Membrane Proteins in Human Neutrophils

Identification and characterization of lipid rafts in subcellular organelles

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ABSTRACT

The human neutrophil is an important effector cell in acute inflammation and in the innate immune response against bacteria and fungi. When immune reactions occur in the tissue in response to antigen challenge, neutrophils are the first cells to enter the site of inflammation. The neutrophil is equipped with a vast amount of receptors that both interact with inflammatory mediators and host tissue as well as with the prey. These receptors are found on the cell surface but are also stored in different types of granules and vesicles in the cell. By mobilizing the granules and vesicles to various extents and thereby upregulate receptors to the plasma membrane, the mature neutrophil can modulate its communication with the environment. One granule type, the azurophil granules, primarily delivers a killing machinery to intracellular organelles containing a prey that has been engulfed. These granules have traditionally been regarded as classical lysosomes, but their membrane is so far largely uncharacterized.

The aim of this thesis was to elucidate details regarding azurophil granule membrane composition in order to further understand their role in neutrophil function. The studies led to identification of so-called lipid rafts in the azurophil and other granule membranes, and a detailed characterization of the azurophil granule lipid rafts with regard to protein composition was thus performed.

One of the proteins identified in azurophil granule membranes was stomatin. This protein was present also in other granule/vesicle membranes and the plasma membrane. Furthermore, the protein was localized to lipid rafts. Apart from stomatin, the azurophil granule membrane rafts contained a vast number of proteins, of possible importance for membrane structure/integrity and fusion. The fact that several cytoskeletal proteins also were identified, suggests that the granule membrane is organized in much the same way as the plasma membrane.

The thesis also includes studies on the neutrophil receptors for galectin-3, a potent activator of extravasated human neutrophils. Since granule mobilization is a prerequisite for galectin-3-induced activation of the cells, the receptors have been suggested to be granule localized. Here, galectin-3-binding proteins from specific/gelatinase granules were isolated, and among these, CD66a and CD66b were identified as the most plausible receptor candidates. The CD66b is a glycophosphatidyl inositol (GPI)-linked protein that was found to be localized to lipid rafts, suggesting that raft-associated signaling may be of importance for the galectin-3-induced neutrophil responses.

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PREFACE

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. The presence of stomatin in detergent-insoluble domains of neutrophil granule membranes.
E. Feuk-Lagerstedt, M. Samuelsson, W. Mosgoeller, C. Movitz, Å. Rosqvist, J. Bergström, T. Larsson, M. Steiner, R. Prohaska, and A. Karlsson
*Journal of Leukocyte Biology (2002); **72:** 970-977*

II. Lipid raft proteome of the human neutrophil azurophil granule
E. Feuk-Lagerstedt, C. Movitz, S. Pellmé, C. Dahlgren, and A. Karlsson
Submitted for publication, 2006

III. Identification of CD66a and CD66b as the major galectin-3 receptor candidates in human neutrophils
E. Feuk-Lagerstedt, E. T. Jordan, H. Leffler, C. Dahlgren, and A. Karlsson
Journal of Immunology (1999); 163:5592-5598.

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THE NEUTROPHIL

Polymorphonuclear leukocytes (neutrophils) are important effector cells in acute inflammation and in the innate immune response against bacteria and fungi [1, 2]. These professional phagocytes are produced in the red bone marrow from the same common haematopoietic stem cell precursor as the other blood cells. After maturation for 5-10 days in the bone marrow the neutrophils enter the bloodstream where they form one circulating and one marginating pool, together making up 60-70 % of the total normal blood leukocytes. The neutrophils spend less than a day in the blood before entering various tissues and organs. Here they may survive several days before they are cleared by apoptosis or lost from mucosal surfaces.

The process of neutrophil activation starts when inflammatory mediators stimulate the upregulation of adhesion receptors like selectins on the endothelial cell lining of the blood vessels, making it possible for the neutrophils in the bloodstream to sense the activation and bind to the blood vessel wall [2]. In parallel, neutrophil adhesion molecules are upregulated and activated by inflammatory mediators and strong binding to the endothelial cells is mediated by integrins like CR3 (identical to Mac-1 or CD11b/CD18) present on the neutrophils [3]. The endothelial cells also produce e.g. interleukin-8 (IL-8), which modulates the adherence of neutrophils and stimulates their chemotaxis [4]. The neutrophils thus traverse the endothelial cell layer and move towards higher concentrations of other potent chemoattractants released from the focus of infection to carry out their task of killing microbes by the process of phagocytosis.

During phagocytosis, the microbe is bound to the surface of the neutrophil, either through opsonin-mediated interactions via phagocyte complement receptors and/or Fc-receptors [5], or through microbial lectins binding to phagocyte glycoconjugate receptors (or vice versa) [6]. When adhesion between the neutrophil and the invader is established, the plasma membrane zip-locks around the prey which thus becomes enclosed in a phagocytic vacuole. In the phagosome, the microbe is exposed to the toxic contents of membrane-enclosed organelles, so-called granules, which fuse with the phagosomal membrane, forming a phagolysosome [7, 8]. Killing of the engulfed invader is accomplished both by oxygen-dependent and oxygen-independent mechanisms. Oxygen-dependent killing starts with a dramatic increase in oxygen consumption in the neutrophil [7], due to activation of an enzyme system, the NADPH-oxidase, that is present in the membrane of granules as well as of the plasma membrane and, consequently, the phagolysosome. The NADPH-oxidase catalyses a reaction in which oxygen

is reduced to highly reactive oxygen metabolites, which in the presence of granule-derived myeloperoxidase (MPO) can form highly toxic oxygen species such as hypochlorous acid [7]. Oxygen-independent killing takes place through the action of bactericidal granule components, e.g., cathepsin G, bactericidal/permeability increasing protein (BPI) and defensins [8].

In order for neutrophil functions to be properly executed, the cells have to interact with its surroundings as well as with its prey in a regulated and efficient manner. To accomplish this, the cell contains, in addition to toxic molecules and enzymes, a vast array of receptors that recognize inflammatory mediators, adhesion-promoting ligands and bacterial surface structures. These receptors are to large extents stored in the above-mentioned granules that are mobilized not only to the phagosomal membrane, but also to the neutrophil plasma membrane, to deliver new/more receptors to the cell surface upon cell activation. The granules and vesicles are different from each other with regards to size, density, membrane content, matrix composition as well as readiness to be mobilized [9].

DEVELOPMENT AND CHARACTERISTICS OF NEUTROPHIL GRANULES

During neutrophil development in the bone marrow, the neutrophil passes six stages of maturation, and different granules and vesicles appear in the cell at different stages. In the first developmental stages the cells are still proliferating, but throughout the later stages, i.e., from the metamyelocyte stage and on, they are non-dividing but still developing. The original myeloblast is relatively undifferentiated and has no or few granules [1, 10, 11]. The primary or azurophil granules, characterized by their content of the peroxidase MPO, are formed during the promyelocyte stage, while the secondary or specific granules are formed during the myelocyte stage and are peroxidase-negative. Another type of peroxidase-negative granules are the tertiary, or gelatinase granules, which are formed somewhat later during the band cell stage. Finally, when the neutrophil has matured into a segmented cell, the so-called secretory vesicles are formed. Ultimately, the granulopoiesis leads to formation of a mature neutrophil with a lobulated nucleus and a vast amount of granules and vesicles (Fig.1).

