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Endosomal targeting and secretion of lysosomal proteins in U937 cells

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“ I DO NOT KNOW what I may appear to the world, but to myself I seem to have been only like a boy playing on the sea-shore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me.”

Isaac Newton

1 Introduction

Eukaryotic cells contain a variety of specialized organelles surrounded by single or double membrane bilayers. The membranes separate different compartments, in witch varied functions can be performed and regulated. Unlike prokaryotes in which cellular functions are mostly coordinated by diffusion in the cytosol, eukaryotes have to use specific transport mechanisms to direct molecules to distinct locations within the cell. Therefore, it is not surprising that highly specific transport mechanisms are required to direct molecules to defined places and to ensure that the identity and function of individual compartments are maintained. Proteins contain structural information that targets them to their correct destination and many targeting signals have now been defined. The *trans*-Golgi network (TGN) is the place where newly synthesized proteins are sorted into the appropriate vesicles and sent down one of three pathways: transport to endosomes/lysosomes, constitutive secretion and regulated secretion. In addition, proteins synthesized in the cytosol can be target into mitochondria, peroxisomes, nucleus and the extracellular space as well. Finally, C-terminal transmembrane segments and various anchors can be used for inserting and attaching proteins to the endoplasmic reticulum (ER) and other organelles.

1.1 Sorting from the TGN to the endosomal/lysosomal system

Protein transport between the organelles of endosomal pathway is mainly mediated by small, membrane-bound transport vesicles. This process is referred to as vesicular transport (Nakatsu and Ohno, 2003). Budding of transport vesicles and selective incorporation of cargo into the forming vesicles are facilitated by protein coats. These coats are assemblies of proteins that are recruited from the cytosol to the nascent vesicles. They participate in cargo selection and the necessary membrane deformation (Bonifacino and Traub, 2003).

Transport vesicles are classified by the identity of the protein coat used in their formation and also by the cargo they contain. Of those, clathrin-coated vesicles

(CCVs) are responsible for the transport of proteins between organelles of TGN, endosomes, lysosomes and the plasma membrane. CCVs' name derives from the predominant protein of the coat, clathrin (Crowther *et al.,* 1981). Clathrin forms a mechanical scaffold around these vesicles, while it interacts with adaptor proteins (APs), which bind to the clathrin, phospholipids and cargo protein components of donor membranes (Owen *et al.*, 2004) (Fig. 1.1). In the presence of APs clathrin self-assembles into spherical cages *in vitro* (Keen, 1990). However, this does not mean that clathrin coat assembly provides enough energy for bending the membranes. In fact, local deformations involve lipid-binding proteins such as epsin and BAR-proteins (McNiven and Thompson, 2006). Actin polymerization facilitates an invagination of membranes and results in a tension at the vesicle neck where a constricting activity of the large GTPase dynamin appears to promote a fission of membranes and production of vesicles (Lanzetti, 2007). The formation of CCVs is a complex process that depends on and is coordinated by numerous accessory proteins which will not be discussed here in detail.

To achieve correct sorting of lysosomal proteins into the CCVs at the TGN, lysosomal proteins are separated from the non-clathrin trafficking pathway that is used by the secretory and plasma membrane proteins. In the sorting of lysosomal proteins at the TGN at least two types of CCVs are involved, one for the soluble lysosomal proteins and the other for lysosomal membrane proteins.

1.1.1 Adaptor proteins

Adaptor proteins (APs) play a key role in the transport of proteins. They regulate the formation of the clathrin scaffold and mediate the selection of the cargo proteins (Fig. 1.1). Four AP complexes have been characterized to date AP-1, AP-2, AP-3 and AP-4 (Robinson and Bonifacino, 2001). Each of them consists of four subunits: two large subunits (γ/β1, α/β2, δ/β3 and ε/β4), a medium (μ1-4) and a small (σ1-4) subunit. The μ and/or β subunits are involved in cargo selection and recognize distinct sorting signals that are present within the cytoplasmic tail of the cargo molecules (Ohno, 2006). The AP complexes display differences in cellular

localization patterns and mediate distinct vesicle-formation processes. AP-1, AP-3 and AP-4 are believed to function at the TGN and/or endosomes, whereas AP-2 functions at the plasma membrane (Bonifacino and Traub, 2003; Fig. 1.2). AP-mediated protein sorting depends on the recognition of sorting motifs that are present in the cytosolic domains of transmembrane proteins (McNiven and Thompson, 2006). Two major classes of endosomal sorting signals are referred to as "tyrosine-based" and "dileucine-based" (Bonifacino and Traub, 2003).

Lysosomal membrane proteins contain one or more sorting signals in their cytosolic domains and can directly interact with AP-3 at the TGN or early endosomes. It was shown that several lysosomal membrane proteins (Lamp-I/II, CD63, and Limp-II) are routed to the cell surface instead of lysosomes in AP-3 deficient mammalian cells (Le Borgne *et al.*, 1998; Dell'Angelica *et al.*, 1999). Thus AP-3 is believed to traffic lysosomal membrane proteins. In specialized cells, AP-3 has additional tissue-specific functions such as the formation of melanocyte pigment granules and platelet dense core granules. Mutations in AP-3 result in Hermansky-Pudlak syndrome type 2, an autosomal recessive disorder characterized by defects in lysosome-related organelle biogenesis (Dell'Angelica *et al.*, 1999). In neurons AP-3 participates in the biogenesis of synaptic vesicles (Gleeson *et al.*, 2004). AP-1 was initially considered to be responsible for the assembly of CCVs at the TGN and thus for the transport of mannose-6-phosphate receptors (MPRs) and their cargo to the late endosomes. Later, using fibroblasts from μ1 knockout mice, MPRs were found to accumulate in endosomes and not in the TGN (Meyer *et al.*, 2000). AP-1 is now thought to play a role in recycling MPRs from endosomes to the TGN. It might also cooperate in packaging of MPRs into CCVs at the TGN, however, the role of AP-1 in anterograde transport remains unclear (Owen *et al.*, 2004). AP-2 is excluded from the TGN membrane. Its complexes localize at the plasma membrane and mediate the formation of endocytic CCVs which eventually fuse with early endosomes (Owen *et al.*, 2004) and thus participate in sorting of lysosomal proteins *via* an indirect trafficking pathway to lysosomes. Much less is known about the AP-4 complex. It is localized to TGN vesicles and was shown recently to mediate polarized trafficking of dileucine-sorted proteins in epithelial cells (Ohno, 2006).

Several years ago, another family of adaptor proteins, the Golgi-localized gamma-ear-containing ARF-binding proteins (GGAs) were identified. GGAs 1-3 are associated with TGN and mediate transport of proteins from TGN to endosomes. All three GGAs, actually exist and function as part of a single complex at the TGN. Each GGA is recruited onto the same coated vesicles membrane where they bind to each other and function as a single complex (Gleeson *et al.*, 2004). The hinge region of GGAs contains a clathrin-binding region similar to that found in APs (Robinson *et al.*, 2004). GGAs recognize an acidic dileucine sorting motif on MPRs and mediate

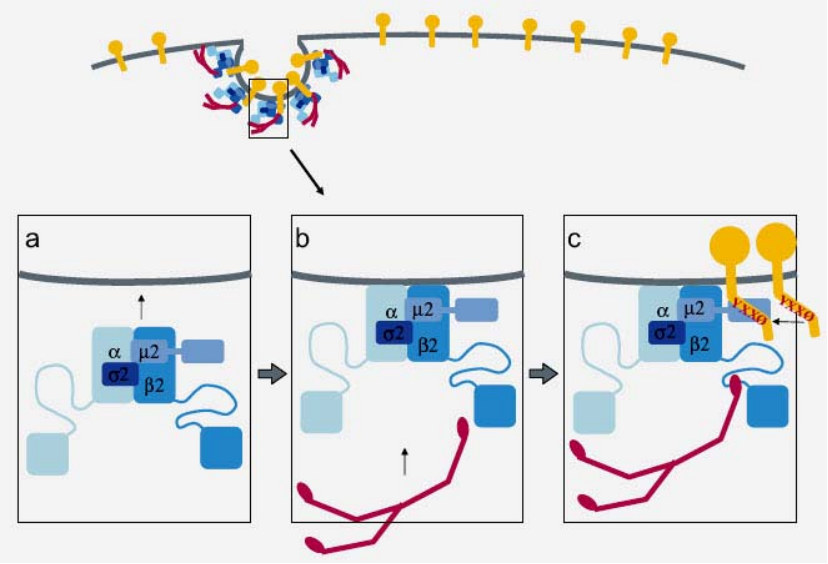


Figure 1.1 Schematic representation of the mechanism of action for the AP complex. Mode of action for the AP complex is depicted by using AP-2 as the representative. AP-2 regulates the formation of CCV and selection of cargo. CCV formation initiates when AP-2 is recruited from the cytosol to the plasma membrane, mainly by the affinity of the α subunit with the lipid components of the membrane (a). Next, clathrin is recruited, again from the cytosol, to the membrane-bound AP-2 (b). Upon binding to AP complexes, clathrin can self assemble to form the clathrin lattice which serves as a mechanical scaffold to bend the membrane. Transmembrane cargo proteins are thought to move relatively freely by diffusion in the membrane. When they diffuse into forming CCVs, they are trapped by coat component to be concentrated for selective sorting. Cargo proteins containing the YXXØ-type sorting signal ("tyrosine-based") in their cytoplasmic region are directly recognized and bound by the C-terminal domain of μ2 (scheme reproduced from Ohno, 2006).

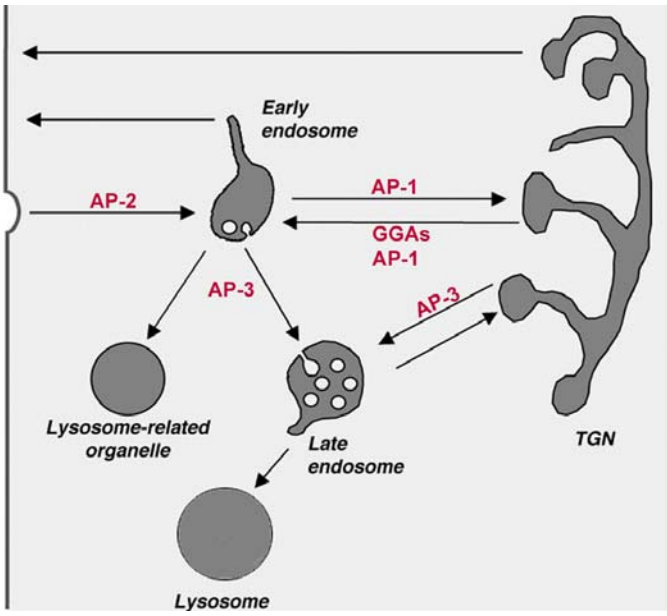


Figure 1.2 Adaptor proteins in sorting of newly synthesized lysosomal proteins. In mammalian cells, three adaptor complexes (AP-1, AP-3 and GGAs) mediate protein transport of newly synthesized proteins to lysosomal compartment. Lysosome-related organelle includes melanosomes, dense core granules, and other organelles that share some biogenetic pathways with endosomes and lysosomes (scheme reproduced from Bonifacino and Traub, 2003).

transport of the receptor-bound soluble lysosomal hydrolases to endosomes. It has been shown, that GGAs and AP-1 actually bind each other, and might cooperate in packing MPRs at the TGN (Doray *et al.*, 2002).

1.1.2 Sorting of soluble ligands at the TGN

CCVs deliver newly synthesized lysosomal hydrolases and lysosomal membrane proteins from TGN to the endosomal compartment. The lysosomal membrane proteins do not require a sorting receptor to leave TGN since their cytoplasmic tails interact directly with AP-3. However, most of the soluble lysosomal proteins require an interaction with transmembrane receptors to leave TGN. Examples are MPRs and sortilin, which enter CCVs to perform multiple rounds of transport (Robinson *et al.*, 2004).

1.1.2.1 Mannose 6-phosphate receptors

Newly synthesized lysosomal hydrolases are modified with one or more mannose 6-phosphate (M6P) residues in a two-step process (Hasilik *et al.*, 1980; Varki and Kornfeld, 1980). In the first step phospho-N-acetylglucosaminyl residues are attached to mannose-rich oligosacharides in *cis*-Golgi and in the second the glucosaminyl residues are removed by hydrolysis. This "uncovering" step occurs in TGN. It is necessary to expose M6P residues at the non-reducing termini of the N-linked oligosaccharide side chains and allows a recognition of the phosphorylated glycoproteins by M6P receptors (MPRs). The cytoplasmic domain of MPRs contains an acidic dileucine sorting motif and the hydrolase-receptor complex is recognized by GGAs adaptor proteins. Within CCVs the hydrolases are transported into endosomes (Ghosh *et al.*, 2004). The MPRs bind M6P residues at a slightly acidic pH in the TGN. The increasing acidity along the endosomal/lysosomal pathway induces a release of the hydrolases from the MPRs. The hydrolases are conveyed to lysosomes while the MPRs return to the TGN *via* AP-1 CCVs for reutilization (Meyer *et al.*, 2000). In the presence of agents that increase the endosomal and lysosomal pH, the lysosomal enzyme sorting is reduced and this results in the constitutive secretion of the precursor forms of lysosomal hydrolases (Hasilik and Neufeld, 1980; Braulke *et al.*, 1987). Indeed, this has led to the notion that pH-insensitive targeting of lysosomal enzymes is due to an M6P-independent transport pathway (Gupta *et al.*, 1984; Capony *et al.*, 1994). Evidence for the existence of alternative sorting receptors has been reported (McIntyre *et al.*, 1993), however, isolation and identification of the alternative targeting system has remained elusive**.**

Two distinct MPRs were identified by their ability to bind M6P-containing ligands. i) The cation-independent MPR (CI-MPR), has an apparent molecular weight of 215 kDa and does not require divalent cations for ligand binding (Sahagian *et al.*, 1981). ii) The cation-dependent MPR (CD-MPR), is predominantly a dimer with subunit apparent molecular mass of 46 kDa and requires divalent cations for high affinity ligand binding (Kornfeld *et al.*, 1985). Both receptors reach the cell surface and are rapidly internalized; however the CD-MPR is not efficient in mediating

endocytosis of extracellular ligands (Stein *et al.*, 1987). The pathway scavenging non-sorted (secreted) lysosomal enzyme precursors depends exclusively on CI-MPR.

In addition to its intracellular role in lysosome biogenesis, the CI-MPR has been implicated in numerous cellular processes, including cell growth, apoptosis, and cell migration, due to its ability to bind a wide range of ligands such as transforming growth factor β, granzyme B, CD26, insulin-like growth factor II (IGF-II), retinoic acid, urokinase-type plasminogen activator receptor and plasminogen at the cell surface (Hancock *et al.*, 2002). The ability of the CI-MPR to interact with many different molecules is mediated by distinct ligand-binding sites that are localized in at least some of its 15 homologous domains (Fig. 1.3).

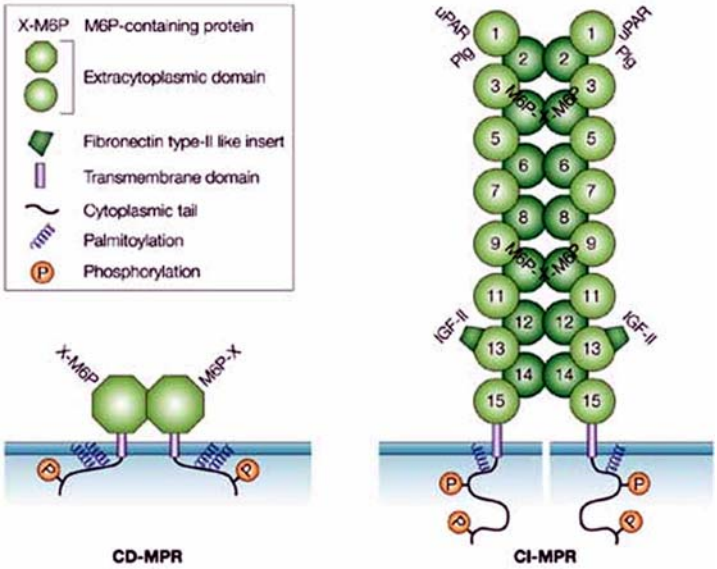


Figure 1.3 Mannose 6-phosphate receptors. The cation-dependent mannose 6-phosphate receptor (CD-MPR) is present predominantly as a stable homodimere in membranes and has a single M6P-binding site per polypeptide. The cation-independent mannose 6-phosphate receptor (CI-MPR) seems to be a dimmer in the membrane, although it tends to act as a monomer in detergent solution. CI-MPR possesses two M6P-binding sites and several non-M6P-binding sites. IGF-II, insulin-like growth factor II; uPAR, urokinase-type plasminogen activator receptor; Plg, plasminogen (scheme reproduced from Ghosh *et al.*, 2003).

1.1.2.2 M6P-independent sorting pathways

The observations on I-cell disease (ICD) patients suggest that, in addition to MPR-dependent mechanisms, MPR-independent mechanisms are likely to exist for the transport of newly synthesized lysosomal hydrolases to the lysosomes. In ICD disorder, deficiency of the phosphotransferase leads to synthesis of lysosomal enzymes that lack M6P markers (Hasilik *et al.*, 1981; Reitman *et al.*, 1981). These lysosomal enzymes fail to bind to MPRs in the TGN and are therefore secreted. As a result, excessive amounts of lysosomal enzymes are found in body fluids such as plasma, cerebrospinal fluid, tears and urine of ICD patients (Kornfeld, 1986). However, in tissues of these patients, such as liver, spleen, kidney and brain, lysosomal enzyme levels are close to normal despite a general deficiency in the phosphotransferase activity (Dittmer *et al.*, 1999). It was therefore proposed that many tissues possess alternative mechanism(s) for delivery of newly synthesized lysosomal enzymes to lysosomes independent of the M6P modification. Subsequently, a number of reports appeared on alternative MPR-independent pathways of sorting of the lysosomal enzymes. A study on knock-downs of CI- and CD-MPR genes in mice showed that the targeting of lysosomal carboxypeptidase cathepsin D into lysosomes was normal in thymocytes despite the lack of MPRs. In contrast, fibroblasts secreted the newly synthesized procathepsin D (Dittmer *et al.*, 1999). In rat hepatocytes, the majority of newly synthesized cathepsin H and cathepsin B was targeted to lysosomes by alternative mechanism(s) distinct from the M6P-dependent pathway (Tanaka *et al.*, 2000; Tanaka *et al.*, 2000). However, the nature of the M6P-independent pathway for sorting lysosomal enzymes is still unknown.

1.1.2.3 Sortilin

As mentioned aboive, several studies have provided evidence for the existence of an alternative, MPR-independent mechanism of lysosomal targeting (cf. also Hasilik *et al.*, 1981; Dittmer *et al.*, 1999). A MPR-independent lysosomal sorting mechanism has been defined in yeast through the identification of the yeast sorting

receptor Vps10p which traffics carboxypeptidase Y to the vacuoles (Marcusson *et al.*, 1994). Recently, a 95 kDa transmembrane glycoprotein sortilin has been found in several mammalian tissues (Petersen *et al.*, 1997) and implicated in the Golgi-endosome transport (Nielsen *et al.*, 2001). The luminal binding domain of sortilin is homologous to that of the yeast sorting receptor Vps10p (Petersen *et al.*, 1997). Furthermore, the C-terminal segment in the cytoplasmic tail of sortilin is closely related to the cytoplasmic localization and sorting sequence of CI-MPR (Johnson *et al.*, 1992).

Analysis of fibroblasts from patients with ICD showed that sphingolipid activator proteins (SAPs) and GM_2 activator protein (GM_2AP) use an alternative targeting mechanism to reach lysosomes (Lefrancois *et al.*, 2003). Inactivation of sortilin by siRNA effectively blocked the trafficking of SAPs to lysosomes in COS-7 cells (Lefrancois *et al.*, 2005) and a dominant negative competition experiment using a truncated sortilin in Sertoli cells impaired the lysosomal trafficking of SAPs and the GM_2AP (Hassan *et al.*, 2004). Recently, it has also been shown, that sortilin is involved in the lysosomal targeting of acid sphingomyelinase in COS-7 cells. Acid sphingomyelinase uses in part MPRs and in part sortilin as alternative receptors for targeting to lysosomes (Ni *et al.*, 2006).

The cytosolic tail of sortilin, similar to that of MPRs, can be recognized and shuttled within CCVs from TGN to lysosomes by the action of adaptor proteins, the GGAs. Dominant negative expression of a truncated form of a GGA results in the absence of the lysosomal targeting of SAPs and cathepsin B. It had no effect on AP-3 dependent sorting of Lamp-II to lysosomes (Hassan *et al.*, 2004).

