Functional characterisation of Rab22a and Munc18b, two proteins regulating vesicle transport in mammalian cells

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Academic Dissertation

To be publicly discussed, by permission of Faculty of Biosciences of the University of Helsinki, in the lecture room 3, Biomedicum Helsinki on 16th April 2004, at 12 o'clock.

Helsinki 2004

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Julkaisija-Utgivare-Publisher:

Kansanterveyslaitos (KTL) - Folkhälsoinstitutet- National Public Health Institute
Mannerheimintie 166
00300 Helsinki
tel. +358-9-47441

Publications of the National Public Health Institute
KTL A4/2004
ISBN 951-740-423-9
ISSN 0359-3584
ISBN 951-740-424-7 (PDF version)
ISSN 1458-6290 (PDF version)

Yliopistopaino

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ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are in the text referred to their Roman numerals. In addition, some unpublished results are presented.

I Kauppi, M., Simonsen, A., Bremnes, B., Vieira, A., Callaghan, J., Stenmark, H. and Olkkonen, V.M. (2001) The small GTPase Rab22 interacts with EEA1 and controls endosomal membrane trafficking. *J.Cell Sci.* 115, 899-911

II Riento, K.*, Kauppi, M.*, Keränen, S. and Olkkonen, V.M. (2000) Munc18-2, a functional partner of syntaxin 3, controls apical membrane trafficking in epithelial cells. *J.Biol.Chem*. 275, 13476-13483

III Kauppi, M., Wohlfahrt, G. and Olkkonen, V.M. (2002) Analysis of the Munc18b-syntaxin binding interface: Use of a mutant Munc18b to dissect the functions of syntaxins 2 and 3. *J Biol. Chem.* 227, 43973-43979

*) Equal contributions

ABBREVIATIONS

AGA	aspartylglucosaminidase
ATP	adenosine triphosphate
BHK-21	baby hamster kidney cell line
Caco-2	human colon carcinoma cell line
CCK	cholecystokinin
CCK-OPE	phenylethylester analogue of cholecystokinin
cDNA	complementary deoxyribonucleic acid
COS-1	African green monkey kidney cell line
EEA1	early endosomal antigen 1
EGF	epidermal growth factor
ER	endoplasmic reticulum
GAP	GTPase activating protein
GDI	guanine-nucleotide dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine-nucleotide exchange factor
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
HA	hemagglutinin
kDa	kilodalton
LAMP-1	lysosomal-associated membrane protein
LBPA	lyso-bis-phosphatidic acid
LLC-PK1	porcine kidney cell line
MDCK	Madin-Darby canine kidney cell line
MINT	Munc18 interacting protein
M6PR	mannose-6-phosphate receptor
MVB	multi-vesicular bodies
NSF	N-ethylmaleimide sensitive fusion protein
PBS	phosphate-buffered saline
PI	phosphatidylinositol
PI(3)K	phosphatidylinositol-3-kinase
PIP	phosphatidylinositol phosphate
REP	rab escort protein
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFV	Semliki Forest virus
SM	Sec1/Munc18
SNAP	soluble NSF attachment protein
SNAP-25	synaptosomal-associated protein of 25 kDa
SNARE	SNAP receptor
tfn	transferrin
tfR	transferrin receptor
TGN	*trans*-Golgi network
wt	wild-type

I REVIEW OF THE LITERATURE

1. Introduction

A cell is capable of synthesising tens of thousands of different molecules. An important challenge in cell biological studies has been to figure out how these molecules are transported to their destinations, such as to the cell organelles where they act to maintain cellular homeostasis. In the early 1950's, electron microscopy provided scientists with the possibility to study the internal structures of the cell and to observe the fine membranes that surround cell organelles. Maintenance of the functional identity of these organelles requires effective as well as selective sorting and delivery of organelle constituents. In addition, many nutrients and signalling molecules are taken into the cell from the extracellular space and a portion of the newly synthesised proteins are transported out of the cell.

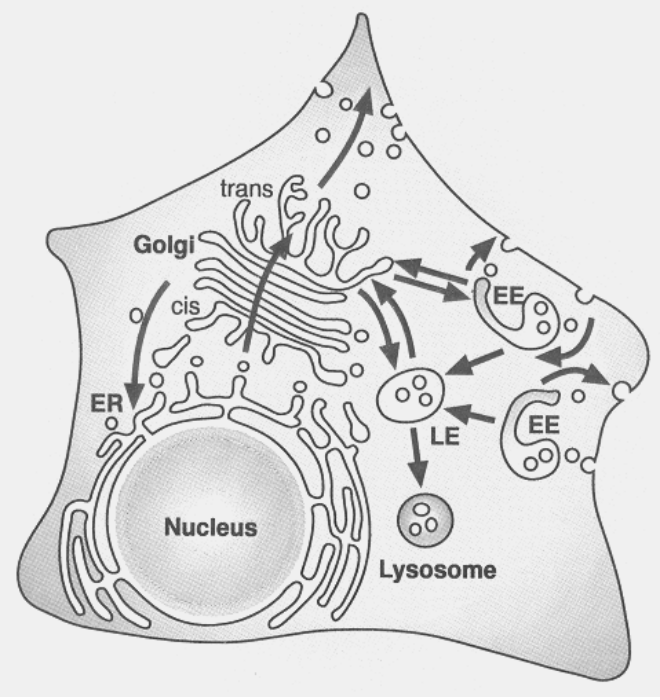

Figure 1. The intracellular compartments of eukaryotic cells involved in endocytic and secretory pathways. Molecules are taken up from the plasma membrane by endocytosis and are transported via early (EE) and late (LE) endosomes to several different intracellular compartments, or recycled back to the cell surface. In the secretory pathway, cargo molecules are transported from the endoplasmic reticulum (ER) through the Golgi to the plasma membrane or to endosomes/lysosomes. Transport is mediated via transport vesicles that bud off one membrane and fuse with another.

Proteins that are destined for the secretory pathway are translated at the endoplasmic reticulum (ER) bound ribosomes. ER is the large membrane organelle responsible for correct folding of proteins and the addition of N-linked oligosaccharides. From the ER a major portion of proteins travel in vesicular carriers to the Golgi apparatus where sugar residues are further modified and O-linked glycosylation takes place. In the last part of the Golgi, the *trans*-Golgi network (TGN), cargo molecules are packaged again into transport vesicles. The vesicles are transported to different cell organelles: to lysosomes (or vacuoles), to endosomes, back to the Golgi or ER, or to the plasma membrane where uptake of material from the extracellular space also occurs. This trafficking is called intracellular membrane traffic or vesicle transport. Only the trafficking to the cell nucleus, mitochondria or peroxisomes (and chloroplasts in plants) is excluded from this concept (Fig.1) (reviewed by Mills et al., 1999; Pfeffer, 2003; Waters and Hughson, 2000).

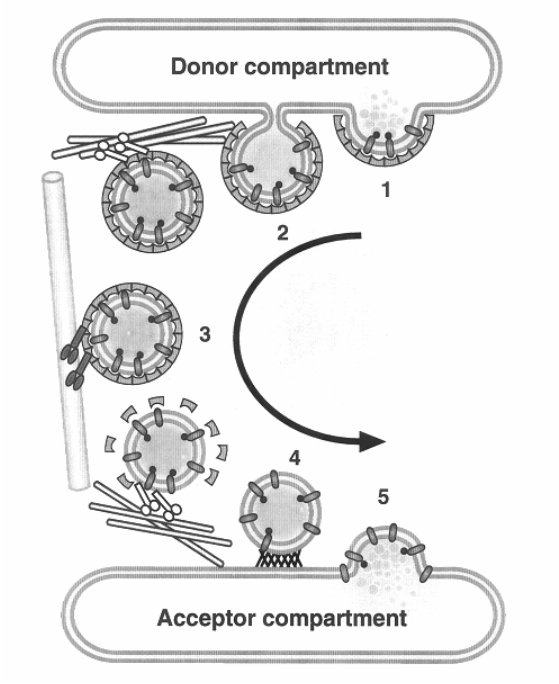

Figure 2. The five stages of a membrane trafficking event. 1. Cargo selection. 2. Coat and vesicle formation. 3. Movement of a vesicle along microtubular elements by motor proteins from the donor to the acceptor compartment. 4. Vesicle tethering and docking. 5. Fusion

In vesicle transport, the specificity of delivery is absolutely crucial for maintaining the proper function of cell organelles. Protein trafficking requires a series of fast events that include cargo selection, vesicle budding at the donor organelle, and finally docking and fusion of the transported vesicles with the target organelle (Fig.2). The secretory pathway (transport from the ER towards the plasma membrane) and the early steps of endocytosis (uptake of material from the plasma membrane and homotypic endosome-endosome fusion) are two well-characterised transport processes (reviewed by Mills et al., 1999; Pfeffer, 2003; Waters and Hughson, 2000). Also, the release of neurotransmitters in synaptic terminals has been intensively studied (reviewed by Burgoyne and Morgan, 2003).

The main features of the membrane trafficking process were already characterised in 1974 when George Palade, Albert Claude and Christian de Duve were awarded the Nobel Prize for their work on the intracellular aspects of the process of protein synthesis. Since then, scientists have been working towards identifying precisely how the protein transport machinery works. The major experimental studies in this field have been made in *Saccharomyces cerevisiae* and nerve cells. In 1993, James Rothman's group proposed a working model, the SNARE (soluble NSF attachment protein (SNAP) receptor) hypothesis, which contained the idea of how one cell can transport such a huge amount of molecules into correct target organelles (Söllner et al., 1993b). SNARE-complexes are still believed to be the central machinery in membrane fusion and SNARE proteins are, at the moment, the most widely studied proteins in membrane trafficking. The main features of the vesicle transport process have mostly been elucidated; however, many mysteries still remain unsolved.

2. Membrane trafficking

2.1 Vesicle formation

Only a subset of proteins and lipids are allowed to enter each transport vesicle. Coat protein complexes assemble on membranes at a site of vesicle formation and participate in cargo selection. During the formation of the transport vesicle, a limited set of coat proteins carry out a programmed set of sequential interactions that lead to

vesicle budding from the parent membrane, and later, vesicle uncoating and fusion with the target membrane. Carrier vesicles can be identified by their coats; COPI (coat protein complex I), COPII (coat protein complex II) or clathrin. There are clear similarities and differences between the behaviour of these coat protein systems. There are also many transport routes with yet unknown carrier vesicles (reviewed by Rothman and Wieland, 1996; Springer et al., 1999).

