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Isolation of caveolae using affinity two-phase partitioning

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Department of Biochemistry
Lund University
Sweden
2004



Akademisk avhandling

som för avläggande av filosofie doktorsexamen vid Naturvetenskapliga fakulteten vid Lunds universitet offentligt kommer att försvaras i hörsal B, Kemicentrum, Getingevägen 60, fredagen den 27e februari 2004, kl. 10:30.

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Electron micrographs on front cover from left to right:

- ✿ Caveolae from rat lung plasma membranes purified by affinity two-phase partitioning (paper I)
- ✿ Caveolae from rat liver basolateral plasma membrane region, obtained by immunoaffinity two-phase partitioning (paper II)
- ✿ Caveolae from rat liver apical plasma membrane region, obtained by immunoaffinity two-phase partitioning (paper III)

Electron micrographs on back cover from left to right:

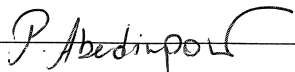
- ✿ None-caveolar high-density fraction from rat liver basolateral plasma membrane region (paper III)
- ✿ None-caveolar high-density fraction from rat liver apical plasma membrane region (paper III)

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Abstract <p>The intention of this work was to establish alternative purification methods to obtain highly purified caveolae from various tissues. In order to isolate caveolae, sufficiently pure plasma membranes are needed. A method is presented for the isolation of plasma membranes from lung tissue using a combination of conventional polyethylene glycol/dextran two-phase partitioning and affinity partitioning using the lectin wheat germ agglutinin as affinity ligand. A caveolae-enriched fraction was purified from lung plasma membranes isolated by this procedure. Caveolae were released from the membranes by Triton X-100 treatment or by sonication. Highly purified caveolae were obtained at low buoyant density by sucrose gradient centrifugation. The affinity method is advantageous to other methods for the isolation of plasma membranes and should be useful for other tissues as well.</p> <p>A method to purify caveolae by immunoaffinity partitioning was developed exploiting the interaction between caveolin and anti-caveolin antibodies. A sandwich approach was used where primary antibodies directed against caveolin interacted with biotinylated secondary antibodies and NeutrAvidin coupled to dextran in a polyethylene glycol/dextran two-phase system. Caveolae were directed efficiently into the immunoaffinity bottom phase of the two-phase system by the anti-caveolin antibody. Immunoaffinity two-phase partitioning has wider applications potentially, as this technique should be useful to purify any type of membrane by selecting appropriate antibodies directed against surface components of the membrane of interest.</p> <p>Caveolae were isolated to a high degree of purity from apical and basolateral domains of liver plasma membranes. The caveolae from the two domains were quite homogeneous as studied by immunoaffinity partitioning and electron microscopy. Analysis showed that the caveolae differed in properties in several respects.</p>			
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Contents

LIST OF PUBLICATIONS	2
1. INTRODUCTION.....	3
1.1 Membrane structure and organization.....	5
1.2 Caveolae and lipid rafts - structure and function.....	7
2. ISOLATION OF PLASMA MEMBRANES.....	14
2.1 Centrifugation.....	15
2.2 Two-phase partitioning.....	16
2.3 Affinity two-phase partitioning.....	19
3. ISOLATION OF CAVEOLAE.....	21
4. THE PRESENT INVESTIGATIONS.....	24
4.1 Isolation of a caveolae-enriched fraction from rat lung plasma membrane purified by affinity two-phase partitioning (paper I).....	25
4.2 Purification of caveolae using immunoaffinity two-phase partitioning (paper II).	28
4.3 Separation of caveolae from apical and basolateral domains (paper III)	33
5. CONCLUSIONS AND FUTURE PERSPECTIVES.....	37
6. POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA.....	39
7. ACKNOWLEDGEMENTS	40
8. REFERENCES	42

List of publications

This thesis is based on the following papers, referred to in the text by their Roman numerals.

I. Isolation of a caveolae-enriched fraction from rat lung by affinity partitioning and sucrose gradient centrifugation¹

Parisa Abedinpour and Bengt Jergil

Analytical Biochemistry 313 (2003) 1-8

II. Purification of caveolae by affinity two-phase partitioning using biotinylated antibodies and NeutrAvidin-dextran

Irene Barinaga Rementeria Ramírez, Parisa Abedinpour and Bengt Jergil

Submitted to Analytical Biochemistry

III. Caveolae isolated from the apical and basolateral domain of rat liver differ in properties

Parisa Abedinpour and Bengt Jergil

Manuscript

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Publications not included in this thesis

Alveolar liquid clearance in anaesthetized, ventilated guinea pigs

Andreas Norlin, Neelu Finley, Parisa Abedinpour and Hans G. Folkesson
Am. J. Physiol. 274 (1998) L235-L243

Dexamethasone and thyroid hormone pretreatment upregulate alveolar epithelial fluid clearance in adult rats

Hans G. Folkesson Andreas Norlin, Yibing Wang, Parisa Abedinpour and Mickael Matthay
J. Applied Physiol. 88 (2000) 416-424

1. Introduction

The pioneer anatomist Vesalius showed the relation between structure and function in animals in 1442. He claimed that the excitation of nerves was the cause of which the vocal cord of pigs became activated (Kramer et al. 1979). Human curiosity soon led to the wish to “see inside” the organisms, which was achieved by the dissection of carcasses. As a result of the invention of light microscopy, Hooke 1665 observed in small box-like compartments in cork and used the word “cells”. Nearly 200 years later, in 1840, Schwann discovered that “the elementary parts of tissues are cells, similar in general but diverse in form and function” (Harrison et al. 1980).

The plasma membrane surrounds all living cells separating components of vital metabolic processes from the external milieu. Eucaryotic cells contain a variety of organelles, which are also enclosed by membranes. The basic structure of the plasma membrane and intracellular membranes is similar but there are many molecular differences as well. There is a direct flow of communication between adjacent cells and also with the basal lamina. Information between distant cells is mediated by a variety of chemical agents like neurotransmitters and hormones. These agents regulate an enormous range of biochemical processes (Harrison and Lunt 1980; Jain et al. 1980) by binding to surface receptors and raising a cascade of reactions inside the cell. Additionally, the plasma membrane contains sites for inward/outward transport mechanisms like passive and active transport, and also mechanisms for vesicular transport such as endocytosis, exocytosis (Jain and Wagner 1980; Albert et al. 2002), or transcytosis (Anderson 1998; Schnitzer 2001). The plasma membrane is a complex structure, encompassing specialized regions of different functions. In polarized cells, the plasma membrane is divided into the apical and basolateral regions, which show a structural and functional polarity. Each region may also have specialized compartments including coated pits, caveolae and lipid rafts. Coated pits have functions for molecular transport, while caveolae and lipid rafts are believed to partition in transport and signal transduction.

Knowledge about plasma membrane structure and function keeps growing, but still many aspects are left to explain. In order to study the plasma membrane and its different compartments, development of rapid and specific purification methods is needed. There are benefits to study the plasma membrane *in vivo* and *in situ* since no membrane isolation methods are required. As a complement to *in vivo* studies it would be beneficial to perform *in vitro* studies to investigate mechanisms of the plasma membrane regions and its compartments in detail.

This thesis is focused on finding new strategies for the isolation of plasma membrane compartments such as caveolae. The plasma membrane along with their compartments constitutes only a minor part of the total membrane contents in the cell. Therefore, no one method would be specific enough to isolate the plasma membrane or its compartments in a one-step procedure. Thus, a combination of various methods is required to obtain highly purified fractions. In this thesis caveolae-enriched fractions have been isolated from rat liver and lung. In both cases plasma membranes were first isolated by different methods. In paper I caveolae were obtained after the prior isolation of plasma membranes from rat lungs by affinity two-phase partitioning. The affinity technique was faster and less invasive to membrane components compared with the cationized silica method used by other groups and resulted in caveolae of a similar purity as other established methods (Schnitzer et al. 1995). In paper II a new immunoaffinity two-phase partitioning method was developed for isolation of caveolae. The method is based on a sandwich technique utilizing primary and secondary antibodies. Primary antibodies are directed against the caveolar protein caveolin, while biotinylated secondary antibodies interacted with one of the polymers of the two-phase system through avidin. In this manner caveolae were efficiently separated from non-caveolar material. This is a new approach for the isolation of membrane structures, and the method should be useful for immunoaffinity purification also of other types of membranes by using other specific primary antibodies. In paper III plasma

membranes were isolated from the apical and basolateral regions of rat liver and caveolae-enriched fractions were then isolated from each region. Caveolae were further separated from non-caveolar material using immunoaffinity two-phase partitioning. The latter material may largely represent lipid rafts.

1.1 Membrane structure and organization

Cell survival is crucially dependent on communication with the external environment, and, in that sense, makes the plasma membrane a particularly important organelle. All biological membranes are composed of proteins and lipids in a bilayer structure. In addition, plasma membranes are particularly rich in carbohydrates present as components in glycoproteins and glycolipids (Harrison and Lunt 1980). The two leaflets of the bilayer differ both in lipid composition and in protein contents (Jain et al. 1977; Simons et al. 1988; Silvius et al. 1996). The carbohydrate moieties are present at the outer leaflet facing the surrounding aqueous medium, while the inner leaflet is associated with the underlying actin-cytoskeleton by attachment through transmembrane proteins.