1.2 Protein secretion from the TGN

The TGN is a distal compartment, in which secreted proteins are subjected to sorting and packaging for their final destinations. Trafficking of secreted proteins from TGN can occur in either constitutive or regulated manner (Burgess and Kelly, 1987). Constitutive secretion (Section 1.1.1) is used to deliver newly synthesized proteins

from the TGN to the plasma membrane and the extracellular space. In contrast, certain specialized secretory cells are able to store selected secretory proteins in vesicles and to secrete them in the presence of distinct stimuli. This process is known as the regulated secretion.

1.2.1 Constitutive secretion

The formation of secretory vesicles at the TGN is incompletely understood. The secretory proteins appear to leave the TGN in tubules with a larger diameter than typical CCVs. It seems that clathrin, APs and GGAs are excluded from the secretory vesicle formation. Blocking the budding machineries involving AP-1, AP-3 and clathrin has been reported to have no effect on constitutive secretion (Keller *et al.*, 2001, Ponnambalam *et al.*, 2003). On the other hand, it has been shown that vesicular stomatitis viral glycoprotein (VSVG) interacts with the AP-3 complex and that AP-3 facilitates VSVG transport from the TGN to the plasma membrane (Nishimura *et al.*, 2002). Dynamin, which is a very well-characterized fission protein in the formation of CCV, has also been implicated in VSVG export from the TGN (Cao *et al.*, 2000). However, the effect of dynamin on VSVG export was found to be cell-type-specific (Bonazzi *et al.*, 2005) and the role of dynamin in the formation of constitutive secretory vesicles remains controversial. Protein kinase D has been proposed to be essential in the formation of the constitutive secretory vesicles (Baron and Malhotra, 2002). This kinase probably acts as a regulator, rather than an effector, of fission. The biochemical identity of a potential coat, accessory proteins as well as the receptors that recognize the signal on cargo molecules is unknown (Bard *et al.*, 2006). However, the importance of fission and of a lipid-based machinery in the TGN-to-plasma membrane transport has been well established (De Matteis and Godi, 2004) as discussed further below.

1.2.1.1 Components regulating membrane fission and secretory vesicles formation

Recently Malhotra and Bard formulated a working model for the generation of constitutive secretory vesicles at the TGN, as shown in Figure 1.4. These authors suggest the existence of a signalling cascade at the TGN with a central role of protein kinase D (PKD). According to the proposed model, specific cargo destined for secretion or plasma membrane would activate a not-yet-identified G-protein-coupled receptor (GPCR) at the TGN to trigger dissociation of Gα and Gβγ subunits. The role

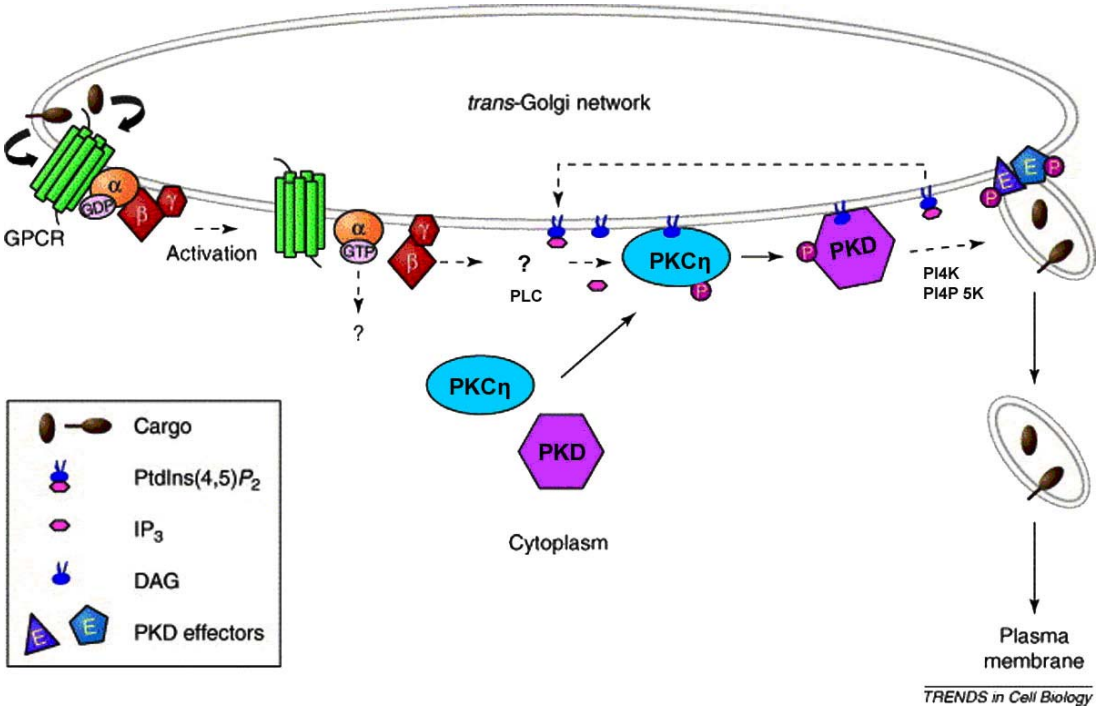


Figure 1.4 A signalling cascade model for secretory vesicles formation at the *trans*-Golgi network. Activation of a not-yet-identified G protein-coupled receptor (GPCR) by cargo induces by an unknown mechanisms, diacylglycerol (DAG) production at TGN. DAG recruits PKCη and PKD to the TGN. PKCη phosphorylates and activates PKD, which in turn phosphorylates effector proteins (E) to drive fission of secretory vesicles at the TGN. Association of PKD with a PI4K and a PI4P 5K could also stimulate fission through production of phosphoinositides that recruit additional effectors as well as acting as precursors for further DAG production by PLCβ, generating a positive-feedback loop for PKD recruitment (dashed line) and subsequent carrier fission. (scheme from Ghanekar *et al.*, 2005)

of Gα is currently unclear, whereas Gβγ complexes induce synthesis of diacylglycerol (DAG) at the TGN by an unknown mechanism. The generated DAG was proposed to recruit the TGN-associated protein kinase Cη (PKCη) as well as PKD from cytosol to the TGN membrane and subsequently to activate PKCη.The activated PKCη is likely to phosphorylate and activate PKD, which is assumed to phosphorylate and activate as-yet unknown target proteins involved in membrane fission (Bard *et al.*, 2006, Ghanekar *et al.*, 2005).

1.2.1.1.1 DAG

The first evidence that DAG plays a crucial role in formation of secretory vesicles came from the observation that mutants of Sec14 (a yeast PI-transfer protein) present defects in the secretory pathway at the level of the Golgi complex. Sec14 binds both phosphatidylinositol (PI) and phosphatidylcholine (PC). Furthermore, in its PC-bound form it negatively regulates the synthesis of PC *via* the CDP-choline pathway. This is the major pathway 'consuming' DAG, and thus an impairment of Sec14 accelerates the consumption of DAG (Kearns *et al.*, 1997).

The strongly conical shape of DAG may affect the composition and curvature of the bilayer and facilitate the budding, fusion and fission phenomena (Lev, 2006). Therefore, a local generation of DAG or assembly of a DAG-rich domain in the outer leaflet of TGN may initiate the generation of secretory vesicles. Its level in the TGN membrane appears to be tightly regulated by several metabolic pathways that are involved in its consumption and production (Matteis and Godi, 2004) (Fig. 1.5).

DAG is an important second messenger that recruits to membranes and/or activates specific proteins that function in membrane transport, such as PKD or PKCs. DAG signaling relies on the presence of conserved 1 (C1) domain in its target proteins. C1 domains also bind the DAG analogue phorbol ester, phorbol-12-myristate-13-acetate (PMA), with a high affinity (Toker, 2005). The structures of DAG and PMA are shown in Fig. 1.6.

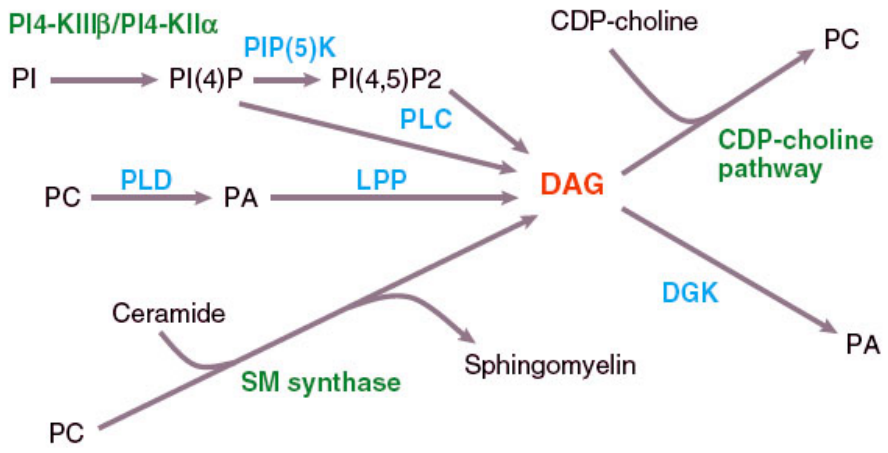


Figure 1.5 Possible mechanisms regulating the levels of diacylglycerol (DAG) at the TGN. DAG is generated transiently in biological membranes. The possible sources of DAG at the TGN are shown. The enzymes thus far localized to the Golgi membranes are shown in green. Other enzymes that play a role in DAG metabolism, for which the Golgi localization is controversial, are shown in blue. Other abbreviations used: DGK, DAG kinase; LPP, lipid phosphate phosphatase; PA, phosphatidic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; PI(4)P, phosphatidylinositol 4-phosphate; PIP 5-K, phosphatidylinositol 4-phosphate 5-kinase; PI(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PLD, phospholipase D; SM synthase, sphingomyelin synthase (scheme from Bard and Malhotra, 2006).

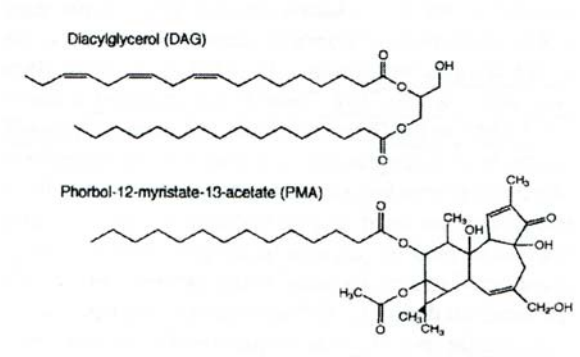


Figure 1.6 Structures of DAG and PMA.

1.2.1.1.2 Protein kinase D

Protein kinase D (PKD) is a serine/threonine kinase and also known as PKCμ. However, PKD is distinct from the PKC family (Ghanekar *et al.*, 2005). It is involved in the transduction of a wide variety of extracellular signals and is important in processes such as proliferation, apoptosis, differentiation and membrane trafficking

(Wang *et al.*, 2006). The initial evidence that PKD is required for the fission of transport carriers from the TGN came from the observation that, when a PKD inactive form was expressed in HeLa cells, the cargo destined for the plasma membrane and secretion was accumulated in tubules witch failed to detach from TGN (Liljedahl *et al.*, 2001).

As mentioned above, PKD is phosphorylated and activated by PKCη at the TGN. PKD has also been shown to bind DAG *via* its first C1a domain and this binding is necessary for its recruitment to the TGN (Baron *et al.*, 2002). PKD is recruited to the TGN membrane to phosphorylate key proteins involved in generation and fission of transport vesicles. Although the precise downstream targets of PKD have not yet been identified, several candidates containing the PKD consensus phosphorylation motif, such as DAG kinase or CtBP3/BARS, were proposed to mediate PKD-dependent fission of transport carriers by a local production of the fission-promoting lipids PA and DAG (Lev, 2006). It was recently found, that PKD phosphorylates and activates a phosphatidylinositol 4-kinase IIIβ (PI4KIIIβ) (Hausser *et al.*, 2005). The increased production of phosphatidylinositol 4-phosphate (PI4P) may be used for further production of DAG through the action of phospholipase C (PLC). Another possibility is that the increased concentration of PI4P may facilitate membrane separation between DAG and PI4P enriched lipid phases and facilitate fission (Bard and Malhotra, 2006). PI4KIIIβ is a substrate of PKD at the TGN. However, intracellular protein targets of PKD are as yet not fully identified.

1.2.1.1.3 Phospholipase D

Phospholipase D (PLD) catalyses the hydrolysis of the membrane lipid phosphatidylcholine (PC) and generates phosphatidic acid (PA). It is commonly accepted that in the intracellular membranes PA is short lived due to a rapid conversion into DAG by the PA phosphohydrolase (Lev, 2006). Hence, PA and DAG may act as second messengers in cellular responses. PLD activity is associated with several functions, including vesicle trafficking to and from the plasma membrane, cell migration and mitosis (Cockcroft, 2001). A unique feature of PLD is its high

preference for primary alcohols over water to produce phosphatidylalcohols instead of PA. This reaction is experimentally used to determine the role and activity of PLD *in vivo* (Chahdi *et al.*, 2002).

Two mammalian PLD genes, PLD1 and PLD2, have been identified (Nakashima *et al.*, 1997). PLD was found to be associated mainly with the plasma membrane, Golgi apparatus and nuclei (Freyberg *et al.*, 2001, Freyberg *et al.*, 2002). However, in mast cells, most of PLD1 is associated with secretory granules and PLD1 activation may take part in early stages of degranulation of mast cells (Hitomi *et al.*, 2004) and neutrophils (Tou *et al.*, 2005). Thus, its activity seems to facilitate a fusion of membranes.

PLD is activated by the small GTPases (such as ARF and RhoA proteins) and by the isoenzymes α and βII of PKC (Becker *et al.*, 2005). The first link between PKC and PLD emerged from studies that showed that phorbol ester, a structural analogue of DAG, could induce hydrolysis of PC by a mechanism involving PLD (Vinggaard *et al.*, 1991).

PC is an abundant lipid component of Golgi membranes. It can be cleaved by PLD thus raising the local concentration of DAG. Treatment with primary alcohols lowers the concentration of DAG in the Golgi. Under these conditions PKD is not recruited to the TGN and cargo transport to the cell surface is impaired (Baron *et al.*, 2002).

1.2.2 Regulated secretion

In multicellular organisms the regulated secretion is highly elaborate. In secretory cells such as neurons, endocrine and exocrine cells it is mediated by secretory granules and in hematopoetic cells by secretory lysosomes. In both cases, the contents of the organelles are released in response to diverse stimuli. The mechanism of sorting into secretory granules from TGN is poorly understood. It is thought to involve a passive aggregation of secretory proteins leading to their retention within maturing granules. The aggregation may be assisted by helper proteins facilitating mutual interactions between the luminal polypeptide species and/or between these and

the lipids of the granule membrane. The storage compartment is thought to progressively evolve and diverge from other post-Golgi sorting compartments (Arvan and Castle, 1998).

1.2.2.1 Secretory lysosomes

A set of immune system cells of hematopoetic origin contain a specialized lysosomal compartment that can release its contents into the extracellular environment in response to external stimuli. These organelles have been commonly referred to as ‘‘secretory lysosomes’’ (Holt *et al.*, 2006).

Secretory lysosomes share lysosomal characteristics such as the acidic pH and the ultimate degradative compartment of the cell. However, they also store secretory molecules that are released in response to an extracellular signal (Tab. 1.1). Hematopoietic cells posses specialized sorting mechanisms, which allow the correct sorting of the secreted products to the secretory lysosomes. However, this mechanism is not clear. It has been shown that many of the soluble basic antimicrobial proteins of haematopoietic cells are sorted to secretory lysosomes along with the proteoglycan serglycin and that serglycin plays a key-role in their targeting (Forsberg *et al.*, 1999, Galvin *et al.*, 1999, Lemansky *et al.*, 2001, Lemansky *et al.*, 2003, Abrink *et al.*, 2004, Grujic *et al.*, 2005). In other cells lysosomal exocytosis may be triggered by the necessity to repair the plasma membrane (Reddy *et al.*, 2001).

TABLE 1.1 - CELLS POSSESSING ‘SECRETORY’ LYSOSOMES
(reproduced from Griffiths, 1996)

Cell type (examples)	Functions	Secreted products
Cytotoxic T lymphocytes and natural killer cells	Target cell killing	Perforin Granzymes
Eosinophils	Defence against parasites	Major basic protein Neurotoxin
Neutrophils	Inflammatory response	Chemoattractants Histaminase
Basophils	Inflammatory response	Histamine
Platelets	Inflammatory response Clotting	Clotting factors Acid and neutral hydrolases

Mast cells	Inflammatory response	Histamine Serotonin
Macrophages	Phagocytosis Antigen presentation	Lysosome 'secretes' into phagosome
Osteoclasts	Bone resorption	Forms lysosome with bone

1.3 Aims of the present study

The aims of this study were to examine:

1. The nature of the M6P-independent pathway of the sorting of lysosomal enzymes. Promonocytic U937 cells target a portion of the lysosomal hydrolase cathepsin D in M6P-independent manner, similar to leukocytes in I-cell disease patients. In addition, the proteoglycan serglycin mediates the delivery of cationic proteins into lysosomes in a M6P-independent manner. A M6P-independent sorting and transport of lysosomal proteins should be characterized in U937 cells using immunofluorescence and cross-linking experiments.

2. The release of lysosomal proteins by cells treated with PMA. In this work the effects of PMA on the trafficking of both the newly synthesized lysosomal proteins and of mature lysosomal proteins should be studied.

3. The PMA-induced activation of major intracellular signaling pathwas known to involve protein kinase C and protein kinase D. To extend our knowledge on the molecular mechanisms of the regulated secretion this work will explore proteins that may become phosphorylated in the presence of PMA. The identification of one or more phosphorylated proteins should be attempted.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Acetic acid	Merck, Darmstadt
Acetone	Merck, Darmstadt
β-N-Acetyl glucosaminidase	Sigma, Taufkirchen
Acrylamide	Serva, Heidelberg
N,N´-Methylen-*bis*-acrylamide	Serva, Heidelberg
β-Aminocapronic acid	Riedel-de Haen, Seelze
Ascorbic acid	Merck, Darmstadt
Bovine serum albumin (BSA)	Serva, Heidelberg
Bromphenol blau	Serva, Heidelberg
Calyculin A	Alexis, Lausen
Cetyltrimethylammonium bromide (CETAB)	Merck, Darmstadt
Coomasie blue (Brilliant blue G 250)	Serva, Heidelberg
Coomasie blue (Roti-blue)	Serva, Heidelberg
Dimethyl sulfoxide (DMSO)	Roth, Karlsruhe
2,5-Diphenyloxasol (DPO)	Roth, Karlsruhe
Dithiotheitol (DTT)	ICN, Heidelberg
ECL detection kit	Amersham, Freiburg
Ethanol	Roth, Karlsruhe
Ethylendiamine (EDTA)	Merck, Darmstadt
Formaldehyde	Merck, Darmstadt
Glycerol	Merck, Darmstadt
HEPES	Serva, Heidelberg
Iodoacetamide	Serva, Heidelberg
Iodonitrophenyl tetrazolium chloride	Sigma, Taufkirchen

Isopropanol	Merck, Darmstadt
Leupeptin	Boehingen, Mannheim
L-Phenylalanine	Gibco BRL, Karlsruhe
Magnesium chloride	Merck, Darmstadt
Magnesium sulfate	Merck, Darmstadt
Methanol	Roth, Karlsruhe
Srimmed milk powder	Fluka, Steinheim
3-(N-Morpholino)-propanesulfonic acid (MOPS)	J.T.Baker, Phillipsburg
p-Nitrophenyl-N-acetyl-β-D-glucosaminide	Sigma, Taufkirchen
p-Nitrophenyl-β-D-glucopyranoside	Sigma, Taufkirchen
Pansorbin (*Staph.* A-Suspension)	Merck Biosciences, Schwalbach
Penicillin/Streptomycin (100x)	PAA, Pasching
Pepstatin A	Sigma, Taufkirchen
Peppermint stick phosphoprotein molecular weight standard	Invitrogen, Oregon
Phenyl methyl sulfonyl fluoride (PMSF)	Merck, Darmstadt
Phosphoric acid	Merck, Darmstadt
PMA (4β-Phorbol 12-myristate 13-acetate)	Sigma, Deisenhofen
Potassium hydroxide	Baker, Deventer
Pro-Q-Diamond phosphoprotein gel stain	Invitrogen, Oregon
Protein molecular weight marker (broad range)	Bio-Rad, Munich
Pyronin Y	Serva, Heidelberg
Roti-Blue (colloidal Coomassie blue)	Roth, Karlsruhe
RPMI- Medium	PAA, Cölbe
Saponin	Sigma, Deisenhofen
Silver nitrate	Roth, Karlsruhe
Sucrose	Roth, Karlsruhe
Sodium azide	Merck, Darmstadt
Sodium carbonate	Riedel-de-Haen, Seelze
Sodium chloride	Sigma-Aldrich, Seelze
Sodium dodecyl sulfate (SDS)	Roth, Karlsruhe

