were later termed nanoclusters (Plowman et al., 2005). Hancock and coworkers showed recently that epidermal growth factor (EGF) stimulates K-ras-GTP loading and the formation of nanoclusters that recruit the kinase Raf1 that further activates the MAPK pathway. In contrast to a graded response that would occur in parallel to ligand binding, nanoclusters act as a switch for full activation of signaling above a certain threshold value. In an *in silico* analysis, the authors were also able to demonstrate that on abrogation of nanoclustering, no response is generated, indicating that this type of domain formation in the plasma membrane is essential for Ras signal transduction (Tian et al., 2007).

1.4.4 MCC – Membrane Compartment of Can1

In *S. cerevisiae*, three lateral distribution patterns of plasma membrane proteins were observed. While some proteins show a rather homogeneous distribution (e.g. Hxt1, Figure 1.5, right), proteins as the H^+-ATPase Pma1 and associated ones localize to a mesh-like pattern (membrane compartment of Pma1, MCP; Figure 1.5, middle; Malínská et al., 2003). A third compartment appears in a spotty distribution (Figure 1.5, left). In adult cells, this compartment forms about 40 – 60 patch-like domains and houses several proteins of unknown function, as well as a number of H^+ symporters. Among the latter the arginine permease Can1 was the first one described (membrane compartment of Can1, MCC; Malínská et al., 2003). Additionally, the

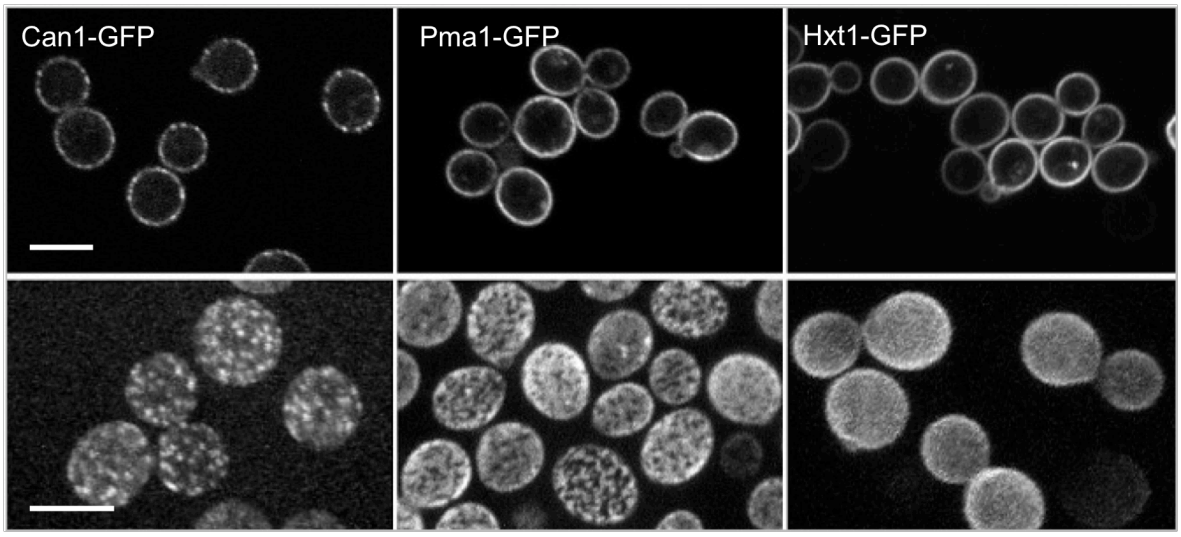

Figure 1.5 Three protein distribution patterns in the plasma membrane of *S. cerevisiae*. Left, Can1-GFP (corresponds to MCC); middle Pma1-GFP (corresponds to MCP); right, Hxt1-GFP. Cross sections (top panel) and superposition of four consecutive optical sections are shown. Scale bars: 5 μm. Figure reproduced from Malínská et al., 2003.

uracil/H^+ symporter Fur4 and Sur7, a protein of unknown function, could be localized to MCC (Malínská et al., 2004).

The domains of MCC are clusters with a diameter of about 200-300 nm. As MCC is filling the "holes" of MCP, these two compartments are completely separated from each other (Figure 1.6). Moreover this lateral protein segregation seems to remain absolutely stable once it has emerged in the young cell. During 90 minutes of measurement, which reflects a whole generation time in bakers yeast, no signif-

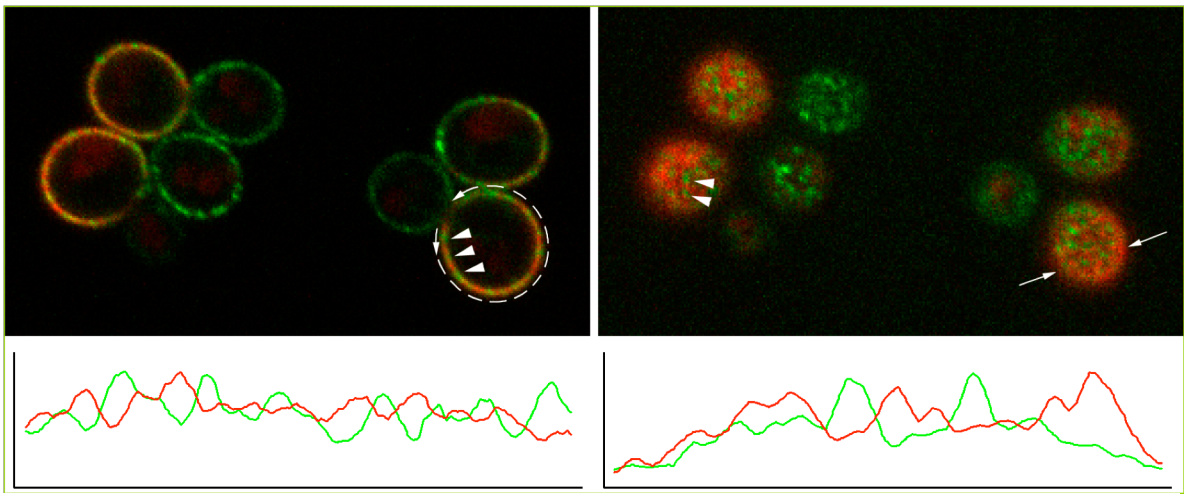

Figure 1.6 Mutual localization of Can1-GFP and Pma1-mRFP; left, cross-section; right, tangential section. The fluorescence intensity profiles, measured as indicated by arrows, show a separation of Can1 accumulations from MCP. Figure reproduced from Malínská et al., 2003.

icant change in the pattern was observed (Malínská et al., 2003). This remarkable domain stability has been shown to be independent of microtubules and actin cytoskeleton (Malínská et al., 2004). What really holds the two compartments in place is still unknown.

The fluorescent labeled Sur7 protein is an excellent marker for this compartment since it is well expressed under all growth conditions, it is extremely concentrated to MCC and it remains stable over time, and it is clearly visible even in the stationary phase (Figure 1.7; Young et al., 2002). This very useful tool was used also by others for colabeling experiments. Walter and colleagues identified a novel protein complex that localizes to the cell cortex at the cytoplasmic side directly be-neath the MCC. Using a GFP-tagged version of Pil1, the main constituent of the organelle together with Lsp1, they found a clear co-localization with Sur7. Since the authors present evidence that the organelle marks static sites of endocytosis, they termed it "eisosome" (from Greek '*eisodos*', meaning '*entry*', and '*soma*', meaning '*body*'; Walther et al., 2006). The authors showed that the deletion of *PIL1* resulted in few enlarged clusters of Sur7 in the plasma membrane, which were interpreted as remnants of eisosomes. The *pil1*Δ mutant leads to a retarded but not abolished endocytosis of the **a**-factor mating pheromone receptor Ste3.

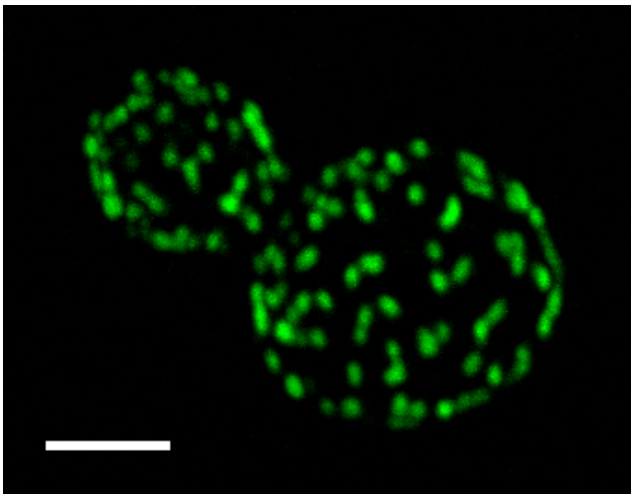

Figure 1.7 3D reconstruction of MCC marked by Sur7-GFP from the top to the central cross section. Scale bar: 2 µm.