The view of membrane organization, including the plasma membrane, has developed during the past 100 years. In the late 19th century Overton discovered that the rate of molecular permeation was directly related to the lipid solubility of a molecule and inversely related to its size (Branton et al. 1984). He proposed that a membrane essentially consists of lipids. Gorter and Grendel found that extracted lipids from erythrocyte membranes when spread as a monolayer occupied approximately twice the total surface area of intact erythrocytes (Gorter et al. 1925). They suggested that the membrane consisted of a lipid bilayer with hydrocarbon chains in its center and with the polar head groups facing the aqueous surroundings. In 1935 Danielli and Davson proposed a sandwich model of lipids arranged in a bilayer covered on both sides of proteins (Danielli et al. 1935). In a later version of the model Danielli suggested that protein-lined pores were spanning the membrane (Danielli 1975). As

late as 1966 Robertson proposed that all membranes are constructed in a similar way as a “unit membrane” (Robertson 1966). This was based on the “trilaminar appearance” of membranes by electron microscopy with two darker outer lines and a lighter inner region. According to the unit membrane model the darker lines represent protein layers and the inner region the lipid bilayer. However, it was clear at the time from various kinds of biochemical evidence that the Danielli-Davson-Robertson model was unsatisfactory and that a new model was required.

In 1972 Singer and Nicolson suggested a more dynamic membrane structure, the “fluid mosaic” model (Singer et al. 1972). They viewed biomembranes essentially as a two-dimensional matrix consisting of a phospholipid bilayer interspaced by proteins floating in this “sea” of lipids. In such a structure the polar groups of lipids and proteins are in contact with the surrounding aqueous medium, while the non-polar residues are facing inwards. The components of the membrane matrix are held together largely by noncovalent forces. In the fluid mosaic model, lipids and proteins of the membrane are supposed to be able to move freely and laterally in the lipid plane.

A further development came in 1993 when Kusumi et al. working on the erythrocyte membrane (Kusumi et al. 1993; Jacobson et al. 1995) proposed the “membrane-skeleton fence” model. They suggested that the membrane was connected through membrane-spanning proteins to the spectrin-like cytoskeleton facing the cytoplasm. The lateral diffusion of proteins in the membrane was thereby limited. In 2002 Fujiwara et al. confirmed and elaborated this model using computer simulations and calculations of diffusion rates of single molecules such as phospholipids in a membrane (Fujiwara et al. 2002). They postulated that the lateral diffusion of phospholipids and transmembrane protein in the plasma membrane proteins depends on the actin-based membrane skeleton (Fig. 1A). Transmembrane proteins

and phospholipids interacting with the actin-based cytoskeleton act as rows of pickets resulting in a limited lateral mobility of membrane components.

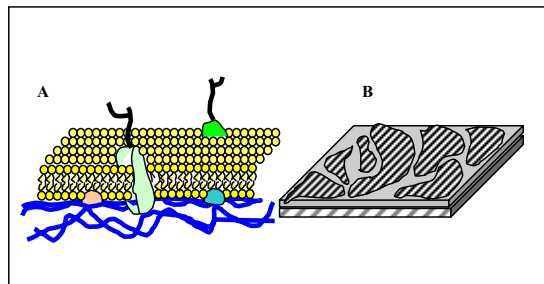


Figure 1. Model structures of plasma membranes. A) Illustrates "the membrane picket fence model" (Kusumi et al. 1993; Fujiwara et al. 2002). The cytoskeleton coupled to proteins on the cytoplasmic surface of the lipid bilayer influences the mobility of components in the membrane. B) The organized and disorganized regions are viewed as plates, each having characteristic properties. The composition of proteins and lipids differs in the bilayer leaflets.

Another aspect of the plasma membrane structure, based on the dynamic clustering of organized and disorganized regions to form patches, was proposed in 1977 (Jain and White 1977) and further developed by Simons et al. (Simons et al. 1997). They suggested that membrane lipids are not randomly distributed in the lipid bilayer, and that organized lipid molecules called lipid rafts, form discrete compartments moving around in the relatively disorganized lipid bilayer (Fig. 1B). The organized and disorganized regions are considered as plates, each having characteristic molecular properties (Simons and Ikonen 1997; Simons et al. 2002). In the liquid ordered state, phospholipids with saturated hydrocarbon chains along with glycosphingolipids pack tightly with cholesterol. In contrast, the liquid disordered phase has a high concentration of phospholipids with unsaturated acyl chains, which together with cholesterol provides a more fluid phase.

1.2 Caveolae and lipid rafts - structure and function

Caveolae are flask shaped invaginations of the plasma membrane of many mammalian cells, which were first observed by electron microscopy of endothelial

and epithelial cells in the 1950s (Palade 1953; Yamada 1955). They were named “small caves” or caveolae by Yamada (Yamada 1955). Caveolae have distinct form and are easily observed by electron microscopy, while the structure of lipid rafts is more elusive (Anderson et al. 2002; Munro 2003). The lipid composition of caveolae and lipid rafts is supposed to be similar making both these membrane compartments resistant to detergent treatment at low temperature (Brown et al. 1998; Galbiati et al. 2001). It is believed that lipid rafts are present in all cells (Simons and Ikonen 1997; Simons and Ehehalt 2002), while caveolae are lacking in some cells such as erythrocytes, platelets, lymphocytes and neuroblastoma cells (Fra et al. 1994; Gorodinsky et al. 1995). It is unclear whether these membrane compartments are related. The exact function of them is not entirely clear (Stan 2002), but caveolae are likely to be involved in endocytosis and signal transduction, while lipid rafts may be involved in signal transduction (Simons and Ehehalt 2002).

Caveolae

Caveolae are characterized as omega shaped “smooth” invaginations from the plasma membrane, 50-100 nm in cross section (Anderson 1998; Fujimoto et al. 1998). They are defined both by morphological observations and biochemical findings. Morphological properties are easy to observe by electron microscopy, while biochemical studies have required the establishment of purification techniques. The observations show that caveolae appear not only as invaginations from the plasma membrane but also as free vesicles in the cytoplasm. Furthermore, caveolae may associate in groups as grape-like clusters or rosettes, or even appear as elongated channels *in vivo* (Razani et al. 2002; Stan 2002). Caveolae are present in many cell types, but their abundance is highly variable. Thus, caveolae are particularly abundant in adipocytes, endothelial cells (10-20 caveolae/ μm^2) and skeletal muscle cells (70-90 caveolae/ μm^2) (Stan 2002).

Caveolae are characterized by their resistance to non-ionic detergent at low temperature (Anderson 1998; Galbiati et al. 2001; Matveev et al. 2001), and to high salt (Rothberg et al. 1992; Anderson 1993) and high pH (Rothberg et al. 1992; Kurzchalia et al. 1994) conditions. They also have a rather defined size and a low buoyant density (Shaul et al. 1998; Hooper 1999; Stan 2002), and are identified by their content of caveolin. The detergent resistance and low buoyant density of caveolae led to the suggestion that cholesterol and sphingolipids might be abundant in these compartments (Parton 1996; Fujimoto et al. 1997; Razani et al. 2002). The accessibility of glycosphingolipids and sphingomyelin to antibodies and specific enzymes on the cell surface suggested that sphingolipids are primarily situated in the outer noncytoplasmic leaflet of plasma membranes (Liu et al. 1995; Fujimoto 1996; Fujimoto et al. 1997; Holthuis et al. 2001; Stan 2002). The presence of cholesterol in caveolae was supported by electron microscopy observations using cholesterol-binding probes (Rothberg et al. 1992; Fujimoto et al. 1997; Razani et al. 2002). This lipid was detected both in the cytoplasmic and the extracellular leaflet of the lipid bilayer. Caveolin presence in caveolae is sensitive to cholesterol depletion. This was examined by treatment of cells with cholesterol binding agents, such as nystatin, cyclodextrin or filipin, which lead to the flattening of caveolae invaginations (Rothberg et al. 1992; Schnitzer et al. 1994; Stan 2002). Additionally, the treatment of cells with cholesterol oxidase, converting cholesterol to cholesterolone, led to the disappearance of morphologically detectable caveolae. Simultaneously caveolin was transported transiently to the ER before moving to the Golgi (Smart et al. 1994; Razani et al. 2001). It appears that caveolin is important both for membrane invagination and for the vesicular transport of newly synthesized cholesterol from the ER to the cell surface (Uittenbogaard et al. 2000).

