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Novel findings on cellular trafficking and targeting for granule storage of neutrophil elastase, a multifunctional effector molecule of innate immunity

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Novel findings on cellular trafficking and targeting for granule storage of neutrophil elastase,

a multifunctional effector molecule of innate immunity

Doctoral thesis

by

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CONTENTS

Original papers

This thesis is based on the following papers. They are referred to in the text by roman numerals:

I. Tapper H, **Källquist L**, Johnsson E, Persson AM, Hansson M, and Olsson I. Neutrophil elastase sorting involves plasma membrane trafficking requiring the C-terminal propeptide. **Exp Cell Res 312:3471-84, 2006**.

II. *Rosén H, ***Källquist L**, Hansson M, Olsson I. Neutrophil pro-elastase sorting involving the plasma membrane is structure dependent. **Manuscript.**
equal contribution

III. **Källquist L**, Hansson M, Persson AM, Janssen H, Calafat J, Tapper H, Olsson I. The tetraspanin CD63 is involved in granule targeting of neutrophil elastase. **Blood 112:3444-3454, 2008**.

IV. **Källquist L**, Hansson M, Calafat J, Persson AM, Olsson I. Neutrophil elastase and proteinase 3 trafficking routes in myelomonocytic cells. **Submitted for publication.**

Selected Abbreviations

AML	acute myeloid leukemia
AP	adaptor protein
BFA	brefeldin A
BLOC	biogenesis of lysosome-related organelles complex
BPI	bactericidal/permeability-increasing protein
C/EBP	CCAAt enhancer-binding protein
CD63	cluster of differentiation 63
cDNA	complementary deoxyribonucleic acid
CN	cyclic neutropenia
CTL	cytotoxic T lymphocyte
ESCRT	endosomal sorting complex required for transport
ER	endoplasmic reticulum
FKBP	FK506 binding protein
G-CSF	granulocyte colony-stimulating factor
GFP	green fluorescent protein
hCAP-18	human 18-kDa cationic antimicrobial protein
Ig	immunoglobulin
IL	interleukin
IP	immunoprecipitation
kDa	kilodalton
LAMP	lysosome-associated membrane protein
LPS	lipopolysaccharide
M6P	mannose 6-phosphate
MMP	matrix metalloproteinase
MPO	myeloperoxidase
MPR	mannose 6-phosphate receptor
mRNA	messenger ribonucleic acid
MVB	multivesicular body
NE	neutrophil elastase
NET	neutrophil extracellular trap
NGAL	neutrophil gelatinase-associated lipocalin
NK	natural killer
PR3	proteinase 3
RAB	Ras-related in brain
RBL	rat basophilic leukemia/mast cell tumor line
RNA	ribonucleic acid
SCN	severe congenital neutropenia

SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SLPI	secretory leukocyte peptidase inhibitor
SNARE	SNAP and NSF attachment receptors
TGN	trans-Golgi network
TLR	toll like receptor
TNF-α	tumor necrosis factor α
VAMP	vesicle associated membrane protein

Introduction

Neutrophil granulocytes evolved as components of the innate immune system for the survival of the host. They are essential in first-line defence, combating invading pathogens, which they engulf and destroy in the process of phagocytosis. Accordingly, a deficiency of the antimicrobial effector molecules of neutrophils may lead to life-threatening infection. Importantly, after their release, the same molecules control immune responses and inflammation, but may damage tissues at the site of inflammation. Among the effector molecules within neutrophil stores (granules) are hematopoietic serine proteases. My thesis concerns this family, especially one member, the multifunctional neutrophil elastase (NE).

Newly synthesized neutrophil effector molecules travel by the secretory pathway to reach the Golgi complex and become subject to sorting, a dynamic process in which proteins are directed to their final destinations by vesicular transport. Along the way, during the early stages of cell maturation, the granules become tightly packed with proteins that are later then unloaded in phagocytic vacuoles and the extracellular environment when needed to protect the host when mature neutrophils encounter microbes and inflammation. As to diseases, mutations of the NE gene can lead to the destruction of neutrophils by apoptosis (programmed cell death) and thus to a shortage of these cells (neutropenia).

NE does not have a known sorting amino acid sequence and its sorting mechanism for granule storage has been elusive. A hypothesis was tested in this study, based on the prediction that a transmembrane carrier protein helps in the vesicular transport of soluble protein to granules. The postulated carrier protein was assumed to offer transport machinery to benefit NE sorting. The reason for pursuing this hypothesis was to increase knowledge of neutrophil biology and to open the way to novel ideas such as stimulating an antibiotic response or inhibiting the detrimental effects of excess serine proteases in inflammation.

Background

Neutrophils and monocytes in innate immunity

The immune system is commonly divided into innate and adaptive subsystems. The innate immune system is evolutionarily older than the adaptive and provides immediate defence against infection. Important features of innate immunity are the recruitment of immune cells to sites of infection, killing of pathogens, removal of apoptotic cells, and presentation of antigens to cells of the adaptive immune system. Specialized white blood cells are important in the innate immunity. Phagocytes, including neutrophils (polymorphonuclear leukocytes) and monocytes/macrophages, are responsible for phagocytosis and killing of invading pathogens. NK cells kill both infected cells and tumor cells (Beutler, 2004; Tosi, 2005).

Development from the common myeloid progenitor into granulocyte/macrophage progenitor is governed by the transcription factor PU.1; C/EBPs are important for final granulocyte maturation and Maf and Jun transcription family members for final monocyte development (Friedman, 2002). Transcription factors are expressed sequentially during neutrophil differentiation, governing the different stages of maturation. Factors important during the myeloblast/promyelocyte stage are thereafter downregulated while factors for myelocyte/metamyelocyte development are upregulated and so on until mature neutrophils emerge (Bjerregaard et al., 2003).

Neutrophils are referred to as the first line of immune defence, being the first to arrive at inflammation/infection sites. These cells are identified by their lobulated nuclei and cytoplasmic granules. The consecutive developmental stages of neutrophils are myeloblast, promyelocyte, myelocyte, metamyelocyte, band cell and mature segmented neutrophil. Four kinds of granules are developed sequentially during maturation. The formation of granules during maturation is shown in figure 1. Azurophil granules are the first to be produced, at the promyelocyte stage.

Granule protein constituents are also produced sequentially during neutrophil maturation. The first proteins, generated in early precursors, are packed in azurophil granules, while proteins produced later during maturation are packed in specific and gelatinase granules (Borregaard and Cowland, 1997). The synthesis of granule proteins is under transcriptional regulation (Borregaard et al., 2001).
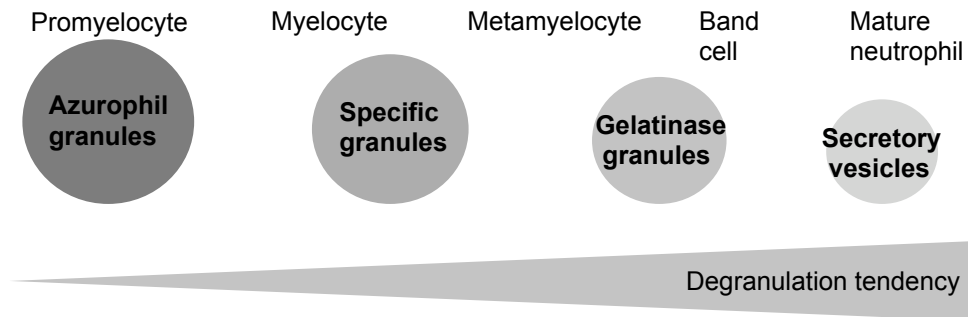
Figure 1. Granule formation during neutrophil maturation and degranulation propensity. Granule constituents are produced sequentially during neutrophil maturation and packed in granules formed at the same stage of maturation (Borregaard and Cowland, 1997). The degranulation tendency (bottom) is opposite of the order of formation.

Neutrophils are fully developed when they are released from bone marrow into circulation. Their release marks the end of a five- to seven-day development and the beginning of the end of the short lived neutrophil, the half-life of which is only six hours in blood. Neutrophils are needed during infection/inflammation. They are first recruited from a marginated pool. In addition, the release of neutrophils from bone marrow is accelerated and neutrophil production is increased to meet the need. (Walker and Willemze, 1980).

Monocytes constitute a second wave, being summoned by neutrophils. They develop into macrophages, which finally clear the inflamed site by phagocytosing apoptotic neutrophils. Monocytes are similar to neutrophils in many ways, but unlike neutrophils they play a role in both innate and acquired immunity. The expression of Toll-like receptors (TLRs) for the recognition of conserved structures on pathogen surfaces is much higher on monocytes than on neutrophils. Monocytes spend two to three days in the blood, surviving much longer than neutrophils. (Kantari et al., 2008).

The monocyte developmental series consists of monoblast, promonocyte and mature monocyte. In contrast to neutrophils, blood monocytes are not fully mature and differentiation into macrophages occurs in tissue. Resident macrophages arise continuously from blood monocytes. During inflammation/infection, monocytes are recruited more slowly than neutrophils, but remain longer in tissue. It has been suggested that two different monocytic subsets are responsible for these two assignments: continuous recruitment into normal tissue and recruitment of inflammatory monocytes (Mobley et al., 2007).

Monocyte granules have a composition partly similar to that of neutrophils and the granules decrease in number during differentiation into macrophages (Kantari et al., 2008; Kargi et al., 1990). Monocytes have a strong capacity for biosynthesizing cytokines, the importance of which is seen in the regulation of hematopoiesis and potentiation of an early inflammatory response. They are also precursor cells to dendritic cells, which are involved in antigen presentation during T-lymphocyte activation (Kantari et al., 2008).

Adhesion to the vessel wall and transmigration

A multi-step cascade of events is needed for neutrophils/monocytes to adhere to the endothelial cell layer, the first step of transmigration. Adhesion molecules called selectins mediate the first steps; capture and rolling. L-selectin (on leukocytes) and E- and P-selectin (on endothelial cells) interact with their ligands to promote tethering between endothelial cells and neutrophils/monocytes. L-selectin also mediates neutrophil-to-neutrophil association, which increases the number of such cells arresting (McEver and Cummings, 1997). P-selectin rapidly appears at the endothelial cell surface when stimulated by inflammatory mediators, while E-selectin appears more slowly, as it demands new synthesis (Lawrence and Springer, 1993). The integrin adhesion molecules are activated by chemokines for the next step; slow rolling and firm arrest of neutrophils/monocytes on the endothelial cell layer. The integrin LFA1 is important for neutrophil binding to its ligand ICAM1 and the integrin VLA4 is important for monocyte binding to VCAM1. Chemokines increase the affinity of integrins, promoting neutrophil/monocyte adhesion (Ley et al., 2007).

Neutrophils/monocytes crawl along the endothelium to find a suitable transmigration site (Phillipson et al., 2006; Schenkel et al., 2004). They must traverse the endothelial cell layer, the underlining basement membrane and the pericyte sheath. Passing the endothelium may take between two and five minutes and crossing the basement membrane may require additional five to 15 minutes. The firm binding of neutrophils to endothelial cells by integrins facilitates transmigration (Ley et al., 2007). Neutrophils usually pass between endothelial cells (the paracellular route), but rapid transcellular migration can occur in thin parts of the endothelial cell layer (Feng et al., 1998). Migration through the endothelium basement membrane preferentially occurs where the density of matrix proteins is low; these sites often coincide with pericyte gaps. Digestion catalyzed by NE and matrix metalloproteinases may facilitate crossing the basement membrane (Ley et al., 2007; Wang et al., 2006). The constitutive migration of monocytes into tissue is dependent upon chemokines and integrins different to those that are upregulated in the inflammatory setting (Kantari et al., 2008).

Phagocytosis and killing

The most important task for neutrophils is engulfing and destroying invading microorganisms. Monocytes also phagocytose pathogens and macrophages engulf apoptotic neutrophils. Phagocytosis is mediated by cell-surface receptors, several of which are shared by both neutrophils and monocytes (Greenberg and Grinstein, 2002). Fcγ receptors bind to IgG-opsonized pathogens, triggering membrane remodelling leading to the particle becoming covered in protrusions. Complement-opsonized particles bind to receptors, inducing a slightly different ingestion, with the pathogen sinking into the cell. This difference in phagocytosis is caused by diverse intracellular signalling through the respective receptors (Caron and Hall, 1998).