In two competing studies, it was also demonstrated that the sphingolipid long-chain base activated protein kinases Pkh1 and Pkh2 regulate clustering of eisosomes and thus MCC formation by phosphorylation of Pil1 (Walther et al., 2007; Luo et al., 2008). Yet, the two studies contradict each other in answering the question whether Pil1 phosphorylation promotes assembly (Luo et al., 2008) or disassembly of

eisosomes (Walther et al., 2007). Furthermore, Pkh kinases are required for endocytosis (Friant et al., 2001). Dickson and colleagues also find in their work a dependence of eisosome assembly on active Ypk kinases (Luo et al., 2008), which are known to be regulated by the sphingolipid-Pkh signaling pathway, as well (Roelants et al., 2002). In both recent studies it was found that the kinases also cluster beneath MCC implicating an *in situ* regulation of eisosomes and, hence, endocytosis (Walther et al., 2007; Luo et al., 2008).

1.5. Goals of the thesis

This thesis focuses on MCC with the aim to further characterize the compartment, to analyze its formation and stabilization and to suggest a biological function of this protein segregation. Many conclusions are based on results obtained by fluorescence microscopy. Since the distance between individual MCC clusters can be up to one micron, they can easily be resolved and studied by confocal laser scanning microscopy (CLSM) with the use of fluorescent protein tags.

When the experimental work for this thesis started, DRMs were only known in animal and yeast cells, although they were already expected to exist in all domains of life. Thus, studying a plant membrane protein regarding to microdomain formation was of great interest. For this purpose, the well characterized hexose/H^+ symporter HUP1 of *Chlorella kessleri* was chosen, to find out whether it behaves as a constituent of DRMs when isolated from *Chlorella* cells and treated with mild detergent. Based on the finding that HUP1 is indeed part of DRMs, the question arose whether its distribution within the plasma membrane is also inhomogeneous as it was reported before for lipid raft proteins in yeast (Malínská et al., 2003). Due to the genetic inaccessibility of *Chlorella*, the localization was intended to be carried out by heterologous expression in *S. cerevisiae* and *S. pombe*, where HUP1 previously had been shown to be fully active (Sauer et al., 1990; Robl et al., 2000). Because of its known affinity to sterols (Robl et al., 2000), it was tested whether the GFP-tagged transport protein is targeted to sterol rich areas in the plasma membrane

of *S. pombe*. In *S. cerevisiae* a potential accumulation of HUP1 into one of the two raft-based membrane compartments (MCC or MCP) should be examined by colocalization experiments. In ergosterol and sphingolipid biosynthesis mutants, the question for a dependence of domain formation on the lipid composition of the membrane was addressed, as well as the question whether a potential inability of clustering in these mutants affects the uptake activity of MCC transport proteins.

Another main goal was the further characterization of MCC regarding its lipid and protein composition. By data mining and own colocalization experiments, new MCC residing proteins were expected to be found. Besides screening the literature, the existing localization database for yeast (Huh et al., 2003) offered a starting point to search for cortically clustered proteins. A similar approach was also considered for proteins of MCP though the mesh-like pattern would be more difficult to be recognized in published results, which usually present localization data as cross sections. Once a comprehensive set of colocalizing proteins would have been obtained, the biological reasons for this protein segregation presumably will become evident.

In addition to the compartmentation of membrane proteins, it was also of great interest to test whether it is possible to visualize also an inhomogeneity of membrane lipids. For this purpose it was planned to elaborate the filipin staining of sterols for bakers yeast.

Though there are theories that lateral sorting and domain formation could occur spontaneously by phase separation within complex lipid mixtures, transferring this concept to the formation and stabilization of MCC and MCP is unsatisfying in view of the positional stability of these compartments. Thus, it was especially interesting to unveil the parameters that affect pattern formation, as well as the mechanisms to immobilize the compartments.

From observations that were reported back in the late seventies by Komor et al. (1979), it was known that deenergized cells exhibit an increased resistance to detergents suggesting that the voltage difference across the plasma membrane might have an influence on membrane organization and stability. Since microscopical techniques are now available to visualize the higher order within membranes, this

yet unsolved phenomenon should be studied again. Therefore, it was intended to test chemical uncouplers and reversible depolarization to study the putative role of the membrane potential on membrane compartmentation.

Biological factors, i.e. enzymes or structural proteins, that are involved in formation and stabilization were expected to be identified by mutant analyses. To test as many mutants as possible, a genome wide approach was conceived, involving high throughput transformation with a fluorescent MCC marker and large scale visual screening. With the help of eventually revealed mutants, it was also hoped to finally disclose the biological significance of the apparent compartmentation of the yeast plasma membrane.

2 Studies on the Compartmentation of the Yeast Plasma Membrane

2.1. Lipid raft-based membrane compartmentation of a plant transport protein expressed in *Saccharomyces cerevisiae*. (P1)

Lateral compartmentation is now believed to be a wide-spread feature of cellular plasma membranes throughout all domains of life. However, this phenomenon was so far studied mainly in animal cells, where the domains were estimated to be too small to be resolved by light microscopy. There was evidence that detergent resistant membranes (DRMs) also exist in plants (Mongrand et al., 2004), but due to the novelty of the plants lipid raft field, no microscopy localization data of such domains were available, so far. In the model organism *Saccharomyces cerevisiae* large, microscopically well resolvable protein clusters had been described, which are related to membrane microdomains (Malínská et al., 2003; Malínská et al., 2004). By heterologous expression in yeast, the association of an individual plant transport protein with such domains was addressed.

Using the classical test based on Triton X-100 resistance, it is shown that the hexose/H^+-Symporter HUP1 from the unicellular alga *Chlorella kessleri* is detergent resistant and after density gradient centrifugation, accumulates in the sterol rich fraction. As *Chlorella* is not accessible for molecular genetics so far, the functional expression of HUP1 in fission yeast and bakers yeast (Sauer et al., 1990; Robl et al., 2000) provided a convenient alternative to study the molecular and biochemical features of this transport protein, as well as its membrane localization. For *S. cerevisiae*, it is shown that GFP-tagged HUP1 is efficiently targeted to the plasma

membrane, where it localizes to the spotty lipid raft-based membrane compartment of Can1 (RMC C, later termed membrane compartment of Can1, MCC). The distribution of HUP1-GFP appeared to be highly dependent on the correct lipid composition of the plasma membrane. In the sphingolipid mutant *lcb1-100* and in the mutants of ergosterol biosynthesis *erg6*Δ and *erg24*Δ, the clustering of HUP1 was abolished. However, also in wild type a clear pattern could be only observed when cells were grown at low glucose concentration – an observation that eventually became important, as reported below.

In fission yeast *Schizosaccharomyces pombe*, sterol-rich membrane areas can be visualized in the polar caps and the septum by filipin staining. When expressed in *S. pombe*, HUP1-GFP also predominantly localizes to these raft-like structures. This is consistent with the previous finding that the HUP1 protein exhibits a high affinity for sterols (Robl et al., 2000). As shown in an *S. cerevisiae* mutant lacking endogenous glucose permeases, the preference for ergosterol appears to be also of functional importance. Uptake measurements of radioactively labeled glucose revealed that the additional deletion of the *ERG6* gene results in a significant loss of the transporter activity as compared to control cells.

Obviously, preferences for certain lipid environments are an intrinsic feature of transmembrane proteins independently of expression system. The information for sorting into specific lipid microdomains appears to be maintained even when a protein is heterologously expressed in a distantly related organism.

2.2. Membrane potential governs lateral segregation of plasma membrane proteins and lipids in yeast. (P2)

This preference of HUP1 for sterol-rich environments in *S. pombe* raised the question whether the same sorting principle might be also responsible for its accumulation in MCC. Thus, it was of great interest to study the distribution of sterols in the plasma membrane of bakers yeast. So far, *in vivo* visualization of the plasma membrane compartmentation was only possible at the level of proteins,