The use of caveolin as a marker opened up new possibilities to identify caveolae. An important finding was that caveolin gives caveolae its characteristic shape (Rothberg et al. 1992; Stan 2002). The overall organization of caveolae is best

observed in rapid-freeze, deep-etch images of the inner plasma membrane surface. Using this technique the surface of each caveolae appears as concentric rings staining with antibodies directed against a 21-kDa protein (Rothberg et al. 1992; Anderson 1993; Fujimoto et al. 1998) assumed to be caveolin. Caveolin is a structural protein of caveolae present in several forms (see below). One form, caveolin-1, was discovered as a 21-kDa phosphotyrosine-containing protein in Rous sarcoma virus (v-Src) transformed fibroblasts screened for tyrosine phosphorylation substrates (Glenney et al. 1989; Glenney et al. 1992). Caveolin was also independently cloned as a vesicular integral protein of 21-kDa, a component of trans-Golgi-derived vesicles (Rothberg et al. 1992). As mentioned before, caveolae are lacking in certain cells such as lymphocytes, which do not express caveolin. However, by transfecting them with caveolin-1 morphologically detectable caveolae were generated *de novo* (Fra et al. 1995).

Caveolin-1 is a member of the caveolin gene family, which also includes caveolin-2 and caveolin-3. Caveolin-1 and -2 are distributed with the highest levels in adipocytes, endothelial cells and fibroblasts, while caveolin-3 occurs in cardiac, skeletal and smooth muscle cells (Rothberg et al. 1992; Anderson 1998; Okamoto et al. 1998; Razani et al. 2002). There is relatively high gene homology between caveolin-1 and caveolin-3 leading to the suggestion that these may have similar structural and functional properties. According to the degree of identity, caveolin-2 is the most divergent of the three proteins (Scherer et al. 1996).

Caveolin-1 is a protein of 178 amino-acid residues incorporated in the caveolar membrane (Song et al. 1997; Schlegel et al. 2000). Both the N-terminal (residues 1-101) and the C-terminal (residues 135-178) are exposed on the cytoplasmic side of the membrane (Rothberg et al. 1992; Anderson 1998; Razani et al. 2002). The lipid spanning part, a hydrophobic 32 amino-acid sequence, is not long enough to form a harpin spanning the entire lipid bilayer (Monier et al. 1995) (Fig 2). This was

confirmed by cell surface biotinylation experiments, which did not result in labeling of caveolin-1, indicating the inaccessibility of the protein from the extracellular side (Sargiacomo et al. 1995). Kurzchalia et al. (Kurzchalia et al. 1994) suggested that caveolin-1 is inserted into the membrane co-translationally via the classical ER translocation apparatus. It is believed that caveolin-1 is combined to highly stable homo-oligomers of approximately 14-16 individual caveolin molecules immediately after synthesis in the ER and prior to Golgi transit (Monier et al. 1995). Adjacent homo-oligomers are thought to pack side by side within the caveolae membrane thereby providing a structural basis for membrane invagination (Monier et al. 1995; Schlegel and Lisanti 2000). Caveolin-1 and -2 can interact with each other forming stable hetero-oligomers, but caveolin-2 is not capable of forming large homo-oligomers in the absence of caveolin-1 (Scherer et al. 1997) and cannot form membrane invaginations alone.

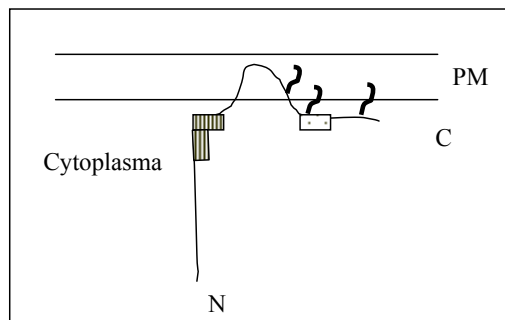


Figure 2. The topology of caveolin-1 in the plasma membrane (PM) shows that caveolin has a loop-shaped membrane domain. Caveolin-1 exists either as a homo-oligomer or as a hetero-oligomer with caveolin-2. The homo-oligomerisation is mediated by residues 81-101, and is known as N-MAD (hashed black box). It is believed that caveolin-1 is tightly associated via N-MAD and C-MAD (white box) to the plasma membrane. The curved short bold dark lines show the sites of acylation close to the C-terminal end.

The interest in caveolae and caveolin has increased over the past several years and caveolae were thought to be essential to life. However, knockout experiments in animal models becoming deficient in caveolin-1, -2 or -3, showed that these animals were alive and fertile (Drab et al. 2001; Galbiati et al. 2001; Parton 2001; Razani et

al. 2001; Park et al. 2002; Razani et al. 2002). Although these animals did not have any phenotypic abnormalities, various tissue abnormalities were seen (Parton 2001; Razani et al. 2002). For instance, lung abnormalities such as pulmonary fibrosis was observed in caveolin-1 deficient mice, and in adipose tissues effects like resistance to diet-induced obesity were detected (Razani et al. 2001; Razani et al. 2002).

The function of caveolae has been of interest since they were first observed in 1955. They have been implicated in vesicular transport such as transcytosis and endocytosis and in signal transduction (Kamen et al. 1991; Anderson 1993; Anderson 1998; Gumbleton 2001; Razani and Lisanti 2001; Razani et al. 2002). One of the first functions proposed for caveolae was the transcytotic transport of proteins across the capillary endothelial cell (Schnitzer et al. 1994; Predescu et al. 1997; Schnitzer 2001). While endocytosis has been associated primarily with clathrin-coated vesicles it is clear that some extracellular macromolecules such as cholera and tetanus toxins can be internalised by caveolae instead (Montesano et al. 1982; Razani and Lisanti 2001). In addition, albumin is endocytosed by caveolae, a process inhibited by treating endothelial cells with cholesterol-binding agents, but not affecting clathrin-dependent uptake of other proteins (Schnitzer et al. 1994; Gumbleton 2001). Also larger structures such as Simian virus 40 and certain strains of *Escherichia coli* appear to be internalised via caveolae. This happens through the coalescence of several caveolae into larger endocytotic compartments (Shin et al. 2000; Pfeffer 2001).

The function of caveolae in signal transduction is based on the fact that many acylated proteins have a tendency to be attracted by cholesterol. These proteins are inhibited when they come into contact with caveolin (Schnitzer et al. 1994; Razani and Lisanti 2001; Sowa et al. 2001). Many signaling molecules, such as heterotrimeric G proteins and the Src family tyrosine kinases, may become acylated (Parton et al. 1995) either through direct acylation or through the interaction of

glycosylphosphatidylinositol anchors (GPI-anchors) (Brown and London 1998). This tends to compartmentalize them in caveolae. The caveolae signaling hypothesis (Razani and Lisanti 2001) suggests that caveolae act as a platform for signaling proteins facilitating the cross-talk between different signaling pathways including PKC α (Lisanti et al. 1994), Src (Li et al. 1996), G-protein coupled receptors (Dupree et al. 1993; Rybin et al. 2000) and insulin receptors (Yamamoto et al. 1998; Gustavsson et al. 1999; Kurzchalia et al. 1999).

Lipid rafts

Lipid rafts are membrane structures that appear to be widespread in many kinds of cells (Simons and Ikonen 1997; Simons and Ehehalt 2002). In polarized epithelial cells lipid rafts appear to be more prevalent in the apical region than in the basolateral region (van Meer et al. 1988; Simons et al. 2000). As lipid rafts are likely to have a similar lipid composition as caveolae (Galbiati et al. 2001; Anderson and Jacobson 2002) they are resistant to Triton X-100 treatment and focus at a low buoyant density on sucrose gradient centrifugation (Simons and Ikonen 1997; Galbiati et al. 2001; Simons and Ehehalt 2002). Even if lipid rafts are difficult to observe, they may be detected by their content of GPI-anchored proteins (Anderson 1998; Brown and London 1998; Kurzchalia and Parton 1999; Razani et al. 2002). They have been visualized by antibody cross-linking of raft components (Harder et al. 1998; Simons and Toomre 2000). Lipid rafts have a limited size of up to 50 nm in cross section, thus containing only a few thousand lipids and rather few proteins, but after cross-linking they increase in size to 100-1000 nm (Harder et al. 1998; Abrami et al. 2001). Rafts may also be detected using biophysical methods such as fluorescence electron microscopy, and fluorescence resonance energy transfer technique (FRET) (Varma et al. 1998), photonic force microscopy (Pralle et al. 2000), and single fluorophore tracking microscopy (Schutz et al. 2000). These methods monitor the diffusion and dynamics of proteins and lipids in the plasma membrane.

According to the lipid raft theory (Simons and Ikonen 1997) assemblies of sphingolipids and cholesterol provide the basis for an association of signaling components to lipid rafts. Thus rafts are platforms with a high concentration of signaling molecules such as GPI-anchored 5'-nucleotidase (Varma and Mayor 1998), Src, α subunits of heterotrimeric G proteins, certain cytoskeletal proteins, and eNOS (Simons and Ikonen 1997; Varma and Mayor 1998; Simons and Ehehalt 2002). As several of these signaling molecules also have been reported to reside in caveolae, the distribution of them between these compartments seems unclear presently. One problem is the uncertain relationship between caveolae and lipid rafts (Kurzchalia and Parton 1999; Abrami et al. 2001; Anderson and Jacobson 2002; Simons and Ehehalt 2002; Lai 2003). A current hypothesis is that lipid rafts represent a precursor of caveolae and play a central role in many cellular events including membrane sorting, cell polarization and signal transduction processes (Simons and Ehehalt 2002). Caveolae may primarily serve in vesicular transport and to accumulate and down regulate raft proteins (Janes et al. 2000; Cherukuri et al. 2001; Simons and Ehehalt 2002; Dykstra et al. 2003). This hypothesis would explain the presence of the same proteins in lipid rafts and caveolae.