The membrane-bound vesicle containing the pathogen is called the phagosome. It matures into a late phagosome following fusion with late endosomes, after which a phago-lysosome is formed by fusion with lysosomes/azurophil granules. The fusion steps are controlled by Rab and SNARE proteins and may be complete or of the "kiss-and-run" kind, when endosome content is delivered, without merging of the two vesicles. Material also leaves the phagosome through small vesicles, travelling in a retrograde fashion (Haas, 2007).

LAMPs are important regulators in the maturation of phagosomes. In mice lacking both LAMP-1 and LAMP-2, phagosome maturation halts before fusion with late endosomes and bacteria killing is thus not achieved (Binker et al., 2007). Neutrophils isolated from LAMP-2 knock-out mice showed impaired fusion between phagosomes and lysosomes and decreased killing capacity, indicating that LAMP-2 is particularly important for phagosome maturation (Beertsen et al., 2008).

Neutrophils use both oxygen-dependent and oxygen-independent mechanisms to kill microorganisms. The NADPH oxidase complex is assembled after phagocytosis to generate O_2-, which is converted into highly reactive oxygen intermediates to kill bacteria (Burg and Pillinger, 2001). The oxygen-independent mechanism relies on a repertoire of preformed granule proteins, such as proteases and antimicrobial peptides, which are released into the phagosome. Oxygen-dependent and oxygen-independent mechanisms overlap; for example, MPO from azurophil neutrophil/monocyte granules helps in the conversion of hydrogen peroxide (H_2O_2) into highly toxic hypochlorous acid (HOCl) (Klebanoff, 2005). It was recently shown that neutrophils are also capable of trapping and killing bacteria in the extracellular environment by expelling long threads consisting of chromatin and proteases such as NE. These are called NETs, and their production kills the neutrophils in a manner that does not resemble either apoptosis or necrosis (Brinkmann et al., 2004; Fuchs et al., 2007).

Neutrophils are the first cells to arrive at an infected site and ideally they enter tissues, phagocytose and destroy invading pathogens, commit suicide, and in turn are phagocytosed by monocytes/macrophages to prevent tissue damage and prolonged inflammation. Macrophages attracted to the infection site produce anti-inflammatory agents (e.g. IL-10, IL-13, and TGF-β) to phase out the inflammatory response. They also delay further neutrophil recruitment and stimulate monocyte chemotaxis (Kantari et al., 2008). Protectins and resolvins, which are fatty acid-derived lipid mediators, influence the resolution of inflammation (Bannenberg et al., 2007).

Apoptosis and the removal of neutrophils are important in order to minimize damage to surrounding tissues. Many bacteria have been shown to induce the death of neutrophils and other phagocytes upon phagocytosis (Kennedy and Deleo, 2008). Apoptotic neutrophils expose cell surface phosphatidyle serine, which is recognized by macrophages the receptors of which either directly or indirectly bind to the apoptotic cells and initiate their removal (Taylor et al., 2005). Macrophages switch from producing pro-inflammatory to anti-inflammatory cytokines upon the phagocytosis of apoptotic neutrophils, promoting resolution of the infection (Fadok et al., 1998). Thus, even though neutrophils are crucial in host defence against infection, neutrophil apoptosis is a key event in the resolution and maintenance of homeostasis. Under some circumstances the exclusion of neutrophils from the scene in injury/inflammation may therefore be beneficial for healing, provided that pathogens are absent such as in aseptic surgery (Dovi et al., 2003).

Secretory lysosomes/Lysosome-related organelles

Secretory lysosomes are organelles containing both secretory proteins and lysosomal constituents including hydrolases and lysosomal membrane proteins such as LAMPs. They combine the functions of lysosomes and secretory organelles. Secretory lysosomes are found in hematopoietic cells—such as CTLs, NK cells, platelets, basophils, and neutrophils—and in melanocytes (Dell'Angelica et al., 2000). Melanocytes originate from the neural crest and store melanin in secretory lysosomes called melanosomes (Orlow, 1995) Melanin gives rise to hair and skin colour and protect against UV damage (Brenner and Hearing, 2008).

The secretory lysosomes of CTLs and NK cells, called lytic granules, are acidic and electron-dense and store lysosomal enzymes, granzymes and perforins (Bossi and Griffiths, 2005). Granzymes cause cell death in target cells, gaining access with the aid of perforin (Cullen and Martin, 2008). Platelet dense granules containing serotonin,

ATP and ADP, which are essential for platelet aggregation, are also regarded as secretory lysosomes (McNicol and Israels, 1999). Genetic disorders that affect melanosomes/lysosomes also affect these granules (Dell'Angelica et al., 2000). Basophil granules contain LAMPs, agents released during allergic reactions, and hydrolases, qualifying them as secretory lysosomes (Dell'Angelica et al., 2000).

The primary/azurophil granules of neutrophils may also be called secretory lysosomes or lysosome-related organelles as they contain lysosomal enzymes and other agents, which are secreted into phagosomes and extracellularly. These granules contain LAMP-3 (Cham et al., 1994) but lack LAMP-1 and LAMP-2 (Cieutat et al., 1998; Dahlgren et al., 1995), making their classification difficult. However, the function of azurophilic granules is also affected in disorders of secretory lysosomes such as *Chediak-Higashi syndrome* (see below).

Mature secretory lysosomes usually contain an electron-dense core. They arise from intermediate multivesicular endosomes (Bossi and Griffiths, 2005) with many internal vesicles formed by invaginations of the limiting membrane. These invaginations are dependent on the escorting protein ESCRT. The sorting of ubiquitylated proteins into these vesicles and the dissociation of newly formed MVBs from endosomes require specific factors (Gruenberg and Stenmark, 2004). The core becomes denser as newly synthesized lysosomal and cell-specific proteins accumulate at the same time as the number of internal vesicles decreases.

BLOC protein complexes are connected to the formation and trafficking of lysosome-related endosomal compartments. They may also regulate SNARE-mediated membrane fusion, which is important in vesicle trafficking (Huizing et al., 2008). LAMP-1 and LAMP-2 are found in the limiting membranes of MVBs while LAMP-3 (CD63) is concentrated to inner vesicles (Escola et al., 1998). The Lyst protein is involved in the fusion of secretory lysosomes with the plasma membrane. Rab27a seems to be exclusively associated with secretory lysosomes and appears to play a role in secretion (Blott and Griffiths, 2002).

Internal vesicles are secreted as so-called exosomes. These exosomes contain a limited variety of lysosomal proteins, annexins and Rabs (which are important for membrane fusion in the endocytic pathway), heat-shock proteins, and cell-specific proteins. Exosomes may be involved in intercellular communication, membrane exchange between cells, the elimination of degradation-resistant proteins, and antigen transfer to DCs (Thery et al., 2002).

Human disorders of secretory lysosomes.

Understanding of the biogenesis of, and sorting to, secretory lysosomes has been facilitated by studies of disorders that affect them. A brief summary is given here, but a complete review is available by Huizing et al. (Huizing et al., 2008). A distinctive feature of disorders of secretory lysosomes is hypopigmentation, which is caused by abnormal melanosomes of melanocytes.

Patients with *Hermansky-Pudlak syndrome* show hypopigmentation because of impaired melanosome formation. A lack of dense granules in platelets gives rise to a bleeding tendency. Eight different gene products are associated with this syndrome, all belonging to the BLOC-2 and BLOC-3 protein complexes, which have a role in the formation or trafficking of secretory lysosomes.

Hermansky-Pudlak syndrome type 2 is characterized by hypopigmentation, neutropenia, and immunodeficiency due to impaired cytolytic activity in CTLs. The cause of this syndrome is a mutation in the AP-3 complex that leads to abnormal sorting of lysosomal transmembrane proteins.

Patients with *Chediak-Higashi syndrome* also show decreased pigmentation and a bleeding tendency and have typical giant lysosomes. This syndrome is caused by mutations of the gene coding for CHS1/LYST, which has a role in membrane trafficking.

Griscelli syndrome features hypopigmentation and immunological impairment but not a bleeding tendency. Mutations in the gene coding for the actin-associated myosinVa motor protein gives rise to *Griscelli syndrome type 1*, mutations of Rab27a to type 2, and mutations of melanophilin, a Rab effector protein, to type 3. All of these mutations lead especially to defects in melanosome transport and lytic granule targeting in CTLs.

Neutrophil granules—contents and functions

Neutrophil granules are divided into subtypes based on their contents and appearance (Borregaard and Cowland, 1997). Different degrees of sensitivity to Ca^{2+} elevation may explain the specific order of degranulation. Secretory vesicles are released first, then gelatinase, specific and at last azurophil granules; the granules are, in this order, decreasingly sensitive to Ca^{2+}. Certain members of the SNARE family are believed to

be necessary for vesicle fusion; neutrophil granules contain, for example, the SNARE homologs Syntaxin 4 and VAMP-2. The granules only need to fuse with membrane once, unlike other vesicles in the endosomal machinery, and may therefore not need all the SNARE homologs. The order of degranulation may be affected by the level of VAMP-2, which is highest in secretory vesicles and lower in gelatinase and specific granules (Borregaard et al., 2007; Ligeti and Mocsai, 1999; Tapper, 1996).

Figure 2 shows the contents and function of the neutrophil granules. Peroxidase-positive *primary/azurophil granules*, which have an affinity for the basic dye azure A, are the first to develop in myeloblasts/promyeolocytes. They can be subdivided into smaller defensin-poor granules, which appear first, and larger defensin-rich granules, which are produced later (Borregaard and Cowland, 1997). Membrane proteins such as CD63 and CD68 are found in these granules along with a number of soluble antibacterial proteins and proteases (Borregaard et al., 2007). Among the granule proteins are MPO, which is involved in the conversion of H_2O_2 into HOCl; defensins, which are small antimicrobial and toxic peptides; and BPI, which kills Gram-negative bacteria and can bind to LPS on a bacterium's surface in order to damage the membrane and promote phagocytosis. The serine proteases NE, cathepsin G, and PR3, discussed in depth below, are very effective against a broad range of bacteria. Azurophil granules preferentially fuse with phagosomes but they may fuse with the plasma membrane as well (Faurschou and Borregaard, 2003; Nathan, 2006).

	Azurophil granules	Specific granules	Gelatinase granules	Secretory vesicles	Functions
Membrane proteins		CD11b/CD18 CD66, CD67	CD11b/CD18 CD67	CD11b/CD18 CD67	**Adhesion**
		Gp91phox/p22phox	Gp91phox/p22phox	Gp91phox/p22phox	**Bacterial killing**
			MMP25	MMP25	**Digestion**
				IFN-αR1, TNFR1	**Immune response**
	CD63 CD68	SNP-23, VAMP-2	SNAP-23, VAMP-2	SNAP-23, VAMP-2	**Vesicle fusion** / **Other**
Matrix proteins	NE, PR3, Cathepsin G	Collagenase, Gelatinase	Gelatinase, Arginase 1		**Bacterial killing, immune response**
	Defensins, BPI, MPO, Lysozyme	hCAP18, NGAL, Lysozyme, Lactoferrin	Lysozyme		**Bacterial killing**
	Azurocidin	SLPI		Plasma proteins	**Other**

Figure 2. Major contents of neutrophil granules are shown, along with the general functions of the proteins mentioned. Adapted from (Borregaard et al., 2007)

Peroxidase-negative *secondary/specific granules* develop in myelocytes and metamyelocytes (Borregaard and Cowland, 1997). The membranes of specific granules contain adhesion molecules and members of the NADPH-oxidase complex. Among the soluble matrix proteins are metalloproteinases and a number of bactericidal proteins including iron-binding lactoferrin, NGAL, and lysozyme (figure 2). Specific granules are released after gelatinase granules, when the neutrophils have entered tissue, and the antimicrobial effects are the most important. (Borregaard et al., 2007; Faurschou and Borregaard, 2003; Nathan, 2006). The carboxy-terminal part of hCAP18 from specific granules is cleaved off to generate the antimicrobial peptide LL-37, which effects both Gram positive and Gram-negative bacteria as well as being chemotactic for neutrophils (Faurschou and Borregaard, 2003). This cleaving occurs extracellularly and is catalyzed by PR3, demonstrating the necessity of the joint release of azurophil (for PR3) and specific granules (for hCAP18) in the generation of an antimicrobial peptide (Sorensen et al., 2001).

Peroxidase-negative *tertiary/gelatinase granules* are produced during the band cell stage of neutrophil maturation (Borregaard and Cowland, 1997). The membrane contains adhesion molecules, elements of the NADPH-oxidase complex, receptors, and metalloproteinases. The matrix contains various antimicrobial proteins (Borregaard et al., 2007). Gelatinase granules are released after secretory vesicles, when the neutrophils are crossing the endothelial cell layer. Thus, gelatinase (MMP-9) may help

in transmigration by degrading basement membrane collagen. Aiding matrix degradation during transmigration and the transfer of important receptors to the surface are the most important tasks of gelatinase granules (Borregaard and Cowland, 1997; Faurschou and Borregaard, 2003).