The formation of neutrophil granules (but not secretory vesicles, see below) is believed to occur by aggregation of transport vesicles, which bud off from the *trans*-Golgi network during the promyelocyte stage and from the *cis*-Golgi network during the myelocyte stage [11]. The sorting of different proteins into granules thus takes place in the Golgi network, as does the glycosylation of these proteins. The heterogeneity of the neutrophil granule population can at least in part be explained by the hypothesis "sorting by timing" [12, 13], stating that the time of biosynthesis of each protein during granulopoiesis determines the localisation of the

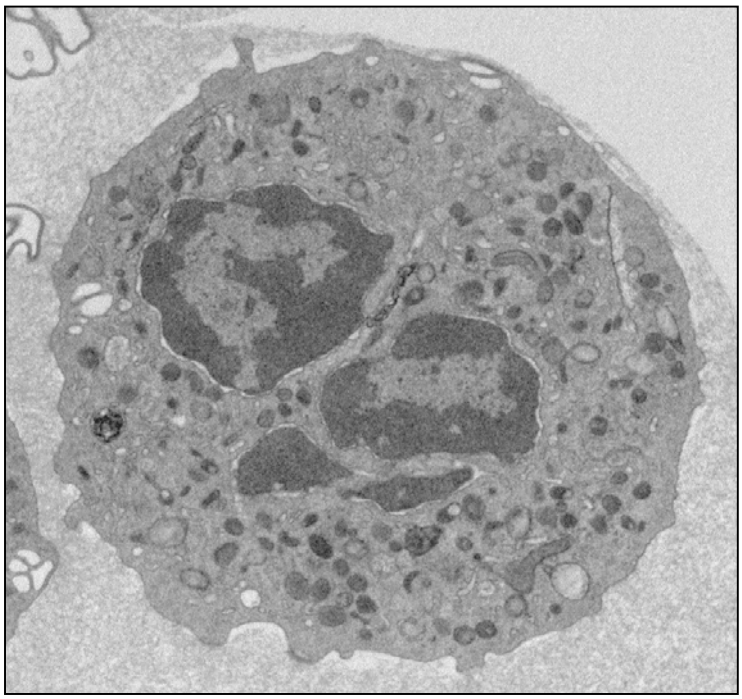

Figure 1. *Transmission electron microscopy picture of a resting mature neutrophil. (60 nm section, 10,000 times magnification) Courtesy of Sara Pellmé.*

protein to the organelle being formed at that same time. Hence, azurophil granule proteins are synthesized in the early, promyelocyte stage, while specific granule proteins are synthesized later, during the myelocyte stage.

The separation of different proteins between different subpopulations of granules may have more than one function [13, 14]. Some constituents may not be able to coexist with certain enzymes without being activated or inactivated, and separation of these into different compartments can prevent premature activation/inactivation. Furthermore, the proteins in the different granules may be needed at different times and/or at different places during the inflammatory process and should thus be located in an organelle that is mobilized at that particular time or place.

Azurophil granules

The azurophil granules are traditionally defined as peroxidase-positive granules since they contain the unique neutrophil peroxidase MPO. These granules comprise approximately one third of the total number of granules in the neutrophil. Azurophil granules can be further divided into two groups; the larger, defensin-rich granules and the smaller, defensin-poor granules. Most of the azurophil granule matrix content is comprised of proteolytic and other enzymes like sialidase, lysozyme and MPO. In addition, they contain antibacterial agents such as defensins and bactericidal/permeability increasing protein (BPI). Until a few years ago, the only known membrane components in the azurophil granules were CD63 and CD68 (Table 1) [11].

Specific granules

The peroxidase-negative granules are unique for the neutrophils and are identified by their matrix content of lactoferrin and vitamin B_{12}-binding protein [15, 16]. They also contain proteases, e.g. gelatinase, and cytochrome b_{558}, the membrane-localized component of the NADPH-oxidase. The membrane of the specific granules also contains many different receptors for inflammatory mediators, bacterial components, as well as adhesion-mediating receptors (Table 1). Two decades ago, a subpopulation of these granules was identified, the gelatinase or tertiary granules [17]. The two subpopulations differ somewhat regarding both membrane receptors and matrix proteins, and the gelatinase granules can be identified by their content of gelatinase in combination with lack of lactoferrin [15].

The differences in composition between the specific granules and gelatinase granules indicate some differences in their respective tasks (Table 1). The gelatinase granules are more readily mobilized than the specific

granules, probably starting already in the blood stream during the extravasation process. That these granules are more important for interaction with the extracellular matrix and migration into the tissue than the specific granules, is mirrored by that they to a higher extent than the specific granules contain tissue degrading enzymes and essential adhesion molecules designed for these purposes. The specific granules are probably mobilized first at the site of inflammation trigger, where their content of receptors are needed for the interaction with an invader. Specific granules are also mobilized to the phagosome to which they deliver the membrane-bound components of the NADPH-oxidase [11].

Secretory vesicles

In the still developing band cells and segmented cells an organelle called the secretory vesicle is found [16, 18]. This compartment contains alkaline phosphatase (ALP) that is localized on the luminal side of the membrane, and its activity can thus be measured only in presence of detergent. The ALP is a marker also for the plasma membrane, but here the enzyme activity is measured in the absence of detergent. The secretory vesicle matrix contains plasma proteins not synthesised by the cell itself, indicating that the vesicle is formed by endocytosis [16, 18]. Similar to the gelatinase granule, the secretory vesicle is an important storage organelle for membrane-bound receptors (Table 1).

Granule mobilization

The mature neutrophil contains all four types of granules and vesicles and can communicate with its environment by mobilizing them to various extents [16]. In correspondence to the sequence in which the granules/vesicles are formed (see above) the mobilization of the organelles occur in a specific order, however starting with the last formed organelles, the secretory vesicles, followed by the others in reverse order of formation, i.e., the gelatinase granules, the specific granules and finally the azurophil granules. The mechanism behind this sequential granule exocytosis is not fully understood but an attractive hypothesis has been put forward suggesting that all granules and vesicles are attached to the cytoskeleton and upon stimulation with e.g. elevated levels of cytosolic Ca^{2+} will move along the microtubule system towards the plasma membrane. The lighter and smaller the organelles are, the faster this movement would be accomplished [16].

When stimulating neutrophils *in vitro*, with e.g. ionophores or chemo-attractants, the secretory vesicles and gelatinase granules are relatively easy to fully mobilize to the plasma membrane. Also the specific granules are

quite mobilizable while plasma membrane fusion of azurophil granules is more difficult to achieve. This may reflect the function of the azurophil granule, which lies in emptying its tissue-destructive enzymes and molecules into the phagosome, and not releasing them to the extracellular milieu and thus risking to damage host tissue [16]. These differences in sensitivity to stimulation between the different granules/vesicles is also reflected in *in vivo* experiments [19].