2. Isolation of plasma membranes

Plasma membranes, or regions of the plasma membrane, constitute a minor part of the total membranes in a cell homogenate. The conventional procedure for isolation of plasma membranes consists of a combination of centrifugation techniques based on particle size and density. An alternative is partitioning in aqueous two-phase systems, where fractionation is based on surface properties of the material (Albertsson 1986). Affinity two-phase partitioning offers a further possibility in which biospecific affinity ligands are utilized to increase the selectivity (Persson et al. 1995).

2.1 Centrifugation

Centrifugation is the most common procedure used for the fractionation of subcellular membranes. There are two general approaches for such a fractionation, differential centrifugation separating particles mainly according to their size, and density gradient centrifugation separating primarily on the basis of buoyant density. Differential centrifugation is the most widely used fractionation technique, particularly as a preliminary step. In a mammalian tissue homogenate the low speed so called nuclear pellet, consisting mainly of nuclei and unbroken tissue, is utilized for isolation of the apical region of rat liver plasma membranes. Centrifugation at high speed (100000 g) sediments the microsomal fraction containing mainly ER, plasma membranes and Golgi membranes and serves for isolation of the basolateral region (Aronson et al. 1974).

In density gradient centrifugation the material is focused in the gradient where its density equals that of the gradient medium. The most widely used gradient medium for fractionation of membranes is sucrose, as it is easily available to a low cost (Gennis 1989). Other commercially available density gradient media are Ficoll, Percoll and non-ionic iodinated compounds like Nycodenz and OptiPrep. Differential and density gradient centrifugations are often combined to isolate plasma membranes and to separate them from intracellular components. Although the size of membrane particles often depends on parameters such as homogenisation procedure and ionic strength (Rickwood 1983), it is clear from Fig.3 that there are limitations to the degree of separation attainable by centrifugation methods. Thus plasma membrane fragments may overlap particularly with Golgi membranes and ER on differential centrifugation, but are partly resolved from these membranes by density gradient centrifugation.

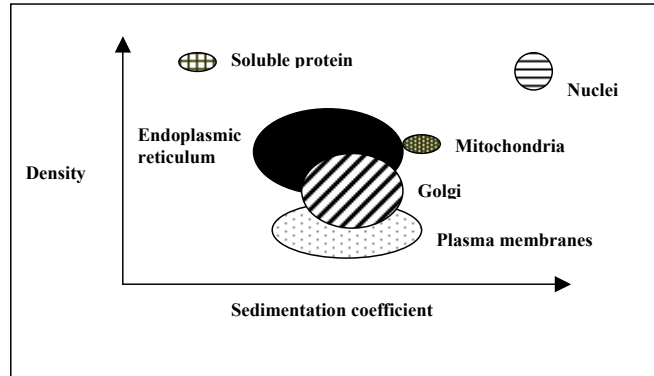


Figure 3, Sedimentation coefficient and density of various subcellular fractions. This plot shows the limitation of centrifugation methods in the isolation of the membranes. Adapted from (Mircheff 1983; Gennis 1989).

Since there are difficulties to separate plasma membranes from other membranes by centrifugation alone, it is useful to combine centrifugation with other techniques based on other separation parameters.

2.2 Two-phase partitioning

The separation of biological material by partitioning in aqueous two-phase systems was originally developed by Albertsson (Albertsson 1986). The partitioning behavior of the material is based on surface properties. Aqueous two-phase systems are obtained by mixing solutions of two immiscible water-soluble polymers above a certain concentration. The mixture spontaneously separates into two liquid phases, each phase being enriched in one of the polymers. The phases have a high water content (80-95%) and salts and buffers can be added to give a desired ionic strength and pH (Albertsson 1986; Albertsson 1989).

The two-phase system is described by a phase diagram (Fig. 4), where the concentration of polymer **P** is plotted as abscissa and the concentration of polymer **S** as ordinate. The curved binodial line separates two areas; the area above the binodial, for instance point **I**, represents phase separation, while that below, like

point **R**, represents a one-phase system. In a system with the total polymer composition **I**, point **A** describes the composition of the top phase and **B** that of the bottom phase. Pairs of points like **A** and **B** are called *nodes* and they are joined by the *tie line* (Albertsson 1986). Any total polymer composition represented by points on the same tie line will result in phase systems with the same phase composition, but with different volumes of the two phases (Albertsson 1986). The volumes are given by the distance **IA** (top phase) and **IB** (bottom phase). The partition of a substance is usually described by the partition coefficient **K**, which is the ratio of the concentration of the substance in the top phase (C_t) over that in the bottom phase (C_b).

$$K = C_t / C_b$$

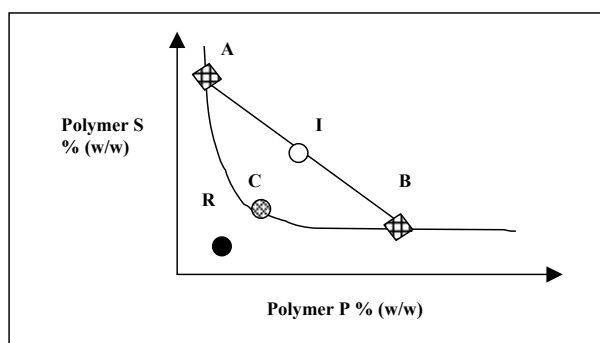


Figure 4. Phase diagram of polymers P and S. Polymer mixture **I** and other mixtures above the binodal give rise to two phases, while mixture **R** and other mixtures below the binodal represent one phase systems. Point **A** describes the composition of the top phase of a system with the total composition **I**, while point **B** describes the composition of the bottom phase. **C** is the critical point between a one-phase and a two-phase system.

The most commonly used two-phase polymers for separation of biological material are polyethylene glycol (PEG) and dextran. PEG is an unbranched polymer of ethylene oxide units available in a wide range of molecular weights (Albertsson 1986; Kroner 1986), while dextran is a polysaccharide consisting of glucose units also having a wide molecular weight range (from 10,000 to 2,000,000) (Kroner 1986). In a PEG/dextran two-phase system PEG is enriched in the top phase and dextran

in the bottom phase. There are a number of other two-phase systems such as polymer/polymer, polymer/salt or polymer/detergent systems (Hustedt et al. 1982; Piculell et al. 1992; Persson et al. 1999). Many of these systems are not suitable for purification of membranes.

Biological material, especially membranes, often partition between the interface and one of the phases. Basically, membranes tend to partition in the PEG-rich phase due to its more hydrophobic character in a PEG/dextran two-phase system. Many other factors, however, modulate the partitioning including the concentration and molecular weight of the phase polymers and also the presence of salts (Albertsson 1986; Kroner 1986; Albertsson 1989). An increase in polymer concentration in a PEG/dextran system will favour membranes to partition in the interface or in the lower phase (Albertsson 1989). Also a decrease in the molecular weight of one of the phase polymers, tend to increase the partitioning of membranes and particles into that phase. The partitioning behaviour of membranes is particularly sensitive to the presence of salts, as salts create an electrostatic potential across the interface. Positively charged ions cause the particles to favour the upper phase in the order $\text{Cs}^+ \sim \text{K}^+ < \text{Na}^+ < \text{Li}^+$, while negatively charged ions have the same effect in the order $\text{Cl}^- \cong \text{H}_2\text{PO}_4^- < \text{HPO}_4^{2-} \cong \text{SO}_4^{2-} \cong \text{citrate}$ (Johansson 1970; Albertsson 1989). As cells and organelles usually have a negative surface charge, Li_2SO_4 would be efficient to distribute them maximally in the top phase. Borate has also been useful to partition membranes into the PEG-rich top phase (Persson et al. 1991; Ekblad et al. 2001). To avoid too much membranes collecting in the interface polymer concentrations should be kept close to the critical point (Persson et al. 1991; Persson et al. 1992). A change in temperature causes relatively large changes in the systems near the critical point. This makes temperature control during partitioning important (Albertsson 1986).

Aqueous two-phase partitioning has been used for separation of macromolecules, membranes and cells (Albertsson 1958; Albertsson 1989; Persson and Jergil 1995). In particular, it has been used for the purification of cellular organelles and membranes from plants (Larsson 1983; Albertsson 1989), and has become the method of choice for isolation of plant plasma membranes (Larsson et al. 1987). In comparison, there are relatively few studies on animal membranes. Two-phase partitioning has not turned out to be selective enough for extensive purification of animal plasma membranes, although the method is useful for a crude fractionation (Persson and Jergil 1992). It has been possible, however, to subfractionate isolated plasma membranes as well as ER by the technique using counter-current distribution (Gierow et al. 1986).