Sodium hydroxide	Merck, Darmstadt
Streptomycin	Gibco-RBL, Karlsruhe
Scintillation cocktail (Rotiszint eco plus)	Roth, Karl
TEMED	Sigma, Deidenhofen
Tris(hydroxymethyl)-aminomethan	ICN, Meckenheim
Triton X-100	Merck, Darmstadt
Urea	ICN, Ohio
ZFF (Z-Phe-Phe-diazomethyl ketone)	Bachem, Heidelberg

2.1.2 Antibodies

Primary antibodies	**Organism**	**Source**
Anti-Lamp-II	mouse	our laboratory
Anti-β-Hex	goat	our laboratory
Anti-CD	rabbit	our laboratory
Anti-CI-MPR	goat	Proff. Kurt von Figura, Göttingen
Anti-CI-MPR	mouse	our laboratory
Anti-CD-MPR	goat	our laboratory
Anti-NE	rabbit	Merck Biosciences, Schwalbach
Anti-IRAP	mouse	Proff. M. Birnbaum, Philadelphia
Anti-Golgin-97	mouse	Molecular Probes, Leiden
Anti-Serglycin	goat	Santa Cruz Biotechnology, Heidelberg
Anti-Serglycin	rabbit	Dr. C. U. Niemann, Copenhagen
Anti-Sortilin	rabbit	Dr. C. M. Petersen, Aarhus
Anti-AP-3	rabbit	Dr. S. Hönig, Göttingen
Anti-saposin C	rabbit	Dr. K. Sandhoff, Bonn

Secondary antibodies	**Organism**	**Source**
Anti-Mouse IgG (HRP conjugate)	goat	Bio-Rad, Munich
Anti-Rabbit IgG (HRP conjugate)	goat	Bio-Rad, Munich
Anti-Mouse IgG (Cy2 conjugate)	goat	Dianova, Hamburg

Anti-Mouse IgG (Cy3 conjugate)	donkey	Dianova, Hamburg
Anti-Mouse IgG (Cy3 conjugate)	goat	Dianova, Hamburg
Anti-Rabbit IgG (Cy2 conjugate)	goat	Dianova, Hamburg
Anti-Rabbit IgG (Cy3 conjugate)	goat	Dianova, Hamburg
Anti-Goat IgG (Cy2 conjugate)	donkey	Dianova, Hamburg
Anti-Goat IgG (Cy3 conjugate)	goat	Dianova, Hamburg
Anti-Goat IgG (Alexa546 conjugate)	donkey	Invitrogen, Karlsruhe
Anti-Rabbit IgG (Alexa 488 conjugate)	chicken	Invitrogen, Karlsruhe

2.1.3 Radiochemicals

[^{35}S]Methionine (70%) and [^{35}S]cysteine (30%)	Hartman Analytica, Braunschweig
[^{35}S]H_2SO_4	Hartman Analytica, Braunschweig
[^{32}P]Orthophosphate	Hartman Analytica, Braunschweig
[^{14}C]Methylated protein standard	Sigma, Deisenhofen
[^{125}I]Protein standard	made in our laboratory

2.1.4 Instruments

Blotting chamber	TRANS-BLOT^{R}	Bio-Rad, Munich
Confocal Microscope	Axiovert 200 M	Zeiss, Jena
Axiovert Microscope	Axioplan 2 MRm	Zeiss, Jena
ELISA-Reader	MRX	Dynatech, Denkendorr
Rotor for ultracentrifuge	SW 40	Beckman, Munich
Ultracentrifuge	L8-70 M	Beckman, Munich
Centrifuges	5415C	Eppendorf, Hamburg
	Super T21	Sorvall, Bad Homburg
Watter bath	Thermomix BC	Braun, Melsungen

2.2 Methods

2.2.1 Cell culture

U937 promonocytic cell line (Sundstrom and Nilsson, 1976) was grown in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin and incubated at 37 °C in 5% CO_2 atmosphere. The culturing was performed in 25 cm^2 or 75 cm^2 flasks and the cells were sub-cultured every 3-4 days in a ratio of 1:4.

2.2.2 General biochemical methods

2.2.2.1 Estimation of protein concentration using the Bradford assay

The method is based on the proportional binding of the Coomassie Brilliant blue dye to proteins. Samples were diluted in 0.25 M NaOH/0.025% (v/v) TritonX-100 to 20 µl. After adding 300 µl of Bradford reagent, absorbance was measured at 595 nm. Bovine serum albumin (BSA) was used as a standard.

Bradford reagent:	0.1 g/l	Coomassie brilliant blue
	150 mM	H_3PO_4
	5% (v/v)	Ethanol

2.2.2.2 Assays of enzymatic activities

2.2.2.2.1 Assay of β-hexosaminidase (EC 3.2.1.52, von Figura 1977)

β-Hexosaminidase is a soluble enzyme and a convenient marker of late endosomes and lysosomes. Samples were diluted in 0.9% NaOH to 50 µl, mixed with 50 µl of substrate buffer and incubated for 60 min at 37 °C. Product was detected as *p*-nitrophenolate after adding 250 µl stopp solution at 405 nm.

Substrate buffer:	10 mM	*p*-Nitrophenyl-2-acetamido-2-deoxy--β-D-glucoside
	100 mM	Citrate, pH 4.6
	0.2% (w/v)	BSA
	0.2% (v/v)	TritonX-100
	0.04% (w/v)	Sodium azide
Stop solution:	0.4 M	Glycine/NaOH, pH 10.4

2.2.2.2.2 Assay of succinate dehydrogenase (EC 1.3.5.1, Diettrich *et al*., 1996)

Succinate dehydrogenase, as the component of respiratory chain, was used as a mitochondrial marker enzyme. The sample was made up to 50 µl with 0.9% NaCl and mixed with 82 µl of the freshly made substrate solution. Reaction was allowed to proceed for 4 h at 37 °C and was terminated by the addition of 150 µl of stop solution. The product was extracted with 400 µl of extraction solution by vortexing and after a centrifugation at 14.000 rpm for 10 min its concentration was determined by absorbance at 490 nm.

Substrate solution:	122 mM	Sodium succinate
	61 mM	Sodium phosphate, pH 7.4
	0.09% (w/v)	Iodonitrophenyl tetrazolium chloride
	0.05% (w/v)	Sodium azide
Stop solution:	10% (w/v)	Trichloroacetic acid
Extraction solution:	50% (v/v)	Ethyl acetate
	50% (v/v)	Ethanol

2.2.2.2.3 Assay of alkaline phosphatase (E.C.3.1.3.1, Taute *et al.*, 2002)

Alkaline phosphatase was used as a marker of the plasma membrane. Samples were diluted with 0.9% NaCl to 50 µl, mixed with 250 µl substrate solution and incubated at 37 °C for 1 h. Product *p*-nitrophenolate was measured at 405 nm.

Substrat solution:	5 mM	*p*-Nitrophenyl phosphate
	250 mM	Sucrose
	5 mM	$MgCl_2$
	0.1% (w/v)	TritonX-100
	50 mM	Tris-HCl, pH 9

2.2.2.3 SDS-PAGE

For sample characterization, proteins were separated by SDS-PAGE according to Laemmli (1970) with modification as described below.

2.2.2.3.1 Preparation of acrylamide gels

The gels with a thickness of up to 1 mm, length 13.5 cm and width 14 cm were used for the protein separation. Plastic combs with 22 or 33 wells were used. Gels were casted with a low percentage stacking gel on the top and high percentage gel on the bottom.

Table 2.1 Acrylamide gel solutions

Stacking gel		
Acrylamide (%(w/v))	3%	4.8%
N,N´-Methylene bisacrylamide (%(w/v))	0.11%	
Tris-HCl, pH 8.8	0.125 M	
SDS (%(w/v))	0.1%	

TEMED (%(v/v))	0.1%
APS (%(w/v))	0.1%

Separation gel		
Acrylamide (%(w/v))	10.6%	15%
N,N´-Methylene bisacrylamide (%(w/v))	0.11%	0.16%
Tris-HCl, pH 8.8	0.375 M	
SDS (%(w/v))	0.1%	
TEMED (%(v/v))	0.1%	
APS (%(w/v))	0.1%	

The separation gel mixture was prepared and poured in between the glass plates and separation gel overlay (0.375 M Tris-HCl, pH 8.8; 50% (w/v) isopropanol) was applied on the top. After 30 min, the overlay was washed off. The stacking gel mixture was poured on top of the separation gel and well combs were inserted. The polymerisation was completed within 30 min. The gel was used fresh or stored at 4°C.

2.2.2.3.2 Sample preparation

After protein determination according to Bradford, the sample was mixed with 1.5X solubilization buffer in 1:2 ratio. After adding the solubilizer, samples were kept at 37 °C with gentle shaking for 30 min. Iodoacetamide was added to a final concentration of 125 mM. Along with the samples, the molecular weight standard was prepared.

1.5X Solubilization buffer:	1.5% (w/v)	SDS
	15 mM	Dithiothreitol
	0.25 M	Tris-HCl, pH 6.8
	15% (v/v)	Glycerol

2.2.2.3.3 Electrophoresis

The electrophoresis was performed at 15 mA during the migration though the stacking gel and at 30 mA during the migration though the separation gel. The separation took approximately 3.5 h. Afterwards the gel was removed from the system and used for analysis.

2.2.2.3.4 Silver staining according to Heukeshoven (modified)

Electrophoretically separated polypeptides can be visualized by silver staining method as described in Table 3.2. For each step 100 ml reagent was used. After stain, the gel was dried between two layers of cellophane membrane.

Table 2.2 Staining of proteins by silver nitrate

Step	Reagent	Duration
Fixing	Fixative	30 min
Reduction	Thiosulfate reagent	30 min
Wash	deionised water	3 x 10 min
Staining	Silver nitrate solution	25 min
Wash	deionised water	30 sec
Development	Developer	3-5 min
Stop	7-8 ml concentrated acetic acid	5 min
Wash	deionised water	2 x 30 min

Fixative:	30% (v/v)	Ethanol
	10% (v/v)	Acetic acid
Thiosulfate reagent:	0.1 M	Sodium acetate, pH 6
	30% (v/v)	Ethanol
	0.1% (w/v)	Sodium thiosulfate

Silver nitrate solution:	0.1% (w/v)	Silver nitrate
	0.01% (w/v)	Formaldehyde
Developer:	2.5% (w/v)	Sodium carbonate
	0.02% (w/v)	Formaldehyde

2.2.2.3.5 Coomassie blue staining of proteins

Protein staining with colloidal Coomassie blue solution was applied in the case of preparative gels, as silver staining is not compatible with mass spectrometry protein identification. As shown in Table 2.3, for each step 100 ml reagent was used.

Table 2.3 Staining of proteins by colloidal Coomassie blue

Step	Reagent	Duration
Fixing	Fixative	1 h
Staining	Staining solution	Over night
Wash	Destainig solution	5 x 30 min

Fixative:	40% (v/v)	Ethanol
	10% (v/v)	Acetic acid
Staining solution:	20% (v/v)	Roti-blue (concentrate)
	20% (v/v)	Methanol
Destainig solution:	1% (v/v)	Acetic acid

2.2.2.4 2D-CETAB/ SDS-PAGE diagonal electrophoresis

2D-CETAB/ SDS-PAGE is a two dimensional gel electrophoresis technique that is especially suited to separate membrane proteins. CETAB (N-Cetyl-N,N,N-trimethylammonium bromide), as a cation detergent supported by an acidic pH, was used for the solubilization and separation of proteins in the first dimension followed by SDS-PAGE in the second dimension (Table 2.4 and 2.5).

Proteins were concentrated by precipitation with 4 volumes of cold acetone. The pellet was solubilized with 30 µl 1.5X solubilization buffer and diluted with 15 µl water. An effective solubilization was achieved with sample incubation at 60 °C for 5 min. The solubilized material was centrifuged 5 min at 14.000 rpm to remove aggregates and undissolved debris.

Anode and cathode buffers were pre-warmed to RT. The electrophoresis was started at an electric current 10 mA per gel and increased to 20 mA once the pyronin Y dye reached the separation gel. After electrophoresis, the sample lanes were cut out and incubated in equilibration buffers I and II each for 20 min at 37 °C.

1.5X Solubilization buffer:	4.5 M	Urea
(pH 5.1)	1.5% (w/v)	CETAB
	141 mM	Acetic acid
	96 mM	KOH
	15 mM	DTT
	7.5% (v/v)	Glycerol
	0.01% (w/v)	Pyronin Y

Table 2.4 The gel composition for CETAB electrophoresis

Components	Stacking gel (pH 5.1)	Separation gel (pH 4.0)
Acrylamide	4% (w/v)	7.5% (w/v)
N,N´-Methylene-*bis*-acrylamide	0.34% (w/v)	0.26% (w/v)
KOH	64 mM	43 mM
Acetic acid	94 mM	280 mM
Urea	6 M	6 M
CETAB	0.1% (w/v)	0.1% (w/v)
Ascorbic acid	4 mM	4 mM
$FeSO_4$	4.25 µM	8 µM
H_2O_2	0.0015%	0.001%

Table 2.5 The gel buffer composition for CETAB electrophoresis

Components	Stacking gel buffer (anode buffer)	Separation gel buffer (cathode buffer)
KOH	43 mM	64 mM
Acetic acid	280 mM	94 mM
CETAB	0.1% (w/v)	0.1% (w/v)
Isopropanol	50% (w/v)	-

2.2.2.5 Western blotting and detection

The electrophoretically separated protein can be detect immunochemically after a transfer onto an immobilizing membrane. The gel was equilibrated in washing buffer for 20 min. The PVDF membrane was soaked in 100% methanol for a few seconds and was washed in washing buffer. Two pairs of blotting paper with the size of gel were soaked, in the anode buffer and the cathode buffer for 20 min, respectively. The gel was transferred into the blotting apparatus which was already prepared for the blotting: The lower, anionic electrode was covered with two layers of blotting papers soaked in the anode buffer and the PVDF membrane. The gel was carefully lowered onto the immunoblotting membrane and was covered with two layers of blotting papers soaked in the cathode buffer. A test tube was rolled over the layers to remove the excess liquid and air. The electrophoretic transfer was performed for 2 h at 1 mA per cm^2 gel surface. Along with the samples, broad range molecular weight standard was used. The standard line was cut out and stained with Coomassie blue staining.

After the transfer, the labeled PVDF membrane was placed in a blocking buffer for at least 1 h at room temperature. After saturating the membrane with protein, the primary antibody was added directly into blocking buffer. Binding was allowed to take place overnight at 4 °C. Excess antibody was washed away using the TBS buffer containing 0.1% TritonX-100. This was repeated for 3 times with each

solution. The secondary antibody, an HRP conjugate diluted in the blocking buffer was poured on the washed membrane and incubated for another 2 h at room temperature. The washing step was repeated.

The blot was developed with ECL detection kit. The membrane was kept on a thin layer of plastic on the top of with ECL mixture was poured and spread uniformly. Within 2 min, the blot was transferred in to a cassette and exposed to a photographic film for a specific time. The gel was stained with silver.

Washing buffer:	23 mM	Tris
	384 mM	Glycine
Anode buffer:	25 mM	Tris
	192 mM	Glycine
Cathode buffer:	40 mM	ε-Amino caproic acid
	192 mM	Glycine
Blocking buffer:	7% (w/v)	Skim milk powder in TBS

2.2.2.6 Pro-Q Diamond Phosphoprotein staining (Agrawal and Thelen, 2005)

Pro-Q Diamond is a commercially available stain that binds the phosphate moiety of phosphoproteins and allows fluorescent detection of phosphoproteins directly in polyacrylamide gels.

Samples to be analyzed by Pro-Q Diamond Phosphoprotein staining were first separated on 2D-CETAB/SDS-PAGE. PeppermintStick phosphoprotein molecular weight standard with phosphorylated ovalbumin and α-casein was used in the second SDS-PAGE dimension. Once the run was completed, the gel was fixed for 30 min and overnight in two aliquots of the fixing solution while gently agitated. The gel was washed twice for 10 min with ultra pure water and stained with Pro-Q diamond staining solution for 2 h. The volume of the staining solution was equivalent to 10 times the volume of the gel. Staining and following steps were protected from light.

The gel was destained with destain solution 4 times for 30 min and washed with ultra pure water twice for 5 min.

Stained gels were visualized with Typhoon gel-scanning instrument with excitation source 532 nm and emission filter 560 nm. After scanning the gel was stained with colloidal Coomassie blue solution for total-protein stain (2.2.2.3.5). The stained phosphoproteins were excised, digested with trypsin and identified by mass spectrometry.

Fixing solution:	50% (v/v)	Methanol
	10% (v/v)	Acetic acid
Destain solution:	20% (v/v)	Acetonitrile
	50 mM	Sodium acetate, pH 4

2.2.2.7 Identification of proteins by mass spectrometry

For the identification of proteins, matrix assisted laser desorption ionisation-time of flight spectroscopy (MALDI-TOF) of samples digested with trypsin using a Bruker Daltonics Ultraflex™ mass spectrometer was carried out with the help of Dr. Julius Nyalwidhe, Biology department, Philipp University Marburg. The evaluation of the data was performed using Mascot analysis tools. The digestion was carried out in 10 µl gel digestion solution containing 10% CH_3CN, 40 mM NH_4HCO_3 pH 8.1 and 200 ng trypsin (trypsin modified sequence grade, Promega).

2.2.2.8 Cell fractionation using linear sucrose density gradient centrifugation

U937 cells (100 – 200 x 10^6) were washed once with PBS and resuspended in 0.5 ml cold MOPS-buffer with proteinase inhibitors and phosphatase inhibitor calyculin (Table 3.6). Cells were disrupted by N_2 cavitation, keeping them for 15 min under a pressure of 30 bars at 4 °C. The sudden release of the pressure resulted in

rupture of the plasma membranes, and cell organelles remained intact. After removal of undisrupted cells and nuclei by centrifugation at 600 × *g*, the cavitate was resuspended in 67% (w/w) sucrose to final concentration of 50% and layered under linear sucrose gradient.

Linear sucrose gradients were prepared with a two chamber gradient mixer. Sucrose solutions I and II (4.7 ml each) were mixed and filled in centrifuge tube to generate a gradient of 18-47 %.

Gradients were centrifuged overnight at 220,000 x g_{max} for 16 h. Intact organelles floated into the gradient until they reached the region of their inherent density. Cytosolic material and the content of ruptured organelles remained at the bottom of the tube. After the centrifugation, 12-13 fractions, 870 µl each were collected and analyzed for the protein content (2.2.2.1), marker enzyme activities (2.2.1.2), Western blots (2.2.2.5) and phosphoprotein staining (2.2.2.6).

MOPS-buffer:	70 mM	MOPS/NaOH, pH 6.8
	10 mM	NaCl
	1 mM	$MgCl_2$
	1 mM	EGTA

Table 2.6 Proteinases and phosphatases inhibitors and concentration

Inhibitors	Concentration	Inhibits
Phenyl methyl sulfonyl fluoride	1 mM	Serine proteinases
Leupeptin	10 µM	Serine and cysteine proteinases
Z-Phe-Phe-diazomethylketone	1 µM	Cathepsins
Pepstatin A	1 µM	Aspartic proteinases
Calyculin A	50 nM	Phosphatase

Sucrose solution I:	18% (w/w)	Sucrose in MOPS-buffer

Sucrose solution II: 47% (w/w) Sucrose in MOPS-buffer

2.2.3 Metabolic radiolabeling, isolation and detection of labeled macromolecules

2.2.3.1 Incorporation of [^{35}S]-labeled amino acids and sulfate

Metabolic labeling was performed with [^{35}S]methionine/[^{35}S]cysteine and [^{35}S]H_2SO_4 in methionine/cysteine- and sulfate- deficient RPMI 1640 medium, respectively. Before labeling, the cells were washed three times in deficient RPMI medium and kept in this medium for at least 1 h up to 3 h. Both labeling media contained 10% heat-inactivated fetal calf serum, which was dialyzed against 0.9% NaCl. Sulfate-deficient medium contained 0.1 mg/mL ampicillin as antibiotics. The amount of radioactivity was varied and will be specified in section Results.

After metabolic labeling with [^{35}S]methionine/[^{35}S]cysteine, the culture medium and the cell extracts were subjected to immunoprecipitation and the immunoprecipitates were separated on SDS-PAGE. The gels were washed three times for 20 min in DMSO and incubated overnight in 16.8% (w/v) DPO in DMSO at 37 ºC. The treated gels were dried, exposed to KODAK BioMax XAR-5 films at -80 ºC and the radioactivity was visualized by fluorography (Laskey and Mils, 1975).

In pulse-chase labeling experiments, cells were labeled for 10 min with sulfate-free RPMI medium containing 400 µCi/ml [^{35}S]H_2SO_4. The chase period was initiated by washing and incubating the cells in RPMI containing 10 mM $MgSO_4$. Afterwards the cells were lysed and polypeptides from corresponding aliquots of cell lysate and the medium were precipitated with TCA and analyzed by liquid scintillation counting.