Table 1. Content of neutrophil granules

Azurophil granules	Specific granules	Gelatinase granules	Secretory vesicles	MVB	MLC
Membrane:	***Membrane:***	***Membrane:***	***Membrane:***	***Membrane:***	***Membrane:***
CD63	CD11b	CD11b	Alkaline phosphatase	LAMP-1[36, 101]	LAMP-1[36, 101]
CD68	CD15 antigens	Cytochrome b_{558}	CR1	LAMP-2[36, 101]	LAMP-2[36, 101]
V-type H^+-ATPase	CD66a (CEACAM1)[81]	Diaglycerol-decylating enzyme	Cytochrome b_{558}	Mannose-6-P-receptor[1]	
Stomatin[39]	CD66b (CEACAM8)	FPR	CD11b		
	Cytochrome b_{558}	FPRL-1[100]	CD14		
	FPR	SCAMP	CD16		
	Fibronectin-R	Stomatin[39]	FPR		
	G-protein$_\alpha$-subunit	Urokinase-type plasminogen activator-R	FPRL-1[100]		
	Laminin-R	VAMP-2	SCAMP		
	NB 1 antigen	V-type H^+-ATPase	Stomatin[39]		
	19- kDa protein		Urokinase-type plasminogen activator-R		
	155-kDa protein		V-type H^+-ATPase		
	Rap1, Rap2		VAMP-2		
	SCAMP		CD10, CD13, CD45		
	Stomatin[39]		$C1_q$-receptor		
	Thrombospondin-R		DAF		
	TNF-R				
	Urokinase-type plasminogen activator-R				
	VAMP-2				
	Vitronectin-R				
Matrix:	***Matrix:***	***Matrix:***	***Matrix:***		
Acid β-glycerophosphatase	β_2-Microglobulin	Acetyltransferase	Plasma proteins (including tetranectin)		
Acid Mucopolysaccharide	Collagenase	β_2-Microglobulin			
α_1-Antitrypsin	Gelatinase	Gelatinase			
α-Mannosidase	HCAP-18	Lysozyme			
Azurocidin/CAP37/heparin binding protein	Histaminase				
Bactericidal permeability increasing protein	Heparanase				
β-Glycerophosphatase	Lactoferrin				
β-Glucuronidase	Lysozyme				
Cathepsins	NGAL				
Defensins	Urokinase-type plasminogen activator				
Elastase	Sialidase				
Lysozyme	SCP28				
Myeloperoxidase	Vitamin B_{12}-binding protein				
N-Acetyl-β-glucosaminidase					
Proteinase-3					
Sialidase					
Ubiquitin-protein					

References, unless given, are found in [11] from which the table is adapted

MEMBRANES AND MICRODOMAINS

Neutrophils contain a rich array of internal membranes enclosing different organelles, and these are characterized by their specific protein, lipid and carbohydrate compositions [11, 20]. All membranes have a common general composition, consisting of large amounts of phospholipids and cholesterol and smaller amounts of glycolipids, all arranged in a double layer. The cholesterol and the sphingolipids, a lipid subgroup comprising both phosphosphingolipids and glycosphingolipids, are mainly localized to the extracellular leaflet [21]. Protein molecules are embedded in the bilayer, either as integral or transmembrane proteins, extending across the lipid bilayer or attached only to one or the other side of the membrane, being so-called peripheral or membrane-associated proteins, respectively. As a general rule, the lipid bilayer determines the basic structure of the membrane while the proteins are responsible for membrane functions and serve as e.g. receptors, enzymes, channels, or transporters. Both lipids and proteins are able to diffuse in the plane of the membrane. Yet, many cells have ways to immobilize these molecules, confining them to particular microdomains. One type of microdomain is the lipid raft [22-26], originally defined biochemically as detergent-resistant membrane fractions (DRMs) since they are insoluble in the non-ionic detergent Triton X-100 at 4^{o}C.

General properties of lipid rafts

At least two different types of DRMs have been isolated. Depending on the presence or absence of the protein caveolin in the DRMs, they are today referred to as “caveolae” or “flattened lipid rafts” (“lipid rafts”), respectively (Fig. 2) [24]. These rafts are enriched (3-5 fold) in cholesterol and saturated phospholipids. They also contain sphingolipids, which have long, largely saturated, fatty acid chains. Sphingolipids and cholesterol are mainly localized to the outer leaflet of the membrane. Due to the chemical structure of these different lipids, they will pack tight together to form a liquid-ordered (l_o) phase. The composition of the surrounding parts of the plasma membrane differs, containing lesser amounts of cholesterol and glycosphingolipids but more unsaturated phospholipids, together constituting a liquid-disordered (l_d) phase. These phase differences give rise to a lateral phase separation and thereby formation of lipid domains. The differences in composition between the l_o and l_d phases is also the basis for the differences in resistance to detergent, a useful characteristic for the isolation of the l_o domains (the lipid rafts) from the rest of the membrane. Such isolation can be achieved by allowing the l_o domains to floatate during density gradient centrifugation [22-26].

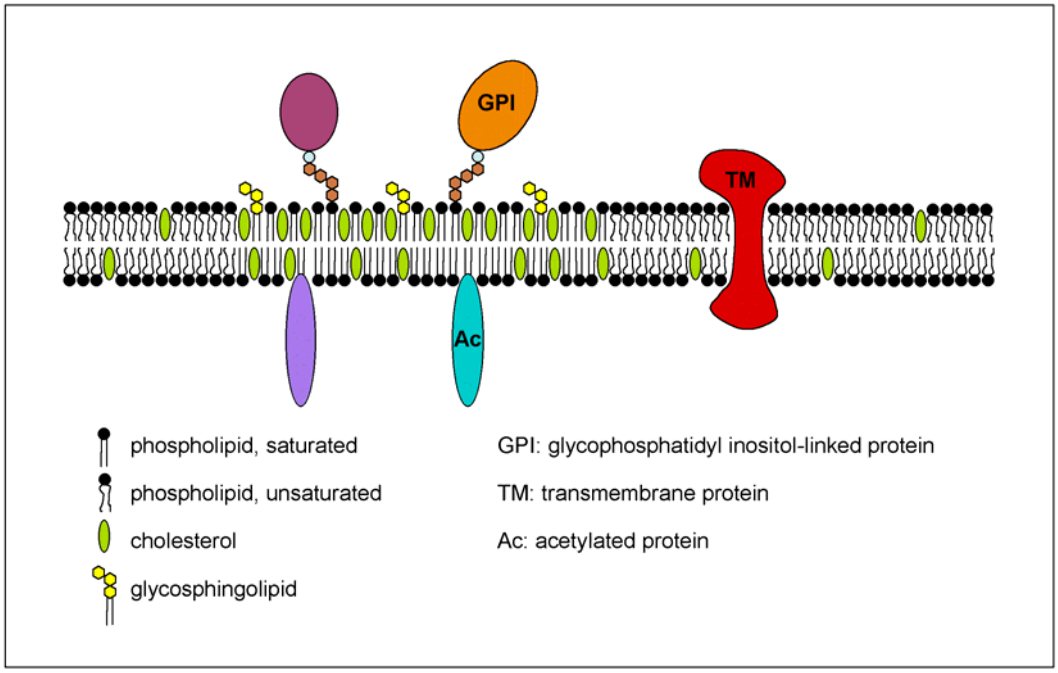

Figure 2. *The plasma membrane is organized into regions, of which some are enriched in saturated phospholipids and cholesterol, i.e., lipid rafts. Sphingolipids, including both sphingomyelin and glycosphingolipids, associate with cholesterol to form a tightly packed domain. The regions rich in unsaturated phospholipids are less densely packed, and form fluid regions outside the raft microdomains. Lipid rafts are enriched in GPI-anchored proteins at the external surface, and acylated proteins at the cytoplasmic surface. Transmembrane proteins are generally excluded from rafts, and are found in the more fluid phospholipid-rich regions of the membrane.*

Lipid rafts have been shown to be of importance for cellular processes such as sorting of membrane components as well as in secretory and endocytic processes [22, 23, 25, 26]. They also appear to function as anchor structures for intracellular signalling molecules [26] (see below).