2.3 Affinity two-phase partitioning

To increase the selectivity of the two-phase partitioning technique, specific ligands can be introduced to one of the phase polymers to affinity partition the material of interest. Affinity partitioning is designed to achieve efficient fractionation of the desired material by a single extraction step, or perhaps including one or a few re-extractions, thus avoiding counter-current distribution common in conventional two-phase partitioning. High yield, fast isolation procedure and the ease of scaling up the method are additional benefits of affinity partitioning (Persson and Jergil 1995; Ekblad and Jergil 2001; Abedinpour et al. 2003). Affinity partitioning was originally introduced by Flanagan for isolation of proteins and membranes (Flanagan et al. 1975; Flanagan et al. 1975). A variety of molecules can be coupled to either of the phase polymers. These include antibodies, lectins and receptor agonists with a specific affinity to proteins or carbohydrates present on the surface of membranes (Johansson 1970; Kroner 1986; Persson and Jergil 1995). Dye ligands have also been used in a few cases (Muino Blanco et al. 1986). Generally, affinity partitioning has been restricted to PEG/dextran systems, and ligands can be coupled to either of the phase polymers (Flanagan 1985; Kroner 1986).

Membranes represented by postsynaptic specializations in muscles contain a high density of nicotinic acetylcholine receptors. These have been affinity partitioned by using trimethylammonium coupled to PEG (Johansson 1970; Flanagan et al. 1975; Flanagan et al. 1976). The same PEG-ligand/dextran affinity system has also been applied to partition synaptic membranes from bovine brain containing opiate receptors (Olde et al. 1985). Immunoaffinity partitioning in two-phase systems was first attempted by isolating red blood cells from a blood cell mixture using a primary antibody against a red blood cell surface antigen coupled directly to PEG (Karr et al. 1986; Sharp et al. 1986; Delgado et al. 1991). Another approach was when the Fc region of a secondary antibody was coupled to PEG and used to attract erythrocytes previously incubated with the primary antibody (Karr et al. 1988; Stocks et al. 1988).

Ligands coupled to dextran rather than PEG have not been utilized to a great extent in affinity two-phase partitioning. This might be due to the more complex coupling chemistry required for dextran. Biospecific ligands used include ATP to purify plant chloroplast phosphoglycerate kinase (Persson et al. 1988) and procion yellow to partition synaptic membrane domains (Muino Blanco et al. 1986). A particularly useful ligand has been the lectin wheat-germ agglutinin (WGA). WGA binds N-acetylglucosamine and sialic acid residues on the extracellular face of the plasma membrane efficiently separating these membranes from intracellular membranes (Persson et al. 1991; Persson and Jergil 1992; Ekblad and Jergil 2001). As a conventional PEG/dextran two-phase system is useful for a crude fractionation, this system can be combined with WGA-dextran affinity partitioning (Fig. 5) to isolate highly purified plasma membranes from a rat liver homogenate (Persson and Jergil 1992; Persson and Jergil 1995). Using this same approach it was possible to isolate plasma membranes from rat lung (paper II).

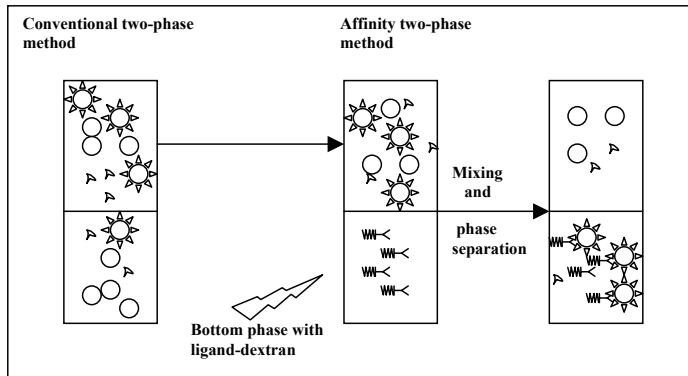


Figure 5. Affinity partitioning of membranes enriched in specific binding sites by utilization of ligands attached to dextran. A phase system is chosen so that all membranes partition in the top phase in a system containing un-substituted dextran. Binding to the ligand mediates a specific transfer of membranes into the ligand-dextran containing bottom phase.

Although WGA-dextran is an efficient ligand for the isolation of plasma membranes, it has not been possible to use other ligands, for instance insulin, for affinity partitioning (Persson and Jergil 1995). To examine conditions required for affinity partitioning of membranes, model experiments utilizing biotinylated liposomes and NeutrAvidin-dextran were therefore conducted (Ekblad et al. 1998; Barinaga-Rementería Ramírez et al. 2000). The experience gained from the model experiments was used to develop immunoaffinity partitioning as a membrane preparation method where caveolae was separated (paper II).

3. Isolation of caveolae

Despite a large number of articles on caveolae and lipid rafts, few have focused on the separation of these compartments. Methods for the isolation of caveolae rely on the fact that caveolae first are detached from isolated plasma membranes and then purified by density gradient centrifugation. They can be detached either by Triton X-100 treatment, which solubilizes most of the plasma membrane but leaves caveolae and lipid rafts, or by sonication. Sonication is usually performed at high pH in carbonate buffer. Sucrose is the most commonly used gradient medium, and

caveolae are collected at a low buoyant density (Sargiacomo et al. 1993; Schnitzer et al. 1995; Liu et al. 1998). It is also possible to focus caveolae at low buoyant density using OptiPrep as a gradient medium (Smart et al. 1995). In principle, caveolae can either be obtained from isolated plasma membranes or directly from tissue or cell homogenates. In the latter case caveolae are rather impure, as was the case in earlier studies (Sargiacomo et al. 1993; Lisanti et al. 1994) judged from the electron micrographs presented. A fraction highly enriched in caveolae was first isolated from lung endothelial plasma membranes, where the membranes had been purified by a silica coating technique (Jacobson et al. 1992; Schnitzer et al. 1995). The endothelial cell membrane was isolated by perfusion of the rat lung with cationized silica followed by homogenisation of the tissue, filtration, extraction of caveolae and density gradient centrifugation. Subsequently highly purified caveolae have been isolated from plasma membranes of several tissues such as lung (Oh et al. 1999; Stan et al. 1999; Abedinpour and Jergil 2003), smooth muscle (Chang et al. 1994), adipocytes (Gustavsson et al. 1999), and liver (paper III). It is apparent that highly enriched caveolae can be obtained from purified plasma membrane fractions either after detergent treatment or sonication, but not directly from crude homogenates.

The assessment of the purity of caveolae is not straightforward. One approach is to follow the purification process through the enrichment of certain proteins or enzymes, and in particular of caveolin which is a suitable marker for caveolae. This has been done only occasionally (Jacobson et al. 1992). As caveolae are not equally distributed in all tissues and it is not known whether the caveolin content is the same in different caveolae, the specific caveolin content cannot be used to judge the purity of preparation. The best way presently is to examine the purity by electron microscopy and to identify caveolae using labeled antibodies against caveolin.

Caveolae and lipid rafts have similar physical properties whether they are prepared by detergent treatment or by sonication. Since they focus at the same low density

upon gradient centrifugation it is apparent that caveolae might be contaminated by lipid rafts in the low buoyant density fraction. Taking advantage of the most obvious difference between them, i.e. the presence of caveolin, offers the possibility to separate rafts from caveolae by immunoisolation. Immunoisolation protocols are based on the immobilization of antibodies on a solid support such as magnetic beads (Schnitzer et al. 1995; Stan et al. 1997; Oh and Schnitzer 1999; Stan 2002) or through interactions with a non-solid support such as two-phase polymers (paper II). The affinity technique using magnetic beads was used first for isolation of Golgi vesicles (Saucan et al. 1994) and was later modified to isolate “true” caveolae (Schnitzer et al. 1995; Oh and Schnitzer 1999). The separation of caveolae and rafts by this technique has not been shown, however.

One problem when analyzing purified caveolae has been the conflicting results often obtained. This may be due to differences in preparation methods, for instance whether caveolae are detached from plasma membranes by sonication or by detergent treatment. Also subtle differences in the preparation protocol in caveolae preparations have resulted in caveolae preparations with different properties. One example is endothelial caveolae prepared by the silica coating method and immunoisolation using magnetic beads. Several proteins involved in signal transduction such as PKC α , eNOS and α subunits of heterotrimeric G proteins found enriched by one group (Oh and Schnitzer 1999) were not detected by another group (Stan et al. 1997; Stan 2002). One difference between the preparations was the incubation time with the immuno-beads indicating that the preparation protocol is crucial. The cationized silica used in the preparation of endothelial plasma membranes may itself cause relocation of negatively charged membrane proteins (Stan 2002), thereby influencing caveolae composition.