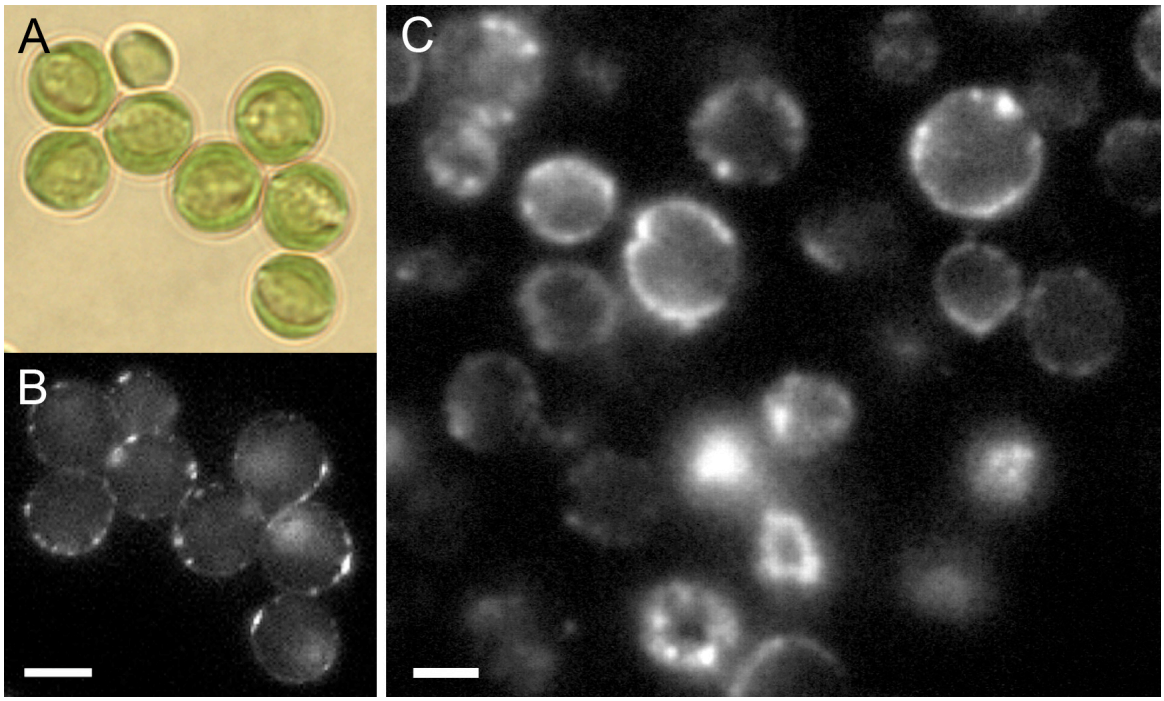

Figure 2.1 Imaging of the unicellular alga Chlorella kessleri. (A) Bright-field image. (B) Same cells stained with filipin. (C) Embedded and sectioned cells immunostained with a specific anti-HUP1 antibody (reproduced Fig. 1 from Grossmann et al., 2006). Note that the inhomogeneous distribution of HUP1 in the plasma membrane resembles the patchy filipin distribution in (B). Scale bars: 5 µm.

either by immunostaining or tagging with fluorescent proteins. However, it was assumed that this domain formation might be based on lateral segregation of lipids. By using low concentrations of the sterol-specific dye filipin, sterol rich domains indeed became visible in the plasma membrane of living yeast cells. Far higher concentrations were previously used to stain large sterol accumulations in polarized cells as *S. pombe* (Wachtler et al., 2003) or mating pheromone treated *S. cerevisiae* cells (Proszynski et al., 2006).

By colocalizing the sterol accumulations with the MCC-marker Sur-mRFP, it was proven that this compartment indeed is enriched in sterols, which supports the hypothesis that the sorting of HUP1 relies on its intrinsic preference for this type of lipids. Interestingly, also filipin-stained Chlorella cells exhibit a clear inhomogeneity in the plasma membrane (Figure 2.1B, unpublished data) that is reminiscent to the localization obtained by antibody staining of HUP1 in sectioned Chlorella cells (Figure 2.1C).

If MCC is the sterol-rich compartment, accordingly, the surrounding compartment of the H^+-ATPase Pma1 (MCP) must be low in sterols. Though there is no direct evidence yet, from the published data of others, it can be predicted that MCP is rather enriched in sphingolipids due to the resident Pma1, which requires them for its correct targeting and stability (Gaigg et al., 2005).

In addition, another H^+-symporter could be found to reside within MCC: the tryptophan-tyrosine permease Tat2. The targeting of this protein had been previously shown to be highly dependent on ergosterol (Umebayashi and Nakano, 2003). This protein-lipid interaction obviously remains stable until the protein reaches the plasma membrane similarly as shown for Pma1 and sphingolipids.

However, the stabilization of Tat2 and the other H^+-symporters Can1 and Fur4 within MCC is quite susceptible to changes of the membrane properties, and the same holds true for the heterologously expressed HUP1. As described before, the proper lipid composition is crucial to maintain the clustering (Malínská et al., 2003; Grossmann et al., 2006). However, as mentioned above, also in wild type cells, the HUP1 pattern becomes best visible when the cells are grown in low-glucose containing medium. An earlier analysis of glucose dependent patchiness revealed that HUP1 highly accumulates within MCC when the glucose concentration in the medium falls below 2.5 mM (Grossmann, 2004). Due to the heterologous expression, a substrate dependent regulation of HUP1 that would lead to the differences in localization could be largely excluded. Thus, the uptake activity of the high-affinity transporter itself was considered to be responsible for its lateral movement within the plasma membrane. Strikingly, upon the addition of glucose to HUP1 expressing cells, a transient depolarization of the membrane potential can be measured (Miroslava Opekarová, personal communication). This fact brought the membrane potential into play, which had been assumed already before to be linked to the membrane organization. Komor et al. (1979) found that cells, loaded with a radioactive compound do not release the radioactivity upon treatment with detergent as fast as control cells if an uncoupler was added before. The authors stated that *„a general change within the bordering membrane or at the membrane surface has to*

take place when an energized cell becomes a nonenergized one" (Komor et al., 1979).

This effect was reproduced by loading yeast cells with radioactively labeled α-amino-isobutyric acid and their subsequent permeabilization by sodium dodecyl sulfate. If the cells were treated with FCCP prior to detergent addition, the leakage was significantly decreased. The changed susceptibility towards detergents can be best explained by a redistribution of lipids. Especially sterols are known to rigidify the plasma membrane and thus, their potential release from MCC could increase the average membrane stability. Although the lateral movement of lipids is difficult to be visualized directly, the reorganization could be followed by observation of the sterol-linked H^+-symporters. Utilizing modern visualization possibilities, it was now possible to confirm the predictions made thirty years ago and to show that the transmembrane potential indeed plays an important role in lateral sorting. As soon as the plasma membrane is depolarized by an uncoupler or an electrical pulse, the patchy arrangement of the transporters is dissipated within seconds. The advantage of the depolarization by an electrical shock is the option to study reversibility of the effect. This allowed the demonstration that the proteins eventually reassemble into the same pattern. Interestingly, the non-transporting Sur7 is not affected by a loss of the membrane potential but maintains its position within MCC. Likewise the MCP seems not to be disturbed by depolarization. Obviously tight interactions stabilize the compartments, while large movements of components occur upon de- and re-polarization. To date, the membrane potential was thought to be mainly the driving force for nutrient uptake. These results described above implicate its novel, important role in lateral organization of the plasma membrane.

2.3. Evidence for coupled biogenesis of yeast Gap1 permease and sphingolipids: essential role in transport activity and normal control by ubiquitination. (P3)

Previously, it was suggested that the association of integral yeast plasma membrane proteins with either MCC or MCP is a result of their presence in DRMs (Malínská et al., 2003). Proteins like Hxt1 that appear homogenously distributed were thus considered as non-raft proteins. However, also Hxt1 was later found to be associated with DRMs when isolated from highly purified plasma membranes (Lauwers and André, 2006). Hence, all integral yeast plasma membrane proteins examined so far, turned out to be to some extend insoluble in Triton X-100 and thus, they behave as raft constituents. The fact that proteins of DRMs can occupy different membrane compartments indicates the existence of different raft types. This is consistent with the lipid shell model that suggests tightly associated lipid molecules generating an individual environment for each membrane protein.

The transport proteins examined so far all exhibit a more or less high affinity for sterols which is considered to be crucial for their localization to MCC. Hence, it was necessary to examine a protein that is attracted by sphingolipids (SL) and independent of sterols. Therefore, the localization of the general amino acid permease Gap1 was investigated, as well as the influence of its immediate lipid shell on its function, ubiquitination and membrane trafficking. It was already known that Gap1 fractionates with DRMs and that its stable accumulation at the plasma membrane requires neosynthesis of SL but not sterols. In the thermosensitive mutant *lcb1-100*, defective in the first step of SL biosynthesis, Gap1 is targeted to the cell surface but immediately undergoes rapid and unregulated degradation in a ubiquitin-dependent manner (Lauwers and André, 2006).

When Gap1 is blocked at the plasma membrane in a mutant affected in endocytosis, its activity and modification can be studied even in the *lcb1-100* strain. Uptake measurements showed that in the absence of newly synthesized SL, the transporting activity is abolished. Being outside SL-enriched domains, Gap1 also looses its detergent resistance properties. This effect could be compensated by the

addition of external dihydrosphingosine in the course of Gap1 synthesis. A later addition, when Gap1 had already accumulated in the membrane, did not restore its activity or association with DRMs. This demonstrates that neosynthesis of both SL and Gap1 has to proceed in parallel. Once the permease has reached the cell surface, the protein-lipid interaction cannot be established anymore.

Interestingly, due to the altered lipid environment, three additional lysine residues that normally are close to the membrane become now available for the ubiquitination machinery and therefore promote degradation of Gap1. Obviously, outside of SL-enriched domains, the protein is less well enclosed in and protected by its lipid microenvironment, presumably through an altered protein conformation. This was also confirmed by an increased susceptibility of Gap1 to proteases.

The high importance of SLs for the protein function and conformation resembles the dependence of Pma1 on SLs (Gaigg et al., 2005). Thus, the question arose whether Gap1 shares one of the mutually exclusive compartments MCC or MCP or whether it accumulates in certain domains at all. To answer this question, cells expressing Gap1-GFP and either Sur7-mRFP or Pma1-dsRed were grown under conditions, where the two measured proteins were associated with DRMs. However, the test for both compartments was negative: an enrichment of Gap1 was neither detectable in MCC nor in MCP. Also, no independent Gap1 domains could be resolved. Its homogenous distribution did not change when biosynthesis was induced in the absence of SLs, neither when, in addition, endocytosis was blocked.