The lipid components of the microdomains in the inner leaflet are much less characterized than those of the outer leaflet and there appears to be a great heterogeneity among them. This heterogeneity appears to be of importance for the ability to attract proteins with characteristic targeting motifs, which take part in membrane/cytoskeleton interactions [27]. The inner leaflet contains phosphoinositides, which are important for regulating the dynamics of the actin filament network in the cell, e.g., many of the membrane skeleton proteins that crosslink actin with transmembrane proteins are activated by the phosphoinositide PIP_2.

Ceramide-enriched membrane platforms

One sphingolipid, sphingomyelin, which is mainly present in the outer leaflet of lipid rafts, can rapidly be hydrolysed to ceramide by sphingomyelinase, an enzyme also present in the outer leaflet [21]. Studies in model membranes implicate that ceramides push cholesterol out from lipid rafts, creating ceramide-rich rafts [28]. Ceramides that are present in the lipid rafts change the biophysical properties of the raft in a striking way, resulting in clear differences between ordinary rafts and ceramide-rich rafts [21]. The ceramide molecules are able to associate with one another, and in that way form small ceramide-rich microdomains. These domains spontaneously merge with one another into patches, and then into larger ceramide-rich membrane platforms. In this way, very small non-signalling rafts can be transformed into large signalling platforms. These platforms seem to restrict receptors and signalling molecules into the platforms, giving high receptor density and providing favourable conditions for effective transmission of signals into the cell. These platforms may also help in the recruitment of soluble intracellular signalling molecules, and may exclude molecules that negatively interfere with the signal transduction from the receptor [21].

Do lipid rafts exist?

During the last decade, there has been a vivid discussion concerning the existence of lipid rafts *in situ* [29, 30]. Much effort has been put into identifying rafts in intact cell membranes, and a number of investigations have contributed to an increased understanding of various membrane heterogeneities. Still, there is a debate concerning the formation of rafts, their functions, size and size control mechanism. The disagreement regarding the "true" existence of lipid rafts probably remains mainly because the lipid rafts are too small to be seen by vital microscopy, while the presence of larger structures such as caveolae is well documented. Studies in different types of model membranes have been important for the understanding of lipid rafts and it has been confirmed that the l_o and l_d domains without any doubt can co-exist in the same bilayer in such membranes [29, 31]. Although the outcome of the debate more or less seems to be that model membranes will always be just that, a thorough analysis of the recent literature taken together is convincingly clear regarding the *in situ* existence of lipid rafts.

How proteins can be associated with lipid rafts

The structural diversity among rafts is mainly determined by the proteins associated with them. This is not solely due to differences in protein structure but also to the way by which the proteins are associated with the rafts. Many non-integral proteins are targeted to the rafts via GPI-anchors, often modified

by myristoyl and/or palmitoyl group(s). Another way of targeting is modification by fatty acylation of proteins lacking GPI-anchors, adding either one myristoyl and one palmitoyl group or two palmitoyl groups to the protein [24]. Transmembrane proteins are usually detergent-soluble, unless they are linked to the cytoskeleton [22]. The transmembrane proteins present in rafts, i.e., detergent-insoluble proteins, are often modified with palmitoyl groups, have amino acid residues that interact with the outer leaflet of the membrane or have an extracellular domain interacting with raft components [24].

Three models for raft structure

Based on experimental studies of rafts isolated by both detergent- and non-detergent methods (see below), three different models for raft structure have been suggested. In the "lipid shell model" the raft is built from layers of lipids. In the centre, there is a well-ordered phase enriched in cholesterol and glycosphingolipids. Outside the centre there are areas with less order, which gradually end up in the disordered structure of the surrounding plasma membrane [32]. In the "thermal Lego model", raft proteins like GPI-anchored proteins together with only a few raft lipids constitute short lived small rafts, which are dynamic structures that can associate temporarily to form stable rafts [33]. In the "heterogeneous system model", a variety of rafts with different compositions of lipids and proteins co-exist in the same membrane [30].

NEUTROPHILS, AZUROPHIL GRANULES AND LIPID RAFTS - AIM OF THE THESIS

It has been shown that dynamic areas involved in cell adhesion and migration, so-called podosomes, in the bovine neutrophil plasma membrane are organized into lipid rafts [34]. These structures are attached to the underlying cytoskeleton and contain a vast amount of cytoskeletal proteins (actin, myosins I and II, α-actinin and supervillin). Lipids rafts have been identified also in human neutrophil plasma membrane, containing raft-organizing proteins, signalling proteins like the Src family kinases, and heterotrimeric G proteins [35].

However, prior to the studies on which this thesis is based, there was no knowledge on the suborganization of the neutrophil granule/vesicle membranes. All the granules and vesicles in the neutrophil have the same basic structure [9]. The matrices contain proteins designed for delivery to the phagosome or secretion to the extracellular milieu, and the membranes are phospholipid bilayers just as the plasma membrane. The membranes of the specific/gelatinase granules and the secretory vesicles store important receptors, which are delivered to the plasma membrane during exocytosis. It is thus reasonable to believe that these receptors are organized similarly in the granules and in the plasma membrane, i.e. in lipid rafts. The azurophil granule membrane is even less characterized than those of the specific/ gelatinase granules and the secretory vesicles. The azurophil granule can with some effort be mobilized to the plasma membrane but primarily merges with the phagosome [9]. It is, however, plausible that the fusion process for the azurophil granule is similar to that of the other granules, and that the membrane organization therefore would be somewhat alike.

The aim of this thesis was to elucidate details of azurophil granule membrane composition and to investigate the presence of lipid rafts in granule membranes in order to characterize these with regard to protein composition. The aim is based on my hypothesis that azurophil granules have a just as elaborate set of membrane proteins as the other granules. Further, in correspondence to the plasma membrane, the granule/vesicle membranes are hypothesized to be organized with part of their protein and lipid components localized in lipid rafts.

Azurophil granules are not classical lysosomes

The azurophil granules were earlier considered to be classic examples of primary lysosomes. Lysosomes are today defined as vesicular compartments that fulfil all four of the following criteria: high concentration of lysosome

associated membrane glycoproteins (LAMPs), full set of dephosphorylated lysosomal enzymes, lack of the cation-independent mannose-6-phosphate receptor, and an acid pH in the matrix [36]. On at least two points the azurophil granules deviate from this definition; the localization of LAMPs and the dephosphorylation of enzymes.