Secretory vesicles are produced in mature neutrophils and are thus the last of the neutrophil organelles to be produced, although they are the most easily released. Secretory vesicles may already fuse with the cell membrane upon rolling, possibly due to selectin stimulation (Borregaard and Cowland, 1997). Their membrane proteins include integrins—to mediate the firm adhesion of neutrophils to endothelial cells— and various receptors for the recognition of complement, bacterial structures and inflammatory mediators. All these membrane proteins prime the neutrophil for transmigration and subsequent pathogen encounter. The secretory vesicle matrix contains various plasma proteins, a reflection of the likely endocytic origin of these organelles (Borregaard et al., 2007; Faurschou and Borregaard, 2003).

Sorting mechanisms for granule proteins of neutrophils

Sorting of transmembrane proteins

The granule targeting of transmembrane proteins may occur via a direct pathway from the TGN to late endosomes or via an indirect pathway with a detour over the plasma membrane and endocytosis. Transmembrane proteins require amino acid sorting signals at cytoplasmic carboxy-terminal tails for targeting (Bonifacino and Traub, 2003). On lysosome tyrosine-based targeting signals, see the discussion on lysosome-associated membrane proteins and tetraspanins below. Sorting involves clathrin-coated vesicles originating from both the cell surface and the TGN (Kirchhausen, 2000).

Adaptor proteins bind to the carboxy-terminal sorting motifs of transmembrane proteins, after which a clathrin coat is recruited to form transport vesicles for sorting. AP-1, AP-3 and AP-4 are found at the TGN and endosomes and AP-2 at the plasma membrane. APs are heterotetramers with four subunits, two large, one medium, and one small. The larger subunits each have a hinged domain that resembles the "ears" of the "head" of the larger core subunits (Robinson, 2004; Robinson and Bonifacino, 2001). AP-3 can be found on a tubular endosomal compartment but not on the same buds as AP-1. AP-3 colocalizes with clathrin in early/sorting endosomes, although not to the same degree as AP-1 (Peden et al., 2004). AP-1 and AP-3 seem to mediate sorting into distinct vesicles and lack of clathrin affects all four adaptor proteins (Chapuy et al., 2008). AP-3 seems to be responsible for trafficking proteins from the

TGN directly to endosomes (Dell'Angelica et al., 1999). Alternatively, AP-3 may be responsible for trafficking from an early endosomal compartment and lack of AP-3 would thus increase the recycling from early endosomes to plasma membrane (Starcevic et al., 2002). From there, endocytosis would occur, a process probably dependent on AP-2, which recognizes YXXØ sorting signals (Evans and Owen, 2002). AP-2 and clathrin are needed for the delivery of LAMPs to lysosomes, which is consistent with the involvement of an indirect pathway requiring endocytosis at the plasma membrane (Janvier and Bonifacino, 2005).

Sorting of soluble proteins

The gene expression of the content of each granule type is activated in succession, thereby ensuring that proteins synthesized during the same time frame are delivered into the same granules. The forced expression of NGAL (which is normally found in specific granules) in the promyelocytic cell line HL-60 leads to sorting to azurophil granules, the only granule type found in this cell line (Le Cabec et al., 1996). Thus the different granule proteins might not need individual sorting signals; the time difference in expression may allow them to share sorting mechanisms.

Sorting of lysosomal enzymes is dependent upon the MPR, which binds to M6P (Kornfeld and Mellman, 1989) Granzymes A and B of CTLs have been shown to depend on M6P sorting (Griffiths and Isaaz, 1993). Sorting by retention has been proposed for neutrophil granules, where condensation or aggregation of proteins in the TGN would ensure sorting, for example by interaction with negatively charged molecules such as proteoglycans. Aggregation would allow proteins without sorting signals to be sorted with or without transmembrane contact (Gullberg et al., 1997). This could also be a passive process, with many proteins flowing en masse into immature granules budding off from the TGN. During granule maturation proteins not belonging there will be removed. The sorting-for-entry hypothesis instead claims that the principal sorting takes place at the TGN, where proteins need to interact with receptors or other proteins already attached to sorting receptors in order to be transferred into maturing granules; aggregation could also be part of this process (Arvan and Castle, 1998).

Hematopoietic serine proteases—control of inflammation

The neutrophil serine proteases include NE, PR3, cathepsin G, and the catalytically inactive azurocidin (Korkmaz et al., 2008b). The genes for NE and PR3 are expressed

in the same cluster as azurocidin on the short arm of chromosome 19 (Zimmer et al., 1992), and have a sequence similarity of between 50 and 60 % (Hajjar et al., 2007; Korkmaz et al., 2008b).

Biosynthesis and processing of NE and PR3

The biosynthesis of NE/PR3 starts in early promyelocytes, at the same time as the formation of azurophil granules (Garwicz et al., 2005), and tapers off during cell maturation (Cowland and Borregaard, 1999). The transcription factors c-Myb, C/EBP-α and PU.1 may be involved in transcriptional regulation of the NE promoter (Bjerregaard et al., 2003; Lausen et al., 2006). NE is also produced in promonocytes, and can be found in mature monocytes (Campbell et al., 1989). The outline of the NE protein is shown in figure 3. Translation gives rise to a preproprotein containing an amino-terminal signal peptide, a prodipeptide, and a carboxy-terminal propeptide (Takahashi et al., 1988). The amino-terminal signal peptide is cleaved off in the ER. The remaining proNE is glycosylated and the oligosaccharides are converted to complex forms in the Golgi. Some proNE is released by constitutive secretion. The amino-terminal and carboxy-terminal propeptides are removed in pregranule/granule structures, generating mature 29 kDa enzymatically active NE for storage (Gullberg et al., 1997; Lindmark et al., 1994). The carboxy-terminal NE propeptide does not seem to be necessary for granule targeting or for the activation of proNE into NE (Gullberg et al., 1995). PR3 is also produced as a preproenzyme, with an amino-terminal signal peptide, a prodipeptide, and a carboxy-terminal propeptide (figure 3). Newly synthesized 32 kDa glycosylated proPR3 is processed in the Golgi into 35 kDa proPR3, which is stored as mature 32 kDa PR3 after propeptide cleavage (Garwicz et al., 1997).
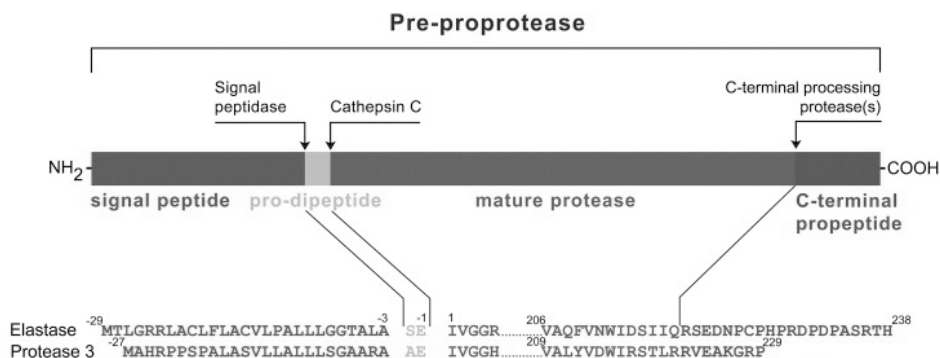
Figure 3. Outline of NE and PR3 protein including propeptides. The signal peptide is removed in ER. The pro-dipeptide is removed by cathepsin C to generate enzymatically active NE/PR3 in pregranule/granule structures. The carboxy-terminal propeptide is also removed before final granule targeting. Adapted from (Korkmaz et al., 2008b).

The enzyme responsible for removing the amino-terminal prodipeptide in order to produce mature protease is cathepsin C (McGuire et al., 1993). The carboxy-terminal propeptide is removed by a hitherto unknown enzyme or enzymes. In *Papillon-Lefèvre syndrome*, mutations of the cathepsin C gene are, as expected, associated with a lack of catalytically active proteases in neutrophils and monocytes (Hart et al., 1999; Toomes et al., 1999). This syndrome is distinguished by palmoplantar hyperkeratosis and early-onset periodontitis, illustrating the pathophysiological consequences of a lack of hematopoietic serine protease activity. The periodontitis is linked to a lack of normal production of the antimicrobial and chemotactic LL-37 peptide, whose production is catalyzed by PR3 (de Haar et al., 2006; Sorensen et al., 2001). Thus, patients with *Papillon-Lefèvre syndrome* have no LL-37 because they lack enzymatically active PR3.

Biochemistry of NE and PR3

Mature NE/PR3 is folded so that the catalytically active domain is located in a crevice between two β-barrel domains and the three-dimensional structure is secured by four disulfide bridges. The folding then brings together the amino acids of the catalytical triad, His57, Asp102, and Ser195 (see figure 4). NE cleaves peptide bonds on the carboxy side of hydrophobic amino acids like glycine, alanine, and valine. A multistep reaction dependent on Ser195 takes place when the protease binds to substrate. The substrate peptide is cleaved and the enzyme restored. The amino acids at specific subsites surrounding the catalytical triad determine the substrate preferences of NE and PR3 (Korkmaz et al., 2008b).

17
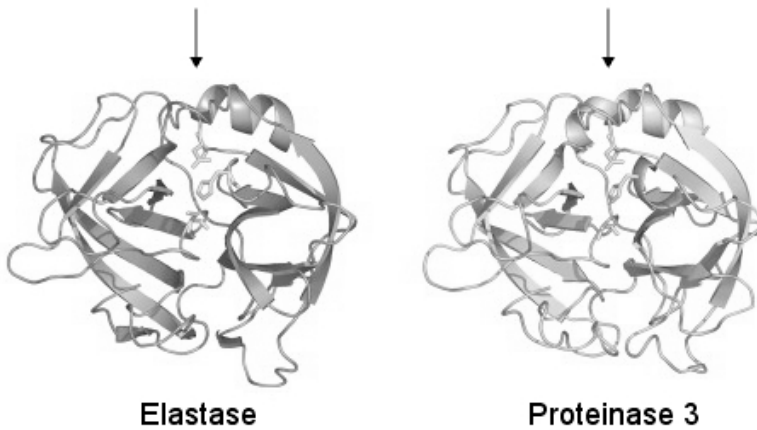
Figure 4. Three dimensional structure of NE and PR3. The catalytical triad resides in the centre (arrows). His57, Asp102, and Ser195 are brought together by folding and conformational changes after pro-dipeptide removal, activating the proteases. Adapted from (Korkmaz et al., 2008b).

Hematopoietic serine proteases are highly cationic proteins. Their cationic nature depends on a positively charged cluster, which is established in NE (figure 5) by conformational changes during the conversion of proNE into NE. This implies that catalytically active proteases, released by degranulation, have higher charge than the corresponding precursor proteases (Korkmaz et al., 2007). Thus, secreted NE may bind to the sulfate groups of chondroitin sulfate-containing and heparan sulfate-containing proteoglycans on the surface of neutrophils (Campbell and Owen, 2007). In contrast, the cell surface-bound mature PR3 of neutrophils (Witko-Sarsat et al., 1999) seems to be associated with the plasma membrane not by charge but by a hydrophobic surface patch on the protease (Korkmaz et al., 2008a). This association is important, as the membrane-bound PR3 of neutrophils is thought to be the target antigen of anti-neutrophil cytoplasmic antibodies, which may activate neutrophils and provoke the perivascular inflammation of Wegener's granulomatosis (van der Geld et al., 2001).
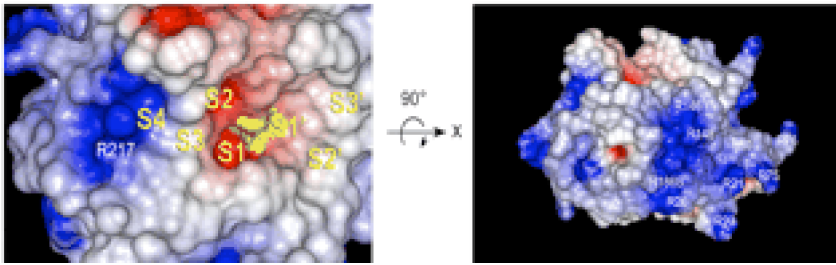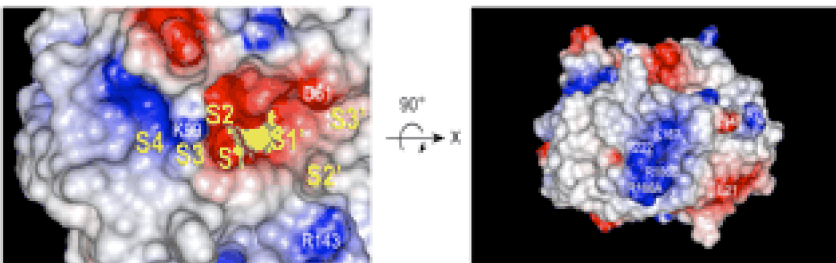
Figure 5. **Positively charged sites of NE and PR3.** The positively charged cluster of NE (top) and PR3 (bottom) is shown in blue, opposite of the catalytic binding site (yellow). The charged cluster of PR3 is much smaller than for NE as it is disrupted by negatively charged residues (red). Adapted from (Korkmaz et al., 2007).