These data suggest that Gap1, and maybe also Hxt1 and other homogenous membrane proteins, either do not distinguish between MCC and MCP or define their own visually overlapping compartment. The possibility exists that conventional fluorescence microscopy is just unable to resolve these novel microdomains. In any case, the data clearly show the importance of a coupled biosynthesis of SLs and a transport protein to create an individual microenvironment that is essential for function, conformation and stability of the protein in the membrane.

2.4. Plasma membrane microdomains regulate turnover of transport proteins in yeast. (P4)

Since its discovery, the MCC within the plasma membrane of bakers yeast has been used as a microscopically accessible model for domain formation in eukaryotic membranes. However, the biological relevance of this protein segregation still remained obscure. The fact that four H^+-symporters (Can1, Fur4, Tat2, and HUP1) all localize in patches that are apart from the H^+-ATPase meshwork, implied a necessity of separation of currents. However, since at least since the H^+-symporter Gap1 and some others did not fit into this pattern, the theory had to be abandoned. Thus, it was hoped that a comprehensive set of further colocalizing proteins would finally unveil the function of MCC.

By screening the localization database of GFP-tagged yeast proteins (Huh et al., 2003), several proteins were identified to form apparent cortical clusters. Subsequent colocalization experiments revealed further eleven MCC associated proteins. Including the localization data of other groups (Roelants et al., 2002; Young et al., 2002; Fadri et al., 2005; Walther et al., 2006 and 2007; Luo et al., 2008), a total set of twenty-one proteins can now be shown to cluster within or – in case of twelve soluble proteins – underneath MCC. What stands out is that besides the transporters, which all contain twelve transmembrane domains, all other integral MCC members are predicted to contain four transmembrane spans. This resembles the protein family of tetraspanins that are implicated in domain formation in various organisms (Hemler, 2005). However, no sequence homology of the corresponding MCC proteins with tetraspanins was found (data not shown). Also the molecular functions of these MCC proteins are still largely unknown. Among the eleven proteins, which are concentrated beneath MCC at the cytosolic side, some are well studied enzymes. The two redundant serine/threonine protein kinases Pkh1 and Pkh2, that are involved in the regulation of endocytosis (Friant et al., 2001), cluster here next to the two redundant phosphoinositide binding proteins Slm1 and Slm2, which are known parts of the TORC2 and calcineurin signaling pathways (Fadri et al., 2005; Bultynck et al., 2006). Interestingly, also Pil1 and Lsp1, the major protein components of

eisosomes, locate beneath MCC. Based on a retarded internalization of Ste3 in the *pil1*Δ and *lsp1*Δ mutants, it was suggested that the novel organelle marks sites of elevated endocytosis (Walther et al., 2006), thus implicating MCC with areas of high protein turnover and degradation. All six proteins have in common that their expression responds to stress conditions and that their functions are linked to regulation of endocytosis (deHart et al., 2002; Zhang et al., 2004; Bultynck et al., 2006). As stated before, a functional importance of colocalization of Pkh kinases with eisosomes has been shown independently by the groups of Walter and Dickson.

Despite the published indications of a link between MCC and endocytosis, it is still an open question, how the compartment is formed and whether the conclusions made by following Ste3 degradation also apply to other membrane proteins. To approach an answering, proteins that are required for MCC formation should be identified in a genome-wide visual screen. By addressing all viable single-deletion mutants regarding the localization of various MCC markers (HUP1-GFP, Can1-GFP, Sur7-GFP, and filipin stained sterols), 28 mutants were identified that show significant alterations in the protein distribution pattern. The phenotypes varied from more diffuse patches to complete homogeneity. Also not all markers were affected to the same degree by the same mutation. While the patterns of the two transporters appeared to be most susceptible to changes in membrane composition, there were only few mutations affecting the distribution of Sur7 or sterols. But whenever the deletion of a particular gene resulted in a changed distribution of these two markers, this had dramatic effects on the MCC accumulation of transporters.

The first analysis of the mutant set revealed a significant over-representation of genes involved in lipid metabolism and vesicle mediated transport. A simple explanation for the involvement of vesicle transport could be that an important factor is not correctly targeted to fulfill its function in MCC formation. The high impact of lipid biosynthesis genes indicates that the modified lipid composition of the plasma membrane or, as in the case of Gap1, an insufficient protein-lipid interaction during trafficking can explain the mislocalization of proteins, as well. As both pathways are functionally interconnected (Opekarová, 2004; van Meer et al., 2008), the

consequence is an abnormal lipid environment in any case, which should directly result in an altered detergent extractability. As shown here in mutants, where Can1 is not concentrated in MCC, it is indeed less stabilized in the membrane and thus susceptible to extraction by lower detergent concentrations. The fact that Can1 looses the lipid connection also under depolarizing conditions (when it already has reached the plasma membrane), favors the hypothesis that the protein-lipid link is established within the plasma membrane and not during earlier steps in the secretory pathway.

Two genes, *PIL1* and *NCE102*, which were found in the screen to be crucial for all markers to form the proper distribution pattern, attracted particular attention. Besides the eisosomal Pil1 protein, also Nce102, which is an integral membrane protein, colocalizes with MCC. In contrast to other proteins with a comparably strong mutant phenotype, e.g. various ergosterol biosynthesis mutants, Nce102 and Pil1 participate directly *in situ* in the formation of the membrane compartments. It is interesting that also in these mutants, the lipid environment of Can1 is changed although these two genes are not known to be involved in lipid biosynthesis. This is consistent with the previous finding that MCC is enriched in sterols indicating a higher rigidity of this part of the membrane.

Strikingly, in young buds Nce102 is not concentrated in patches. Only when the bud grows to approximately one third of the diameter of the mother cell, the Nce102 clusters arise. In contrast, the cytosolic Pil1 and the integral MCC marker Sur7 form patches as soon as a GFP signal can be detected. This does not necessarily mean that Nce102 is not involved in MCC formation from the beginning. In fact, as far as it is resolvable by light microscopy, the homogenous distribution only indicates that the protein might be literally everywhere, both outside and inside the emerging compartment. Once it accumulates within MCC, the protein remains stable within. Membrane depolarization does not result in dispersion, as it is the case for the transport proteins. The same effect was already found for Sur7 (Grossmann et al., 2007). The finding that both, Nce102 and Sur7, physically interact (Loibl, 2008), might explain their similar behavior upon membrane depolarization, but, however, still cannot clarify how they are stabilized within MCC. Doubtless, further protein-

protein or protein-lipid interactions can be postulated and probably will be unveiled in the near future.

The question regarding the physiological importance of lateral membrane compartmentation was addressed by a detailed mutant analysis of the *nce102*Δ and *pil1*Δ strains. However, various changes of growth conditions did not result in a growth rate different to wild type (Loibl, 2008). Also no significantly altered enzyme activity of the arginine permease Can1 could be measured when the protein was localized outside MCC (Miroslava Opekarová, personal communication). According to the finding of Walter and colleagues that deletion of *PIL1* resulted in decreased endocytosis of the **a**-factor receptor Ste3, their hypothesis was also tested on the stability of Can1 in the membrane, by following substrate induced endo-cytosis of the transporter. Contrary to expectations, no decreased but rather an increased internalization of Can1 was found in both the *nce102*Δ and *pil1*Δ mutants. Unlike Can1, Ste3 is homogenously distributed in the membrane (Oestreich et al., 2007). Thus, it was postulated that the differential localization pattern could affect the protein properties regarding stability and turnover. Following the localization of marker proteins for endocytosis, including Ede1, Rvs161 and Sla2, resulted in a clear separation of endocytic sites and MCC. This finding suggests that MCC is an area of retarded protein internalization, implying that Can1 first needs to leave the compartment prior to endocytosis. To check this hypothesis, the microscopical images of the internalization measurements were resurveyed concerning the patchiness of Can1. Indeed, the wild type cells, treated with external arginine, showed a clear reduction of patchiness. In these cells the Can1 distribution became homogenous to a similar extend as observed in the *nce102*Δ and *pil1*Δ mutants.

Hence, it is concluded that MCC can serve as a "shelter", where certain proteins are maintained and stabilized by keeping them away from the endocytic machinery. Nce102 serves as an anchor for Can1 and additional H^+-symporters within MCC, while Pil1 presumably plays an important role in the "sheltering" mechanism by locally downregulating signaling pathways essential for endocytosis (Zhang et al., 2004). The exclusion of endocytic events from MCC can serve as an example of spatially confined regulation of a biological process.

3 Publications

Note:

For reasons of copyright, reprint versions of the publications are not included in the online version of this thesis. Please follow the hyperlink to download the PDF file directly from the publisher's website.

P1

Lipid Raft-Based Membrane Compartmentation of a Plant Transport Protein Expressed in *Saccharomyces cerevisiae*.

Guido Grossmann, Miroslava Opekarová, Linda Nováková,
Jürgen Stolz and Widmar Tanner

Eukaryotic Cell **5**(6), 945-953 (2006)

http://ec.asm.org/cgi/reprint/5/6/945

P2

Membrane Potential Governs Lateral Segregation of Plasma Membrane Proteins and Lipids in Yeast.

Guido Grossmann, Miroslava Opekarová, Jan Malínsky,
Ina Weig-Meckl and Widmar Tanner

The EMBO Journal **26**(1), 1-8 (2007)

http://www.nature.com/emboj/journal/v26/n1/pdf/7601466a.pdf

P3

Evidence for Coupled Biogenesis of Yeast Gap1 Permease and Sphingolipids: Essential Role in Transport Activity and Normal Control by Ubiquitination.