COPII vesicles transport proteins from the ER to the Golgi. The COPII coat system was initially identified in *S. cerevisiae*, but the homologous protein components have also been identified in mammalian cells, in which the same nomenclature as in yeast is used. The COPII coat consists of the small GTPase Sar1p and the heterodimeric protein complexes Sec23/24p and Sec13/31p. These proteins are necessary for COPII vesicle formation and work together with other cytosolic factors or membrane proteins required for targeting and fusion (Barlowe et al., 1994). The Sec23p-Sec24p complex is the component responsible for cargo recognition. Sar1p-GTP facilitates the association of the Sec23p-Sec24p complex with cargo proteins and also activates Sec23p to bind SNARE proteins involved in the specificity of vesicle targeting (Springer and Schekman, 1998). The Sec13/31p complex is presumably involved in membrane curvature and vesicle budding (Antonny et al., 2001).

The transport route that moves proteins from the Golgi back to the ER is one of the intracellular retrograde transport routes. This route is operated by COPI vesicles. COPI coatomer is a complex of seven proteins. COPI coated vesicles capture proteins that carry a sorting signal in their cytoplasmic carboxyl-terminal domain (KKXX, KXKXX, RRXX where X is any amino acid) (Cosson and Letourneur, 1994). Soluble ER resident proteins contain a short retrieval signal at their C-terminal end, called KDEL-sequence (Munro and Pelham, 1987). COPI vesicles use another small GTPase, ARF1, for budding of the vesicle. In the GTP-bound state, ARF1 is myristoylated and becomes membrane bound. The hydrolysis of GTP to GDP releases ARF1 from the membrane and acts as a timer (as with Sar1p and COPII vesicles), triggering the release of other coat components and preparing the vesicle for fusion (Goldberg, 1999). COPI vesicles also transport cargo molecules within the Golgi complex (Orci et al., 1997).

Clathrin coated vesicles bud from the plasma membrane and are transported to endosomes. Vesicles transported from the TGN to endosomes also have clathrin coats.

Many membrane proteins with their ligand, like the LDL (low density lipoprotein) receptor (Bos et al., 1995), are internalised by the clathrin pathway (Payne and Schekman, 1985). Yeast cells also use a clathrin-based pathway, although the use of clathrin in the secretory pathway appears more prominent than in the endocytic pathway (Tan et al., 1993). Clathrin is the most abundant protein in the coat of endocytic vesicles and more than 25 proteins have been identified as its partners in the endocytic pathway alone. So, in contrast to COPI and COPII vesicles, clathrin coated vesicles have a large variety of associated proteins. Clathrin coated vesicles from the TGN carry AP-1 (adaptor protein complex 1), and vesicles from the plasma membrane carry AP-2 (adaptor protein complex 2). AP-1 and AP-2 adaptors bind to specific sorting signals found in the cytosolic tails of a large number of membrane proteins (reviewed by Lafer, 2002). Adaptor protein complexes 3 and 4 are also found and located in the TGN and on endosomes. AP-3 also associates with clathrin (Dell'Angelica et al., 1998). AP-4 does not bind clathrin and recognises cargo that has tyrosine-based motifs at the TGN (Hirst et al., 1999). In the plasma membrane, arrestins are a second type of adaptor in clathrin coated pits. β-arrestin couples directly with clathrin and recruits seven-transmembrane-helix G-protein-coupled receptors together with AP-2 (Goodman et al., 1996).

2.2 Movement of vesicles from the donor to the acceptor membrane

The cytoskeleton is a crucial component of the membrane trafficking machinery. Organelles and vesicular transport intermediates are localised along microtubules. Microtubules are formed via head-to-tail association of tubulin proteins that form polar filaments. The tubulin subunit is itself a heterodimer and has a binding site for GTP at both ends of the dimer. At one end, GTP is physically trapped and is never hydrolyzed or exchanged, while at the other end the GTP is exchangeable to GDP, which has an important effect on microtubule dynamics. Each protofilament in a microtubule is assembled from subunits that all point in the same direction to create two ends, the minus and the plus end. Protofilaments themselves are aligned in parallel. In non-polarised cells, the relatively stable minus ends of microtubules are located at the cell center near the centrosomes, whereas the dynamically growing plus ends extend to the cell periphery (reviewed by Kamal and Goldstein, 2000).

In polarised epithelial cells, the plus ends of microtubules are localised at the basolateral terminus of the cell and the minus ends at the apical side. In non-polarised cells, the ER and early endosomes are usually dispersed towards the plus ends of microtubules at the cell periphery, whereas the Golgi apparatus, late endosomes and lysosomes are often clustered near the minus ends at the cell center. Actin filaments are localised at the cell surface and act as actin cables within the cell. This distribution of organelles is thought to promote efficient vesicle transport by using cytoskeletal motor proteins (reviewed by Kamal and Goldstein, 2000).

Kinesins and cytoplasmic dyneins are microtubule-based motor protein families that transport vesicles by using ATP hydrolysis as an energy source. Both kinesin- and dynein-driven vesicle motility have been shown to require additional protein accessory factors (Schroer and Sheetz, 1991). Kinesin superfamily members play an important role in post-Golgi transport (Rahman et al., 1999) and in neurons with fast anterograde transport that moves cargo from the cell body to the synapse (reviewed by Goldstein and Yang, 2000). Cytoplasmic dynein is a minus-end directed motor that facilitates the inward movement of endocytic vesicles from early to late endosomes by associating with the dynactin complex (Aniento et al., 1993; Holleran et al., 1998). Myosins are motor proteins responsible for actin-based movement. The myosin superfamily consists of 15 families of related proteins in eukaryotic cells, and some of them have been localised on endocytic organelles (Raposo et al., 1999). Several motor protein binding partners that are involved in vesicle cargo selection or in linking the cytoskeleton to organelles have been found (see Rab proteins in chapter 3). Many key issues, however, must be solved: How is the motor activity regulated, and are specific motor proteins required for each membrane sorting step (reviewed by Kamal and Goldstein, 2000)?

2.3 Tethering, docking and priming

While motor proteins transport the vesicle along cytoskeletal tracks close to the target membrane, numerous proteins and protein complexes also direct different classes of vesicles to their appropriate target membrane. Attachment to the membrane before fusion requires several layers of protein-protein interactions. When talking about this issue, scientists often use terms that are somewhat puzzling. Tethering means that a vesicle already has a connection to the target membrane through tethering proteins or

protein complexes but is still quite far from the membrane (>25nm). Docking means that the vesicle is closer (<5-10nm) and tightly associated with the target membrane. This type of interaction may still be reversible. Stable docking probably represents several distinct molecular states before fusion (reviewed by Pfeffer, 1999). The term priming is used in neural and neuroendocrine systems where docked vesicles are not intrinsically fusion competent. Priming events are considered to be those that render docked vesicles competent for Ca^{2+} -triggered fusion (reviewed by Klenchin and Martin, 2000).

Vesicle tethering is a SNARE-independent event and requires several protein-protein interactions. These interacting proteins are called tethering factors and seem to be coordinated by Rab-GTPases (Zerial and McBride, 2001). Different tethering/docking factors are localised on various organelles inside the cell, mediating the specificity of each transport route. Tethering is typically mediated by long helical proteins or large protein complexes that are recruited from the cytosol to the site of vesicle–target membrane contact (reviewed by Guo et al., 1999). EEA1 (early endosomal antigen 1) is a well-characterised tethering/docking factor. EEA1 is a large endosomal coiled-coil forming protein, which regulates endosomal trafficking together with Rab5 and its other effector proteins (see chapter 3.1.2.). The association of EEA1 with the endosomal membrane requires Rab5-GTP and the activity of phosphatidylinositol-3-kinase (PI(3)K). PI(3)Ks are a subfamily of lipid kinases that catalyse the addition of a phosphate molecule specifically to the 3' position of the inositol ring of phosphatidylinositol (PI). EEA1 contains binding domains for both the Rab5-GTP and the PI(3)K lipid product phosphatidylinositol-3-phosphate (PI(3)P). The interaction with GTP bound Rab5 involves the N-terminal C_2H_2 zinc-finger motif of EEA1. There is also another binding site for Rab5 in the C-terminal part of EEA1. The PI(3)P binding domain is called the FYVE finger domain and this creates an important link between membrane trafficking and cell signalling, a role of PI(3)P that is well characterised (Simonsen et al., 1998).

In the Golgi apparatus, the tethering factor p115 links COPI coated vesicles to the Golgi membrane. p115 also links together several other Golgi proteins (like GM130, Giantin, Rab1) and stimulates the specific assembly of the Golgi SNARE complexes (Shorter et al., 2002). There are several identified Golgi tethering complexes in yeast *S. cerevisiae*. The transport protein particle (TRAPP) complexes are conserved tethering complexes in the secretory pathway of yeast. TRAPP I is a

specific receptor for ER-derived COPII vesicles. After the vesicle binds the complex, the small GTPase Ypt1p (a Rab protein) is activated by TRAPP I and then other tethering factors are recruited. TRAPP II contains an additional three subunits and acts within the Golgi transport (Sacher et al., 2001). Another tethering complex, called exocyst, is an eight-subunit complex involved in tethering of exocytic vesicles to the plasma membrane. It is found in both yeast and mammalian cells (in mammalian cells the exocyst is also called the Sec6/8 complex). The exocyst is regulated by five different GTPases, which are members of Rab, Rho and Ral families. At the cell surface, the exocyst is localised to the regions of active exocytosis, like the bud tip in yeast or the tip of growing neurites (reviewed by Lipschutz and Mostov, 2002). In addition, the conserved oligomeric Golgi (COG) complex (also known as Sec34/35, GTC or ldlCp) and the Golgi-associated retrograde protein (GARP) complex (also known as Vps52/53/54 or VFT) act as Golgi tethering complexes in yeast. The COG complex is involved in the recycling of Golgi elements and the GARP complex is required for retrograde transport from endosomes to the Golgi (reviewed by Whyte and Munro, 2002).

2.4 Fusion

N-ethylmaleimide sensitive fusion protein (NSF) (Block et al., 1988) and soluble NSF attachment proteins (SNAPs) (Clary et al., 1990) were found to be important factors in membrane trafficking. The secretory defects observed earlier in *sec18* and *sec17* mutant strains, respectively, are due to mutations in the homologous yeast genes (Clary et al., 1990; Novick et al., 1980; Wilson et al., 1989). Characterisation of these proteins led to the identification of a set of membrane proteins called SNAP receptors (SNAREs). SNARE proteins not only served as a binding site for NSF and SNAP but were also thought to mediate the specificity of vesicle docking and fusion. SNARE proteins, together with NSF and SNAP, formed a complex (the 20S fusion particle) that seemed to be a core element of the membrane fusion machinery. These findings led to the SNARE hypothesis, a theory in which SNAREs are responsible for the specificity of vesicle trafficking and are possibly involved in the actual fusion process (Söllner et al., 1993b). Distinct SNAREs are localised to distinct membrane compartments and domains in all eukaryotic cells. Individual SNARE proteins form specific complexes in the organelle where they are located. Complexes are formed

between the single transport vesicle and the target membrane. With the help of Rab and Sec1 proteins (and other regulatory proteins) SNAREs can make the transport highly specific for one transport route.