The serine proteases are regulated by inhibitors that are present in blood and tissues to prevent improper extracellular enzyme activity. One family of natural inhibitors is the serpins (serine protease inhibitors), of which the α1-protease inhibitor is an example. Serpins form covalent bonds with the serine proteases. The reactive centre loop of the inhibitor binds to the active site of the protease and cleaves off its loop, which stays inside the active pocket, thereby inactivating both the protease and the inhibitor. The α1-protease inhibitor preferentially inactivates NE but also inactivates PR3. Another protease inhibitor, SLPI, inhibits NE and cathepsin G (Korkmaz et al., 2008b). The complexity of the interaction between protease and inhibitor is exemplified by NE and SLPI; neutrophils both produce and inactivate SLPI, which protects itself by suppressing the neutrophil respiratory burst, and SLPI blocks tissue

proteolysis, making the extracellular presence of NE by necessity a highly regulated occurrence (Nathan, 2006).

Role in infection and inflammation

NE has the ability to kill bacteria in phagosomes such as *E. coli and P. Aeruginosa* by means of degrading their outer membranes (Belaaouaj et al., 2000; Hirche et al., 2008). NE has also been found to cleave virulence factors of the enterobacteria *Shigella, Salmonella* and *Yersinia* (Weinrauch et al., 2002). These functions mainly take place inside cells, when phagosomes fuse with azurophil granules, but NE can also be deposited extracellularly, by degranulation. Secreted NE must overcome serine protease inhibitors or make close enough contact with its target to exclude inhibitors. Secreted NE can also be concentrated to the surface of the neutrophil by the binding to proteoglycans (Campbell and Owen, 2007). If not inhibited, secreted NE can degrade extracellular matrix components such as laminin (Heck et al., 1990), collagen (Gadher et al., 1988) and proteoglycans (Janusz and Doherty, 1991). NE is also involved in coagulation (Allen and Tracy, 1995). Proinflammatory cytokines such as TNF-α and its receptor are also targets of NE. Furthermore, NE targets integrins, leading to an increase of neutrophils in inflammatory sites; modifies the activity of chemokines; increases platelet aggregation; and activates TLR4 (Pham, 2008; Wiedow and Meyer-Hoffert, 2005).

PR3 is perhaps best known for its involvement in *Wegener's granulomatosis.* PR3 is found on the surface of neutrophils in patients of this disease, who have autoantibodies directed against PR3/neutrophils (van der Geld et al., 2001). PR3 is also present on the surface of activated neutrophils. Surface-bound PR3 is quite resistant to protease inhibitors (Campbell et al., 2000). The CD177/NB1 protein seems to be responsible for the attachment of mature PR3 (Bauer et al., 2007; von Vietinghoff et al., 2008). Like NE, PR3 can degrade extracellular matrix components such as fibronectin, laminin, vitronectin, and collagen type IV (Rao et al., 1991); activate IL-8 (which attracts neutrophils) (Padrines et al., 1994); and modify the activity of chemokines and cytokines (Pham, 2008). Importantly, as noted above, PR3 catalyzes the conversion of hCAP18 into LL-37 (Sorensen et al., 2001). ProPR3 has been implicated in the regulation of granulopoiesis (Skold et al., 1999).

In conclusion, NE/PR3 mainly influences the inflammatory response by activating specific receptors and proteolytically modifying cytokines and chemokines rather than by killing microorganisms or as a degradative enzyme. In effect, both NE and PR3 have strong effects on cell recruitment and cell migration, prolonging or curtailing the inflammatory response, activation of lymphocytes, and induction of apoptosis (Pham, 2008).

Serine protease knockouts

Mice lacking NE have an increased susceptibility to sepsis when challenged with Gram-negative bacteria such as *K. pneumoniae* and *E. coli*, but not with Gram positive bacteria (*S. aureus*). Neutrophils are still attracted to the infection site and migrate there normally, but NE is obviously needed for the efficient elimination of the bacteria (Belaaouaj et al., 1998). An increased susceptibility to fungal infections has also been discovered in NE knockout mice (Tkalcevic et al., 2000).

Tissue damage by NE

When the balance between NE and protease inhibitors is disrupted, the surrounding tissues can be severely affected, as can be seen in, for example, cystic fibrosis. This inherited syndrome is characterized by lung disease caused by a faulty innate immune response, abnormal mucus obstructing the airways, chronic infection with Gram negative bacteria, and a persistent neutrophil inflammation of the airways. NE seems to exacerbate the disease by promoting inflammation, increasing mucin secretion, damaging cilia which is supposed to clear excess mucus, remodelling airways, and impairing innate and adaptive immune response (Voynow et al., 2008).

NE mutations and neutropenia

Severe congenital neutropenia (SCN), originally called *Kostmann syndrome*, presents in childhood with a severe deficiency of mature neutrophils. The maturation of promyelocytes/myelocytes is arrested and there is a high risk of severe infections. Over 90 % of SCN patients respond to treatment with high doses of G-CSF, which elevates the neutrophil count, but some patients still succumb to infections. The disease displays autosomal-dominant inheritance or arises spontaneously and is associated with an increased incidence of myelodysplasia (MDS) and acute myelogenous leukemia (AML).

Cyclic neutropenia (CN) also presents in childhood, but the infections are often not as severe as in SCN. CN is also inherited in an autosomal-dominant manner. In CN, the neutrophil count oscillates in (typically) 21-day cycles. Monocytes cycle in an opposing phase, displaying almost normal counts when the neutrophils are at the nadir of their cycle (when the risk of infection is largest) and vice versa. Treatment

with G-CSF in smaller doses than is required to treat SCN increases the amplitude of the neutrophil oscillations and shortens the cycle length. Patients with CN do not seem to have an increased risk of developing MDS or AML (Ancliff, 2003; Berliner et al., 2004). G-CSF therapy of SCN patients does not restore NE, MPO or cathepsin G and antimicrobial activity is not fully functional (Donini et al., 2007).

In most patients, CN is caused by mutations in *ELA2*, the gene coding for NE (Horwitz et al., 1999). SCN is caused by *ELA2* mutations in between 50 and 60 % of cases (Dale and Link, 2009; Dale et al., 2000). Those SCN patients with *ELA2* mutations have the most severe neutropenia and are at higher than average risk of develop MDS and AML, compared to other SCN patients (Bellanne-Chantelot et al., 2004). Recently, mutations of the gene coding for the mitochondrial protein HAX1 have been discovered to be responsible for Kostmann disease, an autosomal recessive form of SCN. HAX1 was shown to protect the neutrophil against apoptosis (Klein et al., 2007), and an increase in apoptosis of hematopoietic precursor cells was evident in Kostmann patients (Carlsson et al., 2004). Furthermore, a reduction in the LEF-1 transcription factor was discovered in SCN, leading to the maturation arrest of promyelocytes (Skokowa et al., 2006).

How mutations in *ELA2* give rise to neutropenia has not been clarified, but apoptosis caused by the unfolded protein response induced by mutated NE is one plausible explanation (Grenda et al., 2007; Kollner et al., 2006) and apoptosis of differentiating cells due to *ELA2* mutation has been shown (Massullo et al., 2005). No mouse models of SCN or CN have been established. NE knockout mice do not display neutropenia, even though they are sensitive to infection (Berliner et al., 2004), and mice expressing NE with a mutation from SCN patients have normal neutrophil counts (Grenda et al., 2002).

Neutropenia associated with an AP-3 mutation lacking NE

The autosomal recessive disease *canine cyclic hematopoiesis*, also known as *gray collie syndrome*, is similar to the human CN but the gene mutated is not *ELA2* but *AP3B1*, coding for the AP-3 β-subunit (Benson et al., 2003). When *AP3B1* is mutated in humans it leads to *Hermansky-Pudlak syndrome type 2*, the characteristics of which includes neutropenia. The AP-3 deficiency leads to missorting of lysosomal proteins LAMP-1, LAMP-2 and LAMP-3 (CD63) to the cell membrane, demonstrating the necessity of AP-3 in sorting to lysosomes (Dell'Angelica et al., 1999; Huizing et al., 2002; Jung et al., 2006). Intracellular NE is also reduced in neutrophils in *Hermansky-*

Pudlak syndrome type 2 (Fontana et al., 2006), indicating an essential role for AP-3 in NE targeting.

Benson et al. proposed that the AP-3 complex establishes contact with NE in the TGN, aiding its sorting to the azurophil granules. This hypothesis requires that NE adopts a transmembrane configuration (Benson et al., 2003; Horwitz et al., 2004), despite the fact that NE is considered a soluble protein.

NE sorting and serglycin

A proteoglycan with chondroitin sulfate side chains, now called serglycin, was discovered early on in hematopoietic cells (Olsson, 1969a) and was suggested to form ionic complexes with cationic proteins in developing granules for protection against toxic damage (Olsson, 1969b). Many neutrophil granule proteins besides NE are highly cationic (Olsson and Venge, 1974). The idea of serglycin functioning as an early matrix scaffold for the cationic granule proteins of hematopoietic cells has been further developed (Kolset and Tveit, 2008) and is established for mast cells and cytotoxic T-cells (Abrink et al., 2004; Grujic et al., 2005). However, the role of serglycin in granulopoietic cells has been elusive. Chondroitin sulfate degradation occurrs during maturation of granulopoietic cells (Olsson, 1969b) and only minor amounts of serglycin proteoglycan seem to be left in mature granules (Niemann et al., 2004). Nevertheless, serglycin is reported to be required for the sorting of NE to granules (Lemansky et al., 2007; Niemann et al., 2007); disruption of the serglycin gene in mice blocked the granule targeting of NE (Niemann et al., 2007). During the later phase of the research project this information was born in mind in trying to identify a role for serglycin in a concept for the granule targeting of NE.

Lysosome associated membrane proteins and tetraspanins

The most abundant lysosomal membrane proteins are the LAMPs (Eskelinen, 2006). LAMP-2 is involved in lysosome biogenesis and in autophagosome/phagosome maturation. Impaired phagosomal maturation in neutrophils resulted in periodontitis in LAMP-2 knockout mice due to a reduced ability to kill bacteria (Beertsen et al., 2008). Curiously, LAMP-2 was present in the vesicular structures of wild-type neutrophils but not in azurophil granules, which appeared normal in LAMP-2 knockout mice. These recent findings are consistent with previous reports on lack of LAMP-1 and LAMP-2 (Cieutat et al., 1998; Dahlgren et al., 1995) in the azurophil

granules of human neutrophils. Thus, in neutrophils, LAMP-2 is lacking in the azurophil granules but is still required for the maturation process of phagosomes.

The tetraspanin family consists of a large number of membrane proteins, each with four transmembrane domains featuring one small and one large extracellular loop. The large loop contains a conserved core along with a variable portion that distinguishes tetraspanins from each other and contains the protein–protein interaction site. The tetraspanin proteins take part in many cellular processes, for example cell migration, adhesion, signalling, activation, and tumor invasion. Some tetraspanins form homodimers (Kovalenko et al., 2004) and others form heterodimers with other members of the tetraspanin family and associated proteins, creating tetraspanin-enriched microdomains (TEMs), or webs, on the cell surface. The created proximity of molecules in the TEMs promotes the many and diverse functions of the tetraspanins (Hemler, 2005; Levy and Shoham, 2005). Post-translational modifications such as the addition of palmitate to membrane-proximal cystein residues on the cytoplasmic side are crucial for the ability to generate TEMs with associated proteins (Berditchevski et al., 2002; Hemler, 2005; Levy and Shoham, 2005).