Another factor which may affect the molecular composition of isolated caveolae is the method for detaching caveolae from plasma membranes, i.e. sonication or Triton

X-100 treatment (Kurzychalia et al. 1995; Smart et al. 1995; Oh and Schnitzer 1999; Stan 2002). It is not obvious however, how detergent treatment affects the caveolar membrane composition (Brown and London 1998; Simons and Toomre 2000). Anderson and colleagues compared caveolae prepared by sonication and detergent treatment and found no differences in content of several proteins, heterotrimeric G-proteins being an exception as these were only found in caveolae prepared by sonication (Smart et al. 1995). Other groups have found that receptor proteins present in caveolae prepared after sonication are not present after detergent preparation (Liu et al. 1998; Gustavsson et al. 1999). One conclusion to be drawn is that caveolae obtained by different preparation protocols also differ in composition, at least to a certain extent (Jacobson et al. 1992; Stan et al. 1997; Shaul and Anderson 1998; Stan et al. 1999). Furthermore, it is not known to what extent caveolae from various tissues differ, not only in molecular composition but also in their precise function.

4. The present investigations

The initial aim of this thesis work was to isolate lung caveolae in order to study caveolae in a more applied manner. It had been found that compounds such as thyroid hormone and dexamethasone affect liquid clearance in animal lungs (Folkesson et al. 2000). As caveolae seem to be involved in transcytosis of material in endothelial cells (Schnitzer et al. 1995), they are also likely to be involved in liquid clearance. As no non-invasive purification method yielding highly enriched caveolae existed at the time, we started to develop an alternative method for purification of lung caveolae. Because of several reasons the project was then refocused to find alternative methods for the purification of caveolae also from other tissues.

4.1 Isolation of a caveolae-enriched fraction from rat lung plasma membrane purified by affinity two-phase partitioning (paper I).

In the first paper a new approach for the isolation of caveolae was explored. Lung plasma membranes were purified by a combination of two-phase partitioning and affinity partitioning and were used to isolate a caveolae-enriched fraction. Earlier, plasma membranes from rat liver had been purified by affinity two-phase partitioning in a PEG/WGA-dextran system (Persson et al. 1991) and also in combination with conventional two-phase partitioning (Persson and Jergil 1992). The question was if this approach also was applicable for other tissues. Rat lungs were therefore homogenized in a PEG/dextran two-phase system. After phase separation the PEG-rich top phase was subjected to affinity two-phase partitioning with WGA bound to dextran as a ligand. Plasma membranes were recovered in the affinity bottom phase (I, Tab. 1).

The yield of the plasma membranes by this procedure was high as measured by the plasma membrane marker enzyme, 5'-nucleotidase. The degree of purification was 8-fold with minor contamination of other membranes like ER and lysosomes. However the distribution of caveolin and 5'-nucleotidase during the fractionation did not follow each other in any of the two-phase partitionings. Only 4% of the caveolin present in the homogenate remained in purified plasma membranes. One possible explanation could be that caveolae in rat lungs may participate actively in transcytosis therefore only partly fractionating with plasma membranes. Alternatively caveolae may detach from plasma membranes during the preparation procedure. This seems less likely, however, as homogenisation in an aqueous two-phase polymer mixture and two-phase partitioning are rather gentle procedures. The recovery of caveolin compares favourably with that seen when plasma

membranes are isolated by the silica coating method when less than 1% is recovered (Stan et al. 1997).

Caveolae were detached from plasma membranes either by Triton X-100 treatment or sonication at neutral pH and purified by sucrose gradient centrifugation. A peak of low buoyant density was observed containing both caveolin and 5'-nucleotidase (I, Fig. 2). The enrichment of the caveolae marker in the peak material was 20-fold compared to detergent-treated plasma membranes and that of 5'-nucleotidase 5-fold. However much caveolin-containing material remained in the bottom of the centrifuge tube after the gradient centrifugation together with 5'-nucleotidase and protein. This could be due to the formation of complexes between non-solubilized membranes and associated cytoskeleton as actin was also present in these fractions. The yield of caveolae-enriched material of low buoyant density after sonication and gradient centrifugation was very low, although the specific content of caveolin was similar as after detergent extraction.

Electron micrographs after negative staining of the caveolin-enriched fraction showed homogeneous clusters of small 50-150 nm vesicles of the same size as caveolae together with a few larger ones. These small vesicles together with occasional larger ones became labeled after incubation with anti-caveolin and gold-tagged secondary antibodies (Fig. 6 D). A conclusion is that these small vesicles represent caveolae and that they were obtained at a high degree of purity. As larger vesicles above 150 nm occasionally became labeled it is possible that some of these also represent caveolae. Electron microscopy analysis showed that caveolae isolated after Triton X-100 and those isolated after sonication were similar in size (Fig. 6 C, D, F). This is in agreement with earlier reports on lung caveolae (Schnitzer et al. 1995; Stan et al. 1997).

In summary, the affinity two-phase partitioning technique was suitable for isolation of plasma membranes, not only from liver but also from lung. Caveolae of high purity could be prepared from these membranes and the affinity approach should be useful when purifying caveolae from other tissues as well. The affinity method is an alternative to a silica coating method (Schnitzer et al. 1995; Stan et al. 1997) for isolation of plasma membranes from lung tissue. The considerably higher yield using the affinity partitioning method might be due to the fact that both the apical and basolateral regions of the plasma membrane were recovered in this case, whereas apparently only the luminal (apical) region was isolated by the silica coating technique.

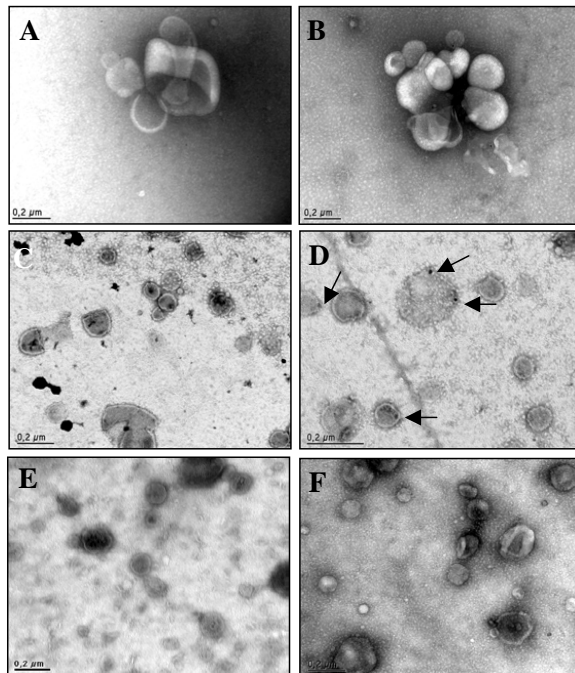


Figure 6. Electron micrograph images of Triton X-100 insoluble material (A and B) show vesicles of different sizes between 50-400 nm in diameter. After sonication of plasma membranes (E), much smaller membrane fragments are seen. The caveolae enriched fractions obtained by gradient centrifugation after detergent treatment (C and D) or sonication (F), show bilayer vesicles mainly with a diameter of 50-150 nm, together with a few larger vesicles (D). Image D shows caveolae-enriched fraction immunolabeled with anti-caveolin bound to gold-labeled secondary antibodies (arrows).

4.2 Purification of caveolae using immunoaffinity two-phase partitioning (paper II).

The aim of this work was to explore the possibility to purify caveolae by affinity partitioning using the interaction between caveolin and anti-caveolin. Affinity partitioning would be advantageous to purification by gradient centrifugation as it is much faster and also might be more selective. The affinity method exploits the high affinity between biotin and NeutrAvidin, a deglycosylated form of avidin with a neutral pI and minimized non-specific binding. A sandwich approach was used where primary antibodies directed against caveolin interacted with biotinylated secondary antibodies and NeutrAvidin coupled to dextran (Fig. 7; II, Fig. 1) in a PEG/dextran two-phase system. The sandwich approach, rather than using primary antibody alone, was chosen as earlier preliminary experiments in our laboratory indicated that primary antibodies coupled directly to dextran did not interact sufficiently well with target membranes to obtain a satisfactory separation.

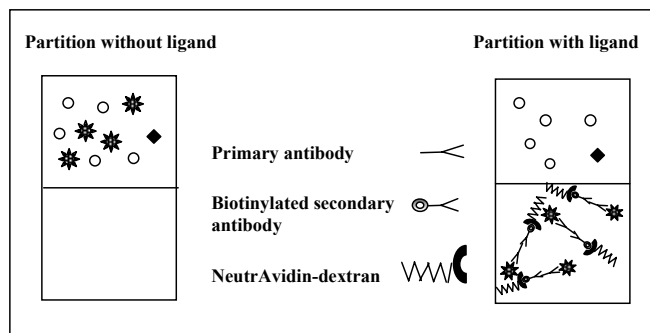


Figure 7. Membrane affinity partitioning using an immunoaffinity sandwich technique. Specific primary antibodies are directed towards components in the star-formed membranes. These interact with biotinylated secondary antibodies and NeutrAvidin-dextran to redistribute the membranes specifically into the bottom phase.