2.2.3.2 Labeling with [^{32}P]orthophosphate

U937 cells (5 x 10^6) were metabolically labeled with [^{32}P]orthophosphate in the phosphate-deficient RPMI medium. Prior to labeling, the cells were washed three times in phosphate-deficient RPMI medium and kept in this medium for 30 min. Cell suspension (600 µl) was labeled with 0.6 mCi [^{32}P]orthophosphate for 20 min. The cells were labeled in the presence or absence of 50 nM PMA. PMA was added to the cells in the same time as radioactive label. Labeled cells were subjected to cell fractionation in sucrose density gradient as described above.

2.2.3.3 Precipitation of proteins with TCA

Samples containing sulfated proteins were mixed with 1 volume 20% TCA and incubated on ice for 30 min. The precipitated proteins were pelleted in an Eppendorf centrifuge at the maximum speed for 1 min and were washed two times with 5% TCA. Samples in form of pelleted proteins were solubilized with 50 µl 1.5X solubilization buffer (2.2.2.3.2).

2.2.3.4 Immunoprecipitation

The pelleted metabolically labeled cells (1-2 x 10^6) were resuspended in immunoprecipitation lysis buffer and the collected medium was made up to 1X immunoprecipitation lysis buffer by adding 10X immunoprecipitation lysis buffer. Nonspecific background was reduced by preclearing the 400 µl (800 µl) sample with 50 µl (100 µl) of *StaphA*-suspension (Pansorbin). The samples were incubated 30 min at 4ºC with rocking. Nonspecific immunocomplexes were centrifuged in a microcentrifuge at maximum speed for 2 min and the supernatant was transferred to a fresh tube. The samples were mixed with 2-4 µl of antibody against protein of interest and incubated overnight at 4 ºC. Next, 10 vol 20% *StaphA* per vol antiserum was added and incubated for 30 min at 4 ºC with rocking. The *StaphA*-antibody-antigen complexes were collected by sedimentation in a microcentrifuge at maximum speed

for 2 min. The supernatant was carefully removed. All precipitates were washed twice with IMM, twice with IMM/2 M KCl, twice with Neufeld-buffer and once with 1/10 TBS. The pellets were treated with 30 µl 1.5X solubilization buffer (2.2.1.3.2) at 37ºC.

Immunoprecipitation lysis buffer:	50 mM	Tris/HCl, pH 7.4
	0.5% (w/v)	TritonX-100
	10 mM	Iodoacetic acid
	1 mM	PMSF
IMM:	10 mM	PBS pH 7.4
	0.5% (w/v)	Sodium desoxycholate
	0.5% (w/v)	BSA
	1% (w/v)	TritonX-100
Neufeld-buffer:	10 mM	Tris/HCl, pH 8.5
	600 mM	NaCl
	0.1% (w/v)	SDS
	0.05% (w/v)	NP-40

2.2.3.5 Cross-linking of pCD and pSap

The labeling with [^{35}S]methionine/[^{35}S]cysteine was performed in the presence of 10 mM NH_4Cl and 0.1 µM PMA to enhance the secretion of proCD and proSap. After labeling for 15 h, the cells were pelleted by centrifugation in Eppendorf centrifuge for 20 s, washed twice with PBS and resuspended in PBS containing freshly prepared 1 mM cross-linking reagent dithio-*bis*-succinimidyl-propionate (DSP). Both the cells and the medium were incubated with 1 mM DSP for 10 min at 37 °C. A stock solution of DSP, 50 mM, was dissolved in DMSO. The controls were incubated with 2% DMSO. After cross-linking, the cells were washed once with PBS and resuspended in immunoprecipitation lysis buffer. The cells and medium samples were then processed for immunoprecipitation (2.2.3.4). The immunoprecipitated proteins

were solubilized in the presence of 20 mM DTT which was used to reductively cleave the cross-links. To compare the cross-linking effect, samples were separated by SDS/PAGE as described above and visualized by fluorography (2.2.3.1).

2.2.4 Immunofluorescence microscopy

2.2.4.1 Indirect immunocytochemistry

U937 cells were washed once in PBS and transferred on a poly-L-lysine coated glass slide (0.3 x 10^6 cells in 50µl PBS per slide). The slide was covered with the cover slip for approximately 2 min to allow the cells attach to poly-L-lysine. The cover slip was carefully removed and 200 µl 4% PBS-paraformaldehyde was added onto each slide and incubated for 30 min at RT. After fixation, the slides were washed 3 times for 5 min each in the coupling jar containing PBS. The cells were permeabilized in PBS/0.3% TritonX-100 for 3 min, washed once in PBS and blocked with PBS/3% BSA for 30 min.

The primary antibodies were dissolved in PBS (1:100 or 1:200). 100 µl of antibody solution was added onto each slide and all the slides were covered with the cover slip and incubated overnight in an airtight humid chamber at 4 °C. After washing 4 times 15 min each, Cy2- and Cy3-labeled secondary antibodies, diluted 1:200 or 1:1000, respectively, in PBS, was applied for 45 min in dark at RT. The slides were washed 4 times for 15 min each in PBS. The excess solution from the sides of the slides was wiped off and the slides were air dried for 5 min in dark at RT. A drop of Fluoromount-G was spread on the sample and the slides were covered with cover slips. The specimens were stored at 4 °C in dark.

Images were taken using an Zeiss Axiocam MRm under the 65X objective and Zeiss Axiovert 200 M confocal laser scan microscope with a LSM 510 meta confocal

laser unit. The images using processed with Adobe Photoshop 6.0 (Adobe Systems) and Huygens 3D deconvolution software.

2.2.4.2 Indirect immunocytochemistry of plasma membrane antigens

Indirect immunocytochemistry was used to detect antigens at the plasma membrane using non-permeabilized cells. All steps in the staining were performed at 4ºC. U937 cells were washed once in cold PBS and incubated with PBS/3% BSA for 30 min. The primary antibody, 1 µl, was diluted in 100 µl PBS and added into the tube containing 1 x 10^6 of cells. After 30 min, the cells were washed 4 times and incubated with Cy2- or Cy3-labeled secondary antibody, diluted 1:200 or 1:1000 in PBS, respectively, for 30 min. The cells were washed 4 times with PBS and finally attached to a poly-L-lysine coated slide and fixed as mentioned above.

3. Results

3.1 Sorting and transport of lysosomal proteins in U937 cells

3.1.1 Mannose 6-phosphate receptors (MPRs)

In order to examine the localization of CD-MPR and CI-MPR in U937 cells a double immunoflurescent staining was performed. Both CD-MPR and CI-MPR showed a pattern of dense staining near the nucleus, which is representative of the TGN and endosomes. This result illustrates that the CD-MPR and CI-MPR strongly colocalize in U937 cells (Fig. 3.1). However, a few small vesicular structures of the CI-MPR appear not to overlap with the staining for CD-MPR.

Cathepsin D (CD), is a lysosomal proteinase which is commonly used as a lysosomal marker. It is known that CI-MPR (unlike CD-MPR) plays a major role in targeting of this protein to lysosomes (Ludwig *et al.*, 1994). When U937 cells were co-stained for CI-MPR and CD, a few small vesicles showed an overlap, whereas most of the CD was localized in compartments not containing CI-MPR (Fig. 3.1). Due to the higher concentration of CD in the late endosomes and lysosomes as compared to TGN, CD brightly marks mainly the lysosomes, whereas CI-MPR marks the TGN and the endosomes.

3.1.2 Sortilin

In order to compare the distribution of sortilin with that of CI-MPRs in U937 cells, a coimmunostaining was performed. Sortilin was detected in small vesicles distributed through the cytoplasm, whereas, CI-MPR was distributed in large vesicles and tubular structures reminiscent of TGN and endosomes. Thus, sortilin and CI-MPR showed different sub-cellular distributions. Nevertheless, as shown in Figure 3.2, there was a partial colocalization of the two receptors. This indicated the existence of a TGN-lysosomal sorting mechanism of sortilin independent of MPRs.

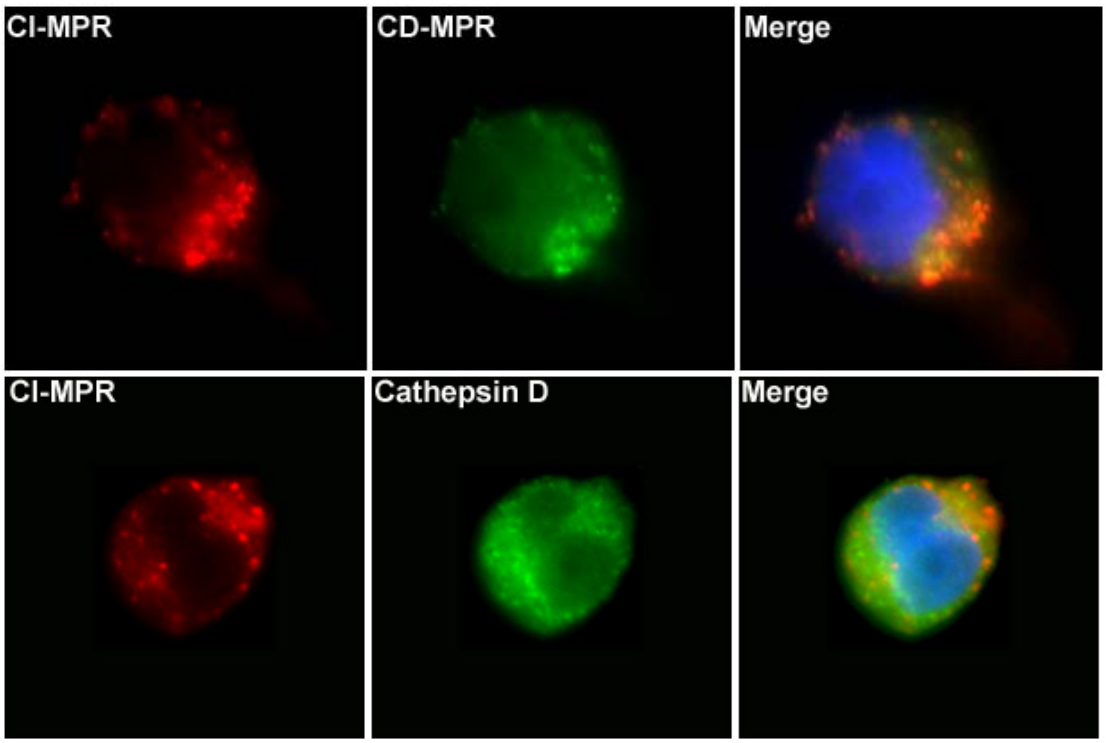


Figure 3.1 Subcellular localization of MPRs and cathepsin D in U937 cells. (A) CI-MPR and CD-MPR highly colocalize in U937 cells. CI-MPR (red), CD-MPR (green) and DNA (blue). The yellow spots in the overlay indicate a colocalization. (B) A portion of the lysosomal marker cathepsin D partially colocalize with CI-MPR. CI-MPR (red), cathepsin D (green) and DNA (blue).

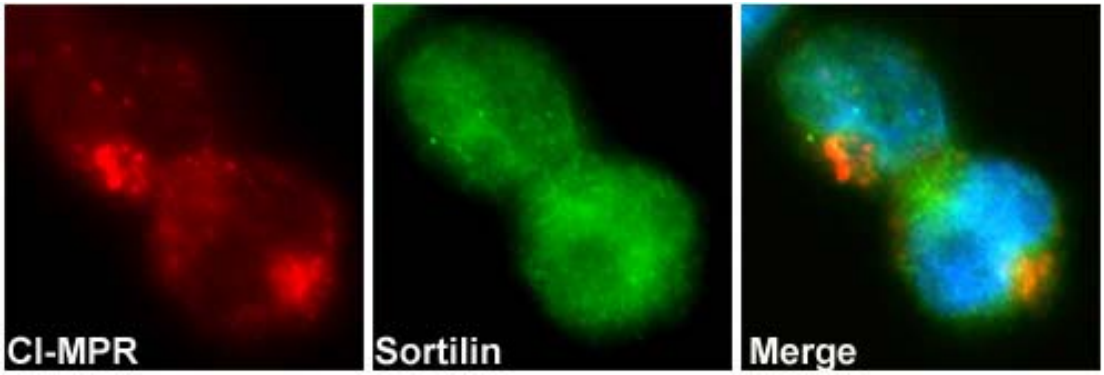


Figure 3.2 Subcellular localization of CI-MPR and sortilin in U937 cells. The vesicles of CI-MPR and sortilin partially colocalize. CI-MPR (red), sortilin (green) and DNA (blue).

3.1.3 M6P independent targeting of procathepsin D to lysosomes

Cathepsin D (CD) is an aspartic lysosomal proteinase expressed in all tissues. In Golgi, CD exists as a precursor molecule – procathepsin D (pCD). A small amount of pCD is constitutively secreted and the remainder is sorted in the TGN by MPRs.

The maturation of pCD results in the conversion of the precursor first into an intermediate form iCD in the endosomes. Subsequently, iCD is processed into mature chains (mCD) by cysteine proteinases in lysosomes (Gieselmann *et al.*, 1985). The targeting of pCD to the lysosomes was found to be partially independent of MPRs, for example in macrophages and HepG2 cells (Rijnboutt *et al.*, 1991, Diment *et al.*, 1988). Results from our laboratory suggested a role of pCD-pSAP interaction in the MPRs-independent targeting. These two molecules form complexes and are likely to travel together to acidic compartments, independent of MPRs (Gopalakrishnan *et al.*, 2004).

In an experiment using dithio-*bis*-succinimidyl propionate as a cross-linking reagent, a portion of pCD was cross-linked with pSAP in U937 cells. After metabolic labeling, the complex pCD-pSap was identified by immunoprecipitation (Fig. 3.3). When secretion of lysosomal proteins from TGN was stimulated with 10 mM NH_4Cl and 50 nM PMA the pSAP-pCD complex was identified in the medium. Saposin, which is a spingolipid activator protein, is delivered to the lysosome in a sortilin dependent mechanism (Hassan *et al.*, 2004). Therefore, it is likely that pSAP-pCD complexes are delivered into lysosomes in a MPR independent manner using sortilin and sortilin receptors.

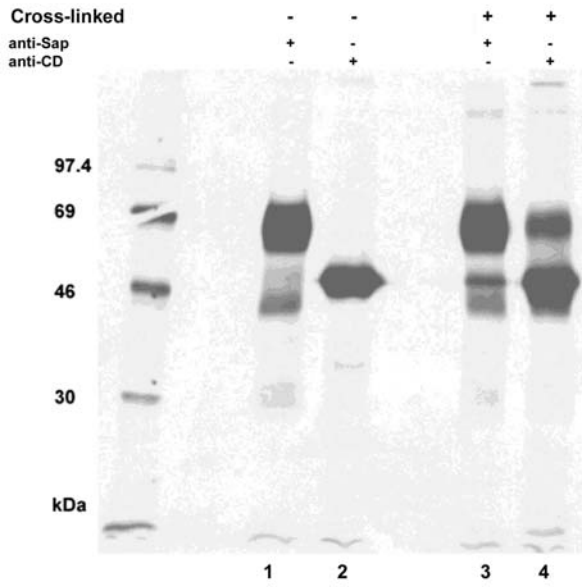


Figure 3.3 The presence of cross-linkable pCD-pSap in secretions of NH_4Cl and PMA-treated cells. U937 cells were metabolically labeled in the presence of [^{35}S]methionine and [^{35}S]cysteine, 10 mM NH_4Cl and 50 nM PMA for 15 h. Aliquots of the medium from 6 x 10^6 cells were either left

untreated (-) or incubated with 1mM dithio-*bis*-succinimidyl propionate cross-linking reagent for 5 min at room temperature (+) and processed for immunoprecipitation with anti-saposin C (line 1 and 3) and anti-CD antibodies (lane 2 and 4). The labeled precursors were separated by SDS/PAGE under reducing condition and visualized by fluorography (exposure time 3 days).

3.1.4 Neutrophil elastase is delivered to the lysosomes in association with proteoglycan serglycin

Several examples of M6P-independent targeting of lysosomal enzymes have been reported (Glickman *et al.*, 1993, Dittmer *et al.*, 1999, Tanaka *et al.*, 2000). U937 promonocytic cells synthesize a high amount of proteoglycan serglycin (Lemansky *et al.*, 2001). Negatively charged sufate side chains of proteoglycans are known to mediate the delivery of positively charged molecules such as proteinases and hormones to secretory granules in several hematopoietic cell-types (Forsberg *et al.*, 1999, Galvin *et al.*, 1999, Lemansky *et al.*, 2001, Lemansky *et al.*, 2003, Abrink *et al.*, 2004, Grujic *et al.*, 2005). In our laboratory, it was shown that serglycin is involved in the lysosomal transport of the positively charged protein lysozyme in U937 cells (Lemansky *et al.*, 2001).

Neutrophil elastase (NE) is another cationic lysosomal protein that is synthesized predominantly as a soluble glycoprotein in U937 promonocytes. Recently, cross-linking experiments have proven that within cells NE interacts with serglycin. In U937 cells it is delivered as a 34 kDa pro-form in association with serglycin to lysosomes (Lemansky *et al.*, 2007b).

To examine the intracellular localization status of NE in comparison with serglycin, double immunostaining with corresponding antibodies was performed using fixed in U937 cells. In Figure 3.4 a partial colocalization of serglycin and NE is shown. The colocalization is apparent in vesicles near the nucleus that may represent early endosomes and TGN. Since serglycin is subjected to a rapid degration upon reaching endosomes, the vesicles staining for NE alone most likely represent late endosomal and lysosomal compartments. Serglycin stains mostly TGN, secretory vesicles and early endosomes. The half-life of serglycin is approximately 1 h (Lemansky *et al.*, 2001). The present result is compatible with the possibility that the transport of NE to lysosomes is mediated by the proteoglycan serglycin.

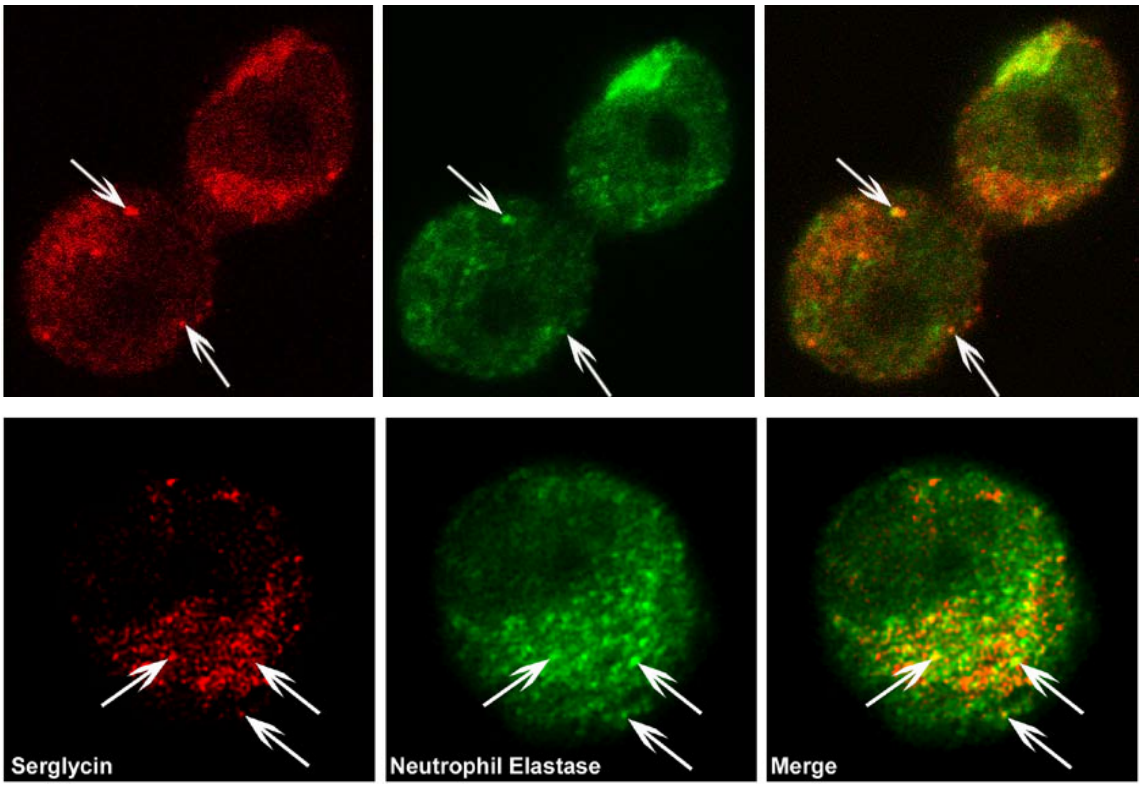


Figure 3.4 Subcellular localization of neutrophil elastase and serglycin. Two examples of confocal laser scan imaging at a single plane (50 µm) examining the localization of serglycin (red) and neutrophil elastase (green) in U937 cells. The arrows point to examples of colocalization of serglycin and neutrophil elastase.