The LAMPs belong to a class of proteins present in the lysosomal membranes of many cell types. Two members of this family, LAMP-1 and LAMP-2, are highly glycosylated transmembrane proteins with a molecular weight of about 120 kDa, of which approximately 40 kDa comprise the polypeptide core [37]. The majority of the molecule is pointing into the matrix of the lysosome, leaving only a short tail in the cytoplasm. A third protein, LAMP-3, or CD63, is classified into this family mainly because it shares the same cytoplasmic structure as the two other LAMPs. CD63 is localized exclusively to the neutrophil azurophil granules, while interestingly, LAMP-1 and LAMP-2 are not found in these organelles [38]. Instead, they are localized to multivesicular bodies (MVB) and multilaminar compartments (MLC), suggested to correspond to prelysosomal structures (Table 1) [36]. The functions of these organelles in the mature neutrophil are not yet known, but it has been shown that they are mobilized to the phagolysosome following engulfment of a prey [36, 38].

Prior to formation of “true” lysosomes, newly synthesized lysosomal enzymes are modified to contain mannose-6-phosphate during the transportation from the endoplasmatic reticulum to the Golgi complex [36]. In the Golgi, the enzymes are bound to mannose-6-phosphate receptors, which enable their further transport to the lysosome. When the enzyme reaches the mature lysosome the mannose-6-phosphate/mannose-6-phosphate receptor complex is dissociated and the lysosomal enzyme is rapidly dephosphorylated. Azurophil granules are formed during the promyelocyte stage, from the *trans-Golgi* network according to the general pathway for lysosome formation [11]. However, the azurophil granule enzymes such as MPO are still phosphorylated, supporting the LAMP data in that the azurophil granules do *not* belong to the classical lysosomes [36].

Presence of lipid rafts in neutrophil azurophil granules

During granule mobilization, different fusion processes take place in the cell and the membranes of the different organelles are integrated into the plasma membrane or the phagosomal membrane. An interesting issue for us was to explore if lipid rafts exist in the membrane bilayer of the organelles in conformity with the plasma membrane and to gain knowledge on the possible restriction of components to these structures. With the objective to shed more

light primarily on the membrane of the so far poorly characterized azurophil granule, we developed a detergent-dependent technique to isolate lipid rafts and combined this with subcellular fractionation and proteomics.

During our work, we found the presence of DRMs not only in azurophil granule membranes but also in the membranes of specific/gelatinase granules and secretory vesicles/plasma membrane. In the lipid rafts of all granule types, the protein stomatin was present (Paper I; [39]). Further investigations of the azurophil granule lipid rafts showed that, apart from stomatin, these structures contained a vast number of proteins (Paper II). Some of them, in addition to stomatin, are highly interesting in relation to membrane structure and fusion, e.g., the flotillins, which are considered structural proteins in lipid rafts, and dysferlin, a protein participating in membrane fusion events. These three examples of azurophil granule lipid raft proteins are described in more detail below.

We also found proteins associated with all three types of cytosolic fibers composing the cytoskeleton, i.e., microfilaments, intermediate filaments, and microtubules, in the DRM preparations from neutrophil azurophil granules (Paper II). This indicates the presence of highly complex cytoskeletal structures associated also with these granules, suggesting similarity of the granule membranes with the, so far thought, more complex cytoplasmic membrane. The implications of the presence of all these microfilament proteins in the azurophil granule DRMs with regard to azurophil granule movement, phagolysosome formation, and signaling processes involving the granules, has to be further studied.

Some proteins involved in membrane structure, fusion and maintenance

Stomatin

Stomatin, earlier known as band 7.2b, is a 31 kDa integral membrane protein that exists in abundance in different mammalian tissues and cell types, as well in plants and prokaryotes [40]. Absence of the protein from the red cell membrane in humans is observed in the rare hereditary hemolytic anaemia called overhydrated hereditary stomatocytosis. In such anaemia, erythrocytes have a major defect in membrane permeability to Na^+ and K^+, making the cells leaky to these ions. The protein is a member of the SPFH-family (stomatin, prohibitin, flotillin homolog) [41], designated by the presence of a prohibitin like domain (PHB) in the N-terminus, a conserved entity even in prokaryotic members of the family [42].

The specific functions of stomatin are still not quite clear. In red blood cells, stomatin is a major component of the lipid rafts [43]. As mentioned

above, lipid rafts called caveolae are maintained by the structural protein caveolin [24]. However, red blood cells, neutrophils, and all other hemopoietic cells, lack caveolin [25, 44, 45]. As a consequence, they have to depend on other structural proteins to stabilize the raft structure in their membranes, and stomatin has been suggested to fill this function. This protein, like caveolin [25, 46], is anchored in the membrane by a hairpin loop, which leaves both the N and C termini exposed to the cytoplasm for interaction with other molecules. They also both form SDS-stable hetero-oligomers [46, 47] like many other proteins playing a structural role in the plasma membrane.

Overexpression of stomatin leads to reduction of the glucose transport rate through the Na^+-independent glucose transporter GLUT-1, and to a higher grade of association between these two proteins [48]. This indicates that stomatin can be a modulator of glucose homeostasis. Recently, stomatin homologues have been studied in both prokaryotic cells as well as in *Caenorhabditis elegans* [40]. The results indicate that stomatin is part of a trafficking system, playing a role as partner protein for a membrane-bound protease. Another possible function of stomatin is as cytoskeletal anchor. In the human epithelial cell line UAC, stomatin colocalizes with cortical actin microfilaments even after treatment with cytochalasin D, a toxin that disrupts the actin filaments, leading to collapse of the actin network [49]. It has been suggested that the potential functions of stomatin as ion channel regulator and cytoskeletal anchor may work in concert if the protein acts as an information relay between sensors of stretch in the membrane and the ion channels which are embedded in the lipid bilayer, perhaps to influence channel stability and organization in the plasma membrane [50, 51].

Flotillins

The flotillins, earlier called reggie-1 and reggie-2, are two 48 kDa members of the SPFH-family [41] having N terminal regions with relatively high homology to stomatin and prohibitin. Just like stomatin and caveolin [46, 47], flotillins form homo-oligomers [41] and flotillin-2 also shares the property with stomatin of being functionally associated with the actin cytoskeleton [49].

The literature contains contradictory information on the structure of the flotillins. From the beginning it was suggested that flotillin-1 was an integral membrane protein [52]. More recent data emphasize that the protein does not contain any transmembrane domains [41, 52]. Instead, the protein appears to be associated with the cytoplasmic face of the plasma membrane via a palmitoyl group attached close to its N-terminus [52, 53]. Current studies

suggest a similar topology of flotillin-2 [52]. This protein contains both a myristoyl and a palmitoyl group, also close to its N-terminus [53].

In the membrane, the flotillins are suggested to function as scaffolds for the creation or organization of multimolecular complexes in the lipid rafts and for communication across the plasma membrane [53]. The proteins have been proposed to play a role in cellular processes such as endocytosis and phagocytosis [54-57]. Flotillin-2 contains several putative phosphorylation sites, which makes it a possible candidate as a signalling protein, capable of regulating multiprotein complexes involved in transmembrane signalling. The flotillins have recently been shown to be present in plasma membrane lipid rafts of bovine neutrophils [34], but have not earlier to our study (Paper II) been identified in lipid rafts of neutrophil granule membranes.