SNARE proteins are classified in two groups: SNARE proteins on transport vesicles (v-SNAREs) and those on target membranes (t-SNAREs). They are integral membrane proteins with a single transmembrane domain at the C-terminus. They contain the characteristic SNARE domains of α-helical repeats ~60 amino acids in length that form coiled coil structures. Through these coiled-coil structures, SNARE proteins form extremely stable complexes that bring vesicle and target membranes close together (Rothman, 1994). SNAP-25-like t-SNARE proteins form an exception to other SNARE proteins in their structure. These proteins are anchored to membranes via palmitic acid modification and yet still participate in the SNARE complex formation by using two α-helical segments, whereas other SNAREs participate only with one segment (Hess et al., 1992). In the SNARE complex, the four α-helical segments are in register so that in a highly conserved layer of amino acid residues there are three glutamine (Q) residues and an arginine (R). Based on this structural definition, the SNARE proteins can also be classified as Q- or R-SNAREs. The fusion competent SNARE complexes consist of four-helix bundles composed of three Q-SNARE helices and one R-SNARE helix (Fasshauer et al., 1999; Fasshauer et al., 1998). The same structural features have been observed in SNARE complexes of different membrane compartments even though the complex in some cases consists of four SNARE proteins instead of the more common three proteins (Antonin et al., 2000). After fusion, NSF and SNAP are needed for the disassembly of the so-called *cis*-SNARE complex arising during fusion (Söllner et al., 1993a).

SNARE complex formation is most widely studied using neuronal SNARE proteins. Three SNARE proteins are involved in this process: one v-SNARE on the synaptic vesicle membrane (VAMP-2) and two t-SNAREs on the target membrane (SNAP-25 and Syntaxin1A). SNAP-25 contributes two SNARE domains in complex formation, while Syntaxin1A and VAMP-2 both contribute one domain. Due to coiled-coil formation of SNARE motifs, the synaptic vesicle is pulled closer to the presynaptic plasma membrane, thereby enabling it to fuse (reviewed by Lin and Scheller, 2000) (Fig.3). When liposomes containing VAMP-2 are mixed with liposomes containing SNAP-25 and syntaxin 1A, membrane fusion is observed

(Weber et al., 1998). While this suggests that SNAREs are the minimal machinery for fusion, if only the rate of fusion *in vitro* is far from the physiological rate in synaptic terminals (Fasshauer et al., 2002). It is now evident that the SNARE complex is capable of overcoming biophysical barriers of fusion. Remarkable structural similarities between the fusogenic state of viral fusion proteins and the core SNARE complex suggest a related function (reviewed by Skehel and Wiley, 1998). *In vitro*, liposome studies also show that the complex is formed and causes fusion when the v-SNARE is on one membrane and the t-SNARE is on the other, indicating specificity of membrane fusion (Parlati et al., 2000).

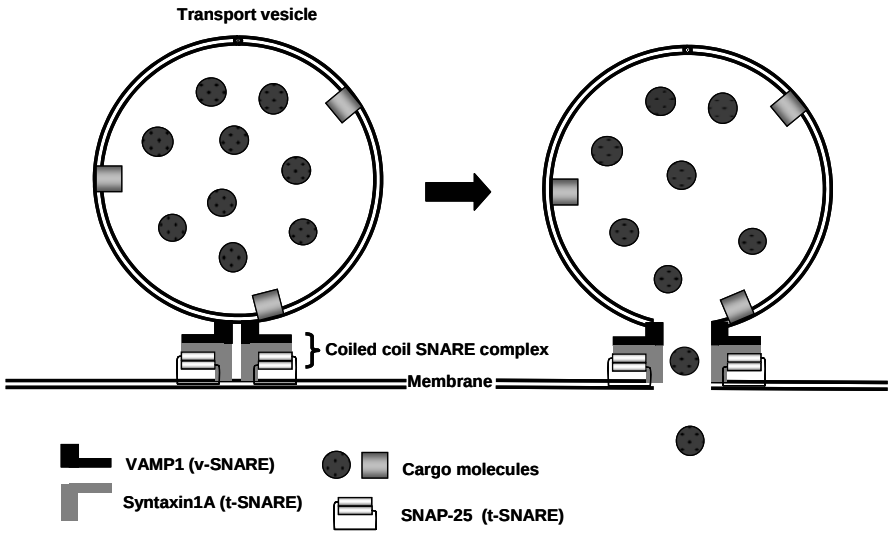

Figure 3. A schematic presentation of SNARE complex function. SNARE complexes are formed from SNARE proteins, which each contribute one or two α-helical subunits of the complex. The subunits form a coiled-coil structure that pulls the vesicle close to the target membrane and allows the membranes to fuse.

Cell biological studies *in vivo*, however, indicate that SNAREs alone cannot be responsible for fusion specificity. Certain SNAREs mediate more than one transport step and incorporate into more than one SNARE complex (Fischer von Mollard and Stevens, 1999). Furthermore, *Drosophila* syntaxin 1A is expressed throughout the axonal plasma membrane, yet synaptic vesicles only fuse with the plasma membrane at the synapse (Schulze et al., 1995). Also *S. cerevisiae*'s syntaxin proteins, Sso1p and Sso2p (Aalto et al., 1992), are located around the yeast cell while the actual secretion site is only a small part of the plasma membrane (Brennwald et al., 1994). According

to Fasshauer and colleagues, cognate and non-cognate SNARE complexes share similarities in their biophysical properties, suggesting the unlikelihood that specificity of membrane trafficking is due to intrinsic specificity of SNARE pairing (Fasshauer et al., 1999). These observations imply that SNAREs alone do not provide the level of specificity that is needed in vesicle trafficking. Additional factors must be required. The main regulatory protein families for SNARE complex formation/function include the Rab protein family and the SM related proteins (see chapters 3.1. and 3.2.). The surface of the SNARE complex provides several binding sites for other binding partners and regulators (Antonin et al., 2002). Proteins that interact with SNAREs, many of which may facilitate complex formation, are still being discovered today.

3. SNARE complex regulators

3.1 Rab proteins

3.1.1 General features of Rab proteins

The term ‘Rab’, meaning ‘Ras-related in brain’, was first introduced in 1987 when four new members of the *ras* gene family were identified in rat brain (Touchot et al., 1987). Today, the Rab GTPase family is considered one of the most important regulatory protein families in membrane trafficking. There are 11 Rab (Yptp/Sec4p) proteins expressed in the yeast *Saccharomyces cerevisiae* and over 60 family members in humans. Rab proteins are widely distributed throughout the cytosolic face of distinct intracellular membrane compartments, and some Rabs are cell type or tissue specific (Fig.4).

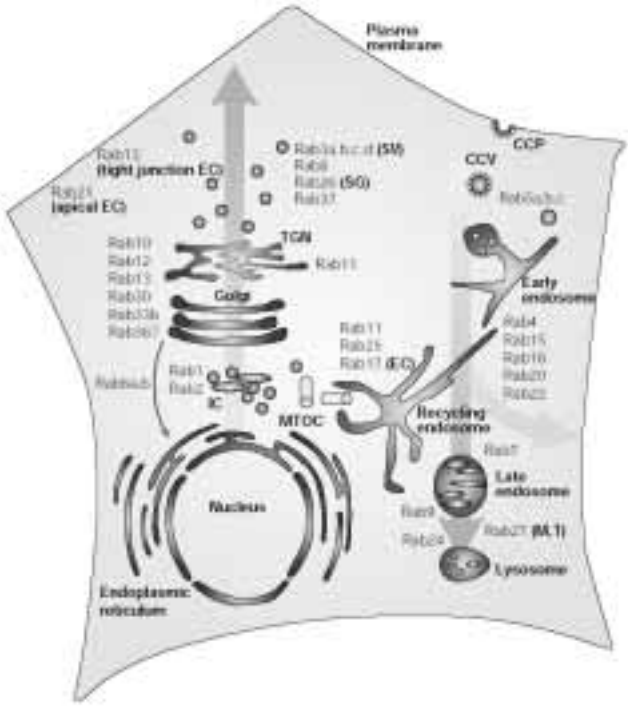

Figure 4. Rab protein localisation in mammalian cells. Some proteins are cell- or tissue-specific (e.g Rab17 in epithelia) or show cell type specific localization (e.g Rab13 in tight junctions). Symbols in the picture are: CCV, clathrin coated vesicle; CCP, clathrin coated pit; EC, epithelial cell; IC, ER-Golgi intermediate compartment; M, melanosomes; MTOC, microtubule-organizing centre; SG, secretory granule; SV, synaptic vesicles; T, T-cell granules; TGN, *trans*-Golgi network (From Zerial and McBride 2001).

Rab proteins regulate membrane trafficking between organelles via their GTP binding/hydrolysis cycle and they oscillate between GTP- and GDP-bound conformations. The GTP-bound form is considered the active form that interacts with downstream effector proteins. The most important feature of GTPases, however, is their ability to cycle between GTP- and GDP-bound states under the regulation of other factors. This cycle imposes the temporal and spatial regulation of membrane transport by Rab proteins, depending on the rates of nucleotide hydrolysis and exchange (reviewd by Zerial and McBride, 2001). A GDP/GTP exchange factor (GEF) in the Rab GTPase cycle catalyses nucleotide exchange from the GDP- to the GTP-bound state. Alternatively, the GTPase activating protein (GAP) facilitates GTP hydrolysis from the GTP- to the GDP-bound form (Fig.5). Changing the nucleotide bound to Rab also changes the conformation of the protein. Rab GTPases can

associate with membranes using their prenylated carboxyl termini. This post-translational modification at the carboxyl terminus requires the initial recognition of a newly synthesised Rab protein by a Rab escort protein (REP). REP presents the Rab to a geranylgeranyl transferase that catalyses the modification with a 20-carbon geranylgeranyl prenyl chain. Rab proteins are membrane associated in their GTP bound form. After GTP hydrolysis, Rab proteins are released from the membrane by Rab GDP-dissociation inhibitor (GDI). GDI retrieves GDP-bound Rab from the membrane and allows for several rounds of the Rab GTP/GDP cycle, and for membrane association of the Rab protein (reviewed by Stenmark and Olkkonen, 2001).