3.1.5 CI-MPR interacts with serglycin during the lysosomal transport

As shown above, some cationic lysosomal proteins are transported to the lysosomes in a serglycin dependent manner. The precise delivery mechanism of how serglycin itself is recognized and targeted to the lysosomes is unknown. We have found that CI-MPR is partially involved in this process (Lemansky *et al.*, 2007a). A portion of serglycin was coimmunoprecipitated with CI-MPR after cross-linking reaction. Immunocytochemical studies showed that serglycin partially colocalize with CI-MPR (Fig. 3.5). However the colocalization is limited. An example of a large endosome labeled in green with CI-MPR and devoid of serglycin is shown in Fig. 3.5B. It may be speculated that serglycin interacts with CI-MPR at an early stage of

the transport, in TGN but not in endosomes, so that the transport of serglycin to lysosomes is only partially dependent on CI-MPR.

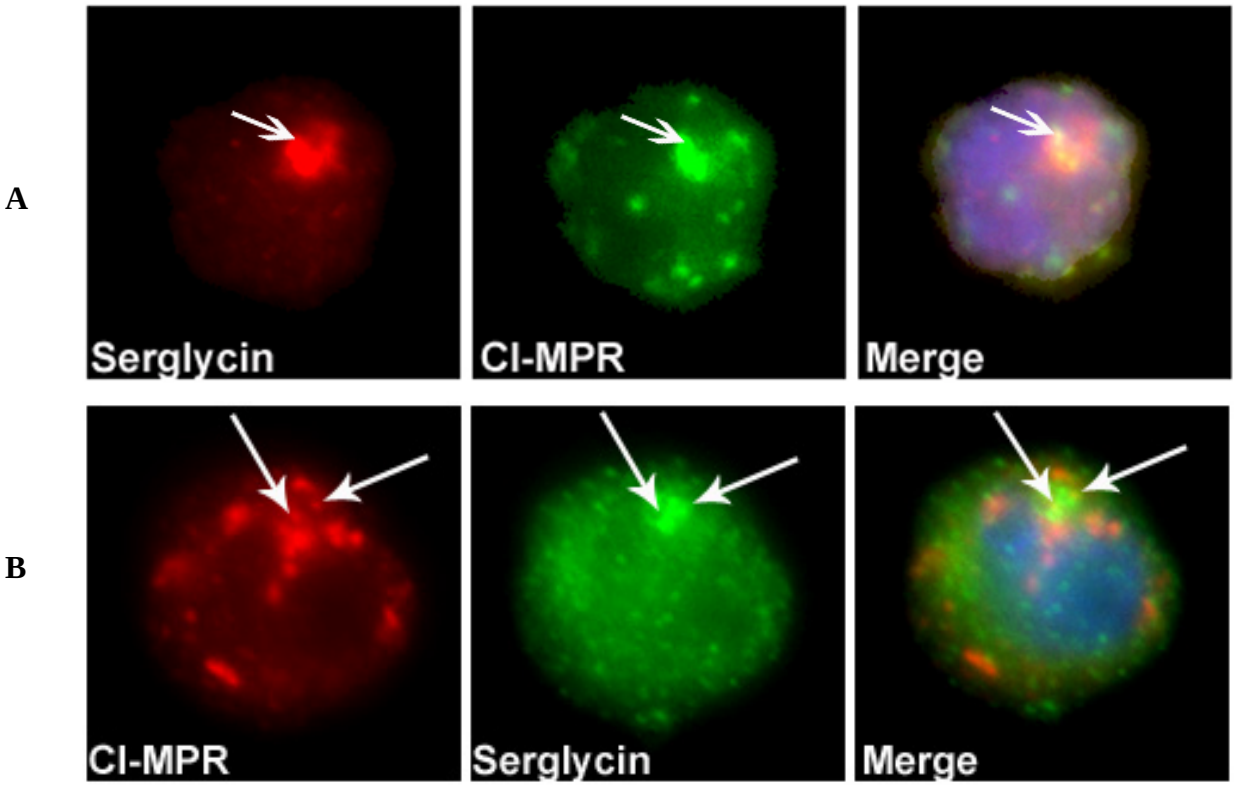


Figure 3.5 CI-MPR partially colocalizes with serglycin. (A) Serglycin (red, rabbit-Ab), CI-MPR (green) and DNA (blue) (B) CI-MPR (red), serglycin (green, goat-Ab) and DNA (blue) in U937 cells. CI-MPR partially colocalizes with serglycin (arrows).

3.1.6 Colocalization of serglycin with AP-3

Integral lysosomal membrane proteins use a distinct pathway to lysosomes different from that of soluble lysosomal proteins. Adaptor protein AP-3 recognizes and mediates the transport of integral lysosomal membrane proteins. In contrast to the CI-MPR, coimmunostaining of serglycin and AP-3 showed that in U937 cells these two molecules extensively colocalize in the vesicular structures probably representating endosomes (Fig. 3.6A). The confocal microscopic image shown below (Fig. 3.6B) illustrates a strong colocalization of serglycin with AP-3.

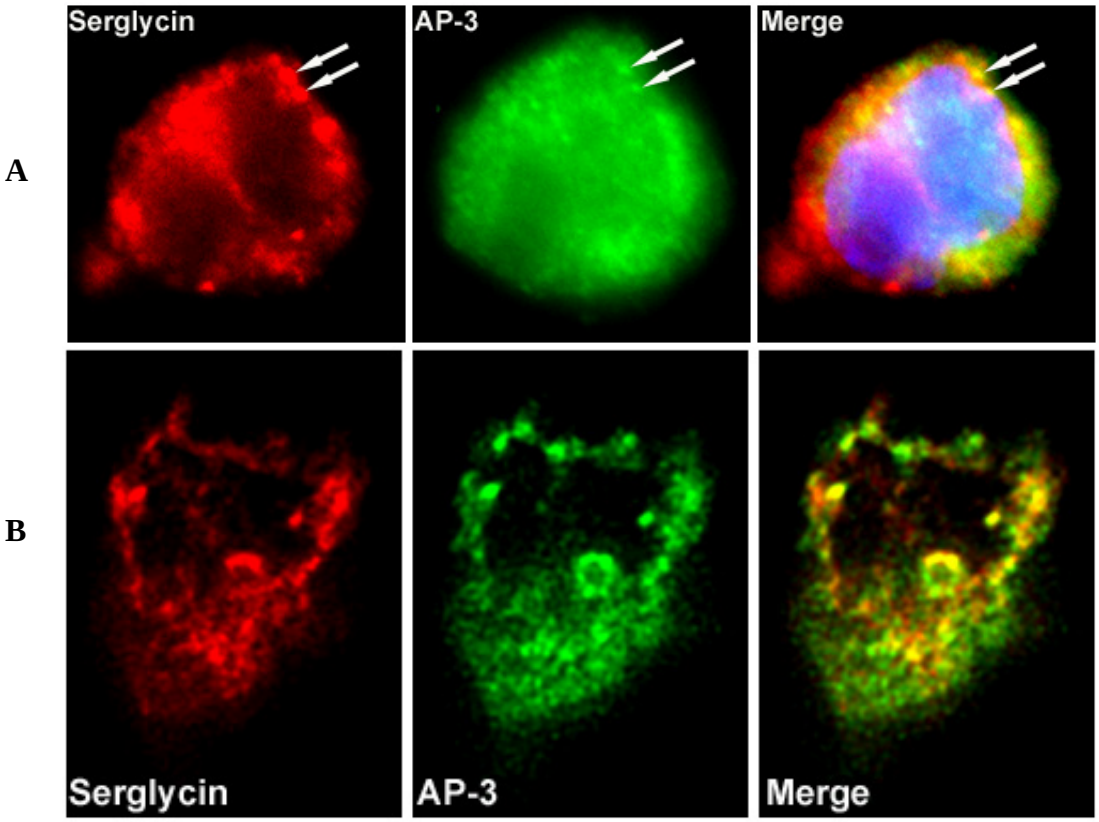


Figure 3.6 Colocalization of serglycin with AP-3 in U937 cells. A) U937 cells stained with serglycin (red), AP-3 (green) and DNA (blue). Serglycin colocalizes with AP-3 (arrows). **B)** A confocal laser scan image showing the staining at a single plane (50 µm), serglycin (red) and AP-3 (green).

3.2 PMA impairs the sorting and transport of lysosomal proteins

3.2.1 PMA induces cell adherence

The precise external signals that control differentiation of peripheral blood monocyte to tissue macrophage are incompletely defined. Monocytes leave the bone marrow and travel through peripheral blood vessels from which they may enter different tissues. Once they reach a tissue, perhaps in response to macrophage colony-stimulating factor (M-CSF), they differentiate into macrophages by growing in size and increasing the volume of the lysosomal compartment. Furthermore, they become adherent and gain the capacity to phagocytose (Valledor *et al.*, 1998). The

promonocytic cell line U937 has been widely used as *in vitro* model for monocytic differentiation. This can be accomplished by exposing the cells to phorbol-12-myristate-13-acetate (PMA) resulting in the generation of macrophage-like cells within approximately one day (Rovera *et al.*, 1979).

When U937 cells are treated with 50 nM of PMA, the cellular morphology changes, the cells adhere to plastic surface within a few minutes of the treatment. After 24 h, filopodial extensions reminiscent of macrophage morphology can be seen (Fig. 3.7) indicating a reorganization of cytoskeleton thin filaments.

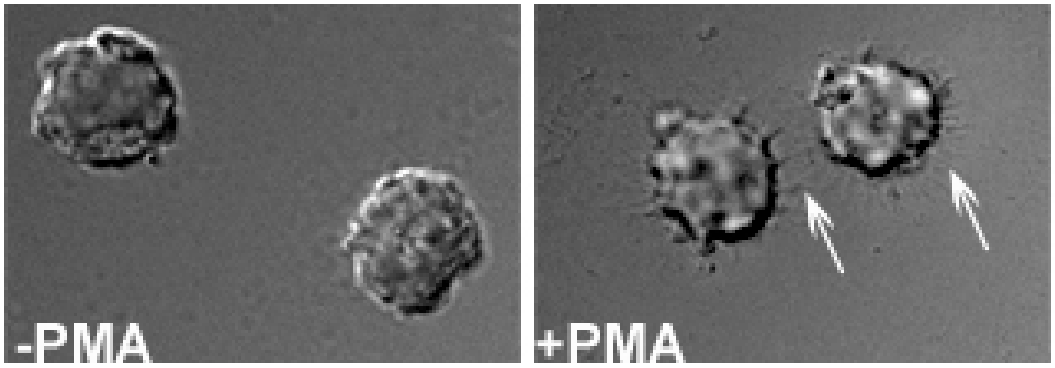


Figure 3.7 Morphology of undifferentiated (-PMA) and differentiated (+PMA) U937 cells. The cells were treated in the presence or in the absence of 50 nM PMA for 24 hours. Undifferentaited cells were sedimented by centrifugation and resusupende in PBS. Differentiated U937 cells adhere to glass coverslip and the filopodial extensions are visible (arrows).

3.2.2 PMA changes processing and targeting of procathepsin D and increases secretion of processed forms

Cathepsin D (CD) is an soluble aspartic lysosomal proteinase suitable as a reporter protein in examining the transport and sorting of soluble lysosomal proteins. Lysosomal proteinases are synthesized as pro-proteins and are processed and sorted to lysosomes *via* the endosomal apparatus by means of the M6P recognition system. During and after the transport to their final destination, lysosomal enzymes are endo-proteinolytically processed to mature active forms.

To analyze the sorting and transport of CD in the presence of PMA, proteins in U937 cells were pulse-labeled with a mixture of [^{35}S]methionine and [^{35}S]cysteine for

15 h in the presence and absence of 50 nM PMA. After the pulse-labeling, immunoprecipitation was performed in order to isolate the different forms of CD.

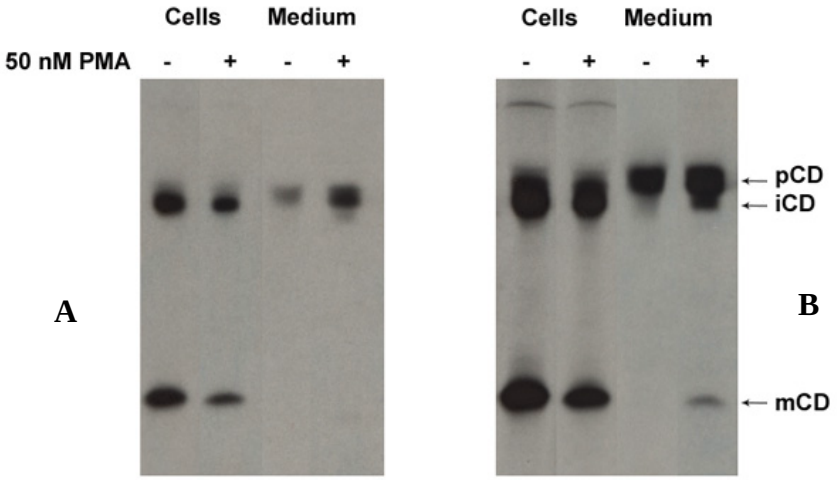


Figure 3.8 PMA increases secretion of cathepsin D. U937 cells were metabolically labeled in the presence of [^{35}S]methionine and [^{35}S]cysteine and in the presence or absence of 50 nM PMA for 15 h. CD was immunoprecipitated with anti-CD antibody from cell extract (cells) and medium. Labeled polypeptides were separated by SDS-PAGE and visualized by fluorography ((A) 25 h exposure time and (B) 70 h exposure time). The relevant polypeptides are labeled with arrows: precursor (p), intermediate (i) and mature (m) chain of CD.

In the cell extract of control cells, two main forms of activated CD bands were visible (Fig. 3.8), i) an intermediate 51.5 kDa form of CD (iCD) and ii) mature 30 kDa form (mCD). The band corresponding to the 53 kDa procathepsin D (pCD) is detectable just above the processed intermediate form iCD. In the medium of control cells, pCD is detected indicating constitutive secretion of a portion of the precursor.

In PMA-treated cells, the secretion of pCD is increased approximately 2-fold (Fig. 3.8 A). Unexpectedly, a considerable amount of the partially processed iCD and the mature form mCD was detected in the medium (Fig. 3.8 B). Since the processing of cathepsin D is considered to occur in endosomes and lysosomes in U937 cells, PMA is likely to induce a secretion of a portion of endosomes or lysosomes.

To examine possible effects of PMA on the distribution of the late endosomal/lysosomal membrane marker Lamp-II, an indirect immunofluroscence staining of Lamp-II in PMA-treated U937 cells was comapared with that in the control. The treatment was performed with 50 nM PMA for 3 h and non-permeabilized cells were stained with anti-Lamp-II antibody at 4°C. This temperature

was used to avoid endocytosis. The PMA treated cells showed a stronger staining of Lamp-II at the plasma membrane (Fig. 3.9) than the control. Since Lamp-II resides in endosomes and lysosomes, this result suggested that PMA may induce a fusion of endosomes and/or lysosomes with the plasma membrane.

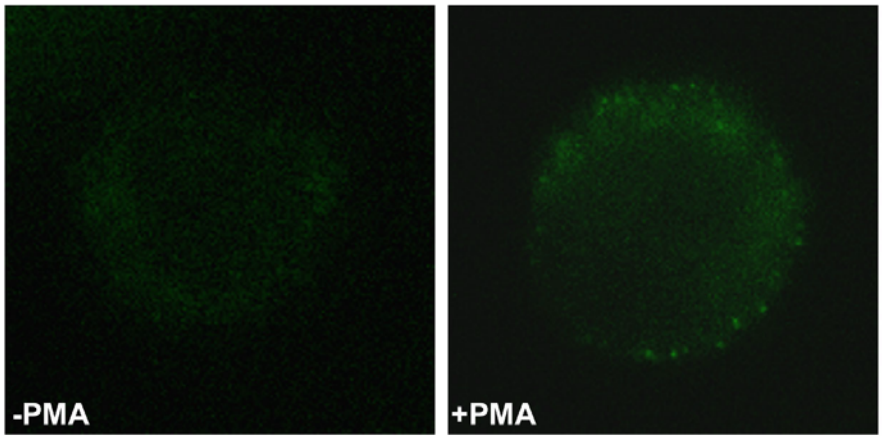


Figure 3.9 PMA increases localization of Lamp-II at the plasma membrane. Indirect immunofluroscence analysis of PMA treated (+PMA) and untreated (-PMA) non-permeabilized U937 cells stained with anti-Lamp-II antibody (green). A single plane microscopic picture of cells incubated for 3 h with 50 nM PMA show Lamp-II at the plasma membrane, whereas in untreated cells no Lamp-II protein was detected at the cell surface.

3.2.3 β-Hexosaminidase is secreted in the presence of PMA

Lysosomes contain several isoenzymes of β-hexosaminidase. The isoenzymes A and B of this glycosidase are missing in Tay-Sachs and Sandhoff disease, respectively. To investigate the secretion of β-hexosaminidase upon PMA treatment, the enzymatic activity of the enzyme was determined in the medium.

The cells were incubated with 50 nM PMA and the activity of β-hexosaminidase was measured in aliquots of the medium through 6 h of the treatment. In the medium an increase in the enzymatic activity of β-hexosaminidase was detected as early as 5 min in the presence of 50 nM PMA. The proportion of the enzyme activity in the medium reached 10% after 1 h. After 6 h 15% of total activity was found in the medium. In contrast, in the medium of control cells, the level of

enzymatic activity was always below 1% (Fig. 3.10). Thus PMA induces a secretion of β-hexosaminidase.

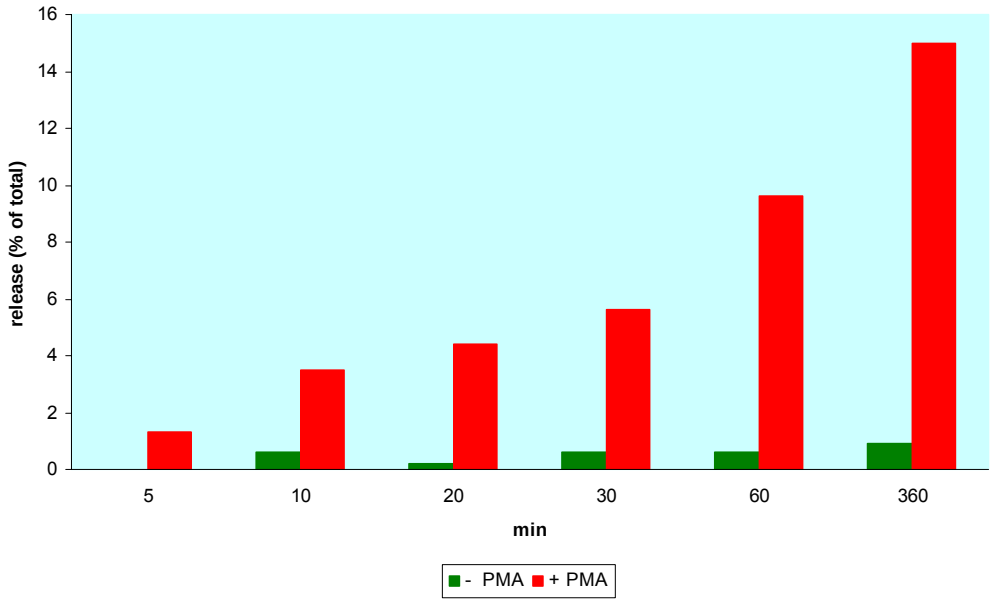


Figure 3.10 PMA induces secretion of β-hexosaminidase. U937 cells were incubated in the presence or absence of 50 nM PMA and release of β-hexosaminidase activity into medium was measured by the determination of the enzymatic activity. The release was expressed as the percentage of the total culture contents.