This immunoaffinity approach was first examined using caveolae-enriched fractions from rat liver and lung prepared by Triton X-100 treatment of isolated plasma

membranes and density gradient centrifugation. In control experiments without NeutrAvidin-dextran, or primary and secondary antibodies, 90 % of the isolated caveolae from lung as well as liver distributed in the top phase. In the complete immunoaffinity system approximately 90% of the caveolin from both sources distributed in the affinity bottom phase (II, Fig. 2), showing the efficiency of the affinity system. In an additional control with both antibodies but without NeutrAvidin a slightly lower distribution than in the first control was found and 70 % of the caveolin was recovered in the top phase. A possible explanation of this lower distribution is that caveolae complexes may form with primary and secondary antibodies and that these partly partitioned in the bottom phase. The selectivity of the method was confirmed by including intracellular membranes such as ER along with caveolae in the complete affinity system. Under these conditions 90% of the ER marker distributed in the PEG phase while caveolin-containing material partitioned in the bottom phase.

The next step was to examine if this technique could be applied to the more impure detergent-treated plasma membrane fractions as well, and whether a similar degree of purification of caveolae could be obtained as with gradient centrifugation. The partitioning results were similar to the caveolae-enriched fraction both in the complete affinity system and in the control systems. As the specific content of marker components could not be determined due to the addition of antibodies and NeutrAvidin-dextran the fractionation was instead followed by the caveolin/5'-nucleotidase ratio together with electron microscopy of the separated material. The reason for calculating the caveolin/5'-nucleotidase ratio is that these markers may exist in different compartments as 5'-nucleotidase has been reported to reside in a non-caveolar fraction (Schnitzer et al. 1995). Therefore this ratio may give a measure of the enrichment of caveolae on affinity partitioning. The caveolae/5'-nucleotidase ratio increased in the affinity bottom phase material (II, Table 1) and indicated an enrichment of caveolae in this phase. Furthermore, the ratio in this

material was similar to that in the caveolae-enriched fraction after gradient centrifugation, showing that the immunoaffinity two-phase partitioning can be used for the partitioning of caveolae as an alternative method.

The electron micrograph images show that caveolae-enriched preparations from rat liver and lung had a similar vesicular morphology, often with pomegranate shaped appearance (Fig. 7; II, Fig. 6).

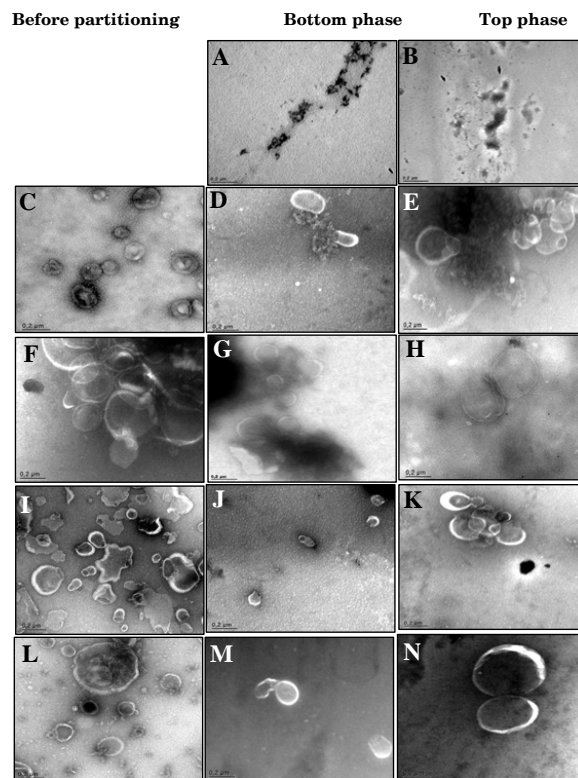


Figure 7. Electron micrographs of immunoaffinity two-phase separations. Samples were stained by negative staining. Each horizontal row (except the first row) shows (from left to right) the material before affinity partitioning, the bottom and the top phase. (A-B), a control system without added membrane particles; (C-E), caveolae-enriched fraction from rat lungs; (F-H), Triton X-100 treated lung plasma membranes; (I-K), caveolae-enriched fraction from rat liver; (L-N), Triton X-100 treated liver plasma membranes.

The vesicles were differently sized, 50-300 nm in cross section, but the large majority of them were in the size range reported for caveolae (50-150 nm) indicating that the material was rather homogeneous. The more impure fraction obtained by Triton X-100 treatment was heterogeneous with many large vesicles. On immunoaffinity two-phase partitioning of both the caveolae-enriched fraction and the Triton X-100 fractions almost all small vesicles (50-150 nm) partitioned in the NeutrAvidin-dextran containing bottom phase, while the distribution of vesicles above 200 nm in diameter mostly was in the PEG-rich top phase. This suggests that the affinity system directs caveolae into the bottom phase, leaving other membrane structures in the top phase. The homogeneous vesicular size in the affinity bottom phase obtained from Triton X-100 treated material indicated that caveolae could also be immunopurified from a non-homogeneous material. Larger vesicles may represent lipid rafts; although lipid rafts are rather small compartments, they seem to fuse into larger structures through the effect of antibodies (Harder et al. 1998; Dykstra et al. 2003) or perhaps during homogenisation. However, as some larger vesicles became tagged with gold-anti-caveolin (paper I), it is also possible that larger vesicles to a certain extent represent caveolae.

Further morphological observations show that the caveolae often contain pores, with or without a neck. Such structures are mostly observed in the immunobottom phase material, but occasionally in the top phase material as well (Fig. 7 D, E, J, K). It is possible that these pores represent the site of attachment to the plasma membrane from where caveolae became detached by Triton X-100 treatment. Background structures were occasionally seen in the electron micrographs (for instance in Fig. 7 D), which probably represent aggregated polymer material as observed in the control without added membrane and antibodies (Fig. 7 A, B).

The benefits of using immunoaffinity two-phase partitioning, apart from its specificity, include that the method is fast and easy to scale up while retaining its

separation properties, and that particles are partitioned in a mild aquatic milieu. The drawback regarding the sandwich method is that there may be difficulties to optimize the partitioning conditions to obtain a good separation. On the other hand, this also offers the possibility to fine-tune the conditions to a specific separation.

The immunoaffinity partitioning approach has wider applications potentially, as this technique should be useful to purify any type of membrane just by changing the biotinylated primary antibody.

4.3 Separation of caveolae from apical and basolateral domains (paper III)

The purpose of this work was to purify caveolae from two plasma membrane domains of the same tissue and to examine whether these differed. For this purpose rat liver was selected, as hepatocytes are highly polarized cells containing apical and basolateral domains. In addition, there are established isolation procedures for these domains (Aronson and Touster 1974). Electron microscopy studies on isolated rat liver plasma membrane fractions (Calvo et al. 2000) have shown an enrichment of caveolae in the basolateral domain, while interestingly enough no caveolae were observed in the apical domain. This would have been expected since caveolae have been implicated in transcytosis (Schnitzer et al. 1995; Oh et al. 1998) involving the apical domain.

Apical and basolateral plasma membranes were prepared by an established method (Aronson and Touster 1974) and the fractionation was followed using various markers. In the isolated apical plasma membrane fraction 5'-nucleotidase, a marker for apical membranes, was enriched 26-fold over the homogenate, while this enzyme was enriched 9-fold in the basolateral fraction. Analysis of the insulin receptor as a marker for basolateral membranes showed a 5-fold enrichment in the isolated fraction of these membranes, but no enrichment in isolated apical membranes. These analyses indicate a limited cross-contamination between the isolated membrane fractions. When the caveolin content was examined it was found that this protein was enriched in both plasma membrane fractions, 25-fold in apical membranes and 38-fold in basolateral ones. It can be concluded that caveolin is present in both these membrane fractions.

The plasma membrane fractions were treated with Triton X-100 to release caveolae. Detergent extraction was preferred to sonication as the yield of caveolae-enriched

material then was several-fold higher. The detergent solubilized most of the protein in both plasma membrane fractions, but also left most of the caveolin and 5'-nucleotidase in the Triton X-100 insoluble material (III, Table 2). In addition, the extraction of Ca-ATPase was followed as this enzyme appears to be present in caveolae (Schnitzer et al. 1995). While nearly all of this enzyme was released from the basolateral membranes, a significant amount was left in the Triton X-100 insoluble material of apical plasma membranes.

The detergent insoluble material from both membrane domains was subjected to sucrose gradient centrifugation to isolate caveolae (III, Fig 1). A peak of low buoyant density was obtained highly enriched in caveolin and 5'-nucleotidase. In addition, caveolin was found together with some 5'-nucleotidase and most of the protein in the bottom high-density fractions of the gradient, indicating an incomplete release of caveolae. Ca-ATPase activity was also found in the low buoyant density peak of the apical material, while the activity was too low to be analysed in the basolateral material.