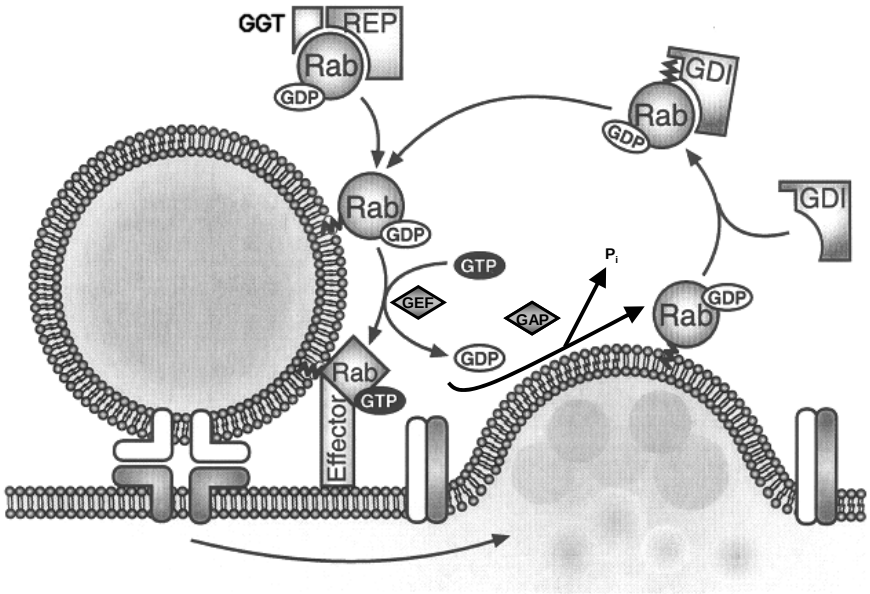

Figure 5. The GTPase cycle of a Rab protein. The newly synthesised Rab is bound by Rab escort protein (REP), which introduces it to the Rab geranylgeranyl transferase (GGT). This enzyme attaches prenyl tails to C-terminal cysteine residues of Rab, enabling Rab protein to attach to membranes. When the Rab protein associates with a membrane, bound GDP is exchanged for GTP by a guanine nucleotide exchange factor (GEF). The GTP bound form of Rab binds to its effector proteins on membranes. GTPase activating protein (GAP) catalyses GTP hydrolysis and Rab-GDP is released as a complex with GDP dissociation inhibitor (GDI) to the cytosol, where it can be recycled back to membranes.

3.1.2 Diversity in the functions and effectors of Rab proteins

Several studies show that Rab proteins function throughout various steps of membrane trafficking: cargo selection, vesicle budding, tethering/docking that leads to membrane fusion. For example, Rab5 has shown to be crucial for cargo selection at the plasma membrane formed vesicles (Seachrist et al., 2002) and *S. cerevisiae* Rab protein Ypt1p is participating in the tethering step (Jones et al., 1995). These findings suggest that the same regulatory machinery may coordinate all steps in vesicle transport. Rab proteins are known to be highly specific regulators at distinct transport steps and this could explain the huge number of Rab proteins found in mammalian cells.

Rab proteins interact also with cytoskeletal elements. They can associate with both microtubule- and actin-based motor proteins. A well-studied example of a cytoskeletally associated Rab protein is Rab27a, which is functionally connected to the actin based motor protein MyosinVa of melanosomes. The first indication of this association came from patients with Griscelli syndrome. Griscelli syndrome is a rare autosomal recessive disorder that is characterised by defective pigmentation of the skin and hair due to an aberrant accumulation of melanosomes in melanocytes, caused by mutations in MyosinVa. A novel and interesting finding was that some Griscelli patients had mutations in Rab27a causing the same defect in pigmentation as the mutations in MyosinVa. This finding indicates that Rab27a is closely associated with the motor protein MyosinVa (Menasche et al., 2000). In retinal pigment epithelial cells, Rab27a forms a complex with myosin-VIIA via a protein called MyRIP (El-Amraoui et al., 2002). MyRIP was found to have a broad distribution at the tissue level and recent studies in pheochromocytoma (PC12) cells show that Rab27a and MyRIP bridge the secretory granules with F-actin and regulate the movement of vesicles within the actin cortex (Desnos et al., 2003). Rab6 in the Golgi interacts with another type of motor protein, the kinesin like protein Rabkinesin-6 (Echard et al., 1998), involved in retrograde vesicular traffic between the Golgi and the ER (White et al., 1999). In addition, Rab8, which has an important role in polarised trafficking, shows association with microtubular elements. Overexpression of Rab8 results in the reorganisation of both actin filaments and microtubules (Peränen et al., 1996).

Proteins that interact with the GTP bound form of Rabs, and mediate the downstream functional effects of Rabs, are called Rab effectors. In addition to the

function of Rab proteins, these effector proteins also show broad heterogeneity. Many of these proteins are docking/tethering factors, which together with Rab proteins, direct vesicles to the correct target membrane. The identification of Rab interacting proteins has revealed a vast complexity of machinery downstream of the early endosomal GTPase Rab5. Rab5 is a key factor in homotypic endosome-endosome fusion and it participates in clathrin dependent transport from the plasma membrane to early endosomes. More than 20 polypeptides from bovine brain cytosol that interact directly or indirectly with Rab5 have been found using an affinity–chromatography method and few of them have been characterised (Christoforidis et al., 1999a) (Table I).

Table I. Rab5 effectors

EFFECTOR	DESCRIPTION
APPL1 and 2	Cell proliferation regulators
EEA1	FYVE-finger protein, tethering factor
Rabaptin-5	Coiled coil protein with several interaction partners
Rabenosyn 5	FYVE-finger protein, tethering factor
Rabip4'	FYVE-finger protein, tethering factor
p85α-p110β	PI(3) kinase
p150-hVPS34	PI(3) kinase

Rabaptin-5 was the first identified Rab5 effector protein (Stenmark et al., 1995) that forms a complex with another protein called Rabex-5. Rabex-5 displays GDP/GTP exchange activity (Horiuchi et al., 1997). This complex also contains NSF, a factor responsible for SNARE complex disassembly (McBride et al., 1999). Rab5 is found to interact directly with two distinct PI(3)Ks, p85α-p110β and p150-hVPS34. p150-hVPS34 has a function in vesicle formation in the Golgi and p85α-p110β may be involved in the formation of clathrin coated vesicles at the plasma membrane (Christoforidis et al., 1999b). In addition to the earlier mentioned EEA1, Rabenosyn 5 is another FYVE finger effector of Rab5. It acts in a way similar to EEA1. Rabenosyn 5 is recruited in a Rab5 and PI(3)P dependent fashion to early endosomes where it works in the docking and fusion process (Nielsen et al., 2000). The latest interesting finding is that Rab5 binds also nuclear proteins APPL1 and APPL2. APPLs are essential factors for cell proliferation and are located in endosomal structures from which they are relocated to the nucleus upon stimulus. This finding indicates a direct link between the plasma membrane signalling, endosomes and nucleus. The trafficking through endosomes may have more a prominent role in nuclear signal transduction than previously thought (Miaczynska et al., 2004). Rab5 also interacts

with angiotensin II Type 1A receptor ($AT_{1A}R$) at the plasma membrane, and ligand activation of the receptor is suggested to affect Rab5 function and thereby the endocytosis process (Seachrist et al., 2002).

Several studies have reported direct molecular links between Rab effector proteins and the SNARE machinery or with the SM protein family. For example, EEA1 binds directly to the t-SNARE proteins Syntaxin 6 and 13 (McBride et al., 1999; Simonsen et al., 1999), and the EEA1-like protein in yeast, Vac1p, interacts with both the Rab GTPase Vps21p and the SM protein Vps45p (Tall et al., 1999). In mammalian cells, the pancreatic β-cell protein granuphilin interacts with both the GTP bound form of Rab3 and the SM protein Munc18a. Granuphilin is a member of the synaptotagmin-like (Slp) protein family and is associated with insulin-containing secretory granules. Granuphilin, like other Slp proteins, has C2-calcium-phospholipid binding domains with a Ca^{2+} sensor function. In addition, it shares structural similarities with other Rab effectors, such as a Zn^{2+}-finger motif involved in Rab binding (Coppola et al., 2002). More of these links and more effector proteins and their oligomeric complexes have to be found to understand in detail the function of Rab proteins in the regulation of membrane trafficking.

3.1.3 Endosomal Rab proteins

A strikingly high number of Rab GTPases have been localised to endosomal membrane compartments (Table II). This most probably reflects the complex organisation and multiple sorting functions of endosomes. Material internalised from the cell surface firstly enters early endosomes, which are responsible for the dissociation and sorting of receptors and ligands in the slightly acidic environment. Many receptors are recycled back to the cell surface. Molecules destined for degradation are sorted and move through the late endosomes to lysosomes. Late endosomes also receive cargo from the TGN network through the mannose-6-phosphate receptor (M6PR) pathway. The M6PR recognises lysosomal proteins in the TGN and transports these cargo proteins to late endosomes. At the low pH of late endosomes, the cargo dissociates from the M6PR and the receptors are transported back to the Golgi for further rounds of transport. All endosomal organelles are widely distributed in cells and they typically consist of vacuolar-shaped and tubular microdomains (reviewed by Deneka and van der Sluijs, 2002).

Table II. Endosomal Rab proteins in mammalian cells

NAME	LOCALISATION	ROLE	EFFECTORS
Rab4	Early/recycling endosomes	Sorting/recycling in early endosomes	Rababtin-5/5β Rabaptin-4
Rab5	Early endosomes, plasma membrane, clathrin-coated vesicles	Clathrin coated vesicle/early endosome fusion, homotypic fusion of early endosomes, ligand sequestration at plasma membrane	See Table I
Rab7	Late endosomes	Early-late endosome trafficking, lysosome biogenesis	RILP, RabRING7, p150-hVPS34
Rab9	Late endosomes	Late endosome-Golgi trafficking	TIP47
Rab11	Early/recycling endosomes	Recycling through perinuclear endosomes	Rab11BP
Rab15	Early endosomes	Early endosome-plasma membrane trafficking	N.D.
Rab18	Early endosomes	Apical endocytosis/recycling	N.D.
Rab20	Early endosomes	Apical endocytosis/recycling	N.D.
Rab22	Early endosomes	N.D.	N.D.
Rab23	Early endosomes/ plasma membrane	Negative regulator of sonic hedgehog signaling pathway	N.D.

N.D., Not determined

The previously mentioned Rab5 is the most thoroughly characterised early endosomal Rab protein (Novick and Zerial, 1997). In addition, Rab4 and Rab11, located on early and recycling endosomes, are also well-studied. These proteins are responsible for the endocytic recycling process. Rab4 is suggested to act at the level of early 'sorting' endosomes (van der Sluijs et al., 1992), whereas Rab11 exerts a function in trafficking of cargo through the perinuclear recycling endosomes (Ullrich et al., 1996). These three Rab proteins have distinct but partly overlapping distributions, forming domains at the surface of endosomes. The group of Zerial showed with fluorescently tagged Rab proteins and fluorescently labelled transferrin (as a cargo) how different Rab proteins label distinct domains on the same endosomes and how the cargo travels through these domains. Three major populations of domains were observed: one that contains only Rab5, a second containing Rab5 and Rab4, and a third one containing Rab4 and Rab11. The distinct nature of these domains is reflected by their differential sensitivity to pharmacological tools, such as brefeldin A (causes Golgi complex disruption) and wortmannin (PI(3)K inhibitor). Transferrin seems to enter the endosomal system via a Rab5 domain and returns to the surface via Rab4/Rab11 domains (Sonnichsen et al., 2000). It is known that some of the effector proteins can interact with two different Rab proteins. Rabaptin-5 is a bifunctional effector of Rab5 and Rab4 (Vitale et al., 1998) and the Rab5 effector Rabenosyn 5 also binds directly to Rab4 (Nielsen et al., 2000). Neither of these effector proteins

binds to Rab11. Rabip4 binds simultaneously to the GTP-form of both Rab4 and Rab5 (Fouraux et al., 2003). It has been suggested that the bifunctional effector proteins participate in communication between different Rab domains and serve as platforms for domain formation (reviewed by Deneka and van der Sluijs, 2002). Several other not so thoroughly characterised Rab proteins are located to early endosomes, like Rab15, Rab18, Rab20, Rab22 and Rab23 (Eggenschwiler et al., 2001; Evans et al., 2003; Lutcke et al., 1994; Olkkonen et al., 1993; Zuk and Elferink, 1999).