3.2.4 Effect of PMA on CI-MPRs

The transport of lysosomal hydrolases to the lysosomal compartment is dependent on MPRs. A portion of CI-MPR is recycled between the plasma membrane and intracellular compartments to facilitate endocytosis of the phosphorylated glycoproteins that may have escaped the sorting. Thus, CI-MPR performs a recapture of secreted lysosomal proteins if endowed with uncovered M6P-residues. As observed above, PMA enhanced the secretion of lysosomal enzymes into the medium. A question may be raised if the steady state of CI-MPR at the plasma membrane can be increased to enhnce the recapture of the secreted lysosomal enzymes. To answer this

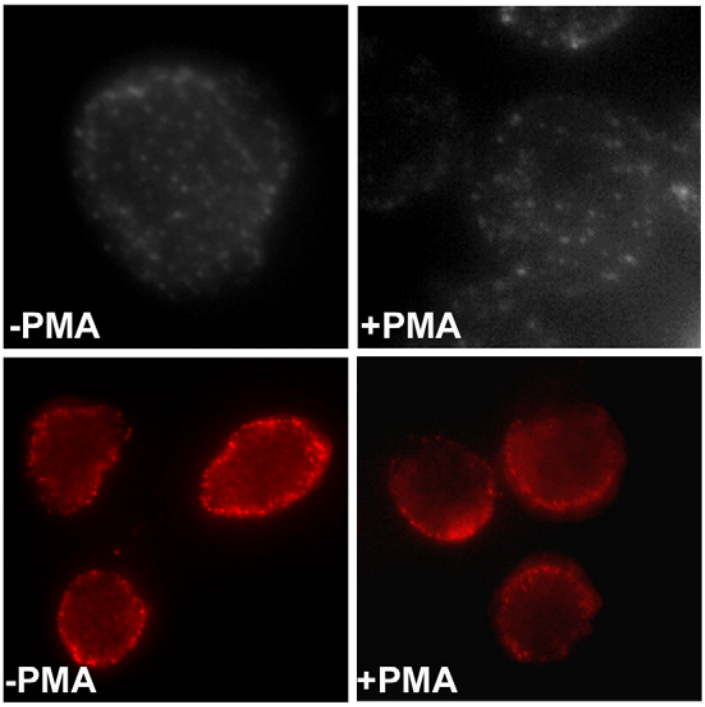


Figure 3.11 Reduced level of CI-MPR at the cell surface upon PMA treatment. Top panel in the grey scale shows tangential sections with images of the surface picture of non-permeabilized U937 cells treated with anti-CI-MPR antibody. Bottom panel shows medial sections with CI-MPR (red) at the cell surface (periphery staining).

question, both PMA treated and control U937 cells were subjected to indirect immunofluroscence staining of the plasma membrane with anti-CI-MPR antibody in the absence of detergents. The incubation in the presence of 50 nM PMA was performed for 3 h. Apparently, in the PMA treated cells the cell surface staining of CI-MPR was reduced as compared to the untreated cells (Fig. 3.11).

3.2.5 PMA increases the secretion of prosaposin

Since PMA enhances the secretion of lysosomal enzymes, it was considered that sorting of sphingolipid activator proteins (saposins) could be affected too. Saposins are synthesized as *via* precursor– prosaposin (pSap). After delivery to lysosomes the precursor is proteinolytically cleaved into its four domains containing saposins A through D. Transport of pSap to lysosomes depends on the sortilin receptor (Lefrancois *et al.*, 2005). Therefore, pSap may be used as a reporter protein to study the transport and sorting mechanism *via* the sortilin pathway.

In order to investigate the effect of PMA on U937 cells with respect to pSap, the cells were pulse-labeled with a mixture of [^{35}S]methionine and [^{35}S]cysteine for 15 h in the absence and presence of 50 nM PMA. After the pulse-labeling, saposin was isolated by immunoprecipitation with an anti-saposin C antibody. This antibody immunoprecipitates the precursor form pSap as well as its fragments containing saposin C. Native saposin C was not detected in cells labeled with [^{35}S]methionine and [^{35}S]cysteine indicating that transport and proteinolytic maturation requires a longer time period than 15 h. In control cells, a major portion of pSap was found in the cell extract while a minor portion was in the medium (Fig. 3.12). In the presence of 50 nM PMA the secretion of pSap into the medium was increased.

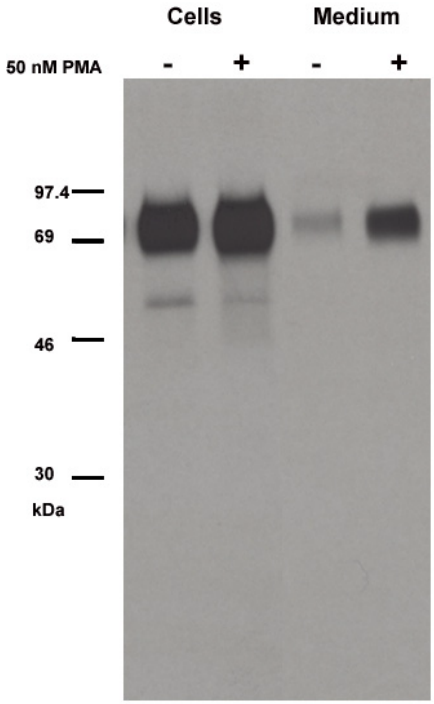


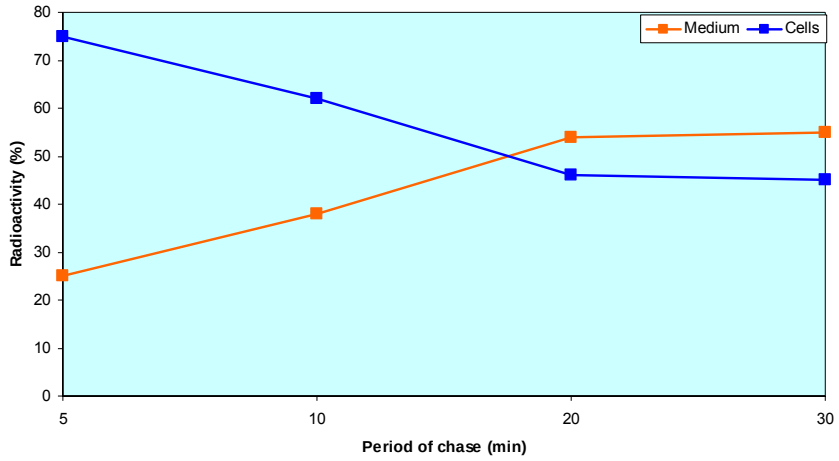
Figure 3.12 PMA strongly increases the secretion of prosaposin (pSap). U937 cells were metabolically labeled in the presence of [^{35}S]methionine and [^{35}S]cysteine and 50 nM PMA for 10 h. pSap was immunoprecipitated with anti-Saposin C antibody from cell extracts and the medium and the labeled precursor was separated by SDS/PAGE and visualized by fluorography (exposure time 4 days).

3.2.6 In the presence of PMA the secretion of serglycin is greatly stimulated

Serglycin is a major proteoglycan in U937 cells (Kolset *et al.*, 1996). It is thought to play a pivotal role in the transport of proteins to the lysosomal compartment

in a mechanism distinct from the usual M6P pathway (Lemansky *et al.*, 2001; Lemansky *et al.*, 2007b). Thus, serglycin has been shown to participate in the lysosomal transport of lysozyme and of neutrophil elastase (NE) in U937 cells.

In previous trafficking studies metabolic labeling with [^{35}S]methionine and [^{35}S]cysteine was used. In this study we used radioactive sulfate taking advantage of the fact that serglycin is strogly sulfated in its glycosaminoglycan moiety. The sulfation takes place in the *trans*-Golgi thus allowing a more precise kinetic analysis of the post TGN events. Another advantage of this method is that 90% of sulfated proteins in U937 cells is serglycin (Kolset *et al.*, 1996, Lemansky *et al.*, 2007a). To examine the release of serglycin, U937 cells were labeled for 10 min with [^{35}S]sulfate and the label was chased for up to 30 min. Polypeptides from corresponding aliquots of a cell lysate and the medium were precipitated with TCA and analyzed by liquid scintillation counting. In control cells, the secretion of sulfated TCA-precipitable material reached a maximum of 54% at 20 min and it remained at this maximum level. In the presence of 50 nM PMA, the secretion of sulfated TCA-precipitable material increased rapidly to 68% of sulfated proteoglycan in the medium within 5 min of chase. At 30 min of chase, the secretion of serglycin reached 90% (Fig. 3.13). This indicates that PMA greatly enhances the rate of the secretion of serglycin.



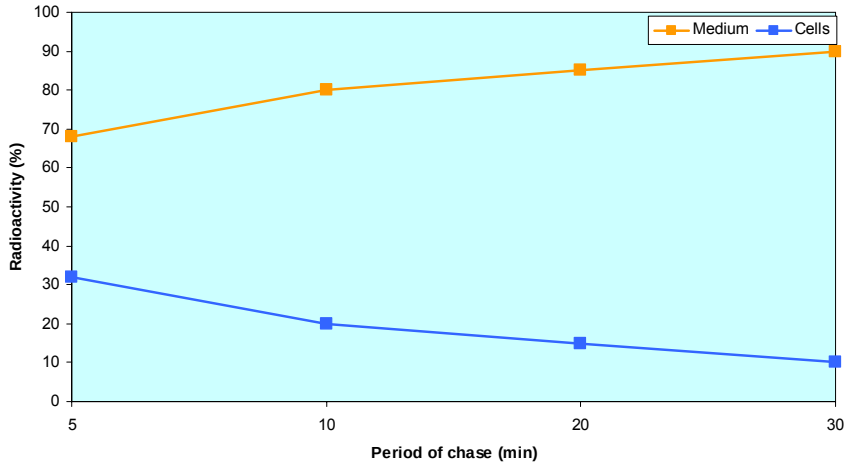


Figure 3.13 PMA greatly enhances the secretion of serglycin. U937 cells were labeled with [^{35}S]sulfate for 10 min and chased for up to 30 min in the absence (top) or presence of 50 nM PMA (bottom). TCA-precipitable polypeptides from cell lysates and media were analyzed by liquid scintillation counting. Values are given as percentage of the TCA-precipitable radioactivity in samples for defined period of chase.

3.2.7 Phospholipase D appears to control the secretion of serglycin

Phospholipase D (PLD) has been shown to stimulate and control the formation of secretory vesicles from TGN (Baron *et al.*, 2002). PLD has a high preference for primary alcohols such as 1-butanol over water in converting phosphatidylcholine (PC) to phosphatidylalcohols instead of phosphatidic acid (PA). This reaction was

experimentally used to determine the role of PLD in the secretion of serglycin. Here, the question was examined if enhanced secretion of serglycin in the presence of PMA could be correlated with an activation of PLD.

The cells were pulse-chase labeled as described above, using a 10 min pulse and up to 30 min chase. In the presence of 50 mM 1-butanol, the secretion of sulfated TCA-precipitable material was reduced. In control cells, the secretion reached a maximum within 20 min of chase corresponding to 54% of total TCA-precipitable radioactivity. In the presence of 1-butanol the secretion of sulfated proteins was slowed down. It took approximately 30 min of chase to reach the secretion level of 50% (Fig. 3.14). In the presence of 50 nM PMA, the secretion of sulfated proteoglycans rapidly increased as expected. After 5 min of chase, 68% of the labeled proteoglycans were detected in the medium. The inhibition of PLD by 1-butanol was accompanied by a marked decrease in the proportion of the secreted sulfated proteoglycan. At 5 min of chase it was down to 33% (Fig. 3.14). These results show that the secretion of serglycin is impaired in the presence of butanol.

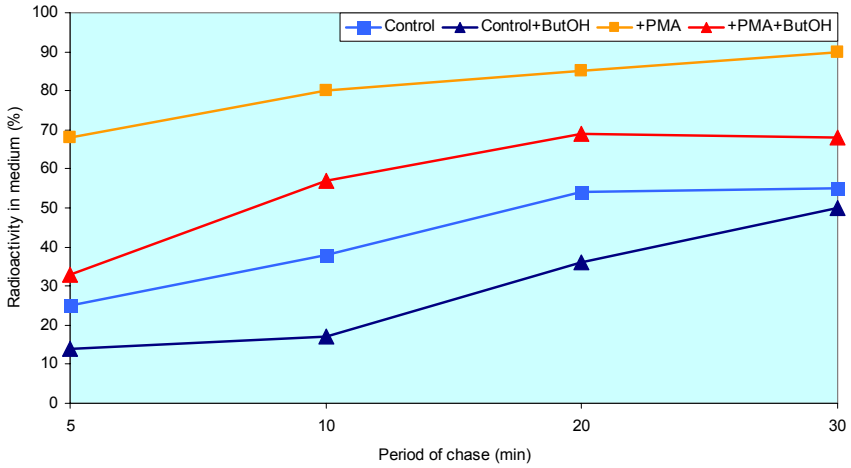


Figure 3.14 1-Butanol inhibits secretion of serglycin. U937 cells were labeled with [^{35}S]sulfate for 10 min and chased for up to 30 min in the absence or presence of 50 nM PMA and 50 nM 1-butanol. TCA-precipitable polypeptides from cell lysates and medium were analyzed by liquid scintillation counting. The secretion of serglycin is expressed as the percentage of total TCA-precipitable radioactivity.

3.3 Examination of protein phosphorylation in PMA treated cells

PMA is a structural analogue to diacylglycerol (DAG) and can activate several regulatory enzymes such as protein kinase C (PKC) and protein kinase D (PKD). The protein substrates of these kinases may be involved in intracellular transport in U937 cells. To examine the substrates of protein kinases responding to PMA, the cell extracts of U937 cells were fractionated by sucrose density gradient centrifugation and analyzed for specific phosphorylation of the proteins.

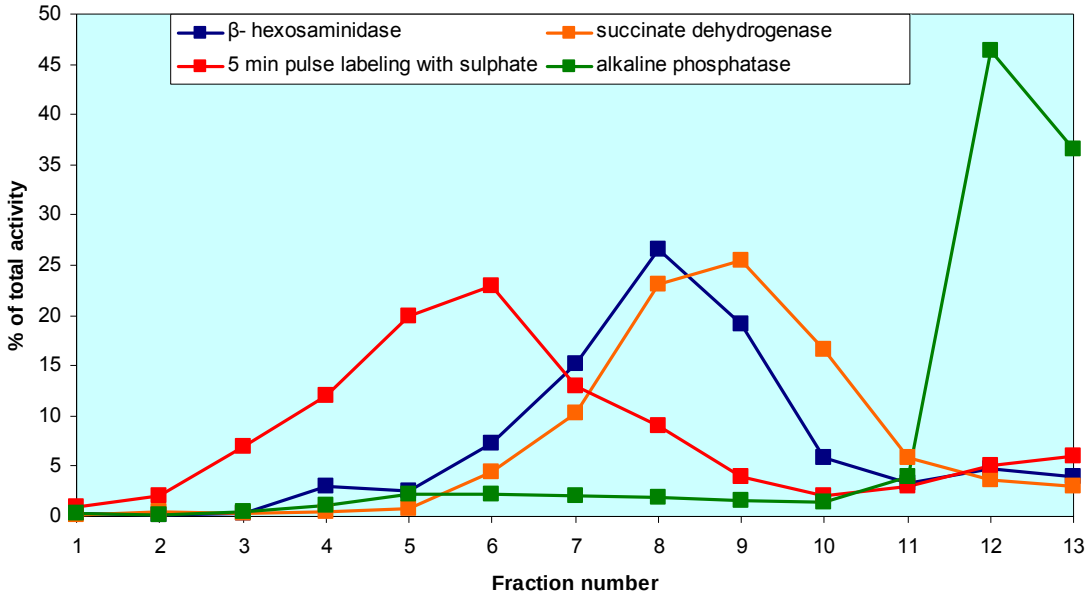
3.3.1 Subcellular fractionation of U937 cells in sucrose density gradients

U937 cells were disrupted by nitrogen cavitation and nuclei removed by low speed centrifugation. The resulting post-nuclear supernatant was mixed with sucrose to obtain a concentration of 50% (w/w) and layered at the bottom of a linear 18-47% (w/w) sucrose density gradient. The samples were subjected to ultracentrifugation at 220 000 x g_{max} for 16 h. Only intact cell organelles were floating in the gradient eventually reaching the matching density. The cytosol remained at the bottom of the gradient and this contained approximately 85% of the total protein amount (fractions number 12 and 13).

The activities of the lysosomal marker β-hexosaminidase, of succinate dehydrogenase (a mitochondrial marker) and of alkaline phosphatase (a plasma membrane marker) were determined in all fractions. After a 5 min pulse labeling with sufate, sulfated glycoproteins and proteoglycans in TGN were labeled. Thus, the TCA-precipitable sulfated material that was detected in the gradient indicated the presence of TGN vesicles. The distribution of relative marker enzyme activities and sulfated proteoglycans in the fractions from sucrose density gradients are shown in Figure 3.15. Due to its lower buoyant density TGN (fraction numbers 5 and 6) was separated from more dense lysosomes (fraction number 8) and mitochondria (fraction numbers 8 and 9). The plasma membrane marker alkaline phosphatase was found at the bottom of the gradient (fraction numbers 12 and 13).

Western blot analysis of endolysosomal antigens (Fig. 3.16 and 3.17) showed that lysosomal antigens such as Lamp-II and neutrophil elastase were enriched in lysosomal fraction (number 8). MPRs were were detected in fractions number 6 and 7 and, thus, partially separated from lysosomes. Golgin-97 that is involved in the retrograde transport from endosomes to TGN was found in the same fractions (numbers 6 and 7). A large amount of the normally membrane associated Golgin-97 (Fig. 3.16) was found in the cytosolic fractions (numbers 12 and 13). This may be due to a disturbance of its interaction with the membrane at a high concentration of sucrose.

The experiments described above were also performed in the presence of 50 nM PMA. However, PMA did not change the distribution of the enzymatic organelle markers β-hexosaminidase, succinate dehydrogenase and alkaline phosphatise. The subcellular localizations of the TGN and endolysosomal antigens (CI-MPR, CD-MPR, Golgin-97, Lamp-II and neutrophil elastase) were not affected (data not shown) either.



bottom

Figure 3.15 Distribution of enzyme markers in sucrose density gradient fractions. Exponentially growing U937 cells were disrupted by nitrogen cavitation and subfractionated on a sucrose density gradient into 13 fractions. Enzyme marker for lysosome (β- hexosaminidase), mitochondria (succinate dehydrogenase), Golgi apparatus (5 min pulse-labeling with [^{35}S]sulfate) and plasma membrane (alkaline phosphatase) were measured in each fraction.

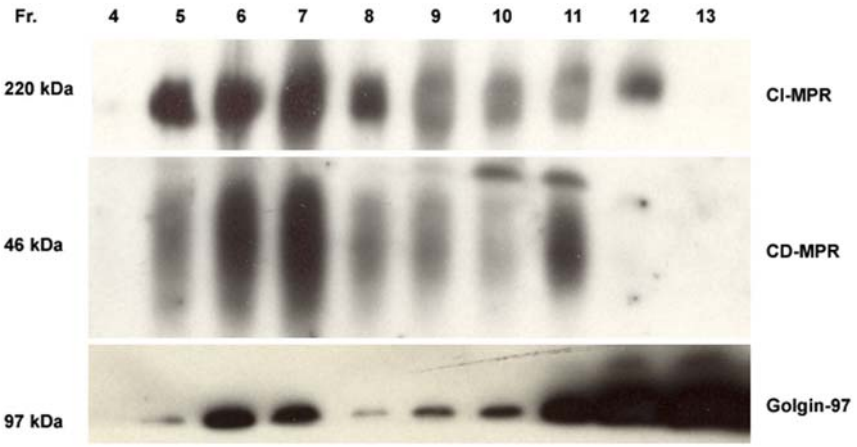


Figure 3.16 Localization of TGN and endosomal antigens in sucrose density gradient fractions. Postnuclear supernatant of 100 x 10^6 U937 cells was subfractionated on a sucrose density gradient into 13 fractions. Proteins contained in 200 µl of the fractions 1-11 and 20 µl of the fractions 12 and 13 were separated by SDS-PAGE and were immunobloted with antibodies to the CI-MPR (220 kDa), CD-MPR (46 kDa) and Golgin-97 (97 kDa). Concentrations included: anti-CI-MPR (1:2000), anti-CD-MPR (1:1000) and anti-Golgin-97 (1:1000). The detection was performed using a chemiluminescence kit.

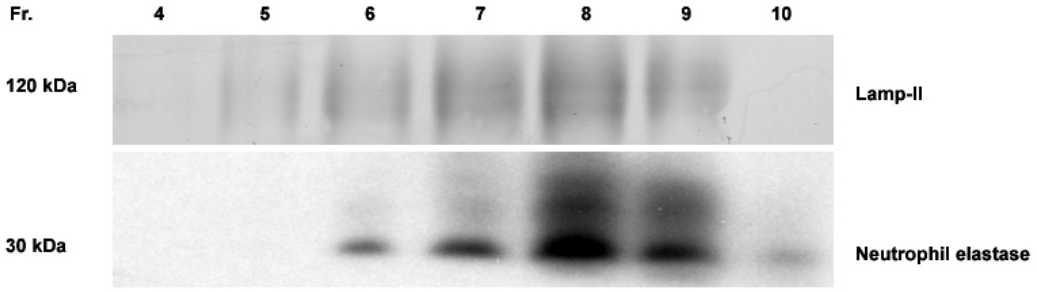


Figure 3.17 Localization of lysosomal antigens in sucrose density gradient fractions. Postnuclear supernatant of 100 x 10^6 U937 cells was subfractionated on a sucrose density gradient into 13 fractions. Proteins from fractions 4-10 were separated by SDS-PAGE and were immunobloted with antibodies to the lysosomal membrane protein Lamp-II (120 kDa) and soluble lysosomal protein neutrophil elastase (30 kDa). Concentrations included: anti-Lamp-II (1:2000) and anti-neutrophil elastase (1:500). The detection was performed using chemiluminescence kit.