The peak material was highly enriched in caveolin and 5'-nucleotidase. Thus, the specific activity of 5'-nucleotidase in this peak from the apical material was 1000-fold higher than in the liver homogenate and 350-fold higher for the basolateral peak. The corresponding enrichment for caveolin was 400-fold and 1200-fold, respectively. Electron microscopy analysis of these caveolae-enriched fractions showed a mixed population of vesicles in size from 100-400 nm in the apical material with a majority below 200 nm (Fig. 8 B; III, Fig. 2 B) and mostly from 50-200 nm in the basolateral material (Fig. 8 F; III, Fig. 2 F). Immunolabeling of material with anti-caveolin and gold-tagged secondary antibodies showed that vesicles below 200 nm become labelled (III, Fig 2 I, J). These vesicles presumably represent caveolae.

The caveolae-enriched fractionated were subjected to immunoaffinity two-phase partitioning to go one step further and try to separate caveolae from non-caveolar larger vesicles. These larger vesicles may constitute lipid rafts as these are detergent resistant and fractionate at low-buoyant density together with caveolae on sucrose gradient centrifugation. The immunoaffinity partitioning approach was the same as in paper II with anti-caveolin as affinity ligand together with biotinylated secondary antibodies and NeutrAvidin-dextran. The partitioning was followed by electron microscopy of the partitioned material, and by 5'-nucleotidase and caveolin analysis, together with analysis of Ca-ATPase in the apical fraction.

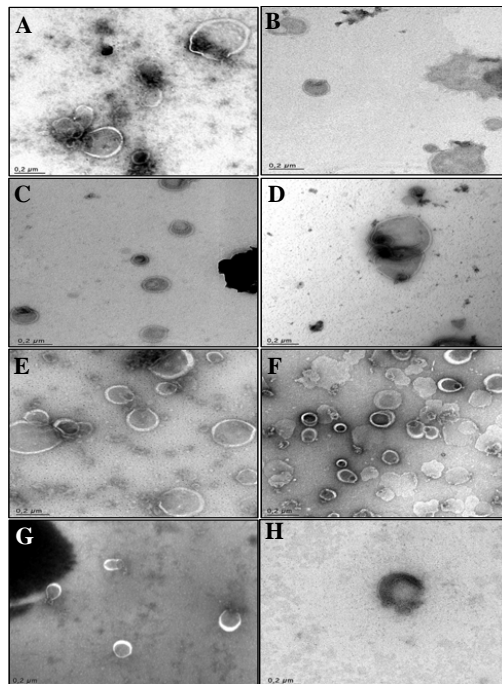


Figure 8. Electron microscopy images from the Triton X-100 insoluble apical domain (A) was also subjected to electron microscopy; B, caveolae-enriched fraction; C, immunoaffinity two-phase partitioning of caveolae-enriched fraction from bottom phase; and (D) top phase. E, detergent insoluble membrane in basolateral domain; F, caveolae-enriched fraction; G, immunoaffinity two-phase partitioning of caveolae-enriched fraction from bottom phase; and (H), top phase.

It was found that the small vesicles to a large extent distributed in the affinity bottom phase, while larger vesicles preferred the top phase. The different markers, 5'-nucleotidase, caveolin and Ca-ATPase all distributed in the bottom phase to the same extent as small vesicles. The conclusions drawn from this experiment are that caveolae were further purified by immunoaffinity partitioning and are separated from large non-caveolar vesicles. Furthermore, not only caveolin but also 5'-nucleotidase seems to be present in caveolae contrary to what has been suggested by others (Schnitzer et al. 1995; Kenworthy et al. 2000; Zajchowski et al. 2002). The measurements showed that the caveolin/5'-nucleotidase ratio differed between apical and basolateral caveolae, and that apical caveolae, but not basolateral ones, contain Ca-ATPase.

Apical and basolateral caveolae obtained in the immunoaffinity bottom phase were quite pure as shown in the electron microscopy images (Fig. 8; III, Fig. 2). They had a particular shape and were of a similar size in both fractions. Basolateral and apical caveolae often had pore-like structures, suggesting the site where they had been attached to the plasma membrane.

The identity of larger vesicles partitioning in the top phase is uncertain. They have a low buoyant density and are resistant to detergent treatment, properties expected for lipid rafts. On the other hand, they are largely devoid of 5'-nucleotidase, which as a GPI-anchored protein would be expected to be enriched in lipid rafts (Simons and Toomre 2000).

5. Conclusions and future perspectives

The purpose of this work was to establish alternative purification methods of caveolae from various tissues. A critical point when isolating caveolae is to obtain sufficiently pure plasma membranes from which caveolae can be detached and isolated in a highly purified state. The approach introduced here with a combination of conventional two-phase partitioning and affinity partitioning resulted in a plasma membrane preparation from lung tissue suitable for the isolation of highly purified caveolae, which should be useful for other tissues as well. The approach has several advantages: it is considerably faster than conventional centrifugation methods; the plasma membrane yield and degree of purification is high; the method can easily be scaled-up and it is supposed to be a mild method for membranes. Caveolae were detached from purified plasma membranes, either by detergent treatment or by sonication. They were isolated by sucrose gradient centrifugation or by a newly developed method for membrane purification, immunoaffinity partitioning. This new method could replace gradient centrifugation to obtain highly purified caveolae or to provide additional purification of the gradient material. In particular, the immunoaffinity method should be able to separate caveolae from material not containing caveolin, for instance lipid rafts, which may be difficult to remove by other methods because of their similar properties. Immunoaffinity partitioning is not restricted to the preparation of caveolae, but is potentially a general method for the preparation of membranes when other specific antibodies directed towards membrane proteins replace anti-caveolin.

In this work methods have been developed to prepare highly purified caveolae from different tissues. These methods offer the possibility to examine isolated caveolae in more detail. In particular, it will be of interest to compare the composition of caveolae from different sources, both regarding their protein and their lipid composition. It is also of interest to compare caveolae released from plasma

membranes by detergent treatment with those released by sonication. A closer view of the protein composition should be obtained by applying proteomics analysis. The specific affinity methods should be useful for a fast preliminary fractionation to remove contaminating material thereby increasing the sensitivity of the mass spectrometry analysis.

From a methodological point of view the affinity methods can be developed further. One development would be to establish conditions combining affinity purification of plasma membranes using WGA-dextran with immunoaffinity isolation of caveolae. Another is to establish conditions to use immunoaffinity partitioning as a general method for the isolation of various membrane fractions using other membrane-specific antibodies than anti-caveolin.

6. Populärvetenskaplig sammanfattning på svenska

Målsättningen för detta arbete var att isolera caveolae för att kunna studera deras struktur och funktion. Caveolae är omegaformade inbuktningar i cellmembranet, ungefär 50-150 nm i diameter. Caveolae finns i många typer of celler, men inte i röda och vita blodkroppar eller nervceller. Man tror att de har flera viktiga funktioner. Exempelvis kan caveolae transportera molekyler mellan olika regioner av cellmembranet och även till membraner inne i cellen. Caveolae är också ett viktigt element i överföringen av signaler från cellens omgivning till dess inre. Än så länge vet man inte om caveolae utför liknande funktioner i olika organ i kroppen eller om funktionerna skiljer sig åt. Den vanliga isoleringsmetoden för caveolae är baserad på att lösgöra dem från isolerade cellmembraner och sedan rena dem genom centrifugering i täthetsgradienter. Caveolae karakteriseras med hjälp av caveolin som är ett protein som finns i caveolaemembranet. Kolesterol och sfingolipider är viktiga lipider i detta membran, vilket gör det olösligt i vissa detergent. Detta utnyttjas för att lösa ut caveolae från andra delar av cellmembranet.

Isolerade cellmembraner används som startmaterial för att rena caveolae. Här har en alternativ metod använts som går ut på att rena membranerna i s.k. tvåfasssystem. Ett tvåfasssystem består av två polymerlösningar i vatten som inte är blandbara. Dessa lösningar bildar två faser ungefär som olja och vatten. Olika membran fördelar sig olika i ett sådant tvåfasssystem och kan separeras från varandra på det sättet. För att förbättra reningen av cell membraner har ett sockerbindande protein, ett s.k. lektin som isolerats från vetegroddar, kopplats till den ena faspolymeren. Eftersom lektiner binder till sockerrester som finns på ytan av cellmembranet styr man cellmembranet till den fas som innehåller lektinet. Proceduren kallas affinitetsrening. Caveolae kan sedan lösas ut från cellmembranet med detergent. För att rena caveolae har en ny metod utarbetats som också bygger på tvåfasseparation och affinitetsfördelning. Här används antikroppar som känner igen caveolinet i caveolae för att styra caveolae till en av faserna i tvåfasssystemet. Affinitetsfördelning är en bra och snabb metod för rening av caveolae. Metoden kan också utnyttjas för isolering av andra membraner genom att byta till antikroppar som är riktade mot ytproteiner i dessa membraner.

Vi har alltså utvecklat alternativa tekniker för att isolera cellmembraner och caveolae för att kunna studera caveolae's exakta roll i celler. Exempelvis kan man studera sådana transportmekanismer i caveolae som utnyttjas av malaria- och sömnsjukesparasiter för att ta sig in i cellen. Även koleratoxin tas upp på samma sätt. Det är därför av intresse att rena caveolae för att kunna studera deras funktioner och därmed försöka lära sig styra sådana transportmekanismer.

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