Dysferlin

Dysferlin is a transmembrane protein mainly known for its presence in the plasma membrane of skeletal muscle and its involvement in the muscular disorder Limb-Girdle Muscular Dystrophy type 2B and Miyoshi myopathy [58, 59]. Its function is believed to lie in the maintenance of the muscle cell membrane and it has been demonstrated to contribute to resealing muscle cell membrane tears that occur with exercise and muscle damage [58, 59]. The exact molecular mechanism by which this takes place is however not yet known. A model has been presented [58] in which the membrane disruption causes influx of extracellular calcium, creating a transient zone of high calcium around the site of the injury. Dysferlin-carrying vesicles are targeted to the injury site where they fuse with each other and with the plasma membrane, adding new membrane to the damaged area. This docking and fusion is suggested to be mediated through dysferlin-dysferlin interactions or interactions with annexins (or some so far unknown protein-binding partner(s)) at the plasma membrane. It is tempting to speculate on whether dysferlin has a similar role in neutrophils as in skeletal muscle cells, i.e., if it is involved in granule-granule fusion or granule-plasma membrane fusion. This is presently under investigation.

RECEPTOR STRUCTURES IN NEUTROPHIL LIPID RAFTS

As stated above, lipid rafts have been suggested to play a role in signal transduction [22, 23, 25, 26], mainly because of their tendency to aggregate GPI-anchored proteins but also due to the fact that they harbour signalling transmembrane proteins, e.g., receptors. Investigations of lipid rafts have shown that they have 10 times higher concentration of signalling proteins as compared to the rest of the membrane [60]. Further, the rafts contain lipid cofactors that operate close to the signalling proteins, increasing the effectiveness, specificity and also the regulation of the signalling cascades. Signalling through lipid rafts has been shown for different types of receptors, and here I will discuss two general groups and one other example.

G-protein coupled receptors

G-protein coupled receptors (GPCRs) belong to a large family of transmembrane proteins, which traverse the plasma membrane seven times. They are expressed in high numbers in certain cell types, among them the neutrophil [61]. Binding of chemotactic factors to neutrophil GPCRs leads to an activation of pertussis-toxin sensitive heterotrimeric G-proteins. A complicated signalling network starts inside the cell leading to migration of the neutrophil in the tissue along a chemoattractant gradient towards the inflammatory site. During this migration process, the neutrophil becomes morphological polarized with a clear difference between the front and the back of the cell [62]. The front of the cell extends into a lamellopodium while at the same time the back of the cell is pulled in, detaching from the contact point. These processes are generally found in combination with local actin polymerization carried out entirely at the front (leading) edge. During cell polarization, the plasma membrane of the neutrophil is reorganized into distinct lipid domains [63]. The protein content in the domains from the lamellopodium does not correspond to the protein content of the domains present in the back of the cell. This reorganization of the membrane into domains facilitates both the enhancement of the chemoattractant gradient and the maintenance of the polarization.

A well-characterized neutrophil GPCR is the chemoattractant receptor N-formyl peptide receptor (FPR) [39]. The FPR is stored in the membranes of the specific/gelatinase granules and secretory vesicles [64] and translocates to the plasma membrane upon cell stimulation. After binding of an activating ligand to this receptor, but before its phosphorylation by GPCR kinases, it has been shown that FPR is translocated and clustered into plasma membrane lipid rafts, which may be a required process to obtain maximal signalling

activity [65]. After completed cell polarization FPR is asymmetrically distributed with the highest concentration at the front of the cell [66].

There is very little information in the literature concerning lipid raft localization of any other neutrophil GPCR than the FPR. However, it has been shown that the two IL-8 (CXCL-8) receptors (CXCR1 and CXCR2) are neither distributed to the lipid rafts in resting neutrophils nor in IL-8 stimulated cells [67]. In the monocytic cell line MonoMac-6, the stromal cell-derived factor 1 (SDF-1) receptor (CXCR4) is not normally present in lipid rafts, but is recruited to the rafts after LPS stimulation [68]. Similar data is found in SDF-1 stimulated T-lymphocytes [69], where the receptor is localized to lipid rafts in the lamellopodium of the polarized cells. Hence, more studies are needed to elucidate the importance of lipid rafts in signalling from different GPCRs in neutrophils.

GPI-anchored receptors

In the neutrophil plasma membrane, there are a number of GPI-anchored receptors, e.g. $Fc_{\gamma}RIII$ (CD16b) [70], CD66b [71], urokinase-type plasminogen activator receptor (uPAR) [72] and GPI-80 [73]. GPI-anchored proteins are normally localized in lipid rafts [74], and signalling through these receptors is triggered without any physical contact with the inner leaflet of the plasma membrane. One hypothesis on how the signal transduction over the plasma membrane instead is carried out suggests a lateral interaction with transmembrane receptors [75]. E.g., the integrin CR3 was reported as a possible partner for $Fc_{\gamma}RIII$, which in resting cells has bee found to localize both within and without lipid rafts. During neutrophil stimulation with an

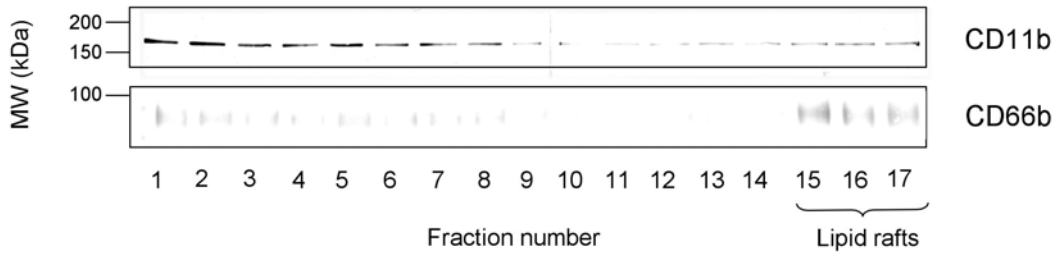

Figure 3. *Distribution of CD11b and CD66b in DRMs of azurophil granule membranes. Isolated membranes from azurophil granules were resuspended in a detergent-containing Percoll solution that was layered under denser Percoll. The DRMs were separated from the detergent-soluble material by flotation during centrifugation. Seventeen fractions were collected from the bottom of the tube and were analyzed by SDS-PAGE and immunoblotting using antibodies towards CD11b and CD66b. Fractions 1-8 correspond to detergent-soluble material, remaining in the lower part of the gradient, while the lipid rafts can be found to have floatated into the upper fractions (12-17).*

anti-CD16b antibody, the $Fc_{\gamma}RIII$ content in lipid rafts increases [76]. By investigating the localization of the CR3 component CD11b in specific granules, we show that the suggested co-receptor in fact localizes to DRMs, but is also found in detergent soluble fractions of the granule membranes (Fig. 3). Whether the increase of lipid raft-localized $Fc_{\gamma}RIII$ in the stimulated plasma membrane is due to incorporation of specific granules with higher concentration of the protein in lipid rafts, or whether the protein diffuses in the membrane into the rafts, remains to be determined.