3.3.2 Detection of phosphoproteins

The role of protein phosphorylation in the constitutive secretion is not well understood. To examine the substrates of kinases that are activated in the presence of PMA, the cells were labeled with [^{32}P]orthophosphate in the presence or absence of 50 nM PMA for 20 min. PMA and [^{32}P]orthophosphate were added into the medium simultaneously. To determine the subcellular localization of the labeled phosphoproteins, postnuclear supernatants were separated on sucrose density gradients. From the gradients 12 fractions were collected. The proteins from each fraction were concentrated by precipitation with 4 volumes acetone, separated by SDS-PAGE and visualized by fluorography (Fig. 3.18). Numerous proteins were phosphorylated throughout the gradient fractions. However, significant changes in protein phosphorylation in the presence of PMA was detectable mainly in the cytosolic fraction (number 12) and the TGN fractions (numbers 5 and 6). In the cytosolic fraction three bands that appeared to be specifically phosphorylated in the presence of PMA were detected (Fig. 3.18, black arrows). These could be either plasma membrane proteins or the associated vesicular membrane proteins after a redistribution into the cytosolic fraction (cf. the distribution of golgin-97 in sucrose density fraction), or genuine cytosolic proteins. Interestingly, a 46 kDa phosphoprotein which is seen in the lysosomal fraction (number 8) in the untreated samples, shifted to the TGN-containing fractions (numbers 5 and 6) in the PMA treated samples (Fig 4.19, white arrows). Furthermore, when the cells were treated with PMA for longer time (2 h), the 46 kDa protein was detected only in the TGN fractions and became absent from the lysosomal fraction (data not shown).

To investigate the phosphorylation of proteins possibly involved in the enhanced secretion of lysosomal proteins in PMA treated cells an additional method of phosphoprotein detection was employed (see below).

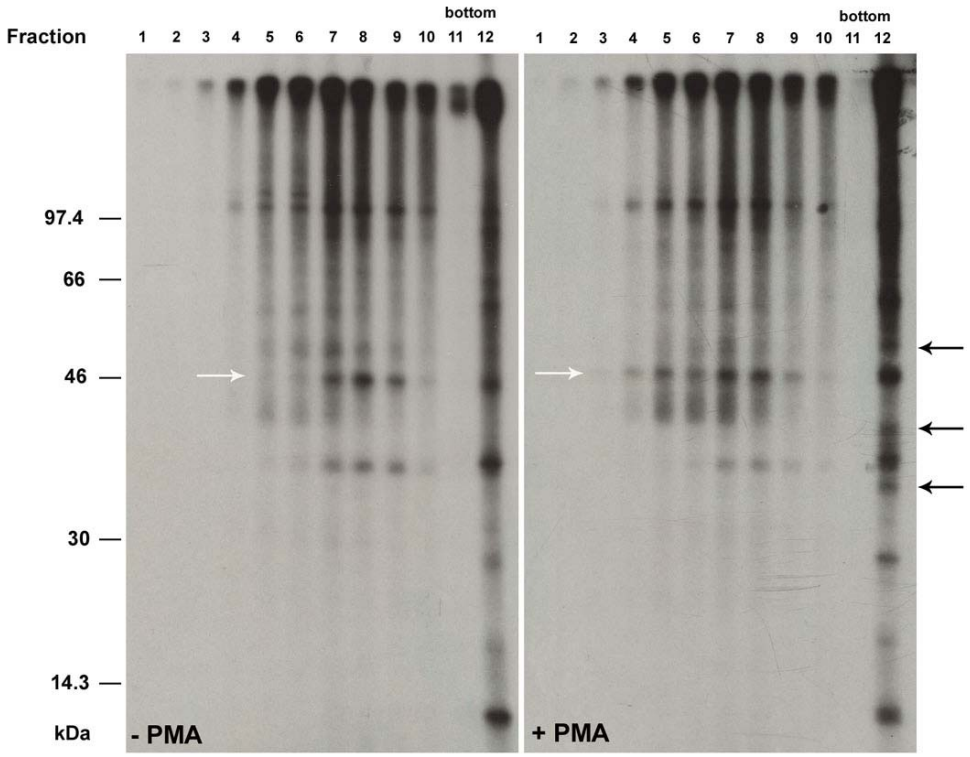


Figure 3.18 Phosphorylation of proteins in response to PMA. Exponentially growing U937 cells were labeled in the presence or absence of 50 nM PMA with [^{32}P]orthophosphate for 20 min. Cells were subfractionated on a sucrose density gradient into 12 fractions. The proteins were concentrated by precipitation with acetone (200 µl of fractions 1-10 and 20 µl of fractions 11-12), and separated by SDS-PAGE and the radiolabeled proteins were detected by fluorography (exposure time 2 days).

3.3.3 Detection of phosphoproteins in fractions of low buoyant density

The secretion of lysosomal proteins from TGN was enhanced in the presence of PMA. Therefore, a specific phosphorylation of proteins at TGN and/or transport vesicles which bud off the TGN could be expected. It was found that secretory and clathrin-coated vesicles that bud off the TGN on the way to plasma membrane and endosomes have a significantly lower buoyant density than TGN. At the here employed conditions in sucrose density gradient separations, TGN was found in fractions 5 and 6 and secretory and clathrin-coated vesicles in fractions 3-5 (Nickel *et al.*, 1994, Lemansky *et al.*, 2007a). Thus, in order to include the transport vesicles that bud off the TGN as well as TGN itself, fractions 3-6 were collected and the proteins were concentrated by precipitation with acetone. An aliquot of 100 µg protein was separated using two-dimensional CETAB/SDS-PAGE and stained with a fluorescent dye (Pro-q-diamond from Invitrogen) specific for phosphoproteins. Subsequently, total protein was stained with colloidal Coomassie blue (Fig. 4.19). The phosphoprotein staining appeared more sensitive than the radioactive labeling. In fractions of PMA-treated samples the relative intensity of several phosphoprotein spots was enhanced. All distinct differentially stained spots of phosphoproteins were excised from the gel, subjected to digestion with trypsin and mass spectrometric analysis of the tryptic fragments. Unfortunately, after Coomassie blue staining, the fluorescence of the phosphoproteins was suppressed. Among the 9 spots selected as encircled in Fig. 3.20, one was identified with insulin-regulated aminopeptidase (IRAP). Its apparent molecular weight was 165 kDa (Fig. 3.20, spot in the dashed circle). The experiment was repeated using more protein (400 µg). However, this resulted in a higher background and a lower sensitivity of the phosphoprotein staining. Therefore, the remaining phosphoproteins could not be identified. In conclusion, IRAP represents one among several proteins that are phosphorylated in U937 cells upon the PMA-treatment.

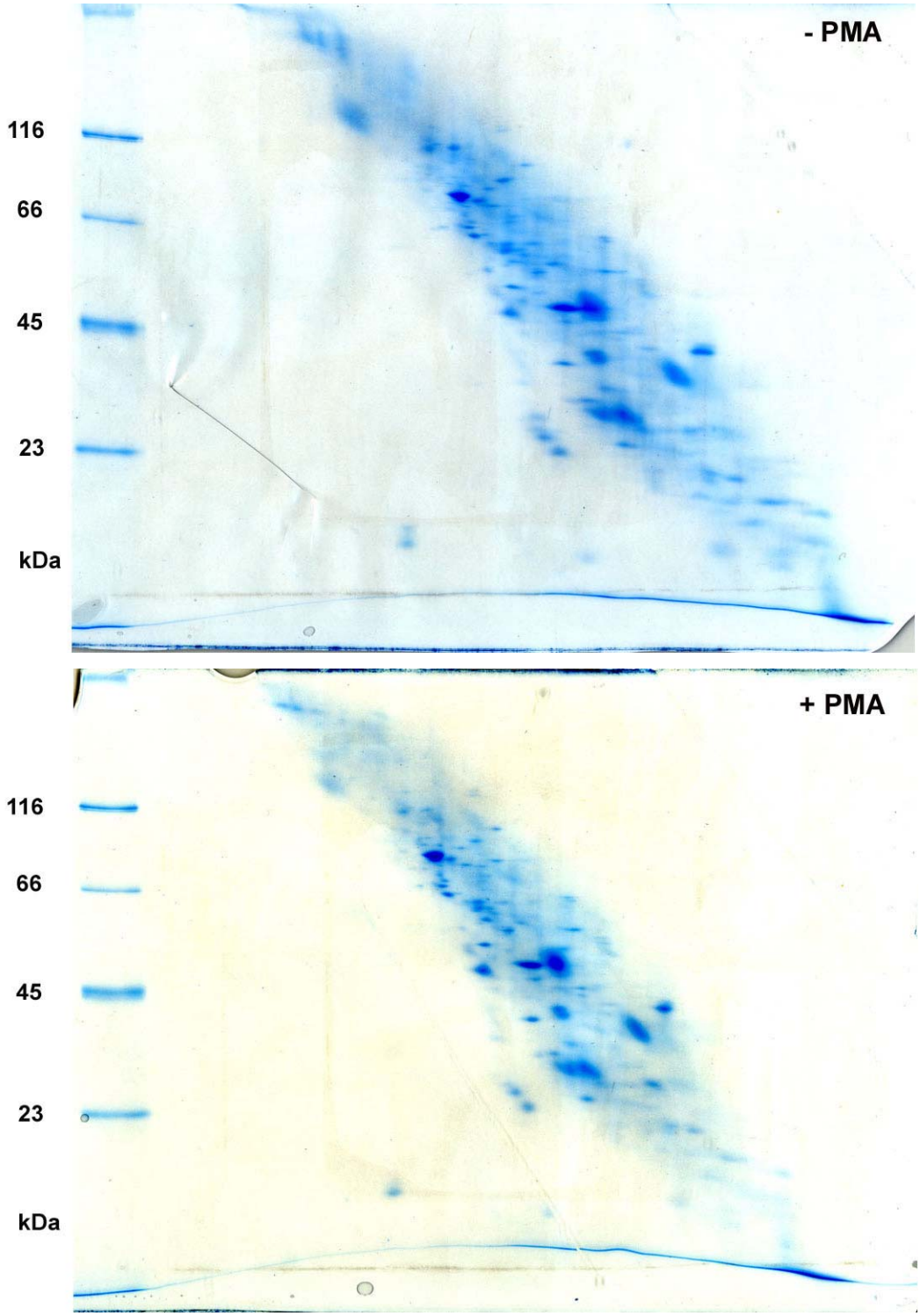


Figure 3.19 Two-dimensional separation of TGN and endosomal and secretory transport vesicles fractions separated by CETAB/SDS-PAGE. The cells were incubated in the absence or in the presence of 50 nM PMA for 20 min and were subsequently subfractionated on a sucrose density gradient into 13 fractions. The proteins from fractions 3-6 were concentrated by precipitation with acetone and an aliquot of 100 µg protein was separated on 2D-CETAB/SDS-PAGE and stained with colloidal Coomassie blue.

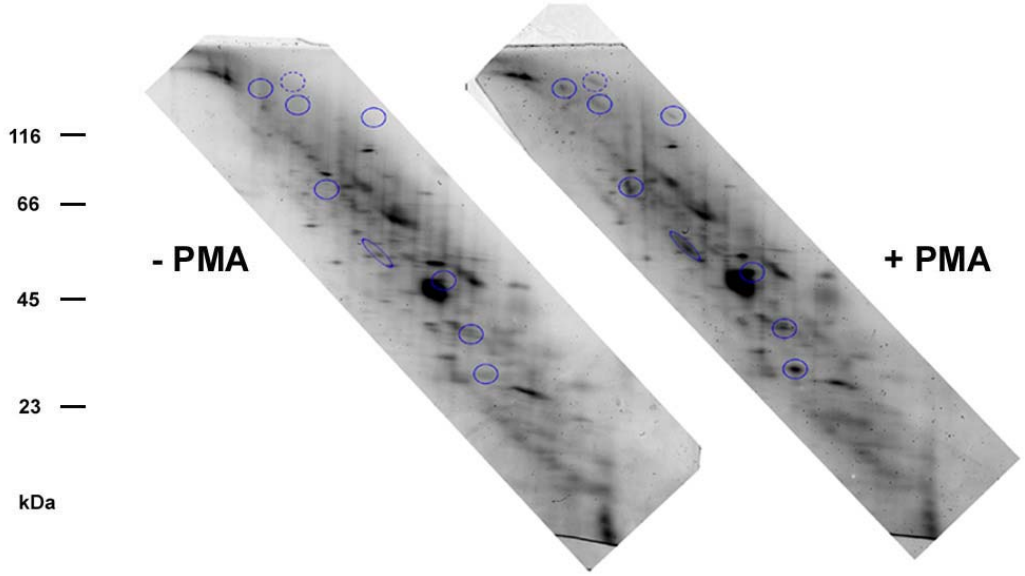


Figure 3.20 Phosphoprotein stain of TGN and endosomal and secretory transport vesicles fractions separated by CETAB/SDS-PAGE. U937 cells were incubated with 50 nM PMA for 20 min (+ PMA). Control cells (- PMA). Cells were subfractionated on a sucrose density gradient into 13 fractions. The proteins from fractions 3-6 were concentrated by precipitation with acetone and an aliquot of 100 µg protein was separated on 2D-CETAB/SDS-PAGE and stained with pro-q-diamond phosphoprotein stain. To reduce high costs, the staining was performed with portions of the gels that contained the separated proteins. Fluorescently stained gels were visualized with Typhon gel-scanning instrument with excitation source 532 nm and emission filter 560 nm. The gels were then stained with colloidal Coomassie blue (Fig. 4.20) and detected phosphoproteins (9 spots in circles) from PMA treated cells were cut out from the gel and were analyzed by Maldi-TOF. The spot marked by the dashed circle was identified as IRAP.

3.3.3.1 IRAP is phosphorylated in the presence of PMA and partially colocalizes with CI-MPR

IRAP was identified as one of proteins phosphorylated upon PMA-treatment in U937 cells. It was shown that this transmembrane protein is phosphorylated *in vitro* by PKCζ (Ryu *et al.*, 2002). The subcellular localization of IRAP was detected in sucrose density gradient fractions using Western blotting (Fig. 3.21). IRAP was detected in fractions numbers 6 and 7, that is those containing TGN and endosomes. IRAP was absent from fractions containing the plasma membrane (fraction numbers 12 and 13). PMA had no influence on IRAP distribution through the gradient (data not shown).

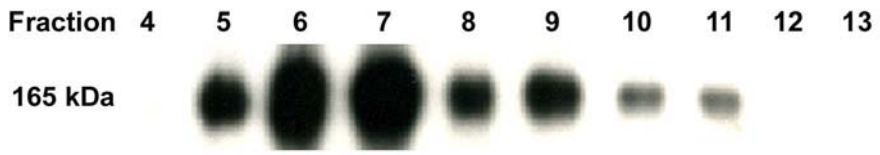


Figure 3.21 Localization of IRAP/leucine aminopeptidase in sucrose density gradient fractions. Postnuclear supernatant of 100 x 10^6 U937 cells was subfractionated on a sucrose density gradient into 13 fractions. Proteins contained in 200 µl of fractions 4-11 and 20 µl of fractions 12 and 13 were separated by SDS-PAGE and were immunobloted with antibodies to the IRAP/leucine aminopeptidase.

To examine the subcellular localization pattern of IRAP in U937 cells, the cells were co-immunostained with IRAP and CI-MPR. IRAP predominantly colocalizes with CI-MPR (Fig. 3.22). The immunostaining shows a colocalization of IRAP with CI-MPR, but neither with the lysosomal marker cathepsin D nor with sortilin (data not shown).

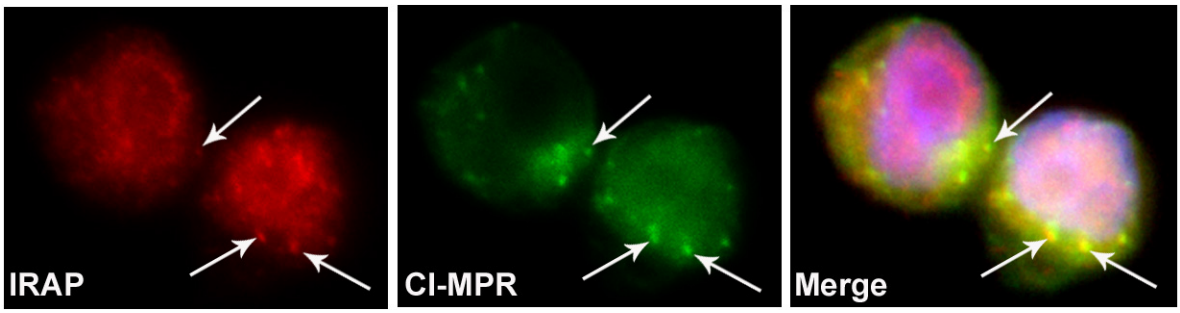


Figure 3.22 IRAP is found in many the same vesicles containing CI-MPR. IRAP highly colocalizes with CI-MPR (arrows). IRAP (red), CI-MPR (green) and DNA (blue).

4 Discussion

4.1 Sorting and transport of lysosomal proteins in U937 cells

Most of the newly synthesized lysosomal hydrolases are modified with M6P residues and are sorted at the TGN by MPRs. The sorting and transport mechanism of lysosomal hydrolases is well characterized. Promonocytic U937 cells synthesize and transport some proteins, such as prosaposin, proteoglycan serglycin, neutrophil neutral proteinases or lysozyme, into lysosomes in a M6P-independent manner. The present results extend our knowledge on M6P-independent sorting pathways in U937 cells.

4.1.1 Sortilin

Sortilin is a receptor protein with a significant homology to the yeast vacuolar sorting receptor Vps10p. The C-terminal segment in sortilin's cytoplasmic tail is closely related to the corresponding and functionally important segment of the CI-MPR (Nielsen *et al.*, 2001). This segment contains a dileucine sorting motif which is recognized by GGA proteins that are shuttled *via* clathrin-coated vesicles from the TGN to endosomes (Jacobsen *et al.*, 2002). Therefore, a similar sorting signal seems to be responsible for the localization of sortilin and the CI-MPR in the same membrane compartments. However, double immunofluorescent staining of the CI-MPR and sortilin in U937 cells showed a significant difference in the subcellular distribution of both proteins (Fig. 3.2) which may be related to a comlex nature of the sorting signals within the cytosplasmic tails. It is possible that sortilin cooperates with different lipid protein-lipid subdomains of the TGN than the CI-MPR. Interestingly, the transport of sortilin´s ligand prosaposin depends on the interaction with sphingomyelin in the TGN membrane (Lefranconis *et al.*, 2002). This interaction is not required for targeting the precursor of cathepsin B, since the latter depends on MPRs in its routing to lysosomes. An explanation were the possibility that sortilin plays a role in sorting of proteins that are associated with detergent-resistant microdomains (DRMs). Sphingolipid-enriched

domains could provide a platform for budding of vesicles enriched in sortilin and depleted of MPRs if an association of the former with DRM is taking place at the TGN. However, the association of sortilin with DRMs is not clear. Another possibility were the presence of additional divergent sorting signals in sortilin and CI-MPR for the anterograde and retrograde transport pathways between TGN and endosomes.

Sortilin is involved in sorting and trafficking of some lysosomally addressed molecules, such as prosaposin, GM_2AP and sphingomyelinase precursors (Hassan *et al.*, 2004; Ni *et al.*, 2006). In addition to its sorting function at the TGN, sortilin could be involved in other intracellular sorting mechanisms, or play a role in signaling. So far, sortilin has been characterized in neuronal and adipose tissues. In neuronal cells, sortilin mediates rapid endocytosis of lipoprotein lipase, neurotensin and nerve growth factor. The ternary complex of sortilin, nerve growth factor and $p75^{NTR}$ plays a role in "death signaling" (Nyborg *et al.*, 2006). In adipose and muscle tissue sortilin is localized in GLUT4 vesicles and participates in the biogenesis of these vesicles. Upon a stimulation with insulin, a fusion of GLUT4 vesicles with the plasma membrane is induced (Hou *et al.*, 2007).