Elsa Lauwers, Guido Grossmann and Bruno André

Molecular Biology of the Cell **18**(8), 3068-3080 (2007)

http://www.molbiolcell.org/cgi/reprint/18/8/3068

P4

Plasma Membrane Microdomains Regulate Turnover of Transport Proteins in Yeast.

Guido Grossmann, Jan Malínsky, Martin Loibl, Wiebke Stahlschmidt, Ina Weig-Meckl, Wolf B. Frommer, Miroslava Opekarová and Widmar Tanner

Journal of Cell Biology, published December 8, 2008
DOI: 10.1083/jcb.200806035

http://jcb.rupress.org/cgi/reprintds/183/6/1075

4 Contribution

P1 **Grossmann G**, Opekarová M, Nováková L, Stolz J and Tanner W (2006) Lipid raft-based membrane compartmentation of a plant transport protein expressed in *Saccharomyces cerevisiae*. *Eukaryot Cell* **5**(6), 945-53

Guido constructed all strains and plasmids, performed all confocal localization studies including mutant analysis and filipin staining. He participated in uptake measurements and detergent extractions.

P2 **Grossmann G**[*], Opekarová M[*], Malínsky J[*], Weig-Meckl I and Tanner W (2007) Membrane potential governs lateral segregation of plasma membrane proteins and lipids in yeast. *EMBO J* **26**(1), 1-8

Guido shares first authorship, constructed strains and plasmids and participated in the first finding of effects of depolarization. He achieved the first visualization of filipin patches and analyzed the colocalization of Tat2 as well as the localization pattern of an MCC marker in respiratory mutants. Together with M.O. he designed the electrical depolarization experiment. He further was involved in revisions to write the final manuscript and he submitted the paper.

[*] These authors contributed equally to the work.

P3 Lauwers E, **Grossmann G** and André B (2007) Evidence for coupled biogenesis of yeast Gap1 permease and sphingolipids: essential role in transport activity and normal control by ubiquitination. *Mol Biol Cell* **18**(8), 3068-80

Guido carried out all confocal localization measurements and contributed the corresponding strains.

P4 **Grossmann G**, Malínsky J, Loibl M, Stahlschmidt W, Weig-Meckl I, Frommer WB, Opekarová M and Tanner W (2008) Plasma membrane microdomains regulate turnover of transport proteins in yeast. *Journal of Cell Biology* DOI: 10.1083/jcb.200806035

Guido contributed plasmids and strains and designed, performed and evaluated the genome wide screen. He conceived and carried out additional confocal measurements including colocalizations, depolarization experiments and microscopical endocytosis measurements. He further evaluated membrane protein patchiness and performed time-lapse measurements of endocytic marker proteins. In addition, he conceived and supervised experiments of W.S. and M.L. He wrote the first draft of the manuscript and was substantially involved in producing the final version and submitted the paper.

Widmar Tanner

5 Working models and future perspectives

When studying a novel phenomenon such as the visible protein clusters in the yeast plasma membrane, one is facing an enormous amount of open questions and possible experiments. Several conclusions could be already drawn, contributing to the elucidation of the pattern formation and stabilization, the domain structure and its biological function, but the phenomenon is still far from being fully understood. In this last part of my thesis I will try to highlight some possible ways how to experimentally proceed with regard to working hypotheses.

5.1. How is the MCC pattern established?

One of the main open questions that will be addressed in the future concerns the initial steps in MCC formation. Comprehensive colocalization data and the genome-wide screen revealed a manageable number of genes that are either associated with MCC or affect its formation at earlier steps in the secretory pathway. Mutant analyses and further protein characterization will be necessary to disclose their immediate influence on domain formation or the mechanisms of their sorting into this plasma membrane compartment.

When following the first appearance of MCC patches in very young buds, it is noticeable that some proteins, including Sur7 and Pil1, right away accumulate within clusters, while Nce102 is still homogenously distributed and present at high amounts as estimated from absolute fluorescence intensities. At first sight, it seems contradictory that Nce102 is supposed to be important in MCC formation but does not participate in this early clustering. However, an equal distribution does not

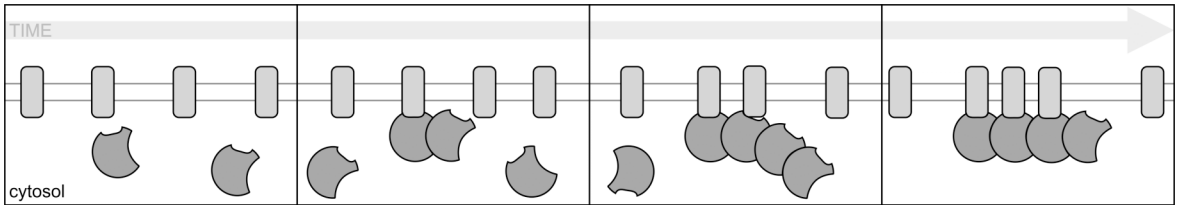

Figure 5.1 Model of how association of a multimerizing cytosolic protein can induce clustering of a membrane protein.

necessarily mean that Nce102 is not involved in the process of domain formation. If new clusters are determined by a random aggregation of cytosolic proteins in the cell cortex, it might be important that a membrane anchor is available wherever a new cluster might eventually create a membrane microdomain. From expression pattern analyses, it becomes evident that *NCE102* is also slightly earlier expressed than *PIL1* during the cell cycle (Spellman et al., 1998). While the peak expression of *NCE102* is determined during M-phase, the peak of *PIL1* is shifted towards the M/G1 transition, which corresponds to the phase of cell separation.

Based on the presented findings, a working model can be suggested that implies such an anchoring function of Nce102 in MCC formation, and that only its cooperation with Pil1 leads to clustering (Figure 5.1). The model further suggests that Pil1 acts as a structural initiator that requires Nce102 as a direct or indirect anchor for its attachment to the plasma membrane. The fact that Pil1 is present in each eisosomal patch in several thousand copies (Ghaemmaghami et al., 2003; Walther et al., 2006), makes it conceivable that it multimerizes to form large clusters. This clustering of Pil1 and an increasing interaction with its anchoring partner at emerging MCC sites will then directly result in an accumulation of Nce102. Yet, the potential interaction between Nce102 and Pil1 or a third mediating protein is still hypothetic (Loibl, 2008). The elucidation of this and other interactions within MCC or between the membrane constituents and eisosomes will be approached by co-immuno precipitation, fluorescence resonance energy transfer (FRET) measurements or by using the split-ubiquitin system (Stagljar et al., 1998).

To elucidate the process of domain formation also *in vitro* experiments will be considered. A reconstitution of fluorescently labeled MCC constituents into giant

unilamellar vesicles is expected to provide insights into the minimal requirements for clustering. Using lipid mixtures that promote phase separation (see Kahya et al., 2003) will further help to determine preferential lipid environments of MCC proteins.

When focusing on the beginnings of patterning, it is also striking that the number of MCC patches is relatively stable among cells, suggesting a limitation in space. Hence, an interplay between MCC and the surrounding compartment MCP is considered to be a determinant in this process. The genome wide screen identified mutants affected in N-glycosylation, which exhibit apparently increased numbers of MCC patches. A thorough characterization of these mutants is a promising approach to identify the lateral limitations for domain assembly.

5.2. What stabilizes MCC and its constituents?

Once the MCC pattern is formed, it remains stable over more than 90 minutes (Malínská et al., 2003). This indicates that the stabilizing connections persist once the proteins are linked during patch assembly. However, the examined transport proteins appear to be only loosely associated with the compartment. It was a very interesting finding that the transmembrane potential is directly involved in the organization of the plasma membrane by stabilizing the MCC association of transporters. Studies on the voltage difference across membranes of living cells reach back to Galvani's[2] experiments on frog legs. However, surprisingly little is known about its influence on the membrane structure. Modern computational power enables now scientists in bioinformatics to simulate the influence of lipid asymmetry on the membrane potential (Gurtovenko and Vattulainen, 2007; Gurtovenko and Vattulainen, 2008), and further the effect of the membrane potential on hypothetical proteins (Delemotte et al., 2008). Where the experimental approaches reach the boundaries of feasibility, such simulations can help to understand molecular mechanisms.

[2] Luigi Galvani (1737 – 1798), Italian physicist.