Galectin-3 binding receptors

One of the GPI-anchored receptors that we have studied is the galectin-3 binding protein CD66b. Galectin-3 is a mammalian lectin with affinity for β-galactosides, especially poly-N-acetyl-lactosaminoglycans [77]. The human galectin-3 is expressed in a variety of cell types but is mainly associated with epithelial and myeloid cells. This lectin is also produced by and secreted from activated macrophages, basophils and mast cells [77]. Because of its saccharide specificity, galectin-3 recognizes and binds to many different glycosylated proteins on the cell surface of a variety of cells and has in this way an influence on many different biological processes. It mediates cell adhesion, cell activation and acts as a chemoattractant [78].

Galectin-3 is evolving into being an important mediator of inflammation [79]. It is involved in numerous parts of the inflammatory process, like recruitment of neutrophils to the inflammatory site and maintenance of the cells in the tissue. Exudated neutrophils have upregulated galectin-3 binding receptors to the plasma membrane from internal storage organelles, and when stimulated with galectin-3, these cells activate their NADPH-oxidase to produce toxic oxygen radicals [80]. We have shown that CD66a and CD66b as well as LAMP-1 and LAMP-2 are potential galectin-3-binding receptors, based on galectin-3 binding studies in neutrophils (Paper III); [81]). We compared the receptor content between neutrophils and differentiated neutrophil-like HL-60 cells that are non-responsive to galectin-3 with regard to NADPH-oxidase activation. The result showed that HL-60 cells lack CD66a and CD66b but contain similar amounts of LAMPs as the neutrophils. We thus concluded that CD66a and CD66b are the more plausible signalling receptors for galectin-3 in neutrophils (Paper III; [81]).

CD66a and CD66b both belong to the carcinoembryonic antigen-related cellular adhesion molecule (CEACAM) family [82]. They differ in their attachment to the membrane; CD66a has a transmembrane domain while CD66b is attached via a GPI-anchor. They are both stored in specific/ gelatinase granules as well as in secretory vesicles (Paper III; [81]). As

CD66b lacks a transmembrane region, the transfer of the signal across the membrane is most probably transduced through an interaction with a transmembrane protein [75]. It has been shown that interactions between CR3 and CD66b take place in the neutrophil during antibody-dependent cytolysis involving the $Fc_{\gamma}RII$ [71]. This process is probably carried out during physical association via lectin-like interactions between CR3 and CD66b. We investigated if CD66b is localized to lipid rafts in granules, and in parallel with other GPI-linked proteins at least part of the specific granule content of CD66b localizes to DRMs (Fig. 3). This suggests that CD66b is in close proximity to CR3 in the membrane and thus could use this transmembrane protein for its signalling.

Since clustering of receptors into lipid rafts facilitates signal transduction, it is not surprising to find that receptors for different kinds of inflammatory mediators translocate to lipid rafts in the plasma membrane during activation. As the result of the neutrophil activation is degranulation and upregulation of receptors from intracellular organelles, the roles played by lipid rafts in the granule/vesicle membranes can only be speculated upon. Localization of receptors to lipid rafts in neutrophil organelles may be a part of a dynamic framework for subcellular organization and regional signalling in the neutrophil and has to be further investigated.

FUNCTIONS OF LIPID RAFTS IN NEUTROPHIL GRANULES

Taken together, it is with little doubt that we conclude that lipid rafts exist *in situ* in the granule membranes, strongly supported by the presence of the lipid raft markers flotillin-1, flotillin-2 and stomatin (paper I; [39], paper II). The azurophil granules are not the only neutrophil organelles containing lipid rafts in their membranes. We found stomatin to be present also in the specific/gelatinase granules and the secretory vesicles and showed that the protein could be mobilized to the cell surface upon exocytosis (paper I; [39]). This is in line with findings reported from epithelial cells showing that DRMs can be isolated from peri-centrosomal vesicles [83], corresponding to late endosomes, and that these structures contain stomatin. Further, the membranes of α-granules in platelets are shown to contain lipid rafts [84], in which flotillin-1, -2 and stomatin are major components.

As stated above, stomatin localized in neutrophil granules and plasma membrane may act as a structural protein as caveolin is not present. Through the interactions between stomatin and the cytoskeleton, stomatin may take part in transport and fusion of the granules with the phagosome and/or the plasma membrane. Whether the protein also has channel-regulating features in the granules can only be speculated upon.

The flotillins may have similar functions to those of stomatin being alternative or complementary proteins used for raft stabilization, as they too are functionally associated with the actin cytoskeleton. They may as well take part in intracellular signalling processes, which could result in granule fusion, either with the phagosome or the plasma membrane. This all has to be further investigated in order to be clarified.

Dysferlin present in the neutrophil granule membranes may play a role in neutrophil granule-granule or granule-plasma membrane fusion. Such granule-granule fusion has in fact been shown for the secretory vesicles [85]. The presence of fusion-mediating proteins other than annexins and synaptotagmins suggest that the fusion processes in the neutrophil are complex events and could possibly be governed by several different activation and signalling pathways.

COMMENTS TO THE TECHNIQUES USED FOR STUDYING LIPID RAFTS IN NEUTROPHIL GRANULE MEMBRANES

Subcellular fractionation and purity

In the papers included in this thesis, neutrophil granules and vesicles are isolated by subcellular fractionation. During this process, the plasma membranes of the cells are broken, leaving the membranes of the organelles intact. The disrupted post-nuclear material, containing the different granules and vesicles, is separated on Percoll gradients according to their buoyant density [86].

We are aware that this technique may allow for minor contaminants in the subcellular organelles. According to the literature, organelles like ER, Golgi, mitochondria and other microsomes end up in the low density fraction together with the plasma membrane during a subcellular fractionation [87-89]. Regarding the fractions containing the azurophil granules used for proteomic studies, we believe that it is as pure as can be achieved with this type of technique. Naturally, small contaminations will always be present in isolations of subcellular organelles, and since LC-MS/MS is such an ultra sensitive technique (see below), proteins from other parts of the cell will most definitely show up in the proteome of one specific part of a cell, even if they are present in extremely small amounts. Therefore, it is of outmost importance to re-evaluate the material using (semi-) quantitative techniques, as we have done by immunoblotting for interesting proteins.

Lipid rafts and detergents

In the original method to isolate DRMs, cells are extracted with a lysis buffer containing 1 % Triton X-100 at 4^{o}C, the post-nuclear lysate is adjusted to 40% (w/v) sucrose and a 5–30% discontinuous sucrose gradient is layered on top. Upon centrifugation, the DRMs will floatate in the gradient and can then be collected in the top fractions [90].

During the years, new methodologies to isolate DRMs from membranes has been introduced and today includes a selection of different detergents as well as centrifugation methods. Both non-ionic detergents like Triton X-100, Nonidet P40 and different types of Brij as well as the zwitterionic detergent CHAPS have been used, each giving slightly different results [24, 91]. As discussed above, this has raised the question if there is heterogeneity between rafts in the membrane that creates these differences or if DRMs are actually artefacts, formed during the different detergent extraction methods [24]. The

general opinion is that some detergents may extract non-raft lipids and proteins from the periphery of the rafts, explaining some of the differences in result between the techniques. It may also be possible that some detergents selectively extract lipids and proteins from inside the raft, giving DRMs which differ somewhat in composition from the original microdomains in the membrane. Finally, it has been shown that detergent extraction causes fusion of rafts during the isolation process [24].