Late endosomes are characterised by their low lumenal pH (lower than in early endosomes) and extensive internal membranes rich in the unique phospholipid species lyso-*bis*-phosphatidic acid (LBPA). Rab9 is a late endosomal protein that regulates trafficking between late endosomes and the TGN (Lombardi et al., 1993). It has been shown by video microscopy that Rab9-positive vesicles move along microtubules and fuse with the TGN (Barbero et al., 2002). The Rab9 effector protein, Tail Interacting Protein of 47 kD (TIP47), mediates M6PR recycling from late endosomes to the Golgi. TIP47 binds directly to M6PR and Rab9 (Carroll et al., 2001). Also, Rab7 is localised to late endosomes but in domains separate from Rab9 domains (Barbero et al., 2002). Data has been presented for its function in trafficking from early to late endosomes (Feng et al., 1995) and/or in the biogenesis of lysosomes (Meresse et al., 1995). Rab7 overexpression causes several lysosomal defects while early endosomal compartments remain unchanged (Bucci et al., 2000). Rab7-Interacting Lysosomal Protein (RILP) is a Rab7 effector which recruits dynein-dynactin motor complexes to Rab7-containing late endosomes and lysosomes (Jordens et al., 2001). Another Rab7 effector is RabRING7, a cytosolic protein that is recruited to late endosomal and lysosomal membranes by the GTP bound form of Rab7 (Mizuno et al., 2003). Rab7 was also shown to bind to PI(3)kinase, p150-hVPS34, that links Rab7 to signaling pathways (Stein et al., 2003).

3.2 Sec1/Munc18 (SM) related protein family

3.2.1 Members of the SM protein family

Sec1/Munc-18 (SM) genes were first discovered from screens for membrane trafficking mutants in *Caenorhabditis elegans* and *Saccharomyces cerevisiae* (Aalto et al., 1992; Brenner, 1974; Novick and Schekman, 1979). There are four SM proteins

in yeast and seven in mammals (see Table III). They are all 60-70 kDa hydrophilic proteins devoid of transmembrane segments. SM related proteins are highly conserved among species and most of them share the common feature of binding t-SNARE proteins of the syntaxin family with high affinity (reviewed by Toonen and Verhage, 2003).

Table III. Main characteristics of the SM proteins in different organisms

SPECIES	SM PROTEIN	SYNTAXIN INTERACTIONS	SYNTAXIN BINDING MODE[a]	PROPOSED FUNCTION
S. cerevisiae	Sec1p	Sso1p, Sso2p	Core complex	Vesicular transport to plasma membrane
	Sly1p	Sed5p, Ufe1p	N-terminus	ER to Golgi transport
	Vps33p	Vam3p	Indirect	Endosome and vacuole membrane trafficking
	Vps45p	Tgl2p, Pep12p	N-terminus	Golgi to late endosome/vacuole transport
D. melanogaster	ROP	Syntaxin	N.D.[b]	Synaptic vesicle release and general secretion
	Sly1	N.D.	N.D.	N.D.
	Vps45	N.D.	N.D.	N.D.
	Vps33/carnation	N.D.	N.D.	Transport to lysosomes and pigment granules
C. elegans	UNC-18	Syntaxin (UNC-64)	Closed conform.	Synaptic vesicle release
	5 other genes in G.D.[c]	N.D.	N.D.	N.D.
Mammals	Munc18a	Syntaxin-1,-2,-3	Closed conform.	Synaptic vesicle release, chromaffin granule exocytosis
	Munc18b	Syntaxin-1,-2,-3	N.D.	Apical trafficking in epithelial cells, mast cell granule secretion
	Munc18c	Syntaxin-2,-4	N.D.	GLUT4 translocation, platelet granule exocytosis
	mVPS45	Syntaxin-4,-6,-13,-16	N-terminus	N.D.
	mVPS33A&B	N.D.	N.D.	N.D.
	mSly1	Syntaxin-5,-18	N.D.	ER to Golgi transport

[a] The binding modes are illustrated in Fig.7; closed conformation,A; core complex,B; N-terminal,C; Indirect interaction,D; [b] N.D., Not determined; [c] G.D., Genome database (www.wormbase.org)

SM related proteins are also highly specific in their activity. Proteins that act in the same pathway are able to rescue a null mutation in a different species but not in different pathways in the same species. For instance, murine Munc18a rescues the *C. elegans* Munc-18 null mutant phenotype, but Munc18c does not (Gengyo-Ando et al., 1996). X-ray crystallographic structural information on SM proteins is available from

three species: from a complex formed between the rat nervous system SM protein Munc18a and the cytosolic domain of syntaxin 1A (Misura et al., 2000) from the *S. cerevisiae* SM protein Sly1p complexed with a short N-terminal peptide of the yeast syntaxin Sed5p (Bracher and Weissenhorn, 2002), and from uncomplexed squid neuronal Sec1p (Bracher et al., 2000). All three structures (free or complexed) reveal a similar arch-shaped organisation composed of three domains 1, 2 and 3. Domain 3 in the mammalian Munc18a has been further divided into domains 3a and b. The syntaxin binding site is located between domains 1 and 3a (Misura et al., 2000) (Fig.6).

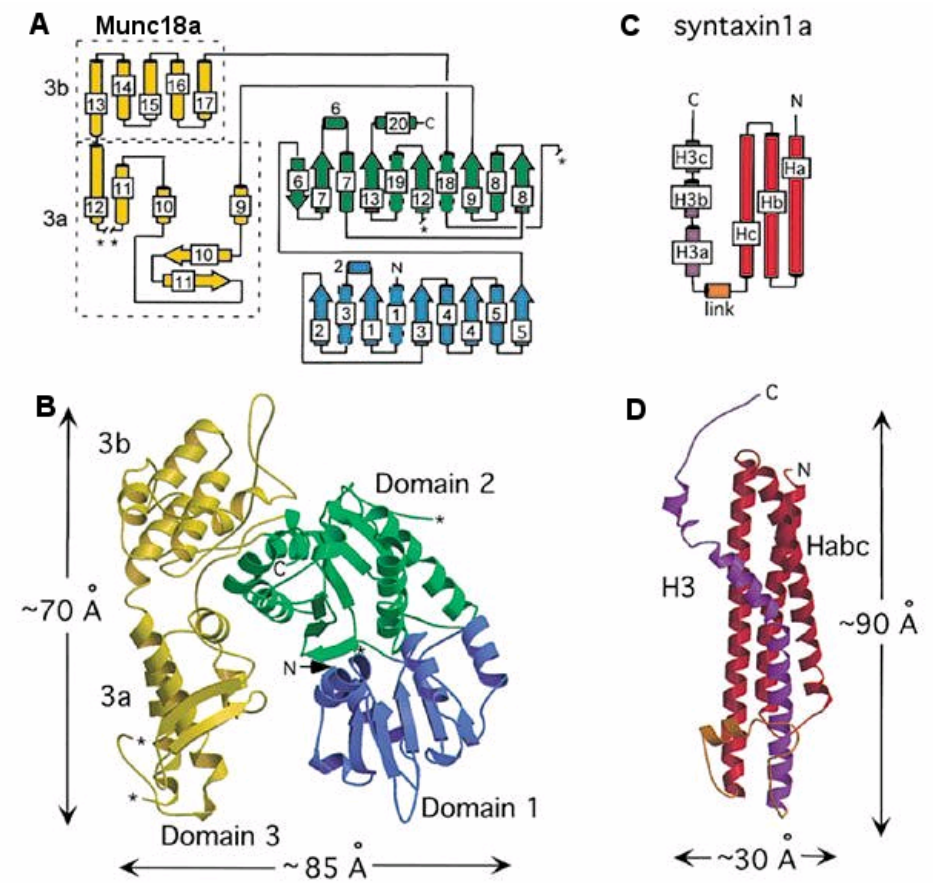

Figure 6. Structure of Munc18a and syntaxin 1a. **A.** A schematic presentation of Munc18a topology. Domain 1 is shown in blue, domain 2 in green and domain 3 in yellow. Helices are shown as cylinders and β-strands as arrows. Asterisks indicate breaks in the structure. Dashed lines denote domains 3a and 3b. **B.** A ribbon representation of Munc18a. Coloured as in A. **C.** Topology diagram of syntaxin 1a. The Habc domain is shown in red, the linker region in orange and the H3 region in purple. **D.** A ribbon representation of syntaxin 1a, with the same colour coding as in C. The conformations are as they appear in the complex, but the two proteins have been separated for clarity. Syntaxin 1a is in the complex located in the cleft between Munc18a domains 1 and 3a (reproduced with permission from Misura et al. 2000; http://www.nature.com/).

3.2.2 Different binding modes of SM proteins

Even though the structure of SM related proteins is conserved they bind syntaxins in very different ways. Four different binding modes can be distinguished (Fig.7).

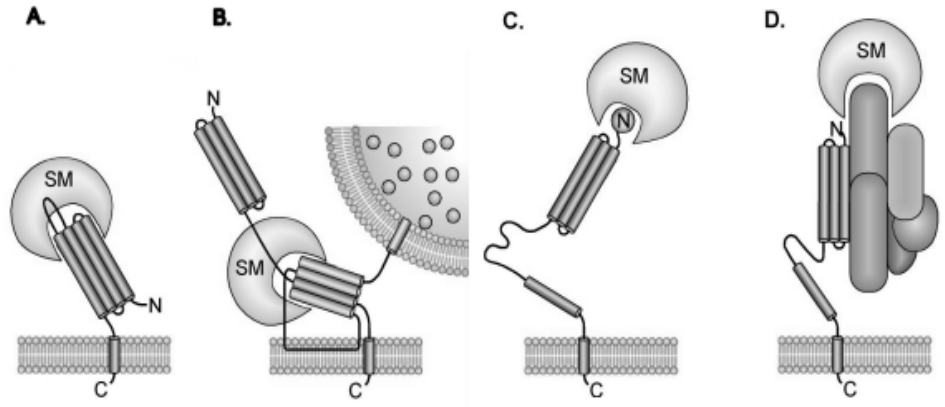

Figure 7. The different modes of interaction between SM proteins and syntaxins. **A.** Binding to a monomeric syntaxin in a closed conformation (Munc18a-syntaxin 1A complex). **B.** Binding to a SNARE complex without affinity for monomeric syntaxin (Interaction of the *S. cerevisiae* Sec1p with a complex of Ssop, Sncp, and Sec9p). **C.** Binding to an N-terminal peptide motif of syntaxin (Sly1p and Vps45p interactions with Sed5p and Tlg2p, respectively). This binding mode allows association with syntaxin, both in a monomeric form and within SNARE complexes. **D.** Indirect association with syntaxin mediated by protein complexes (Binding of Vps33p to Vam3p). Though not graphically depicted in the images, the syntaxin binding surface on SM proteins may vary for the different binding modes.