Until now, functions of sortilin in hematopoietic cells are unknown. This receptor seems to play a role in the differentiation of blood cells. The sortilin mRNA was found to be up-regulated by a factor of 26 during the maturation of U937 cells (Verhoeckx *et al.*, 2004) which is indicative of a prominent function in macrophages. There seems to be a functional overlap of sortilin and the MPRs as indicated by the formation of procathepsin D-prosaposin (pSAP-pCD) complexes in U937 cells (Fig. 3.3). These complexes could be targeted to lysosomes either in a M6P-dependent manner due to the interaction of MPRs with the phosphorylated pCD, or in a M6P-independent manner using sortilin. A M6P-independent targeting of CD in tissues of I-cell disease patients, particularly in leukocytes, has been reported (Glickman *et al.*, 1993). A study with knockdown mice lacking CI- and CD-MPRs has indicated that the targeting of CD to lysosomes is normal in thymocytes despite the lack in MPRs (Dittmer *et al.*, 1999). Hence, it is likely that the formation of the proSap-proCD

complex and its binding to sortilin contribute to M6P-independent targeting of these two proteins to lysosomes. However, it remains unclear how the majority of the lysosomal enzymes not interactig with prosaposin reach lysosomes and related organelles in leukocytes.

4.1.2 Serglycin

Serglycin is a proteoglycan that is abundantly expressed in cells of hematopoietic origin. It consists of a small core protein of 158 amino acid residues (rich in serine/glycine repeats) and up to seven proteoglycan side chains. The repeats are found in the central part of the core protein and carry the side chains, which consist of chondroitin or heparan sulfate (Kolset *et al.*, 1996).

Serglycin is the dominating proteoglycan present in the secretory granule of most haematopoietic cell types (Kolset *et al.*, 2004) and is likely involved in the delivery of some proteins into secretory granules and/or directing the secretion of these molecules. Promonocytic U937 cells synthesize serglycin, but they are not able to elaborate secretory granules. A portion of serglycin is delivered into endosomes and another is secreted (Fig. 3.13). Serglycin mediates the transport of lysozyme to lysosomes in U937 cells (Lemansky *et al.*, 2001). The present results suggest that neutrophil elastase (NE) is delivered to lysosomes in association with serglycin (Lemansky *et al.*, 2007b, Fig. 3.4). Portions of NE and lysozyme are secreted together with serglycin. Both proteins are active at neutral pH and may play a role in degrading the extracellular matrix and in defense mechanisms in U937 cells.

The mechanism of the targeting of serglycin to lysosomes is not known. We have observed that CI-MPR participates in lysosomal targeting of serglycin which, therefore, can be considered as a novel ligand of CI-MPR. An immunocytochemical examination of U937 cells showed that CI-MPR partially colocalized with serglycin (Fig. 3.5). In cell extracts of U937 cells that were treated with a cross-linking reagent, CI-MPR and serglycin were coimmunoprecipitated. When biosynthesis of M6P-bearing N-glycans was blocked in the presence of tunicamycin, the extent of the

coimmunoprecipitation was increased three-fold and this was accompanied with a significant improvement of lysosomal transport of serglycin (Lemansky *et al.*, 2007a) indicating that in the absence of M6P-ligands the availability of CI-MPR to bind serglycin may be enhanced. However, further studies are required to elucidate the molecular basis of the interaction of serglycin and the CI-MPR and to quantitate the contribution of CI-MPR to the lysosomal targeting of serglycin.

The results of the present work indicate that there is an additional lysosomal sorting mechanism for serglycin since immunofluorescent staining of U937 cells showed a strong colocalization of AP-3 and serglycin (Fig. 3.6). In contrast, CI-MPR and serglycin colocalize to a much lesser extend (Fig. 3.5). Since serglycin is a soluble molecule, it could bind to transmembrane proteins with an AP-3 sorting motif. In TGN, AP-3 recognizes the lysosomal transmembrane proteins and deliveres them to lysosomes. A mutation in the β subunit of AP-3 was identified in dogs with cyclic neutropenia. A similar AP-3 defect is found in Hermansky−Pudlak syndrome type 2, a human disorder that is characterized by a partial albinism and neutropenia. In both disorders the contents of neutrophil elastase (NE) in neutrophils is drastically reduced, indicating the lysosomal transport of NE depends largely on carries with AP-3 interacting cytosolic domains. Earlier studies postulated a direct interaction of NE and AP-3 (Benson *et al.*, 2003; Horwitz *et al.*, 2004) but this was challenged by recent studies showing that lysosomal transport of NE relies on serglycin and that NE most likely does not interact with AP-3 directly (Niemann *et al.*, 2007; Lemansky *et al.*, 2007b; Fig. 3.4). If NE is bound by serglycin to affect ist lysosomal transport, it conceivable that a hitherto unrecognized AP-3 dependent lysosomal membrane protein exists that is facilitating this transport.

4.2 PMA enhances the secretion of lysosomal proteins

In the present work, we examined the effect of PMA on sorting and transport of lysosomal proteins in U937 cells. PMA is a structural analogue of DAG and can

directly activate DAG-binding proteins, such as PKC and PKD thus influencing sorting and trafficking of lysosomal proteins. The results described here demonstrate that direct stimulation of cells by PMA caused a missorting of lysosomal proteins towards the plasma membrane and their diversion from the lysosomal transport routes.

The enhanced secretion of lysosomal proteins in the presence of PMA may have its origin at more then one locations in the cell. PMA may (i) cause their default passage into the secretory vesicles at the TGN (the "missorting hypotesis"), (ii) induce a selective fusion of the post-Golgi vesicles containing mainly serglycin ("secretory endosomes hypotesis") and (iii) activate the regulated exocytosis of late endosomes/lysosomes ("secretory lysosomes hypotesis"). The latter possibility is an active process occurring in many hematopoietic cells.

Undifferentiated U937 cells secreted a minor portion of newly synthesized procathepsin D (pCD). Upon exposure to PMA, the secretion of pCD increased and the partially processed and active forms of cathepsin D were also secreted (Fig. 3.8). This indicated that PMA influenced the sorting and/or transport of precursor molecules at the TGN or early post-Golgi compartment due to enhanced secretion. The simplest explanation is that the precursor form of CD is released *via* the constitutive secretory vesicles exiting the TGN towards the plasma membrane. Thus, PMA may enhance the so-called 'constitutive-like' secretory vesicle formation or decrease formation of lysosomal transport vesicles. Furthermore, in the presence of PMA partially and even the fully processed forms of CD were recovered from the medium. Since the processing of lysosomal hydrolases occurs in endosomes and lysosomes, PMA is likely to induce the fusion of a portion of late endosomes/lysosomes with the plasma membrane. This possibility was supported by finding that PMA caused a redistribution of the lysosomal transmembrane protein Lamp-II to the plasma membrane (Fig. 3.9), and also by an enhanced secretion of β-hexosaminidase. (Fig. 4.10). The activity of this enzyme in the medium reached 10% of total after 1 h. The finding of this high proportion of β-hexosaminidase in the

medium indicated a fusion of endosomes or lysosomes, the organelles containing the bulk of the enzyme, with the plasma membrane.

The possibility that PMA may bring about a fusion of endosomes or lysosomes with the plasma membrane in promonocytic U937 cells raises a question about the role of a lysosomal secretion in monocytes and macrophages. The fusion of secretory lysosomes and granules with the plasma membrane is used by cells of the immune system, such as neutrophils, cytotoxic T lymphocytes and mast cells and can be stimulated by PMA and Ca^{2+} to some extent (Griffiths, 1996). Lysosomes of monocytes and macrophages are known to participate in the maturation process of phagosomes. The exocytosis and translocation of lysosomal granules to nascent phagosomes at the plasma membrane has been described following phagocytic stimulation of neutrophils (Tapper *et al.*, 2002). Another study suggested that neuraminidase 1 and cathepsin A pass through lysosomes prior to delivery to the plasma membrane in PMA differentiated monocytic THP-1 cells. However, soluble lysosomal enzymes could not be detected in the cell supernatant in this system (Liang *et al.*, 2006). These studies documented an overlap between lysosomes and secretory granules in promonocytic U937 cells since lysosomal proteinases can be released by inducing the regulated exocytosis by PMA.

The secretion of serglycin and prosaposin (pSAP) was enhanced in the presence of PMA (Fig 3.12 and 3.13). The sorting of pSAP to the lysosomal compartments is mediated by sortilin. At the TGN, the GGA adaptor proteins interact specifically with sortilin and MPRs and direct the trafficking of these receptors to the endosomal pathway. Therefore, the mechanism controlling sorting of the two receptor types may be similar while being sensitive to PMA in distinct ways.

However, the sorting of serglycin at the TGN has its own chartacteristic features. U937 cells secrete a relative large amount of serglycin, whereas soluble lysosomal hydrolases are secreted to a small extent at normal conditions. In the case of serglycin, a treatment with PMA results in an almost complete secretion (Fig. 3.13).

In contrast, a major portion of CD is routed into lysosomes even in the presence of PMA (Fig. 3.8). If CI-MPR is a major TGN receptor of serglycin, the massive secretion of the latter could be explained by a low affinity of serglycin to CI-MPR. This may indicate that PMA caused the massive secretion of the low-affinity ligand serglycin and a moderate miss-sorting of the high-affinity ligand CD. On the other hand, this may indicate the existence of an alternative AP-3 binding carrier that responds more strongly to PMA than do the MPRs.

4.2.1 Possible involvement of PKD and PLD in the secretory effects of PMA

According to the model proposed by Bard and Malhotra, an increased production of DAG in the outer leaflet of the TGN recruits PKCη and PKD to the membrane. The activated PKCη then phosphorylates and activates PKD, which in turn phosphorylates target proteins that are considered to drive membrane fission events which may be involved in the formation of secretory vesicles (for detailed study, see 1.2.1). PKCη posseses a DAG binding domain (Tolker, 2005) and can be activated by PMA. However, in the basal state PKCη is in the cytosol and it is not known whether PMA can recruit PKCη to the TGN membrane. An expression of constituvely activated PKCη caused a fragmentation of the Golgi apparatus and an activation of PKD (Diaz Anel *et al.*, 2005). It is possible that PMA induces a "TGN fragmentation" which may result in a production of secretory vesicles. The increased exit flux of secretory proteins and thus formation of secretory vesicles at the TGN may cause a non-specific capture of lysosomal proteins in plasma membrane-directed secretory vesicles and at the same time decrease the probability (due to time and/or spatial factors) of interaction between lysosomal proteins and sorting receptors.

The inhibition of PLD with 1-butanol impaired the secretion of serglycin in the presence of PMA (Fig. 3.14). Therefore, the enhanced secretion of serglycin appears to depend on PLD. The activation of PKC (Becker *et al.*, 2005) and casein kinase-II (Ahn *et al.*, 2006) in the presence of PMA results in an activation of PLD and

increases the production of PA and DAG in the membranes. The localized occurence of these lipids induces a membrane bending and is probably involved in fusion/fission processes between and in membranes. It is not clear if an activation of PLD occurs at the TGN, plasma membrane or post-Golgi vesicles itself in U937 cells. The increased production of PA and DAG, however, is likely to enhance the rate of membrane fission at the TGN and to facilitate the fusion of post-Golgi compartments with plasma membrane as well.

4.2.2 Effect of PMA on the localization of CI-MPRs

The surface expression of several endocytic receptors can be modulated by hormones, growth factors and receptor ligands. PMA was reported to cause a redistribution of the CI-MPR to the plasma membrane in fibroblasts, where uptake of M6P-containing ligands was increased 2-4-fold (Braulke T. *et al.*, 1990, Damke H. *et al.*, 1992). In contrast to the CI-MPR receptor, ligand uptake and receptor concentration at the cell surface were unchanged for the endocytic transferrin receptor. PMA enhanced the secretion of lysosomal enzymes in U937 cells and the redistribution of CI-MPR to the plasma membrane could be expected to enhance a reuptake of secreted lysosomal hydrolases. However, as shown here the level of CI-MPR at the plasma membrane in U937 cells was reduced in the presence of PMA (Fig. 3.11). Furthermore, in these cells PMA decreased the half-life of CI-MPR (Diploma thesis by Ines Fester, University Marburg, 2006), indicating that the endosomal transport of CI-MPR was probably greatly altered. In fact, when U937 cells were treated with PMA a massive formation of multivesicular bodies (MVBs) was observed within minutes of application (Nilsson *et al.*, 1989). MVBs are considered late endosomes, in which a degradation of membrane proteins takes place. It is possible that MPRs and sortilin transiently accumulate in these structures and this may explain their depletion in other cellular sites and a decreased half-life as well. This may explain the decreaase in the lysosomal sorting of procathepsin D and serglycin reported here.

A recent study showed that differentiation towards macrophages is accompanied by a transcriptional down-regulation of mannose-6-phosphorylation. This regulation is likely to impede the recognition and proper sorting of soluble lysosomal enzymes by MPRs (Lackman *et al.*, 2007). The exposure of the promonocytic cell line THP-1 to *Escherichia coli* causes a macrophage differentiation and secretion of lysosomal precursor molecules. Similar findings were made in human macrophages during their differentiation from peripherial blood monocytes and in mouse marrow-derived macrophages. Thus, monocytes differentiating into macrophages behave similarly to PMA-differentiated U937 cells. The macrophage differentiation pathway may result in an accumulation of lysosomal enzyme precursors outside the cells with a concomittant lack of a recapture *via* CI-MPRs. This possibility implies an extracellular function of lysosomal hydrolases that are secreted in a response to inflammatory agents and may be active at slightly acidic conditions in inflamed tissues. Cysteine cathepsins are active in pericellular enviroments as soluble enzymes or bound to cell surface receptors at the plasma membrane (Brix *et al.*, 2007). However, numerous lysosomal hydrolases are secreted as inactive precursor molecules and need to be proteinolytically activated at the acidic pH. The activation of acidic lysosomal hydrolases and the role of lysosomal proteins in the extracellular environment remain poorly defined. So far few studies have were performed on the function of extracellular lysosomal enzymes. Human macrophages derived from primary monocytes and cultured in an elastin matrix rapidly acidify their interface with the substratum upon recruiting the vacuolar-type H+-adenosine triphosphatase. They appear to form a sequestered acidic microenviroment in which lysosomal hydrolases are degrading varied components of the matrix (Punturieri *et al.*, 2000).

4.3 Examination of protein phosphorylation in PMA-treated cells

Many cellular processes including cell signalling and membrane trafficking are regulated by the coordinated web of phosphoprotein kinases. The present study showed that the differentiation pathway was accompanied by changes in sorting and

trafficking of lysosomal proteins and that PMA enhanced the secretion of newly synthesized lysosomal proteins and serglycin from the TGN. PMA activates DAG-regulated serine/threonine kinases such as PKC and PKD. The analysis of substrates of DAG-regulated serine/threonine kinases was interfered with the basal phosphorylation. The phosphoprotein labeling did not detect a PMA-specific phosphorylation in fractions of low buoyant density, in which the light transport vesicles originating from the TGN membrane and TGN were concentrated (Fig. 3.18). In this work, we tested a novel specific phosphoprotein fluorescent dye Pro-Q-diamond (Agrawal and Thelen, 2005) that was used to detect the phosphoproteome of PMA-activated U937 cells. The proteins in fractions of low buoyant density were separated by diagonal electrophoresis with the cationic detergent N-Cetyl-N,N,N-trimethylammonium bromide (CETAB) which allows a separation of highly hydrophobic membrane proteins in the first dimension. Using Pro-Q-diamond staining, in the low buoyant density fractions nine phosphoproteins were detected that were phosphorylated in response to PMA (Fig. 3.20). Because of the low abundance of these phosphoproteins their identification was incomplete. One of them (Fig. 3.20) was identified as the insulin-responsive amino peptidase (IRAP). The present study shows that the phosphoprotein fluorescent dye Pro-Q-diamond can be used to detect phosphoprotein kinases substrates in PMA activated U937 cells. However, to identify all detected phosphoproteins a larger amount of the material or a more sensitive detection should be used.

4.3.1 IRAP is phosphorylated in the presence of PMA

IRAP was previously characterized as the marker protein of specialized insulin-responsive vesicles containing GLUT4 in muscle and adipose tissue. A phosphorylation of IRAP by PKCζ may play a key role in the redistribution of GLUT4 vesicles to the plasma membrane in response to insulin (Ryu *et al.*, 2002). Unlike GLUT4, IRAP is expressed in many other cell types. Recently, the specific dileucine motif in the IRAP cytosolic tail was found to interact with GGA adaptor proteins, similarly as MPRs and sortilin (Hou *et al.*, 2006). Using subcellular fractionation and

immunocytochemistry, IRAP was found to be localized in vesicles containing CI-MPR (Fig. 3.22) and distinct from those containing sortilin in U937 cells. PMA is known to activate different isoforms of PKC and thus enhanced the phosphorylation of IRAP in U937 cells. However, the physiological function of IRAP in cells of hematopoietic origin is not known. One possibility is that in the presence of PMA a phosphorylation of IRAP results in the exocytosis of IRAP compartments including Lamp-II and various lysosomal-like contents, provided there is a segregation of MPRs, which are not accumutaing at the plasma membrane.

5 Summary

The present contributions on secretion and targeting of lysosomal enzymes are as follows:

1. The previously described strong enhancement in the secretion of lysozyme and serglycin in the presence of PMA and a minor effect on that of procathepsin D is confirmed.
2. PMA enhances the rate of the secretion of prosaposin and this may explain the induced secretion of a portion of procathepsin D, since at least a fraction of the two precursors are engaged in mutual complexes.
3. PMA is shown to induce the secretion of a high proportion of the cellular ß-hexosaminidase indicating exocytosis of an endosomal/lysosomal compartment.
4. PMA is shown to induce secretion of a portion of partially (intermediate) and fully processed (mature) forms of cathepsin D. This and the observation referred to in preceding statement indicate an effect of PMA distally to the sorting at the TGN.
5. An induction of a fusion of a distal compartment is further supported by finding Lamp-II at the plasma membrane in PMA-treated cells.
6. Tunicamycin, which inhibits N-glycosylation and secondarily the endowment of lysosomal proteins with the M6P marker, enhances the lysosomal targeting of serglycin. This effect is strongly pronounced in the presence of PMA. It can be explained by a participation of CI-MPR in the lysosomal targeting of serglycin. The receptor may have a preference for M6P ligands, that may suppress the binding of serglycin.
7. PMA does not enhance the presence of CI-MPR at the plasma membrane.
8. The effects of PMA appear to be mediated by an activation of phospholipase D and the local generation of phosphatidic acid and diacylglycerol in the membrane. It is proposed that these lipids participate in fusion and fission phenomena of different compartments and a selective enhancement of the secretion of lysosomally targeted products.

9. PMA induces a phosphorylation of several proteins in the soluble as well as the vesicular fractions of U937 cells. One of the selectively phosphorylated proteins was identified as insulin-responsive amino peptidase (IRAP). Further experiments are indicated to explore a possible role of IRAP in the selective secretory effects of PMA.

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7 Appendix

7.1 Abbreviations

AP	adaptor protein
BSA	bovine serum albumin
CCV	clathrin-coated vesicles
CD	cathepsin D
CD-MPR	cation-dependent mannose 6-phosphate receptor
CI-MPR	cation-independent mannose 6-phosphate receptor
CETAB	cetyltrimethylammonium bromide
Da	Dalton
DAG	diacylglycerol
DMSO	dimethyl sulfoxide
DRM	detergent-resisitant microdomains
DTT	dithiothreitol
ECL	enhanced chemiluminiscence
EDTA	ethylendiamine tetraacetate
g	gram
GGA	Golgi-localized gamma-ear-containing Arf-binding protein
GPCR	G-protein-coupled receptor
h	hour
IAA	iodoacetamide
ICD	I-cell disease
IRAP	insuline-responsive aminopeptidase
kDa	kilo Dalton
Lamp	lysosomal associated membrane protein
Limp	lysosomal integral membrane protein

Maldi-TOF	Matrix-assisted laser desorption/ionization- Time-of-flight
mA	milli ampere
min	minute
ml	milliliter
mm	millimeter
M6P	mannose 6-phosphate
MPR	mannose 6-phosphate receptor
MVBs	multivesicular bodies
Mw	molecular weight
M	molar
NE	neutrophil elastase
nM	nanomolar
PA	phosphatidic acid
PAGE	polyacrylamide gel electrophoresis
PC	phosphatidyl choline
PI	phosphatidylinositol
PI(4)K	phosphatidylinositol 4-kinase
PI(4)P	phosphatidylinositol 4-phosphate
PKC	protein kinase C
PKD	protein kinase D
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol-12-myristate-13-acetate
PMSF	phenylmethylsulfonylfluoride
rpm	revolution per minute
SAP	sphingolipid activator protein
SDS	sodium dodecyl sulfate
TGN	*trans*-Golgi network
TCA	trichloroacetic acid
2DE	2-dimensional electrophoresis
UV	ultra violet

VSVG	vesicular stomatitis viral glycoprotein
v/v	volume/volume
w/v	weight/volume

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7.3 List of publications

1) Lemansky P., **Smolenova E.**, Wrocklage C. and Hasilik A.
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7.4 Declaration

I declare that I wrote this study myself and carried out the experimental work described in it, without using any other sources and aids than those that are stated.