CD63 is present within the endosomal system, mostly in late endosomes/MVBs/lysosomes but also at the cell surface (Pols and Klumperman, 2008). It is targeted to these destinations via either a direct route from the Golgi complex or an indirect route over the plasma membrane followed by endocytosis. The CD63 carboxy-terminal sorting motif GYEVM contains a lysosome consensus sorting motif corresponding to YXXØ; Y standing for tyrosine, X for any amino acid and Ø for an amino acid with a bulky hydrophobic side chain.

The sorting motif is required for AP contact, clathrin coat recruitment and membrane invagination to create a transport vesicle. The sorting motif allows not only endocytosis but also lysosomal targeting, which requires the glycine (G) residue to precede the crucial tyrosine (Y) of the sorting motif. Furthermore, the entire motif is localized at a distance of six to nine residues away from the transmembrane domain, which seems to be a requirement for lysosomal targeting (Bonifacino and Traub, 2003). The CD63 sorting motif interacts with both AP-2 and AP-3; with AP-2 at the plasma membrane to allow endocytosis and with AP-3 to allow lysosomal targeting within the endosomal sorting system (Janvier and Bonifacino, 2005; Peden et al., 2004; Rous et al., 2002)

CD63 is enriched in the internal vesicles of MVBs/secretory lysosomes formed by inward budding of the limiting membrane. In contrast, LAMP-1 and LAMP-2 remain at the limiting membrane, despite having similar sorting motifs to CD63. This

indicates different regulation of the sorting of LAMPs and tetraspanins (Escola et al., 1998).

A role is also emerging for CD63 as a transporter of proteins in different cell types. For example, CD63 is involved in the recycling of the H, K-ATPase pump between the plasma membrane and the intracellular storage compartment of gastric parietal cells (Duffield et al., 2003) and membrane-associated type-1 matrix metalloproteinase, which is implicated in tumor development, is transported to lysosomes with the aid of CD63 (Takino et al., 2003).

The posssible transporter function of CD63, the observed AP-3-dependent NE sorting (Fontana et al., 2006), and the colocalization of NE and CD63 in azurophil granules suggested the hypothesis of this thesis; that CD63 facilitates the sorting of NE.

The present investigation

Understanding the targeting mechanisms for storage of proteins in neutrophil granules until release has been elusive. Such an understanding would not only lead to new knowledge of neutrophil biology but could also lead to novel ideas for stimulating an antibiotic potential or inhibiting detrimental effects of NE. The overall objective was to find the mechanisms behind localization in the granules of soluble proteins such as NE. The specific aims of the thesis, a discussion of the experimental conditions, and a discussion of the findings follow.

Hypothesis

Transmembrane proteins use carboxy-terminal sorting sequences to recruit adaptor protein in the cytosol in order to assemble coat to create transport vesicles, which deliver cargo to the final destination. ProNE is not known to be a transmembrane protein but still depends on AP-3 for delivery to granules (Fontana et al., 2006) indicating a contact—direct or indirect—between proNE/NE and AP-3. The tetraspanin CD63 is a membrane protein of azurophil granules that depends on AP-3 for granule targeting. We hypothesized that CD63 created an indirect contact between proNE and AP-3 as a transmembrane linker in order to facilitate the granule targeting of NE. According to this hypothesis CD63 may be a proNE transporter for sorting to granules.

Specific aims

I   To examine whether the carboxy terminal part of NE is required for appropriate intracellular trafficking of proNE.

II   To examine the structural requirements for proNE trafficking over the plasma membrane.

III   To examine the role of the tetraspanin CD63 as a possible transmembrane linker in proNE granule targeting.

IV   To examine the endosomal trafficking routes of NE and PR3.

Experimental considerations

The principles, advantages and disadvantages of the most important methods used are covered briefly.

Cell models

The fibroblast-like African green monkey COS cell line (Mellon et al., 1981) was used for transient expression and interaction studies of NE/PR3 and CD63. The rat basophilic cell line RBL was used for transfection of human NE, enabling studies of trafficking. The presence of protease-containing lysosome-related organelles in RBL cells make them suitable for studying granule targeting (Seldin et al., 1985).

The human promyelocytic cell line HL-60 (Gallagher et al., 1979) was used for knock-down of CD63. HL-60 is a particularly suitable cell line for studying NE synthesis and granule targeting because the cells include peroxidase-positive azurophil granules containing NE. The human monocytic cell line THP-1 (Tsuchiya et al., 1982) was also used to study NE/PR3 trafficking, since it produces and stores these proteases in granules. However, all the cell lines we used are leukemic, and even if they can be induced to mature into neutrophils (Collins et al., 1978) and monocytes results from studies made using them may not be fully relevant for the corresponding normal cells. For instance, the THP-1 gene profile during induced maturation is not identical to that of differentiating monocytes (Kohro et al., 2004) and the transcription factor profile activated during HL-60 maturation is not identical with that of normal neutrophil differentiation (Pass et al., 2007). Findings in leukemic cell lines must eventually be confirmed in non-leukemic cells (as was done in **paper I**), but information from studies on leukemic cell lines can be very useful in testing hypotheses.

Transfections

We stably transfected RBL cells with NE cDNA using selection by antibiotic resistence after electroporation. Single clones were selected for expansion and investigation. Electroporation was also used to deliver shRNA into HL-60 cells. Electroporation is cytotoxic and the transfection procedure is time consuming with a low efficiency, but generates stably transfected clones. RBL cells were stably transfected with NE, which was produced at a moderate level in selected clones.

Studies in COS cells involved transient overexpression of NE, PR3 and CD63. The cells were transfected with PolyFect Reagent, which forms complexes with cDNA and assembles it in compact structures that bind to the cell surface and are endocytosed. This is a quick and easy method, but the level of expressed target protein becomes high and may damage the cells. In addition the efficiency of expression vary between individual cells.

Biosynthetic radiolabelling

Biosynthesis and processing of a protein can be monitored using biosynthetic radiolabelling. In this method, the cells are incubated with radioactive amino acids that are incorporated into newly synthesized proteins (pulse), enabling detection. To follow changes of newly synthesized proteins over time (processing) cells from the pulse are resuspended and incubated in medium free of radioactive amino acids (chase).
Biosynthetic radiolabelling is widely used and accurate, but large numbers of cells are needed for effective detection.

Immunoprecipitation and Western blotting—detection of proNE and NE with antibodies

IP was used to pull down proteins of interest after biosynthetic radiolabelling and Western blotting was used to detect proteins after SDS-PAGE. A combination of the two methods (IP-Western) was used to demonstrate the existence of proNE/proPR3 complexes with CD63 (**paper III, IV**). Antibodies against the carboxy-terminal propeptides of both proNE and proPR3 could be used for both IP and Western blotting. Both NE and proNE could be immunoprecipitated by anti-NE, the antibody used, which evidently reacts with a common epitope on NE and proNE. Only proNE, not NE, was immunoblotted by anti-proNE, as this antibody specifically detects the carboxy-terminal propeptide of proNE, which is removed from mature NE. Mature NE could not be immunoblotted by any anti-NE antibody tested in cells used in these studies. Consequently, we cannot detect mature NE by Western blotting, only proNE.

Subcellular fractionation

To show granule targeting, biosynthetic radiolabelling was followed by subcellular fractionation. Cell lysates were centrifuged on a colloidal silica (Percoll) gradient that during centrifugation generated fractions that were identified by examination of organelle markers and electron microscopy. A drawback of the method is that

organelles with identical density cannot be separated. However, organelles such as ER-Golgi can be separated from granules and cytosol. The localization of newly synthesized proteins can thus be followed in the cell. This method requires large amounts of cells in order to detect the protein of interest by IP.

Flow cytometry

Flow cytometry was used to examine cell surface proteins on live cells and intracellular proteins in fixed and permeabilized cells. Flow cytometry offers quick and specific quantitative detection of large numbers of cells, making comparisons between different samples possible. Fluorescently labelled antibodies are used for detection of proteins. The specificity and affinity of the antibodies are crucial. Permeabilization of the cells is a risk factor, since damage may occur, allowing leakage of proteins.

Immunofluorescence microscopy

The staining procedure in immunofluorescence microscopy is the same as in flow cytometry. Looking at individual cells makes it possible to determine subcellular localization and study the colocalization of various proteins. Fewer cells can be examined than can by flow cytometry, but the two methods are complementary.

A drawback of immunofluorescence microscopy is that staining with antibodies requires the binding sites to be unoccupied, which may not be the case when looking at colocalization. For example, anti-CD63 antibodies are directed against the large extracellular loop, which is also the common ligand-binding portion of the tetraspanin (Levy and Shoham, 2005). Thus, complexes such as proNE/proPR3–CD63 may not be easily detectable, due to sterical hindrance, as the antibody binding sites may be occupied by bound ligand. Different pro-proteases may bind differently to CD63, so the accessibility of the binding sites for anti-CD63 antibodies may also vary. This could explain the fact that colocalization of proPR3–CD63 in THP-1 cells was low, despite their interaction in COS cells, compared to the high colocalization of proNE and CD63 (**paper IV**). CD63 may bind to PR3 and NE in different ways, making it more difficult to detect proPR3–CD63 than proNE–CD63 using antibodies.

Immunoelectron microscopy

This method offers high-resolution images of subcellular structures, making it possible to detect the protein of interest in individual organelles. In addition, it allows the colocalization of molecules within compartments to be examined. Ultrathin cryosections of cells are incubated first with specific antibody and then with secondary antibody conjugated with gold particles to make visualization possible. One difficulty is the preservation of cellular structures for observation. Antibody specificity is also important.

Antibody feeding for endocytosis studies

To examine endocytosis, we stained the surfaces of live cells with antibodies at 15°C, then incubated at 37°C to enable internalization of the antibody–protein complex. The cells were then placed on ice and fixed before being examined by immunofluorescence microscopy, allowing the internalization to be visually confirmed. One uncertainty is whether the antibodies themselves provoke endocytosis. Therefore, biotinylation experiments were performed to confirm the immunofluorescence evidence of endocytosis (**paper I, II**). Cell surface proteins were biotinylated at 4°C and incubated at 37°C to allow internalization. After stripping the cell surface of remaining biotin, internalized biotinylated proteins were detected by Western blotting.

General discussion

In this section, I will discuss how the results shed light on neutrophil biology, particularly intracellular trafficking and targeting for granule storage. The reader is referred to the original **papers I-IV** for primary data.

Retention mechanisms for proNE to avoid constitutive secretion

There has been some debate over whether the soluble lumenal proteins of neutrophil granules are subject to active sorting. The MPRs cannot be involved, as the granule targeting of glycoproteins that contain M6P is independent of these receptors in granulopoietic cells (Glickman and Kornfeld, 1993; Neufeld et al., 1975). The finding that as much as 50% of hematopoietic serine pro-proteases like proNE/proPR3 is constitutively secreted (delivered to the outside of the cell without storage) (Garwicz et al., 2005) may indicate a passive process (bulk flow).

The rate of biosynthesis of granule proteins is very high in the granulocyte precursor cells—at such high concentrations, some pro-protease may reach granules in a passive manner. But high constitutive secretion may also indicate the existence of a retention mechanism, with low retention explained by a relatively low efficiency/affinity. In this study, it was assumed that a retention mechanism exists for lumenal proteins in myeloid cells involving membrane association, the failure of which may result in constitutive secretion. Possible alternative mechanisms for retention include selective protein aggregation in mildly acidic compartments such as takes place in neuroendocrine cells (Taupenot et al., 2005); and proteoglycan interactions (see below).

The retention efficiency may vary between lumenal proteins. For example, proMPO is targeted to granules, but propeptide-deleted proMPO is constitutively secreted (Andersson et al., 1998), suggesting that the propeptide plays a role in retention/sorting. Moreover, intracellular retention and decreased constitutive secretion of proNE/proPR3 were discovered when they were over-expresssed together with CD63 in COS cells (**papers III, IV**). However, the physiological relevance of these findings is questionable, because the experiments were performed by over-expression in non-myeloid cells. Importantly, the finding of a complete absence of NE in granules in serglycin proteoglycan knockout neutrophils indicated that an active sorting mechanism is needed for this protease (Niemann et al., 2007). Furthermore, an important example of a soluble lysosomal enzyme requiring a membrane association to avoid extracellular loss by constitutive secretion is found in

non-myeloid cells, where membrane association through LIMP-2 is necessary for retention/targeting of β-glucocerebrosidase (Reczek et al., 2007).