Most of the syntaxins can adopt two conformations,open and closed. In the closed conformation, the N-terminal domain (Habc domain) folds back onto the SNARE motif, preventing the syntaxin from forming complexes with its partner SNAREs. This structure is required for mammalian Munc18a binding (Dulubova et al., 1999; Misura et al., 2000). Contrary, yeast Sec1p binds only assembled SNARE complexes (Carr et al., 1999). The third binding mode is independent of the syntaxin conformation. The SM proteins involved in Golgi membrane trafficking (both in yeast and mammals), Sly1p/mSly1 and Vps45p/mVPS45, bind a very N-terminal motif of their respective syntaxins. This motif is not present in all syntaxins (Dulubova et al., 2002; Yamaguchi et al., 2002). The fourth binding mode involves binding via protein complexes. A yeast SM protein Vps33p is part of a complex, called the HOPS complex, that functions in Golgi-to-vacuole transport and interacts with the syntaxin

homologue Vam3p (Seals et al., 2000). Similar complexes are also observed in *Drosophila* (Sevrioukov et al., 1999).

3.2.3 Functions of SM proteins

The original idea was that SM proteins act as negative regulators of SNARE complex formation (Pevsner et al., 1994; Schulze et al., 1994). Most of the overexpression studies indicate that SM proteins inhibit vesicle transport at some step before fusion. For example, mammalian Munc18c prevents GLUT4 vesicle fusion in adipocytes (Thurmond et al., 1998) and in the Munc18c transgenic mouse model insulin secretion is inhibited (Spurlin et al., 2003). In *Drosophila*, overexpression of ROP inhibits neuro transmission in a dose-dependent fashion (Schulze et al., 1994; Wu et al., 1998) and microinjection of the squid SM protein s-Sec1 into giant squid axons was reported to inhibit evoked neurotransmitter release (Dresbach et al., 1998). In addition, a number of peptide overexpression studies support the idea of an inhibitory role of SM proteins. Expression of Munc18b, its domain 3, or a specific 'effector loop' included in domain 3 were reported to inhibit the IgE-triggered exocytosis of mast cell granules (Martin-Verdeaux et al., 2003) In adipocytes, overexpression of Munc18c or microinjection of a Munc18c 'effector loop' peptide inhibited GLUT4 translocation to the plasma membrane (Tamori et al., 1998; Thurmond et al., 1998; Thurmond et al., 2000). Moreover, corresponding peptides derived from either Munc18a or Munc18c were recently found to inhibit granule exocytosis in permeabilised human platelets (Schraw et al., 2003).

There are some exceptions among the results showing that the role of SM proteins is inhibitory. In cell lines derived from adrenal chromaffin cells, overexpressed Munc18a has no effect on secretion (Graham et al., 1997), and overexpression of Munc18a in primary chromaffin cells and motor neurons enhances fusion of vesicles (Voets et al., 2001). Also in platelets Munc18c peptides corresponding to a syntaxin 4 binding region were found to enhance Ca^{2+}- dependent granule exocytosis (Houng et al., 2003). The discrepancy from overexpression data can be explained by the specialisation of each of the Munc18 proteins to distinct trafficking steps in mammalian cells and the fact that the primary target of the peptides used by Houng et al. 2003 was different than in previous peptide studies. All of the effects caused by SM proteins are consistent with the idea that the ratio between

SM proteins and their cognate syntaxins is crucial for normal membrane trafficking and that an excess amount of SM proteins can lead to inhibition of a specific transport step. Further evidence that the dose of SM proteins is crucial for syntaxin function is that the inhibitory effect of ROP overexpression in *Drosophila* is overcome by simultaneous expression of syntaxin (Wu et al., 1998), and similarly, co-injection of an N-terminal cytosolic fragment of syntaxin into squid giant axons, together with s-Sec1, abolishes the inhibitory effect of s-Sec1 (Dresbach et al., 1998). In addition, syntaxin1A levels are reduced in null-mutant mice (Toonen and Verhage, 2003), and Munc18c protein levels are significantly lower in mice heterozygous for a null allele of the cognate t-SNARE syntaxin 4 (Yang et al., 2001).

SM gene disruption causes severe trafficking defects. In *S. cerevisiae,* four SM related proteins all work in different trafficking steps: between Golgi and plasma membrane (Sec1p), ER and Golgi (Sly1p), Golgi and the prevacuolar compartment (Vps45p) or the prevacuolar compartment and vacuoles (Vps33p). Disruption of these genes causes an accumulation of transport vesicles in the respective trafficking steps that they regulate (Cowles et al., 1994; Ossig et al., 1991; Robinson et al., 1988; Wada et al., 1990). Munc18a null-mutant mice are alive until birth. The gene deletion allows for an apparently normal brain assembly and the synapses develop normally yet the brains are synaptically silent. After assembly is completed, neurons undergo apoptosis even though the normal Munc18b and c genes are present (Verhage et al., 2000). This observation is quite remarkable considering the fact that Munc18a and Munc18b show the same syntaxin specificity.

Several studies suggest that SM proteins are involved in the final steps before fusion yet the biochemical approaches taken thus far are insufficient to understand the exact mechanism. Their characteristic binding to syntaxins indicates a role in SNARE complex modulation, or possibly in the regulation of syntaxin levels. However, high affinity to syntaxins is not necessarily the only or main functionally relevant feature of SM proteins. (reviewed by Toonen and Verhage, 2003). As shown by the effector loop peptide studies, there are other important regulatory elements in SM proteins. One key regulatory mechanism is phosphorylation. PKC stimulates Ca^{2+}-dependent exocytosis in various secretory cell types and it phosphorylates Munc18a in a cell free system. This phosphorylation inhibits Munc18a from interacting with syntaxin 1A, yet PKC does not phosphorylate the assembled Munc18a-syntaxin complex (Fujita et al., 1996) nor does it cause disassembly of the complex. Munc18a is also

phosphorylated by Cdk5. Unlike PKC, Cdk5 seems to be able to phosphorylate Munc18a in a preformed Munc18b-syntaxin heterodimer, resulting in disassembly of the complex (Fletcher et al., 1999).

3.2.4 Interactions of the SM protein family with other components of the vesicle transport machinery

Syntaxins are not the only binding partners of SM proteins. Mammalian Munc18a also interacts with two other families of membrane bound proteins, Doc2 and Munc18 interacting proteins (the Mint proteins). There are three members of the Mint family. Mint1 and -2 are brain specific whereas Mint3 is ubiquitously expressed. Mint proteins have phosphotyrosine binding domains (PTB) at their C-terminal ends that bind specifically phosphatidylinositol phosphates (PIPs). Munc18a binds the N-terminal part of Mint1 and -2, however, as the N-terminal part is missing from Mint3 this protein is unable to bind Munc18a (or Munc18b) (Okamoto and Südhof, 1998). Interestingly, the Mint proteins were reported to regulate trafficking of the Alzheimer disease related amyloid precursor protein, β-APP (Hill et al., 2003).

Syntaxins and Doc2 proteins compete for Munc18a binding. The Doc2 family has two members, the neuronal Doc2A and the ubiquitously expressed Doc2B. They are enriched on synaptic vesicles and contain a double C2 domain capable of binding Ca^{2+} and phospholipids. Doc2 proteins have a role in Ca^{2+}-evoked neurotransmitter release and the entire amino acid sequence of Munc18a is needed for the interaction with Doc2 family members (Verhage et al., 1997).

The pancreatic β-cell protein granuphilin interacts with the GTP bound form of both Rab3 and Munc18a. Granuphilin is a member of the synaptotagmin-like (Slp) protein family and is associated with insulin-containing secretory granules. Granuphilin, like other Slp proteins, has C2-calcium-phospholipid binding domains with a Ca^{2+} sensor function. In addition, it shares structural similarities with other Rab effectors, such as a Zn^{2+}-finger motif involved in Rab binding (Coppola et al., 2002). There is also evidence that SM proteins interact with the cytoskeleton. Munc18b shows a microtubule-dependent localisation and is redistributed after disruption of the microtubule network in mast cells. Together with the microtubule network, Munc18b

seems to have an important role in the regulation of secretory granule exocytosis in this cell type (Martin-Verdeaux et al., 2003).

S. cerevisiae genetic studies of Sec1p have revealed several indirect interaction partners. Overexpression of *SEC1* suppresses defects in most of the exocyst mutants (Aalto et al., 1993; Brummer et al., 2001). A physical interaction has only been documented between Sec1p and a small hydrophilic protein Mso1p. The loss of *MSO1* causes an accumulation of secretory vesicles in the yeast bud (Aalto et al., 1997). So far no mammalian homologues for Mso1 have been characterised. Yeast genetic studies have often led to the identification of SM protein interactions with tethering complexes. Also, in this case loss of *MSO1* results in synthetic interactions between most of the genes that encode components of the exocyst complex regulated by the Rab GTPase Sec4p (Guo et al., 1999; TerBush et al., 1996). No direct interaction partners have been identified for Sly1p (a Sec1 homologue involved in ER to Golgi transport) except from genetic studies where interactions with tethering complexes and a Rab protein, Ypt1p have been shown (Dascher et al., 1991; Sapperstein et al., 1996). The *S.cerevisiae* Vps33p is a component of a hetero-oligomeric protein complex containing the class-C Vps proteins. Mutations in class-C *VPS* genes result in severe defects of vacuolar protein sorting and morphology. The hetero-oligomeric complex consists of four proteins, Vps11p, Vps16p, Vps18p and Vps33p that interact genetically and physically (Rieder and Emr, 1997), and associate with the vacuolar syntaxin homologue Vam3p (Sato et al., 2000). Vsp45p associates directly with Vac1p, which is an EEA1-like zinc-binding FYVE finger protein and regulates trafficking between the Golgi and endosomes. Vac1p also associates with the Rab GTPase Vsp21p, thus forming a physical connection between a Rab and an SM protein (Peterson et al., 1999; Tall et al., 1999).