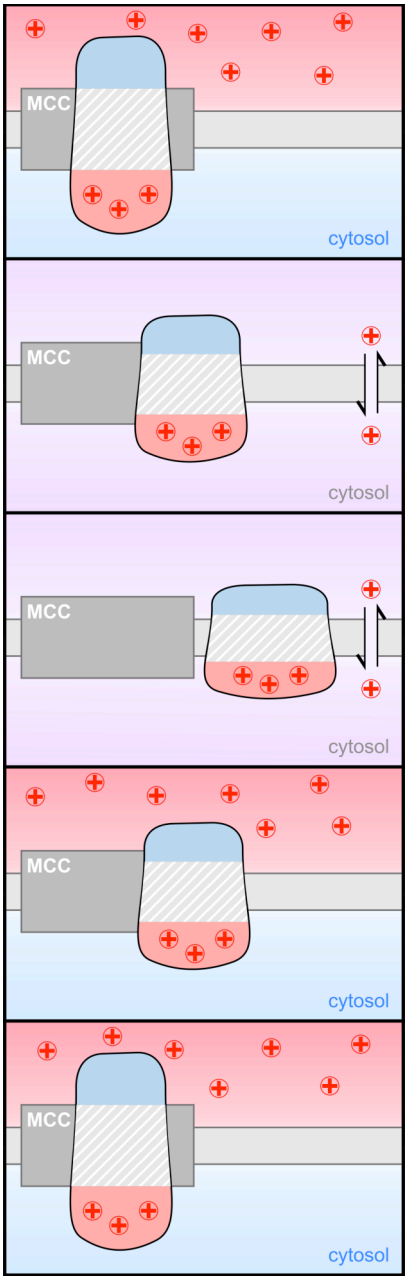

Figure 5.2 Hypothesis of how the membrane potential can influence protein sorting in the membrane. The voltage difference causes stretching of the transmembrane protein, which is conversely charged (top). Upon depolarization the protein collapses and leaves the thicker compartment (middle). Repolarization reverses the protein movement (bottom).

Bioinformatics can also help to predict the structure and conformation of proteins on the basis of previously resolved structures of related proteins. Such an approach will be especially interesting for MCC localized transport proteins to understand their membrane potential dependent accumulation within the compartment.

Also less sophisticated bioinformatical methods like hydropathy analyses can indicate possible solutions. The topology prediction determines twelve transmembrane domains for the two MCC residing transporters Can1 and HUP1. It also reveals a striking asymmetry in the distribution of positively charged residues between the protein regions facing either the cytoplasm and the extracellular space. The published topological model of HUP1 (Caspari et al., 1994) shows the presence of 29 arginyl and lysyl residues inside *versus* nine of them outside. In the Can1 molecule 40 positively charged amino acids are found inside *versus* ten outside, always assuming that the topology predictions reflect the actual number and orientation of the transmembrane spans. This phenomenon of charge asymmetry is known as the "positive-inside" rule in membrane topology (von Heijne and Gavel, 1988).

Considering the fact that the acidification of the extracellular environment leads to an increase of positively charged ions on the outside a simple model for stabilizing the proteins within MCC can

be suggested. The charge difference on the two sides of the membrane, and the converse polarization of proteins could result in a stretching of the integral membrane spans and thus, to an exposure of hydrophobic protein residues to water (Figure 5.2). Assuming that the MCC areas are thicker, as it was shown for sterol rich l_o phases of membranes *in vitro*, they would be attractive for such proteins. In case of membrane depolarization, the stretched protein would collapse and leave MCC. A re-engergization of the membrane reverses the lateral movement back into MCC.

Although the described model does not necessarily require proteins as interaction partners, the presented observations can be also explained by electrostatic (ionic) interactions of the transporters and another protein anchor. One candidate for being such an anchor is Nce102, which is not only similarly distributed as Can1 in young and mature membranes but it is also essential for proper MCC formation, and it physically interacts with Sur7 (Loibl, 2008). Yet, the sites for physical and electrostatic interactions remain to be determined, which will be achieved by a random or site-directed mutagenesis of *NCE102*. The highly conserved C-terminus, which is oriented towards the cytosol, has already been identified to be important for the patchy distribution of Nce102 itself, and for MCC stability in general (Loibl, 2008). Thus, future biochemical studies will focus on this part of the protein.

Besides interactions between proteins, also the interactions between membrane proteins and lipids will be addressed. As shown for Gap1 the immediate lipid microenvironment is crucial for correct conformation, function and stability of the protein within the plasma membrane. It is proposed that individual lipid shells also determine the DRM behavior and sorting of their enclosed proteins into certain membrane subdomains. To disclose the composition of these shells, the encasing lipids will be extracted from purified proteins by organic solvents and subsequently analyzed by electrospray ionization mass spectrometry (Pulfer and Murphy, 2003). This will be done for proteins of MCC and MCP in parallel to unveil the differences in lipid composition of the two compartments. It will also be very interesting to study the changed lipid environment of MCC located transporters either inside or outside the compartment, as it is the case in mutants revealed by the screen.

Another unsolved question is what kind of interactions between MCC and other cellular components account for the remarkable immobility of the sub-compartments. As published previously, cytoskeletal structures as cortical actin or microtubules can be ruled out (Malínská et al., 2004) although there might be still other, yet unknown structural proteins that anchor the compartment from the cytosolic side. Albeit there exists also evidence that MCC domains still persist in protoplasted cells, an anchoring at the cell wall cannot be completely excluded, because patch mobility could not be measured over longer times. Interestingly, in the amino acid sequences of Sur7 and in that of two highly related proteins, as well as in the sequence of Nce102 typical sequons for N-glycosylation can be identified. However, further analyses indicated that no N-glycosylation occurs, and that these sites are of no importance for the ability of the proteins to accumulate within MCC (Stahlschmidt, 2007). Alternatively, proteins and cell wall could be linked *via* O-mannosylation, which is the major type of O-glycosylation of serine and threonine residues in yeast (Lehle et al., 2006). This possibility will be first addressed by an analysis of several protein mannosyltransferase (*pmt*) mutants (Strahl-Bolsinger et al., 1993; Immervoll et al., 1995; Lussier et al., 1995). If these mutants exhibit an affected MCC pattern, the target proteins of glycosylation could be then identified by an altered molecular weight.

5.3. What is the detailed structure of MCC?

Due to the novelty of the phenomenon, the main emphasis of the presented studies on MCC was put on visualization of the compartment, especially by confocal laser scanning microscopy. Also in the future, microscopical approaches will be obligatory to investigate membrane compartmentation in living cells. However, better resolution is required to answer many open questions, as for example the real shape of the domains or the connection between MCC and eisosomes.

A number of sub-diffraction-limit imaging method have been developed in the last decade to circumvent Abbe's law[3] of resolution being limited to approximately half the wavelength of the emitted light (Abbe, 1873; Heintzmann and Ficz, 2006). Just recently, it was achieved to resolve 50 – 60 nm small syntaxin clusters in neuroendocrine PC12 cells by stimulated emission depletion (STED) microscopy (Figure 5.3; Sieber et al., 2007). This method makes use of the phenomenon that a fluorescence signal can be depleted by a second ring-shaped, red-shifted laser pulse. Thus, the originally 200 nm wide, blurred signal spot shrinks in diameter. At least in theory, even atomic resolution is possible. So far, an improvement of about ten-fold (20 nm) has been achieved (Donnert et al., 2006). Using an isoSTED "nanoscope", Hell and colleagues were able to create a nearly spherical focal spot of 40-45 nm in diameter (Schmidt et al., 2008). Thereby, they achieved to resolve TOM20 clusters in the outer membrane of mitochondria not only 5-times better in the focal plane but even more than 10-times better in z.

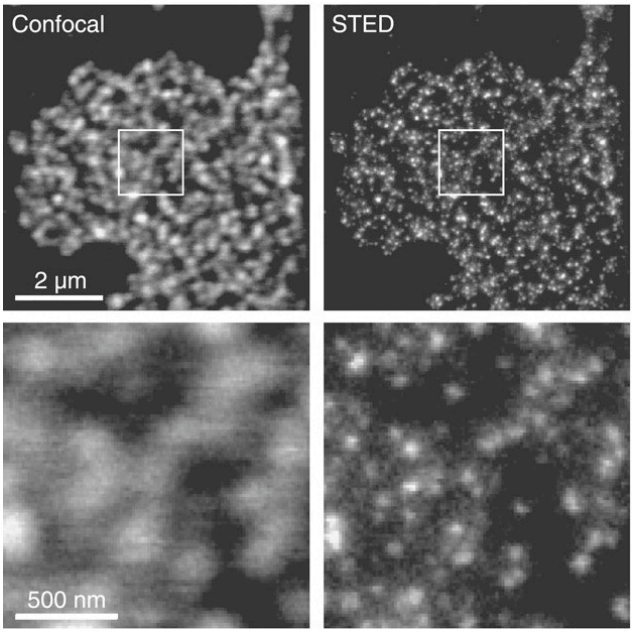

Figure 5.3 Imaging of Syntaxin 1 clusters in PC12 cells by antibodystaining, comparing the results obtained by CLSM (left panels) and STED microscopy. Figure reproduced from Sieber et al., 2007.

The main drawback of STED is that fluorescent proteins or common antibody conjugates cannot be used because special antibody-coupled fluorescent dyes are necessary with both a single very narrow excitation spectrum and an equally well defined emission spectrum. So far, these (Atto-)dyes are also not available in a broad variety, which additionally limits multi-color imaging.