ProNE/proPR3 trafficking may occur along both a direct and an indirect sorting pathway

Lysosomal proteins such as the LAMPs are sorted to lysosomes through both a direct pathway and an indirect pathway via the plasma membrane (Hunziker and Geuze, 1996; Janvier and Bonifacino, 2005). It is not yet clear whether the existence of two sorting pathways serves a function, but it could be a safety mechanism: if so, a saturation of the direct sorting machinery would be compensated for by back-up sorting through the indirect pathway. The presence of proNE on the surface of RBL cells expressing NE (**paper I**) suggested possible indirect sorting to granules or mistargeting to the plasma membrane. Blocking transport through Golgi with BFA abolished surface proNE, confirming that the proNE was newly synthesized.

ProNE was also discovered on the surface of human CD34+ progenitor cells cultured towards granulopoietic differentiation (**paper I**), indicating that the surface expression was not an artefact. Thus, the presence of proNE on the cell surface of granulopoietic cells was demonstrated. Furthermore, the cell surface proNE of both RBL and differentiated CD34+ cells was endocytosed when cells were incubated at 37°C (**paper I**), suggesting a possibility of indirect sorting. These results were also confirmed for PR3, with presence of PR3 on differentiating CD34+ cells (**paper I**) and endocytosis of cell surface proPR3 in the monocytic cell line THP-1 (**paper IV**). For technical reasons, it was not possible to show granule targeting of endocytosed proNE/proPR3, the final step in indirect targeting. However, the presence on the cell surface and endocytosis of both pro-proteases (**papers I, IV**), together with the known existence of indirect sorting of proteins to lysosomes, suggest that indirect sorting of NE/PR3 may occur. Figure 6 outlines sorting by the two different pathways.
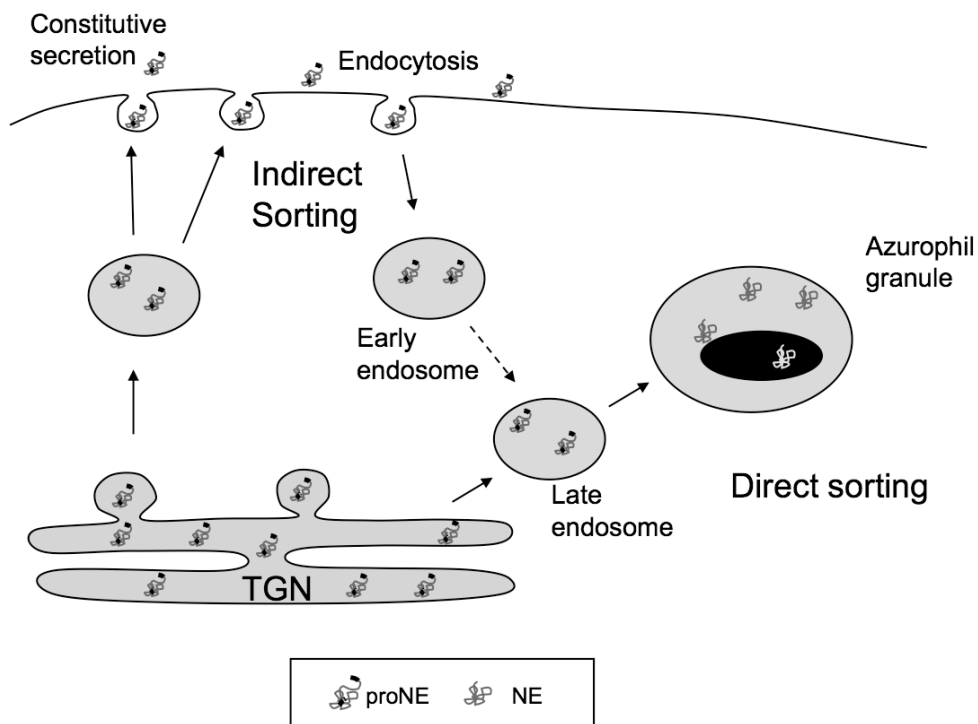
Figure 6. Suggested model of proNE/NE trafficking. Vesicles formed in TGN travelling to late endosomes and directly to granules constitute a direct pathway. ProNE transported to the cell surface is endocytosed. Transfer to early endosomes followed by transport to late endosomes and granules is not proven, but may constitute an indirect pathway.

NE propeptide is required for cell surface trafficking of proNE

Removal of both amino-terminal and carboxy-terminal propeptides is achieved within 60 to 90 minutes of synthesis (Gullberg et al., 1997; Salvesen and Enghild, 1990). The amino-terminal pro-dipeptide is assumed to keep proNE in an inactive conformation; its removal by cathepsin C is required for NE activation (Adkison et al., 2002). Therefore, patients with *Papillon-Lefèvre syndrome* with lack-of-function mutations of the cathepsin C gene, show a lack of enzymatically active NE (Pham et al., 2004).

The carboxy-terminal propeptides of hematopoietic serine proteases are not conserved among species and vary in length, making it unlikely that they have a common specific role. However, it is possible that the carboxy-terminal propeptide of proNE has a specific role, as it is rather long. Removal of this propeptide does not affect activation or granule targeting (Gullberg et al., 1995). We compared

intracellular distribution of NE in RBL cells expressing either a wild type NE construct or an NE construct lacking the carboxy-terminal propeptide. Only the expression of wild type proNE on the cell surface was detected, suggesting that the carboxy-terminal propeptide may affect proNE targeting (**paper I**), given that cell surface expression reflects indirect sorting for granule storage.

Sorting of NE has been shown to depend on AP-3, as lack of AP-3 is associated with neutrophil granules lacking NE (Benson et al., 2003; Fontana et al., 2006). It is possible that the carboxy-terminal propeptide helps to keep proNE in an immature conformation or sterically hinders a sorting signal for direct targeting. Removal of the carboxy-terminal propeptide may, therefore, have caused proNE to assume a conformation normally only seen in granule/pre-granule structures, in which, it is assumed, the carboxy-terminus is cleaved off (Gullberg et al., 1997). This conformation would be more easily recognized by the AP-3–dependent transport machinery, thus avoiding plasma membrane targeting. Alternatively, the carboxy-terminal propeptide may sterically obstruct binding by the sorting machinery, with the result that proNE retaining the carboxy-terminal propeptide slips past TGN sorting, while NE with the carboxy-terminus deleted is effectively directed to granules. It is also possible that targeting from the TGN to the cell surface requires the carboxy-terminal propeptide and without it, proNE is excluded from such targeting.

ProNE/NE are not transmembrane proteins

It has been suggested, based on computational calculations, that proNE adopts a transmembrane conformation to allow contact with AP-3 for correct sorting (Benson et al., 2003), despite the general conception that NE is soluble. According to this theory, the majority of proNE should be present on one side of the membrane— either inside TGN or granules or on the cell surface—and amino-terminal and carboxy-terminal parts should be on the cytoplasmic side (Benson et al., 2003; Horwitz et al., 2004). However, our data contradict such an assumption.

The presence of proNE on the cell surface was shown using an antibody recognizing the carboxy-terminal propeptide and an antibody against a different epitope of NE. Although the latter antibody could not discriminate between NE and proNE, signal from both antibodies indicated the presence of both the carboxy-terminal and other parts of NE on the cell surface (**paper I**). In addition, colocalization with the different antibodies was shown, demonstrating that both the carboxy-terminal propeptide and another epitope of NE were present on the surface. Furthermore, when NE was analyzed for transmembrane sequences and the carboxy-terminal propeptide was included, only a few of the available calculation-programs indicated membrane-spanning sequences (data not shown). Thus, a transmembrane

conformation seems unlikely and, since the carboxy-terminal was clearly on the cell surface, an interaction with cytosolic AP-3 for sorting would not be possible. It also seems unlikely that the protease was alternating directions by membrane flip-flop.

If proNE spans the membrane, with the carboxy-terminus on the inside, direct contact with AP-3 would explain the observed dependency of AP-3 for sorting. Benson et al have suggested a possible binding site for AP-3 in NE, and demonstrated interaction in a yeast two-hybrid assay (Benson et al., 2003). In this thesis, I will not argue that such a binding site does not exist or that an interaction cannot be observed in an experimental setting, but the observation of the carboxy-terminal propeptide being situated on the outside of the plasma membrane would make a direct interaction with AP-3 impossible in vivo (**paper I**). Collectively, the experimental data from this study speak against a transmembrane conformation of proNE/NE. As I suggest in this thesis, an alternative is the existence of a transmembrane linker, linking proNE with AP-3.

ProNE/proPR3 interacted with CD63

We hypothesized that the tetraspanin CD63 acts as a combined linker and transporter, as it requires AP-3 for sorting to lysosomal (and lysosomal-like) structures (Janvier and Bonifacino, 2005; Peden et al., 2004; Rous et al., 2002), and CD63, unlike the other LAMPs, resides in the membrane of azurophil granules (Cieutat et al., 1998; Dahlgren et al., 1995).

To investigate the potential interaction between proNE and CD63—tagged with GFP for convenient detection—we cotransfected both into COS-cells. The results from coprecipitation experiments indicated the formation of a complex (**paper III**). Gel filtration followed by examination with IP-Western showed elution of proNE and GFP-CD63 consistent with their presence in a complex of high molecular weight. Direct visualization of complexes containing both proteins was achieved by silver staining of immunoprecipitated proteins separated by SDS-PAGE.

Cotransfection with CD63 resulted in cellular retention of proNE—according to results from biosynthetic radiolabelling—suggesting cooperation between these proteins. In contrast, cotransfection with CD63 lacking the large extracellular loop (CD63ΔLEL) did not induce cellular retention of co-expressed proNE, indicating that intact CD63 is needed for significant proNE interaction (**paper III**). In addition, immunofluorescence microscopy showed the colocalization of proNE and CD63 but not of proNE and CD63ΔLEL. The large extracellular loop is generally considered to carry the ligand-binding domain (Levy and Shoham, 2005). Thus, the colocalization of proNE and intact CD63 supported our results.

We also wished to investigate whether other serine proteases could form complexes with CD63. We therefore cotransfected PR3 with CD63 in COS cells. ProPR3 coprecipitated CD63 and coexpression led to cellular retention of proPR3 (**paper IV**), suggesting that there is a possible interaction between proPR3 and CD63.

These experiments were performed in COS cells and involved forced overexpression of proNE/proPR3 and CD63. The overexpression and the use of a non-hematopoietic cell line lacking granules may have influenced the results and created an interaction not found in hematopoietic cells. Additional information was thus needed to establish whether CD63 interacted with proNE/proPR3 in situ, but the results suggested such an interaction.

Granule targeting of proNE/proPR3 may require CD63 as a transmembrane linker

To further investigate the role of CD63 in proNE sorting we employed shRNA (or a dominant-negative mutant) to silence the expression of CD63 in promyelocytic HL-60 cells, which synthesize proteins such as NE for storage in their azurophil granules.

Despite the silencing of NE mRNA by CD63 shRNA or dominant-negative CD63 mutant, the steady-state levels of the proNE protein were unchanged. Glycosylation of proNE was also detected. This made it possible to examine the effects of knocking down CD63 on proNE processing and sorting. A role for CD63 was supported by the major finding that proNE was produced but was not processed into mature NE. In addition, no proNE was released by constitutive secretion, which normally occurs in HL-60 cells (**paper III**). These results suggested a block in proNE trafficking. The unchanged steady-state level of proNE could be a result of lack of constitutive secretion, possibly combined with degradation due to the transport block. When cells were examined by immunofluorescence or flow cytometry, the NE level was much lower in CD63-depleted cells than in control cells. Together, these results indicate that proNE was produced, but lacking on the cell surface and elsewhere in the cells, which would explain the drop in fluorescence and the lack of NE signal in immunoelectron microscopy images. The block would prevent granule targeting and, thereby, access to processing enzymes present in endosomes and granules.

Depletion of CD63 in HL-60 cells also gave rise to ultrastructural abnormalities. A question was whether these were due to deficient granule transport or deficient granule formation. The morphological finding of abnormal granules/vacuoles in CD63-depleted cells suggested both possibilities.