An SM protein in *Caenorhabditis elegans*, UNC-18, interacts directly with the priming factor UNC-13 *in vitro*. UNC-13 also binds to the N-terminal part of syntaxin (only the open conformation of syntaxin) and can displace UNC-18 from the syntaxin complex (Sassa et al., 1999). However, an interaction between the mammalian homologues of these proteins, Munc18 and Munc13, has not been shown. Munc13-1 in mammalian cells associates with Doc2A and also with syntaxin (Mochida et al., 1998).

3.3 Other regulatory proteins

The neurotransmitter exocytosis in neurons requires a highly regulated membrane fusion event. A unique feature of synaptic transmission is the tight coupling between the increase in intracellular Ca^{2+}, produced by the arrival of a nerve impulse, and the activation of the exocytotic synaptic fusion machinery. Studies on the synaptic fusion machinery have led to the identification of a number of SNARE complex regulators such as synaptotagmin, GATE-16, LMA1, synaptophysin, tomosyn, amisyn, Vsm1 and complexins. Several of these are also expressed in non-neuronal tissues. These regulators bind directly to the SNARE proteins and regulate SNARE complex assembly. (reviewed by Gerst, 2003). Synaptotagmins are suggested to be calcium sensors that modulate secretion. The synaptotagmin protein family is a large family found exclusively in higher eukaryotes. They localise on membranes and include two Ca^{2+} binding C2 domains. Synaptotagmins bind directly to SNAREs in a Ca^{2+} dependent manner (Earles et al., 2001). Synaptotagmin-like proteins are also expressed in non-neuronal tissues. The earlier mentioned granuphilin is a synaptotagmin-like protein that is expressed in β-cells and the pituitary gland where it controls exocytosis. Interestingly, granuphilin binds both Munc18a and Rab3 but does not bind SNARE proteins (Coppola et al., 2002).

Tomosyn is a regulator of SNARE complexes. It is abundant in the brain where it forms a complex with Syntaxin 1A and SNAP-25 and is capable of dissociating Munc18a from the complex with syntaxin 1A (Fujita et al., 1998). A recent study from adipocytes showed that Munc18c binds the Syntaxin4/tomosyn complex, suggesting that tomosyn and Munc18c operate at a similar stage during the Syntaxin 4 SNARE complex assembly cycle, perhaps by a different mechanism than Munc18a and tomosyn in neurons (Widberg et al., 2003).

If SM proteins are regulating the SNARE complex through their ability to bind to the t-SNARE proteins, syntaxins, one could expect that there are specific v-SNARE regulators as well. Indeed, synaptophysins are shown to interact with v-SNARE proteins (VAMP-like proteins) and prevent their participation in SNARE complex formation (Calakos and Scheller, 1994).

4. Vesicle trafficking in polarised cells

Cell polarity is a reflection of complex mechanisms that establish and maintain functionally specialised domains of different lipid and protein compositions in the plasma membrane and cytoplasm. Polarity is fundamental to the development and functioning of all organisms from bacteria to humans and has been studied in the context of cell differentiation, signalling, cytoskeletal organisation and cell division.

Polarisation of epithelial cells is a complex process directed by cell-cell and cell-extracellular matrix interactions. Epithelial cells form a tight layer whereby the outside edges of the cells cover all free open surfaces of the body, including the skin and mucous membranes that communicate externally of the body. The plasma membrane of an epithelial cell is divided into apical and basolateral domains and three major trafficking pathways help maintain the polarity. Firstly, newly synthesised proteins are transported through the Golgi complex to the TGN where they are sorted to apical or basolateral surfaces. Some proteins are transported to endosomes and only then to the cell surface. Secondly, some proteins are selectively retained at the cell surface and thirdly, components that are not retained at the surface are rapidly endocytosed to endosomes. From endosomes, proteins can be recycled back to the cell surface, delivered to late endosomes or transported across the cell to the opposite surface. This last process is called transcytosis (reviewed by Mostov et al., 2003). Examples of cells in which polarised trafficking has been studied include those from intestine (Caco-2 cells, human) and kidney (MDCK, canine and LLC-PK1, porcine), pancreatic acinar cells, and liver hepatocytes. In neurons, the cell surface is divided into axonal and somatodendritic domains which correspond in some aspects to the apical and basolateral membrane domains of epithelial cells (reviewed by Nelson and Yeaman, 2001). Defects in trafficking pathways that maintain epithelial polarity can cause disease in organs in which epithelial cell polarity is crucial, for example the liver, kidney and intestine (Stein et al., 2002). Finally yeast cells display polarity during budding and mating (reviewed by Chant, 1994).

II AIMS OF THE STUDY

Vesicular transport is one of the most intensively studied research areas in cell biology. By identifying the molecular mechanisms behind the transport reseachers will hopefully uncover the mystery of how cells sort and transport huge amounts of protein and lipid to correct destinations. Hundreds of proteins constitute the vesicle transport machinery, and to elucidate the function of one single protein or to find a new binding partner, will provide us with the important pieces of the puzzle. One of the primary machineries required for membrane trafficking is the SNARE complex. SNARE proteins are located on various cell organelles and mediate vesicle fusion to the target membrane. There are two main regulatory families for SNARE proteins: the Rab protein family and the Sec1/Munc18 (SM) protein family. The Rab protein family, which consists of more than 60 members in humans, regulates membrane trafficking through GTPase activity, and each of these proteins is specialised for one trafficking step. The SM family members are more general modulators of SNARE complex assembly yet their exact function remains unsolved. In the present study the aims below were set to identify the function of a small GTPase, Rab22a, and a member of the SM family, Munc18b.

The specific aims of the study were:

I To characterise the basic properties of Rab22a, in particular, the sub-cellular localisation of Rab22a and its role in endocytic events.

II To gain knowledge on the function of Munc18b by site-directed mutagenesis.

III METHODS

1. List of published methods

All of the published methods used in this thesis are summarised in Table IV below. Further details concerning these methods can be found in the original publications.

Table IV. Published methods used in this thesis

METHOD	ORIGINAL PUBLICATION
General DNA techniques	I, II, III
Cell culture	I, II, III
SDS-PAGE and Western blotting	I, II, III
Immunofluorescence microscopy	I, III
Generation of recombinant SFVs and viral infection	I, II, III
Yeast two-hybrid analysis	I
Protein expression in *E. coli*	I
In vitro assay for Munc18b-syntaxin binding	I, II, III
Analysis of transferrin endocytosis	I
Analysis of EGF uptake and degradation	I
TRITC-dextran uptake	I
Analysis of intracellular trafficking of AGA	I
Site directed mutagenesis	II, III
In vitro translation	II, III
Phosphorimager quantitation	II
Metabolic labelling of proteins	II, III
Immunoprecipitation	II, III
Surface immunoprecipitation	II
Cell fractionation by flotation sucrose gradients	II
Protein expression in insect cells	II
Protein purification using Ni-NTA-agarose	II
Transfection of mammalian cells	I, III
HA trafficking assay by confocal microscopy	III
Generation of a homology model for the Munc18b/syntaxin3 complex	III

2. Unpublished methods

2.1 Transferrin endocytosis and recycling in COS-1 cells

African green monkey kidney (COS-1) cells were plated into 1 cm diameter wells and transfected with Rab22a pcDNA3.1 constructs (wt, mutants or empty vector as a control), together with a human transferrin receptor (tfR) cDNA for 24 h. FUGENE 6 (Roche) transfection reagent was used according to the manufacturer's instructions.

The cells were washed twice with phosphate-buffered saline (PBS) and incubated at +37°C for 1 h in Dulbecco's modified Eagle medium containing 0.1% BSA (DMEMB). The cells were transferred onto ice and 2 µg/ml biotin-holo-transferrin (Sigma) in DMEM was allowed to bind for 1 h. Following this, unbound biotin-transferrin was removed by extensive washing with ice cold DMEMB (four washes), and finally with PBS. The biotin-transferrin was endocytosed at +37°C in DMEMB and the cells were collected at various time points. After each time point, non-endocytosed biotin-holo-tranferrin was washed away two times with ice-cold DMEMB + 200 µg/ml holo-transferrin (Sigma) (shaking on ice) and just before lysis, shortly with ice cold PBS. The cells were lysed in ST-buffer (1 mM EDTA, 50 mM NaCl, 0.1% SDS, 1% Triton X-100, 10 mM Tris-HCl pH 7.5) and the biotin-holo-transferrin was measured from lysates with a solid phase assay. Immuno maxisorb 96-well plates were coated overnight with 2 µg/ml anti-transferrin (Sigma) antibody in 50 mM $NaHCO_3$, pH 9.6, and blocked with 0.2% BSA/ ST buffer for 4 h at room temperature. The cell lysates were incubated on the 96-well plates overnight followed by two washes with PBS. The plate was blocked with 0.2% BSA/ ST-buffer for 5 min at room temperature and washed again two times with PBS. Streptavidin-HRP in 0.2% BSA/ST-buffer (1:5000, Amersham) was added to the wells, incubated for 1 h at room temperature and then washed away with PBS. As a substrate, 0.5 mg/ml 1,2-phenylendiamin (Merck) in 50 mM Na_2HPO_4, 30 mM citrate, pH 5 was used. Fresh substrate was prepared each time and 0.03% (final concentration) H_2O_2 was added to the substrate solution before adding to the wells. The reaction was stopped with 2N H_2SO_4 (¼ of the reaction volume) and the absorbance (A_{490}) was measured.
The recycling assay was carried out in a similar fashion. For this purpose, early endosomes were loaded with biotin-holo-transferrin at 17°C for 2 h.

2.2 Generation of Adenoviruses

Munc18b Adenoviruses were generated according to the Q-BIOgene AdenoVator System instructions. Munc18b wt, S48D, K314L/R315L and E59K cDNAs, with an N-terminal *myc*-tag, were inserted into the BglII site of pAdenoVator-CMV5-IRES-GFP transfer vector. To create recombinant AdenoVator plasmids, the transfer vector constructs were linearised with PmeI and co-transformed with pAdEasy-1 (an adenoviral genome vector) into the competent *E. coli* cell line, BJ5183. Homologous

recombination of the vectors resulted in recombinant plasmids and the correctly recombined clones were confirmed using a restriction digestion with PacI. The recombinant AdenoVator plasmids were transfected into the 293A packaging cell line (human embryonic kidney cell line) and after one week cells were collected, resuspended in PBS and lysed using a freeze-thaw method. Cellular supernatants containing viral particles were used for the first infection of 293A cells. After five days, the cells were collected and lysed in a similar fashion as described previously and the supernatant was used for a second infection. The cells were infected three times to enrich the viral supernatant. Viral particles were then concentrated from a cesium gradient according to the manufacturer's instructions.