[3] Ernst Abbe (1840 – 1905), German physicist; law of limited resolution: $d = \frac{\lambda}{2NA}$ (d, minimal distance; λ, wavelength of emitted light; NA, numerical aperture of the objective)

Another possibility to improve the resolution for visualization of compartments within and beneath the plasma membrane is structured illumination microscopy (SIM) (Gustafsson, 2000). This wide field microscopy technique avails itself of the Moiré effect, which can be always observed when two equal grids are mutually shifted behind each other. The resulting interference pattern contains information

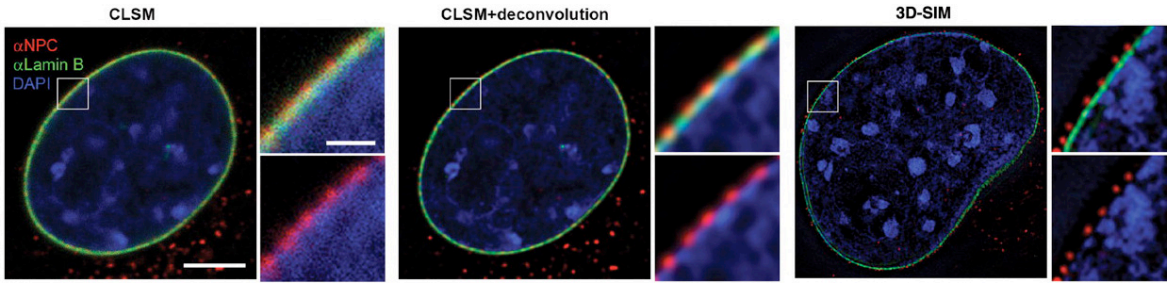

Figure 5.4 Fluorescence micrographs of nuclei in C2C12 cell. The nuclear lamina and noclear pore complexes (NPC) were stained with specific antibodies. DNA was counterstained with DAPI. A comparison of images taken by CLSM (left), after processing by deconvolution (middle), and taken by 3D-SIM (right) is shown. Scale bars: 5 µm and 1 µm (detail). Figure reproduced from Schermelleh et al., 2008.

about the grid structure even if its elements are not directly resolvable. In SIM such interference fringes are created by patterned excitation light and multiple images of the same sample in alternating angles. Although this method allows an enhancement in resolution only by a factor of two, its major advantage to other subdiffraction techniques like STED is the possibility to detect multiple wavelengths, using standard fluorescent dyes. Further optimization of the method for three-dimensional imaging (3D-SIM) accomplished also an enhancement of axial (z) resolution. Recently 3D-SIM provided a detailed visualization of nuclear structures that was not achieved before (Figure 5.4; Schermelleh et al., 2008). Thus it might be also a helpful tool to reveal either an interspace or an interconnection between eisosomes and MCC.

The best resolution results, however, can be achieved by transmission electron microscopy (TEM). New TEM methods already accomplished an atomic, sub-angstrom resolution (O'Keefe, 2008; Smith, 2008). Though, for biological samples that need to be contrasted prior to analysis an efficient resolution of 2 nm is more realistic. A disadvantage of electron microscopy is the requirement for water-free samples. Thus, the sample preparation is prone to produce artifacts, especially when

biological material is chemically fixed. Modern preparation techniques combine high-pressure freezing and freeze-substitution to reach high structure preservation (Sosinsky et al., 2008). Efforts using these intricate and time-consuming but rewarding methods were already made to detect the MCC marker Sur7 by immuno-gold labeling of ultrathin sections, as well as of freeze fracture replicas (Stahlschmidt, 2007). So far, no unambiguous localization data could be obtained but the preliminary results are very encouraging. Being a major task to be resolved in the near future, the ultrastructure of MCC will be addressed in close collaboration with the group of Jan Malínsky (IEM-CAS, Prague).

5.4. What are the molecular mechanisms of the "shelter"?

Besides a detailed image of the MCC architecture, it is also necessary to better understand the molecular mechanisms of its "sheltering" function, and to confirm its general importance. So far, the protective nature of the microdomains is only shown for the arginine permease Can1. A generalization of this effect for other equally distributed transporters requires more experimental evidence. Measuring the turnover of a comprehensive set of plasma membrane proteins, including MCC members and non-members, will provide a broader basis for interpretations.

The demand for mechanistic insights into how endocytosis is actually inhibited will be a prime goal for future investigations. Based on published data of others, a working model of the membrane shelter can already be suggested. The process of endocytosis depends on the Ypk1 pathway (deHart et al., 2002), which is activated by the kinases Pkh1 and Pkh2 (Figure 5.5A; Casamayor et al., 1999). These kinases are activated by long chain (sphingoid) bases (LCB) (Friant et al., 2001). A lack in LCB results in lethality but can be compensated by an overexpression of Ypk1 (Sun et al., 2000). Interestingly, the deletion of *PIL1* upregulates the Pkh and Ypk1 pathways, making the cells independent of LCB biosynthesis (Zhang et al., 2004). Consequently, Pil1 is thought to be a negative regulator of the two pathways and thus also of endocytosis. On the other hand, blocking LCB biosynthesis or the

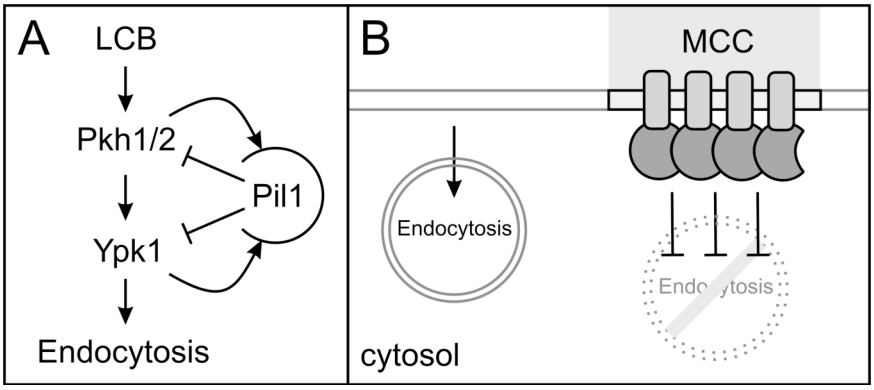

Figure 5.5 (A) The LCB-Pkh1/2 signaling pathway regulates Ypk1, which is important for endocytosis. Pil1 is a negative regulator of both kinases and thus inhibits endocytosis. Conversely both kinases promote clustering of Pil1 within eisosomes. (B) Model for confined regulation of endocytosis. By clustering the negative regulator into microdomains two lateral compartments with different turnover kinetics can exist side by side.

activity of either Pkh kinases or Ypk1 results in the disassembly of eisosomes (Walther et al., 2007; Luo et al., 2008). This indicates that the kinases regulate the assembly of eisosomes and presumably of MCC, as well. For Pkh1/2, it was indeed shown that Pil1 is their phosphorylation target (Walther et al., 2007; Luo et al., 2008).

This interplay of two competing players – Pil1 on one hand and the kinases on the other – could result in a confinement of pathways by a segregation of activators and inhibitors (Figure 5.5B). The compromise could be an area, where endocytosis is efficiently blocked (MCC) being separated from regions, where the internalization can proceed without limitations (MCP). If a transport protein is now supposed to undergo endocytosis, it first has to leave the protective area which indeed is observed for Can1 prior to internalization. This model also explains why the effect of enhanced endocytosis seems to be rather subtle in the compartmentation mutants: the internalization *per se* is not accelerated, only the protein is earlier available for internalization because of a lack of shelter formation.

The fact that the Pkh kinases are structurally and functionally conserved homologs of the mammalian growth factor-regulated serine/threonine kinase PDK1 will eventually help to identify also a homologous structure for MCC in higher eukaryotes. First, more efforts will be undertaken to verify the suggested model in

yeast. However, it is easily conceivable that a stable sub-compartmentation of the plasma membrane into areas of differentiated turnover is a wide-spread feature throughout cellular life.

6 Summary

In the plasma membrane of cells the complex variety of components is sorted into subcompartments, microdomains and nanoclusters. We only begin to understand the principles of this higher order. The heterogeneity of these subunits creates individual microenvironments for various membrane linked processes like substrate transport, signal perception and transduction, or interaction between cells. The yeast *Saccharomyces cerevisiae* is a particularly suitable model organism to investigate plasma membrane compartmentation. In addition to homogenously distributed membrane proteins, two non-overlapping, stable and immobile distribution patterns exist, which can be resolved by light microscopy. This work focuses on composition, formation and stabilization of the spotty membrane compartment of the arginine permease Can1 (MCC) and provides evidence for the biological significance of protein segregation within the plasma membrane.

In adult mother cells, MCC forms 40 to 60 patchy domains that can be studied by tagging MCC protein constituents with fluorescent proteins. In addition to the known members Can1, the protein of unknown function Sur7 and the uracil permease Fur4, eleven further proteins could be localized. From published localization data of other groups additional MCC located proteins could be identified, raising the total number to 21 proteins within or associated with this membrane compartment. Only nine of these proteins are transmembrane proteins, while the other twelve are attached to MCC on the cytosolic side of the plasma membrane. Not only proteins, but also membrane lipids follow this compartmentation. By the finding that Filipin-stained sterols accumulate within MCC as well, an inhomogeneous distribution of lipids in non-polarized cells could be demonstrated for the first time. Consistently, the tryptophan transporter Tat2 was also

localized within MCC when tagged with green fluorescent protein (GFP). For Tat2, a dependence of ergosterol for correct targeting to the plasma membrane had been shown previously. In contrast to the local accumulation of Tat2-GFP, the tagged amino acid permease Gap1-GFP was equally distributed in the plasma membrane under all tested conditions. Gap1 differs from Tat2 in its dependence on sphingolipids for correct conformation, transport activity and stability in the membrane.