Since proNE retention was increased in COS cells when cotransfected with CD63, the depletion of CD63 in HL-60 cells might have been expected to result in the decreased retention of proNE along with increased constitutive secretion. The fact that the constitutive secretion of proNE was blocked in CD63-depleted HL-60 cells may be due to differences in the cell types. COS cells, unlike HL-60 cells, do not have the machinery for normal NE processing. Therefore, experiments in COS cells do not fully reflect the processing and targeting of NE; nevertheless, the results still point to a role for CD63 in the post-Golgi retention and targeting of NE. The results we obtained in HL-60 cells suggested that CD63 depletion resulted in a trafficking block, preventing investigation of post-Golgi retention in these cells. In summary, the data from COS cells may reflect a distal involvement of CD63 in proNE retention, while the HL-60 cell data reflect a proximal involvement of CD63 at the level of the ER/Golgi.

Some aspects of the role of CD63 were tested in monocytic THP-1 cells. Both proNE and proPR3 displayed colocalization with CD63 on the cell surface of these cells, suggesting that CD63 may be involved in possible indirect targeting over the plasma membrane of proNE/proPR3. The clear colocalization of proNE and CD63 was also observed in permeabilized THP-1 cells, while proPR3 showed a much lower degree of colocalization (**paper IV**). This indicated the presence of proNE and CD63 in the same intracellular structures—endosomal vesicles and granules—lending support to the idea that CD63 is a transmembrane carrier of proNE. ProPR3, on the other hand, did not display as strong costaining, suggesting that CD63 is not a carrier for proPR3 to the same degree.

We propose that sorting of proNE into the endosomal pathway may be dependent upon a transmembrane carrier such as CD63, which suggests that exit from the TGN is not a passive process. Binding by a transmembrane carrier might be a prerequisite for exit, which would explain the transport block in CD63-depleted HL-60 cells (**paper III**). The binding could be indirect, according to the hypothesis of aggregation-mediated sorting of both proteins with and without sorting signal being assembled in one aggregate (Gullberg et al., 1997). The data collected from interaction studies in COS-cells and shRNA depletion in HL-60 cells point to a possible direct role for CD63 in proNE targeting.

In conclusion, we examined a possible role for the tetraspanin CD63 in the cellular retention and granule targeting of proNE/proPR3. Coexpression in COS cells showed an interaction between the proteins and proNE and proPR3 were retained by CD63. The depletion of CD63 in promyelocytic HL-60 cells resulted in reduced processing and reduced constitutive secretion of proNE. We propose, therefore, that the targeting of proNE and, perhaps, proPR3 requires the help of the protein-delivery system used by CD63. However, there are still caveats to our conclusions.

The data do not prove that there is a direct interaction between CD63 and proNE—for example, some proNE was present in a high molecular weight complex even when expressed without CD63, according to the gel filtration data. HL-60 cells used for CD63 knock-down are leukemic and studies of them may not be relevant for the corresponding normal cells. However, we regard the transmembrane linker concept as important to explain sorting of soluble proteins in hematopoietic cells and our findings so far are consistent with such a concept.

Mechanisms for the endocytosis of cell surface proNE/proPR3

The colocalization of proNE/proPR3 and CD63 on the surface of THP-1 cells (**paper IV**) supported the interaction suggested for proNE/proPR3 and CD63 (**papers III, IV**). Offering further support, we also found coendocytosis of proNE/proPR3 and CD63 (**paper IV**). Colocalization in endocytic vesicles is consistent with a role for CD63 in endocytosis along the indirect pathway of proNE/proPR3, although the final step of granule targeting could not be demonstrated for technical reasons.

Endocytosis can occur both in a clathrin-dependent and a clathrin-independent manner. Clathrin, aided at the cell surface by AP-2, coats vesicles for endocytosis. When the vesicles have detached from the cell membrane uncoating precedes fusion of the vesicle with various endosomes (Kirchhausen, 2000; Mousavi et al., 2004). Clathrin-independent endocytic pathways include caveolin-dependent endocytosis. Caveolae are special lipid rafts containing caveolin proteins that are essential for this endocytic mechanism (Gong et al., 2008). We discovered the clear colocalization of both proNE and proPR3 with clathrin in THP-1 cells (**paper IV**). Clathrin also coats vesicles originating in the TGN (Kirchhausen, 2000); we therefore concluded that proNE and proPR3 are transported in clathrin-coated vesicles originating at the TGN and/or the plasma membrane.

Interestingly, proNE also colocalized with caveolin-1 to some degree, while PR3 did not. This indicates the possible caveolin-dependent endocytosis of proNE. CD63 is endocytosed by an AP-2-clathrin–dependent pathway (Janvier and Bonifacino, 2005), but it was recently suggested that caveolin-dependent endocytosis may also occur (Pols and Klumperman, 2008), which would be consistent with the possible couptake of complexed proNE.

The endosomal trafficking routes for proNE/proPR3

To shed light on intracellular trafficking, we co-stained proNE and proPR3 in THP-1 cells, with markers for various endosomal compartments. ProNE and proPR3 were found colocalized with Rab7, a marker for late endosomes (Stenmark and Olkkonen, 2001), indicating their presence in this organelle (**paper IV**). Colocalization with Rab5 (early endosomes), Rab9 (TGN—late endosome trafficking) or Rab11 (recycling endosomes) was not detected.

Given the sorting pathways of CD63, dependent upon AP-3 trough the direct pathway and AP-2 for endocytosis along the indirect pathway (Janvier and Bonifacino, 2005; Peden et al., 2004; Rous et al., 2002); the known dependency of NE sorting on AP-3 (Benson et al., 2003; Fontana et al., 2006); and our collective data, we propose a sorting model for NE with two alternative pathways, as outlined in figure 7. ProNE connects with CD63 for transport out of the TGN (**paper III**) and follows a direct sorting pathway, where AP-3 mediates its sorting to late endosomes (**paper IV**), followed by delivery of proNE to granules. Alternatively, proNE can be targeted to the plasma membrane and cointernalized with CD63 (**paper IV**) by an AP-2 dependent mechanism, which is followed by its transfer to endosomes. Final granule targeting along this pathway has not yet been demonstrated. The transport vesicles from the TGN to the late endosomes are coated with clathrin, as are the endocytic vesicles originating in the plasma membrane (Kirchhausen, 2000). As discussed above, proNE may also be internalized via caveolin-dependent endocytosis. The sorting of proPR3 is not as clear, but CD63-dependent sorting via the indirect pathway is conceivable (**paper IV**). Sorting in clathrin-coated vesicles seems quite clear, however, (**paper IV**) but whether these are travelling via the direct or indirect pathways remains to be seen.
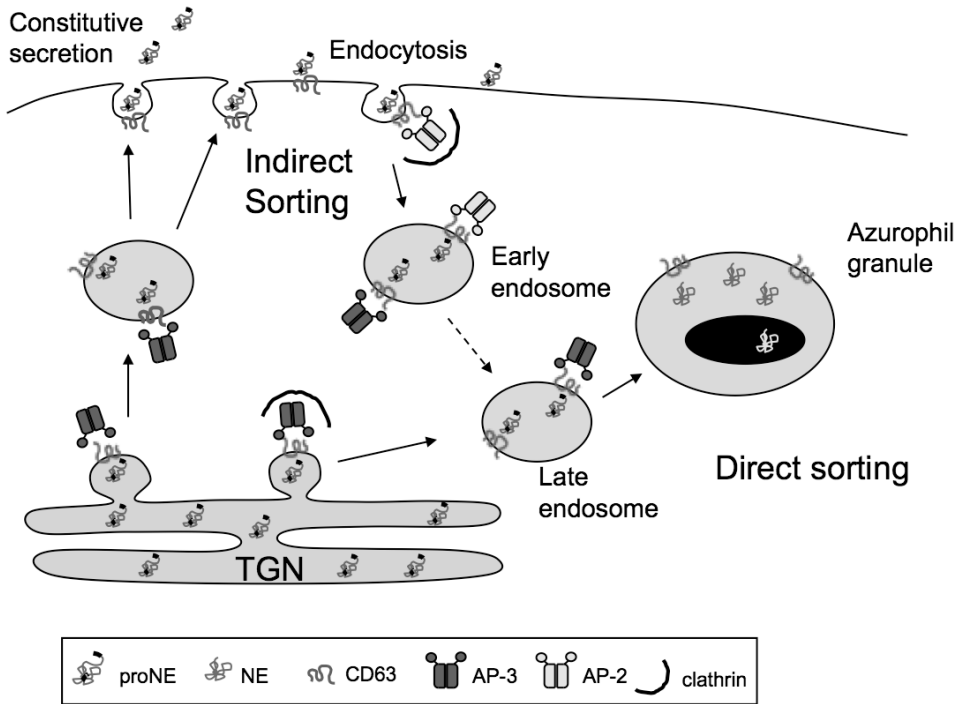
Figure 7. Suggested model of proNE/NE sorting. The tetraspanin CD63, depending on AP-3 and AP-2, is suggested to act as a transmembrane linker, facilitating proNE targeting to late endosomes/granules. Plasma membrane targeted proNE is endocytosed together with CD63; a potential indirect sorting pathway would then lead to late endosomes/granules. Vesicles coated with clathrin appear along both the proposed direct and indirect sorting pathway.

The processing of proNE into NE is involved in sorting for storage in granules

The colocalization of proNE/proPR3 and Rab7 demonstrated that the pro-proteases are still unprocessed when they arrive in late endosomes. Thus, removal of the carboxy-terminal propeptide to yield mature NE/PR3 may take place in late endosomes/granules (**paper IV**).

We blocked the removal of the amino-terminal pro-dipeptide with a cathepsin C inhibitor. Only catalytically inactive proNE could be detected, demonstrating that the inhibitor stopped the cathepsin C-mediated processing and, thereby, enzymatic activation of NE (and PR3) (**paper IV**). This resulted in an increase in the amount of proNE and proPR3 in the inhibited cells, both on the cell surface and intracellularly, as was detected by flow cytometry. The increase of proNE/proPR3—detected by antibodies against the carboxy-terminus—indicated that processing of the carboxy-terminus was also delayed. As final processing is assumed to take place in late

endosomes/granules, the increase suggested that the inhibition of amino-terminal processing also led to inhibition of carboxy-terminal processing, possibly by means of a delay or complete inhibition of delivery to granules.

Serglycin may be involved in the granule targeting of proNE/proPR3

The role of cellular serglycin in sorting for granule storage was not a primary focus of our research. However, recent reports have suggested that proteoglycans are required for the appropriate localization of NE in granules (Lemansky et al., 2007; Niemann et al., 2007) and that mature NE interacts with glycosaminoglycans (Kostoulas et al., 1997). On this basis, we first assumed that proNE also had to interact with serglycin to explain its role in granule targeting. However, proNE did not coprecipitate proteoglycans in lysates of COS cells, suggesting a lack of such an interaction (**paper III**). Furthermore, both proNE and serglycin are in part constitutively secreted, but no proNE–serglycin complexes were detected by gel filtration (**paper IV**), arguing against the existence of such in an isotonic environment.

Nevertheless, our data do not exclude a role for serglycin in sorting of mature NE. The conversion of proNE into NE could take place during sorting and allow an interaction. Early conversion is unlikely, as proNE was widely distributed in various endosomes and on the cell surface (**paper I, IV**). Therefore, our observations were not consistent with a proteoglycan having a direct transporter role for proNE/proPR3.