Lipid rafts have also been isolated using non-detergent methods, some of them involving sonication of membranes [24]. With these techniques, the risk of selectively extracting lipids or mixing membranes is a minor problem as no dissolution of membranes takes place. The results indicate that rafts isolated without detergent are to some extent comparable to some forms of rafts isolated by detergents, i.e. there is some overlap in the type of domains isolated by the two different types of techniques. A major difference between the techniques is however that a larger fraction of the lipids from the inner leaflet of the bilayer is maintained in non-detergent isolated rafts than in preparations where detergent methods have been used. This will give lipid rafts with their inner and outer leaflets coupled together, which suggests that non-detergent isolated rafts more closely mimics the *in situ* situation [29, 92, 93].

Although we are aware of problems associated with isolating lipid rafts using a detergent-based method, we have reasons for choosing to do so. Our goal was not to investigate the exact lipid and protein composition of potential subtypes of rafts in the neutrophil azurophil granules but merely to identify some of the proteins that can be located to the raft population in these granules in order to better understand the functions and characteristics of the azurophil granules.

Sucrose or Percoll density gradients

The methods used in this thesis for subcellular fractionation and isolation of DRMs are based on Percoll instead of sucrose as a medium in the density gradient centrifugation. Percoll is a commercial product (Amersham Biosciences) consisting of colloidal spherical silica particles with a size of 15-30 nm, coated with a firm monomolecular layer of polyvinylpyrrolidone**.** Because of the variety in particle size, Percoll will self-generate a continuous gradient when centrifuged in 0.15 M saline or 0.25 M sucrose [94].

Percoll has advantages over sucrose as a gradient medium. The low osmolality of Percoll will keep the shape of the organelles intact during the centrifugation process while sucrose, which has much higher osmolality,

forms a hypertonic gradient in which the organelles are likely to shrink [95]. Further, the low molecular weight of sucrose makes it possible for the sugar molecules to penetrate into cells and subcellular organelles. Differences in granule membrane permeability has also been discovered while using sucrose as a gradient medium compared to Percoll, resulting in leakage in granule membranes isolated by sucrose gradient centrifugation [95]. It has also been shown that plasma membranes isolated by sucrose gradient centrifugation may seal to form vesicles containing the gradient medium [86]. Taken together, we have used Percoll as gradient medium throughout this thesis to obtain pure isolations of subcellular organelles and membrane rafts in a gentle manner.

Mass spectrometric techniques

During the last decade, a variety of spectrometric techniques have been developed for the analysis of biomolecules; MALDI-TOF MS and Nanoliquid chromatography-tandem MS (LC-MS/MS) are two techniques being used in this thesis.

In both used methods, the proteins are first separated by electrophoresis and the protein bands are submitted to in-gel digestion by trypsin, resulting in a unique set of unmodified peptides for every protein. In the first paper in this thesis (paper I; [39]) we used MALDI-TOF MS that was run on a Micromass Tofspec-2E. Here the peptides are ionized by a UV laser in a MALDI ion source and formed ions pass into the TOF mass analyzer, where their masses are calculated by measuring the velocity for each ion, which is proportional to m/z. By using this method we only succeeded in identifying one protein even though the gel contained several protein bands. This was most probably due to low sensitivity of the technique in combination with the presence of several proteins in each band from the 1-D PAGE (i.e., impure samples).

Since measurement of molecular masses was not enough to identify proteins from the complex mixtures that our samples comprised, a fragmentation technique in combination with a tandem mass analysis (MS/MS) was applied. In the nano-LC-MS/MS technique, we used a LTQ-FT instrument (Thermal Finnigan) coupled to an HPLC. The tryptic peptides were first separated on a C_{18} column and then ionized by electrospray. A certain amount of ions were trapped into the first ion trap situated in the LTQ and a portion of them was passed on to the ion trap in the FT-ICR. In the LTQ the ions are fragmented and the masses of both the parental and the daughter ions are calculated. The FT-ICR calculates the masses of the ions with a measurement accuracy of 1-2 ppm. These procedures give the

technique a higher precision in the mass calculation of each peptide and give a more certain identification of the protein.

The advantage with LC-MS/MS, i.e. the ability to detect nanoscale amounts of proteins is also the disadvantage of the method. Extremely small amounts of contaminants will be likely to show up in the mass spectrometry analysis, and since no quantitative data are given with the identification, the results have to be thoroughly confirmed by e.g. western blot or other (semi-) quantitative assays.

Another reason for finding "wrong" proteins in our azurophil granule proteome, e.g. proteins shown to belong to the specific granules, such as CD11b, is that they may in fact be present also in the azurophil granules. According to the hypothesis of "targeting-by-timing", proteins targeted to a certain granule are expressed at the same time as the corresponding granule is formed during cell maturation [12, 96, 97]. When studying the mRNA content in bone marrow cells in different maturations stages, Cowland and Borregaard have found that a certain amount of overlapping exists between the different granules and vesicles [98]. Hence, the synthesis of specific granule proteins may be initiated before the formation of azurophil granules has ceased. We are also aware that the technique we use to isolate organelles may allow for minor contaminants from other cellular parts like the ER, Golgi and mitochondria.

CONCLUDING REMARKS

During the last decade the knowledge on lipid rafts has increased tremendously. It is confirmed that lipid rafts do exist in model membranes, but researchers still disagree not only about their formation, functions, and size *in situ*, but some even doubt their existence. In my opinion, their existence is difficult to doubt based on my own experiences as well as studies of the literature.

Lipid rafts have been shown to participate in cellular processes such as regulation of secretory and endocytic processes and function as anchor structures for organization of signalling molecules. This is all in great interest from the neutrophil point of view. Prior to the studies on which this thesis is based, lipid rafts have been shown to exist in the plasma membrane of neutrophils. However, there was no knowledge on the suborganization of the membranes in neutrophil organelles. By using a well-defined detergent method combined with mass spectrometric techniques, we think that we have strong evidence for the presence of DRMs in neutrophil granule membranes. Further, the characterization of raftophilic proteins like stomatin and flotillins in our preparations implies that the isolated DRMs actually represents lipid rafts. These rafts contain a number of interesting proteins like annexins and dysferlin, which may be of importance for neutrophil effector functions, such as phagolysosome formation and subsequent bacterial killing. It is therefore of great interest that many of the proteins identified by us correspond to proteins found in mouse macrophage phagolysosomes by Garin and co-workers [57]. Further, the recent publication by Lominadze and colleagues [99] show the presence of some, but not all, of the proteins identified by us in one or more of the other neutrophil granules. This suggests that there are similarities between the lipid rafts of azurophil granules and those of the other granule types. Furthermore, the findings that the azurophil granule membranes are linked to the cytoskeleton, just like the plasma membrane is, has provided us with new insights into the organization and complexity of these granules.

From these data, it is tempting to speculate that lipid rafts in neutrophil granules are of importance for regulating the function and participation of the different granules and vesicles during cell activation. The neutrophil azurophil granules are perhaps not only non-lysosomal secretory organelles delivering matrix components to the phagosome but may in fact be part of a dynamic framework of intracellular organization and regional signalling in the neutrophil cytosol. Hopefully, further research in this field will render us with deeper knowledge on these matters.

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