According to the potentially lipid-mediated sorting of Tat2, also the ergosterol-binding hexose/H^+-symporter HUP1 from *Chlorella kessleri* could be localized within MCC after tagging with GFP and heterologous expression in *S. cerevisiae*. A detailed analysis of the association of HUP1-GFP with MCC unveiled a high dependence of the patchy distribution on the energization of the plasma membrane, which could be confirmed for all MCC located H^+-symporters. As soon as the membrane potential is uncoupled by an ionophor or an electrical pulse, the transporters dissipate from the compartment. Upon repolarization the transporters again accumulate within MCC. However, this behavior was only observed for H^+-symporters, while no movement was detectable for other transmembrane or associated proteins. Nevertheless, this finding demonstrates a previously unknown function of the membrane potential in the lateral organization of the plasma membrane.

In addition to this physical parameter 28 genes could be identified, that are necessary for a correct MCC formation. Using HUP1-GFP as a marker protein a genome-wide, visual screen was performed, addressing all viable single-deletion mutants. Among the identified genes those were significantly overrepresented that affect lipid metabolism (especially ergosterol biosynthesis) or vesicle-mediated transport. By testing the distribution of further MCC marker proteins as well as filipin-stained sterols a group of six most severely affected mutants could be identified, including the deletion strains *nce102*Δ and *pil1*Δ. Both proteins, Nce102 and Pil1, colocalize with MCC and therefore are regulators of domain formation *in situ*. While Nce102 is an integral membrane protein of unknown function, the soluble Pil1 is one of the main components of eisosomes. This novel structure beneath MCC is suggested to mark sites of active endocytosis. However, own localization studies

using the GFP-tagged endocytic markers Rvs161, Ede1 and Sla2 revealed a clear separation of MCC and sites of active endocytosis. Mutant analyses showed that the lack of Pil1 or Nce102 rather accelerates the substrate-induced degradation of the MCC protein Can1. Prior to internalization Can1 leaves MCC which presumably makes it available for enzymes of the endocytic machinery. In both mutants Can1 is equally distributed from the beginning, thereby shortening the initial steps of internalization. Thus, MCC forms a protective “shelter” within the plasma membrane to regulate the turnover of membrane proteins.

7 Zusammenfassung

In der komplexen Vielfalt von Komponenten der Plasmamembran existiert eine höhere Ordnung aus Subkompartimenten, Mikrodomänen und Nanoclustern, deren Prinzipien wir erst allmählich beginnen zu verstehen. Die Heterogenität solcher Untereinheiten erlaubt die Schaffung von individuellen Milieus für verschiedenste Membran-gebundene Prozesse wie Transportvorgänge, Reizweiterleitung und Zell-Zell-Interaktionen. Um Membrankompartimentierung zu untersuchen eignet sich besonders die Hefe *Saccharomyces cerevisiae* als Modellorganismus. Hier existieren neben gleichmäßig verteilten Proteinen auch zwei nicht einander überlappende, stabile und unbewegliche Proteinverteilungsmuster, die sich lichtmikroskopisch auflösen lassen. Diese Arbeit konzentriert sich auf die Zusammensetzung, Entstehung und Stabilisierung des nach dem ersten in ihm identifizierten Protein benannten "Membrankompartiment der Argininpermease Can1" (MCC) und bietet Hinweise für die biologische Bedeutung dieser Unterteilung der Plasmamembran.

In adulten Mutterzellen bildet MCC 40 bis 60 fleckige Domänen in denen in Co-Lokalisierungsexperimenten neben Can1, dem Protein unbekannter Funktion Sur7 und der Uracilpermease Fur4 elf weitere Proteine lokalisiert werden konnten. Durch zusätzliche Übereinstimmungen mit veröffentlichten Lokalisationsdaten anderer Gruppen steigt die Anzahl der co-lokalisierenden Proteine auf insgesamt 21, wobei nur neun davon Transmembranproteine darstellen. Die übrigen zwölf sind offenbar auf der zytosolischen Seite der Plasmamembran mit MCC assoziiert. Jedoch nicht nur Proteine sondern auch Membranlipide folgen dieser Kompartimentierung. Filipin-gefärbte Sterole sind ebenfalls im MCC akkumuliert, wodurch erstmals eine ungleichmäßige Lipidverteilung in nicht-polarisierten Zellen mikroskopisch

nachgewiesen werden konnte. Passenderweise findet sich unter den MCC-ständigen Proteinen auch der Tryptophantransporter Tat2, für dessen Targeting zur Plasmamembran eine Abhängigkeit von Ergosterol bereits nachgewiesen worden war. Im Gegensatz zu dem fleckig verteilten Sterol-affinen Tat2 ist die Aminosäurepermease Gap1 unter allen getesteten Bedingungen gleichmäßig in der Plasmamembran verteilt. Die korrekte Konformation von Gap1, sowie ihre Transportaktivität und Stabilität in der Membran sind abhängig von Sphingolipiden wodurch sich die Permease von Tat2 unterscheidet.

Entsprechend der möglicherweise lipid-vermittelten Sortierung von Tat2 konnte zusätzlich auch der nachweislich Ergosterol-bindende Hexose/H^+ Symporter HUP1 aus *Chlorella kessleri* nach heterologer Expression im MCC lokalisiert werden. In der Spalthefe *Schizosaccharomyces pombe* wurde die bevorzugte Anhäufung von HUP1 in sterol-reichen Membrankompartimenten ebenfalls beobachtet. Eine eingehende Analyse der MCC-Assoziierung von HUP1-GFP offenbarte eine Abhängigkeit der fleckigen Verteilung von der Energetisierung der Plasmamembran, die sich für alle im MCC befindlichen H^+-Symporter bestätigen ließ. Sobald das Membranpotential der Zellen durch ein Ionophor oder einen elektrischen Puls entkoppelt wird, verteilen sich die Transporter gleichmäßig über die Membran. Eine Repolarisierung der Membran führt zu erneuter Akkumulation innerhalb von MCC. Dieses Verhalten konnte nur bei H^+-Symportern, nicht jedoch bei den übrigen Transmembran- oder assoziierten Proteinen festgestellt werden. Dennoch zeigt sich damit eine bisher unbekannte Bedeutung des Membranpotentials in der lateralen Organisation der Plasmamembran.

Neben diesem physikalischen Parameter wurden in einem genom-weiten, Mikroskopie-basierten Screen unter sämtlichen lebensfähigen und mit dem Marker HUP1-GFP transformierten Einzel-Deletionsmutanten 28 Gene identifiziert, die für eine korrekte MCC-Bildung von Bedeutung sind. Unter allen Mutanten waren vor allem solche überrepräsentiert, die Mutationen in Lipidstoffwechselwegen (insbesondere der Ergosterolbiosynthese) oder Vesikeltransportmechanismen tragen. Nach Überprüfung der Verteilung weiterer MCC-Markerproteine sowie filipin-gefärbter Sterole wurden unter den am stärksten betroffenen Mutanten *nce102*Δ und *pil1*Δ

identifiziert, die näher charakterisiert wurden. Beide Proteine, Nce102 und Pil1, co-lokalisieren mit MCC und beeinflussen damit die Domänenbildung vor Ort. Während Nce102 ein integrales Membranprotein mit noch unbekannter Funktion ist, bildet das lösliche Pil1 eine der Hauptkomponenten der Eisosomen, einer neuartigen Struktur unterhalb von MCC, die mit einer Markierung von Orten aktiver Endozytose in Verbindung gebracht wird. Eigene Lokalisationsstudien mit den Endozytose-Markern Rvs161, Ede1 und Sla2 ergaben jedoch eine deutliche räumliche Trennung von MCC und Orten aktiver Endozytose. Mutantenanalysen ergaben, dass das Fehlen entweder von Pil1 oder von Nce102 den Substrat-induzierten Abbau des MCC proteins Can1 sogar beschleunigt. Can1 wandert dabei in Wildtyp-Zellen vor der Internalisierung aus dem MCC aus und wird vermutlich erst damit für die Enzyme der Endozytosemaschinerie zugänglich. In den beiden Mutanten ist Can1 jedoch bereits gleichmäßig verteilt, wodurch der initiale Schritt der Internalisierung abgekürzt wird. MCC bildet daher eine “Schutzzone” zur Regulation des Turnovers von Membranproteinen.

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Abbreviations

ATP	adenosine-5′-triphosphate
CLSM	confocal laser scanning microscopy
DAG	diacyl-glycerol
DHS	dihydrosphingosine;
DRM	detergent resitant membrane
FCCP	carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
GFP	green fluorescent protein
LCB	long chain base
l_d	liquid disordered
l_o	liquid ordered
MCC	membrane compartment of Can1
MCP	membrane compartment of Pma1
mRFP	monomeric red fluorescent protein
PA	phosphatidic acid
PHS	phytosphingosine
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PS	phosphatidylserine
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIM	structrured illumination microscopy
SL	sphingolipids
s_o	solid ordered
SM	sphingomyelin
STED	stimulated emission depletion (microscopy)
TAP	tandem affinity purification
TEM	transmission electron microscopy

Eidesstattliche Erklärung

Ich erkläre hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet. Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Guido Großmann Regensburg, den 15. Juli 2008