Lemansky et al. have shown an interaction between intracellular serglycin and NE by chemical cross-linking (Lemansky et al., 2007), which is a more sensitive technique than our own interaction studies with antibody pulldown. Importantly, the conversion of proNE into NE is accompanied by conformational changes that create a positively charged cluster in NE that is probably lacking in proNE (Korkmaz et al., 2007) (see figure 5). Thus, NE has a higher anionic charge than proNE, which is confirmed by affinity studies on heparin–Sepharose (**paper IV**). In accordance with our hypothesis, proNE may be converted to NE on its release from a transmembrane linker, most likely in late endosomes (as discussed above). We propose that thereafter, serglycin can interact with newly generated mature NE to assist in final granule sorting, storage and protection against proteolytic damage (see figure 8). This seems to be followed by proteoglycan degradation as is observed during the maturation of myeloid cells (Olsson, 1969b), which is also consistent with the detection of serglycin mostly in the Golgi area and in only a few immature granules (Niemann et al., 2004).
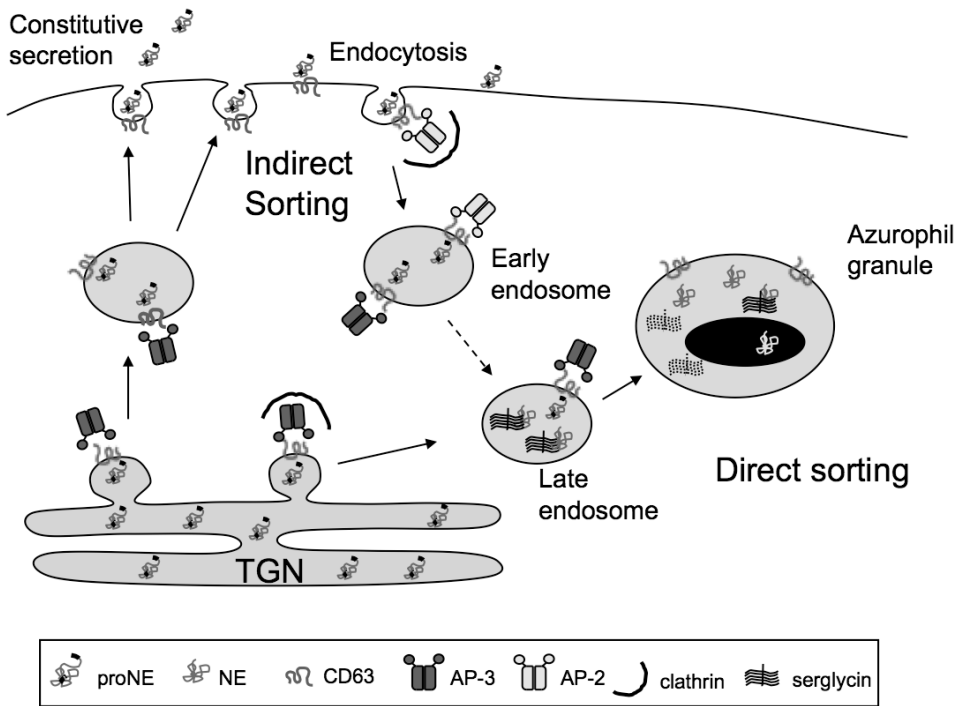
Figure 8. Proposed sorting model of NE including serglycin. Serglycin may play a role in final granule targeting of mature NE from late endosomes.

ProNE has structural requirements for trafficking over the plasma membrane

The importance of aggregation for NE targeting has previously been investigated. To do this a fusion protein—referred to as NE–(FKBP)$_3$—made up of proNE and three copies of the human FK506 binding protein, which can be induced to oligomerize reversibly by addition of derivatives of the cell-permeable drug FK-506, was used. The fusion protein, monomeric or oligomeric, was not sorted for storage but was instead eliminated from cells by constitutive secretion (Rosen et al., 2003). After discovering that proNE was targeted to the plasma membrane and then endocytosed (**paper I**) we also investigated the corresponding targeting of NE-(FKBP)$_3$. The presence of NE–(FKBP)$_3$ on the plasma membrane was confirmed by immunofluorescence microscopy and flow cytometry (**paper II**). Therefore, we used the fusion protein to investigate possible structural requirements of proNE endocytosis. We determined that, unlike proNE, NE–(FKBP)$_3$ was not endocytosed, whether in an oligomerized form or not. On the basis of these results and the fact

that endocytosis is a highly controlled process (Conner and Schmid, 2003), we proposed a possible structural requirement for endosomal uptake of proNE.

Constitutive secretion and cell-surface trafficking for the endocytosis of proNE occur along separate routes

The subcellular trafficking of proNE involves both constitutive secretion and plasma-membrane targeting, followed by endocytosis. An important question was whether the same route is used for both of these pathways. When we investigated the biosynthesis of proNE and NE lacking the carboxy-terminal propeptide in RBL cells, both forms were discovered in the medium in comparable amounts—indicating that they had been constitutively secreted—but only proNE was detectable at the cell surface and endocytosed (**paper I**). Together, theses findings indicate that transport to the cell surface of newly synthesized proNE did not coincide with its transport out of the cell by constitutive secretion.

Cells were treated with BFA to block the exit of newly synthesized proteins from Golgi in order to further investigate trafficking to the cell surface and out of cells. When the block was released, it took 60–120 minutes for proNE to reappear on the cell surface (**paper I, IV**), but constitutively secreted proNE reappeared within 30 minutes (**paper IV**), supporting the theory of separate routes for trafficking to the plasma membrane and constitutive secretion. Possibly, a connection with the sorting machinery before or in TGN is a prerequisite for entry into the indirect pathway. Failure to make contact with the sorting machinery would mean rapid elimination of proNE from the cell through constitutive secretion. Contact with the correct sorting partners would allow a slower passage through TGN—possibly due to the assembly of a larger machinery than that required for constitutive secretion—followed by transport to the cell surface.

One uncertainty was whether constitutively secreted proNE could rebind to the plasma membrane and become endocytosed. However, cells that were depleted of surface-associated proNE/proPR3 by means of a 16-hour BFA block (in order to obtain a surface lacking proNE and thus facilitate the examination of binding) did not show rebinding of these proteins when exposed to medium containing secreted proNE/proPR3 (**paper IV**).

In conclusion, we believe that proNE/proPR3 use separate routes for constitutive secretion and plasma membrane targeting/endocytosis.

Summary and future perspectives

The major findings of this work were the following:

I The results from transfection experiments in a leukemic cell line, which were confirmed in normal hematopoietic cells, show that a population of proNE was targeted to the plasma membrane and endocytosed. This targeting required an intact carboxy-terminal propeptide as lack of the propeptide led to bypass of the plasma membrane and direct granule targeting.

II The results from transfection experiments in a leukemic cell line suggest that cell surface–located proNE–(FKBP)$_3$—a fusion protein made of proNE and the FK506 binding protein, inducible by a cell-permeable ligand to form aggregates—in both monomeric and oligomeric aggregated forms, was excluded from endosomal uptake, in contrast to proNE. This indicates structural and possibly conformational requirements for endosomal re-uptake.

III An association was demonstrated between CD63 and proNE upon coexpression in COS cells. Furthermore, depletion of CD63 in a promyelocytic cell line by RNA interference or a CD63 mutant caused reduced processing of proNE into mature NE and reduced constitutive secretion. We therefore propose that CD63 may be a transmembrane linker that facilitates granule targeting of proNE.

IV ProPR3, like proNE, interacted with CD63 when coexpressed in COS cells. Results in a monocytic cell line suggested that the sorting of proNE/proPR3 was a multistage process including trafficking to the cell surface, endocytosis through coated vesicles and possibly lipid rafts, and possible conversion to mature NE/PR3 in late endosomes. The inhibition of removal of the amino-terminal propeptide was accompanied by the accumulation of proNE/proPR3, suggesting a requirement for precursor processing into mature enzyme before granule targeting.

This research provided new perspectives on the cellular trafficking and sorting of the hematopoietic serine proteases. In this thesis, a hypothesis is put forward on the facilitation of granule sorting by a tetraspanin protein serving as a transmembrane linker and transporter. However, the supportive evidence is based on studies in non-hematopoietic cells and leukemic cell lines. The hypothesis needs further testing in primary cells to acquire firm evidence for the interactions involved.

Biochemical studies are needed to examine the roles of various protein domains for interaction between sorter and target. The timing of processing of proNE, involving cleavage of the amino-terminal and carboxy-terminal propeptides, needs to be integrated into the sorting theory. The hypothesis is also in need of functional evidence based, for example, on gene knockout in mice. The hypothesis may have implications for understanding the pathophysiology of congenital neutropenia, since abnormal NE trafficking may lead to apoptosis.

A functional explanation is still lacking for the involvement of serglycin in the localization of NE in granules. The connection between serglycin and a possible transmembrane linker for sorting needs to be clarified. In addition, when and where proNE/NE interacts with partners of its sorting machinery (CD63, AP-3 and—possibly—AP-2, and serglycin) needs to be investigated.

In a larger perspective, the knowledge of processing and sorting mechanisms, as well as the defects that lead to a lack of mature proteases, will hopefully lead to better treatments for inflammatory disorders. The inhibition of proteases is conceivable, along with the utilization of their storage mechanisms to deliver therapeutic molecules, to inflammatory sites.

Populärvetenskaplig sammanfattning (Summary in Swedish)

Kroppens immunförsvar brukar delas in i det medfödda och det adaptiva immunförsvaret. Det medfödda immunförsvaret reagerar snabbt på yttre hot såsom bakterier. Olika sorters vita blodkroppar är väldigt viktiga för detta snabba svar på infektion och i min avhandling har jag koncentrerat mig på en av dessa; neutrofilen.

Neutrofilen är den vita blodkropp som först kommer fram till en infektion. Den lockas, av olika substanser, att stanna upp och fästa ordentligt till blodkärlet och krypa ut mellan cellerna som utgör kärlväggen. Därifrån kryper den vidare ut i vävnaden tills den får kontakt med de invaderande bakterierna. För att döda bakterierna äter neutrofilerna helt enkelt upp dem och innesluter dem i en membranblåsa kallad fagosom. In i denna membranblåsa levererar neutrofilen sedan olika ämnen som är giftiga för bakterien och bryter ner den, och bekämpar på detta vis infektionen. Samtidigt skickar neutrofilen ut andra ämnen i omgivningen för att locka till sig fler neutrofiler såväl som andra sorters vita blodkroppar. Dessa behövs för att bekämpa infektionen och så småningom städa upp när bakterierna väl har dödats.

Neutrofiler är väldigt kortlivade celler och för att snabbt kunna reagera på bakterier har de redan producerat och lagrat upp alla giftiga ämnen som behövs. Dessa tillverkas under tiden neutrofilerna mognar fram i benmärgen och lagras upp i små blåsor kallade granula. Neutrofiler har flera sorters granula som lagrar olika protein med olika uppgifter. Denna avhandling rör ämnen i den första sortens granula som produceras; de primära.

Det finns många olika ämnen med olika uppgifter i primära granula och jag har koncentrerat mig på neutrofilt elastase (NE). NE kan bryta ner ytterväggen i vissa bakterier och är därför viktigt för bakterie-bekämpning. Dessutom är NE som släpps ut utanför cellen viktig för signalering till andra vita blodkroppar. En för stor mängd utanför cellen kan dock leda till skador på omgivande vävnad. I vissa sällsynta sjukdomar är NE muterat, vilket, av en okänd anledning, kan leda till att neutrofiler helt eller delvis saknas i kroppen. En brist på neutrofiler ger upphov till stor känslighet för infektioner vilket i förlängningen kan leda till döden. Sjukdomar som kännetecknas av brist på neutrofiler kan också uppstå när andra protein är muterade, som verkar behövas för korrekt transport av NE. När protein tillverkas i cellen behövs nämligen olika medhjälpare för att transportera (i det här fallet) NE från

tillverkningsplatsen till upplagringsplatsen i granula. Denna transportväg var inte klarlagd för NE, vilket inte heller alla medhjälpare var. Därför inriktade jag min forskning på att kartlägga transportvägen och medhjälpare.

I mitt första arbete (**I**) upptäckte vi att NE kan transporteras till ytan av celler och sedan tas upp inuti cellen igen, vilket inte var känt tidigare. Transport till ytan var beroende av att NE var intakt. I mitt andra arbete (**II**) undersökte vi återupptaget från cellytan närmare. När vi jämförde normalt NE med en mutant vi framställt fann vi att enbart normalt NE kunde tas upp av cellen, vilket innebär att cellen har krav på vad som får transporteras in.

I mitt tredje arbete (**III**) undersökte vi en eventuell medhjälpare, det membrangenomträngande proteinet CD63, för en roll i NE-transporten till upplagringsplatsen. Vår hypotes var att NE behövde ett protein som fungerade som en länk för indirekt kontakt med olika medhjälpare för transport. Vi fann att NE kunde interagera med CD63. Vidare tryckte vi ner mängden CD63 för att undersöka hur NE–transporten fungerade när CD63 saknades. Vi fann då att NE inte tog sig från tillverkningsplatsen i cellen, utan fastnade där. Inget NE transporterades korrekt till granula utan hjälp av CD63.

I mitt sista arbete (**IV**) undersökte vi transportvägarna av NE och en nära släkting, PR3, i cellen. Vi fann att transporten sker i flera steg.

Sammantaget har mitt arbete gett nya insikter om transporten av NE i cellen och en möjlig medhjälpare för korrekt transport, CD63, har identifierats. Detta kan ha betydelse för förståelse av sjukdomar där transporten av NE är störd och för förståelse av protein-transport i neutrofiler i allmänhet. I förlängningen hoppas vi att kunskapen om denna transport ska kunna användas till att manipulera neutrofiler att lagra upp olika läkemedelssubstanser för att sedan släppa dem på rätt plats i kroppen. Detta scenario skulle vara användbart vid olika inflammatoriska sjukdomar.

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Appendix