2.3 Amylase secretion in pancreatic acinar cells

Pancreatic acinar cells were isolated from male Spraque-Dawley rats according to Gaisano et al., 1989. Dispersed single acinar cells were plated on 12-well plates and infected with Munc18b adenoviruses for 12 h. The infected cells were stimulated for 30 min with 100 pM OPE, a phenylethylester analogue of cholecystokinin (CCK), for secretion. The secreted amylase was measured from the medium using a colorometric assay (Huang et al., 2001). In this assay, α-amylase catalyses the hydrolysis of internal α-1,4-glycan links of polysaccharides containing 3 or more α-1,4-linked D-glucose units, yielding a mixture of maltose and glucose. One unit of enzyme liberates from starch (used as a substrate in this assay) 1 µmol of reducing groups (calculated as maltose) per minute at 25°C in pH 6.9.

IV RESULTS AND DISCUSSION

1. Characterisation of the small GTPase Rab22a

Rab22a cDNA, cloned from the canine epithelial cell line (MCDK II), was previously employed for mRNA expression studies. It was shown that Rab22a mRNA is expressed ubiquitously in mammalian tissues and that the protein localises to endosomal elements in baby hamster kidney (BHK-21) cells (Olkkonen et al., 1993).
To study the localisation and function of Rab22a further, the wild-type (wt) protein and two mutant forms of Rab22a were cloned into mammalian expression vectors pcDNA3.1 and pGEM-1, both of which are compatible with the Vaccinia T7 expression system. For Vaccinia expression, the cells were firstly infected with modified Ankara T7 polymerase recombinant Vaccinia virus, followed by transfection with pGEM-1 constructs (Sutter et al., 1995). Experiments were also carried out using recombinant Semliki Forest viruses (SFV). In the SFV system, the gene of interest is cloned into the viral vector under an SFV 26S promoter and viral particles are produced in BHK-21 cells (Liljestrom and Garoff, 1991). The mutant proteins with defects in the GTPase cycle of Rab22a were created according to the literature (Olkkonen and Stenmark, 1997). Rab22a S19N is equivalent to dominant inhibitory-type mutants of other Rab-proteins and it is expected to show a lower affinity for GTP than for GDP. The Rab22a Q64L mutant represents an activated GTPase deficient mutant.

1.1 Localisation of Rab22a

Localisation of overexpressed Rab22a protein was studied by immunofluorescense microscopy. Rab22a was stained together with marker proteins. Wt Rab22a formed large vesicular-like structures of various sizes. These structures co-localised with the early endosomal marker EEA1 (I, Fig.1 A,B,C), but not with the late endosomal/lysosomal markers lysosomal-associated membrane protein (LAMP-1) (I, Fig.1 D,E,F), and lyso-*bis*-phosphatidic acid (LBPA). In addition, Rab11, a marker of perinuclear recyling endosomes, did not overlap with Rab22a wt staining. The active mutant Q64L formed similar enlarged EEA1 positive endosomal structures to the wt

protein (I, Fig.1 J-L). In cells overexpressing wt or the Q64L mutant form of Rab22a, EEA1-positive early endosomes of normal size were practically absent and most of the EEA1 was collected to large vesicular structures. This phenomenon was similar to that observed with the Rab5a GTPase deficient mutant Q79L. Rab5a is another early endosomal Rab protein that regulates transport between the plasma membrane and early endosomes as well as regulating homotypic early endosomal fusion (Gorvel et al., 1991; Stenmark et al., 1994). When Rab5 Q79L and Rab22a wt were expressed in the same cells they co-localised to the same structures (data not shown). However, Rab5a wt is unable to form enlarged early endosomes as efficiently as wt Rab22, indicating that Rab22a may be a stronger stimulator of early endosomal fusion than Rab5a. The dominant inhibitory mutant of Rab22a, S19N, appeared mainly cytosolic, often with additional bright staining in the perinuclear region. The size and distribution of EEA1 positive early endosomes was normal in S19N overexpressing cells (I, Fig.1 M-O).

1.2 Interaction of Rab22a with EEA1

1.2.1 Yeast two-hybrid assay

Rab5a shows the highest sequence similarity to Rab22 and both proteins share similarities in their cellular localisation and behaviour as well. Rab5 is known to interact directly with EEA1, which is one of the effector proteins of the GTPase. However, EEA1 does not interact with the other endosomal Rab proteins, Rab4 and Rab11. Rab5 interaction occurs at both ends of the long EEA1 molecule (Simonsen et al., 1998). We used the yeast two-hybrid assay to establish whether Rab22a associates with EEA1. To facilitate nuclear transport in yeast, we used Rab22a cDNA (wt and mutants) without the C-terminal double cysteine motif that mediates membrane association. Three EEA1 fragments were used, full length, the N-terminal part (amino acid residues 1-209) and the C-terminal part (amino acid recidues 1275-1411). The N- and C-terminal fragments were both known to contain the Zn^{2+}-binding motif as well as the binding motif for Rab5. Rab22a cDNAs were used as bait and EEA1 cDNA constructs as prey. The active mutant of Rab22a, Q64L, was the only form showing binding affinity to full-length EEA1. The N-terminal part of EEA1 interacted with Q64L whereas the C-terminal part of EEA1 showed no binding at all. Interestingly, wt

Rab22a did not show affinity to any of the EEA1 forms. One possible explanation could be that yeast cells activate GTP hydrolysis by Rab22a more efficiently than mammalian cells. The dominant inhibitory mutant S19N did not show any binding to EEA1. These results indicate only an interaction between the GTP-bound form of Rab22a and EEA1. The binding site is only at the N-terminal region of EEA1, which is in contrast to the binding of Rab5, which occurs via motifs at both ends of the long protein (Fig.8).

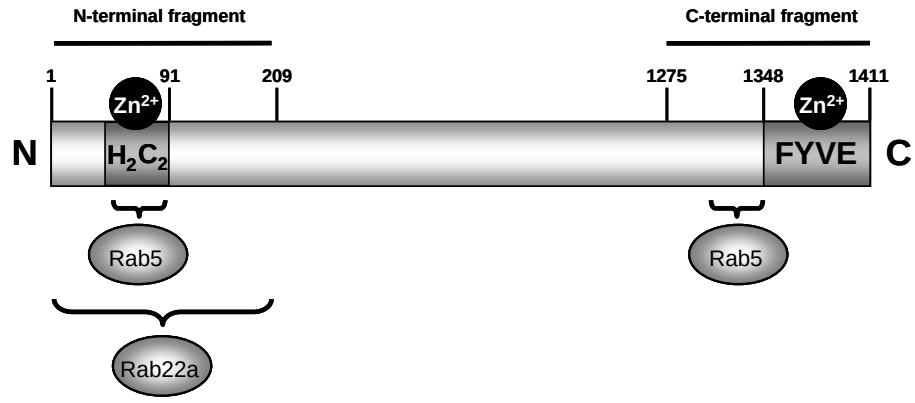

Figure 8. A model of the EEA1 monomer. EEA1 has two zinc binding motifs, one at each end of the protein (H_2C_2 and FYVE finger domain). N-terminal and C-terminal fragments that were used in this study are shown above. Both fragments include a binding site for Rab5.

1.2.2 In vitro binding

An *in vitro* pull-down assay was used to confirm the yeast two-hybrid data. Glutathione-S-transferase (GST)-tagged fusion proteins of Rab22a and Rab5a were expressed in *E. coli* and then coupled to glutathione sepharose beads. The beads were loaded with either GDP or the non-hydrolyzable GTP analogue, GTPγS. The pull-down assay was carried out with the same N-terminal EEA1 fragment that was used in the two-hybrid assay and the results were analysed by Western blotting. Both of the Rab proteins showed a similar binding pattern; GTPγS loaded proteins bound strongly to the N-terminal form of EEA1, whereas the GDP loaded proteins gave only a weak signal (I, Fig.2). However, to obtain the same signal, 5-10 times less Rab22a was required than Rab5, indicating stronger binding by Rab22a.

1.2.3 Phosphatidylinositol-3-phosphate kinase inhibition

To gain further evidence of the interaction between Rab22a and EEA1 *in vivo*, we used the phosphatidylinositol-3-kinase (PI(3)K) inhibitor Wortmannin. EEA1 binds the PI(3)K product, phosphatidylinositol-3-phosphate (PI3P), via a C-terminal Zn^{2+}-binding domain called FYVE-finger. Kinase inhibition by Wortmannin causes detachment of EEA1 from early endosomal membranes. This effect is overcome by overexpressing Rab5 (Simonsen et al., 1998). Rab22a wt was expressed in BHK-21 cells that were treated with Wortmannin. The cells were then fixed and stained with EEA1 antibody. Following Wortmannin treatment, the characteristic small spot-like EEA1 staining was extremely weak in the control cells due to detachment of EEA (I, Fig.3 B,D). However, in Rab22a overexpressing cells, staining of early endosomal membranes remained strong, thus indicating that Rab22a was able to maintain EEA1 on the large early endosomal structures (I, Fig.3 A,C).

The results from the yeast two-hybrid assay, the pull-down assay and the Wortmannin treatment showed that Rab22a binds most likely only to the N-terminal part of EEA1, like Rab5. No binding was observed at the C-terminal part of EEA1 where another Rab5 binding site is located. This indicates that the function of Rab22a on early endosomal membranes is essentially distinct from that of Rab5a. Rab22a is incapable of forming symmetric bridges (via EEA1) between early endosomes the way Rab5a does. *In vitro*, Rab22a showed stronger affinity to EEA1 than Rab5a but it is not known whether Rab22a can compete for EEA1 binding with Rab5a, or whether the N-terminal binding site is the same for these two Rab proteins. It is likely that Rab22a participates in an early endosomal sorting process, together with other early endosomal Rab proteins. Rab effectors that bind the GTP bound form of Rab proteins are suggested to serve as a platform for multiple endosomal proteins and to connect different Rab domains on early endosomal membranes. Rab5 and Rab22a share the same effector protein, EEA1. Rab5 also shares the effector proteins Rabenosyn-5 and Rabip4 with Rab4 (de Renzis et al., 2002; Fouraux et al., 2003). Other effectors of Rab22a are not known but Rab22a may share more effectors with other Rab proteins. All these combinations reflect the complex sorting function of early endosomes.

1.3 Effects of Rab22a overexpression on endocytic functions

1.3.1 Transferrin endocytosis

Rab5a wt and its GTPase deficient mutant, Q67L, enhance transferrin (Tfn) endocytosis and at the same time the Q67L mutant inhibits Tfn recycling back to the plasma membrane (Bucci et al., 2000; Stenmark et al., 1994). We studied the effects of Rab22a on this Tfn pathway. HeLa cells were chosen for their abundant Tfn receptor expression. Rab22a or its mutant forms were expressed by using Vaccinia T7 infection followed by Rab22 cDNA transfection. Alexa labelled Tfn was internalised into the cells and visualised by confocal microscopy. In HeLa cells, Rab22a only rarely causes abnormal vacuole-like endosomes. In this cell line, early endosomal staining remained more evenly distributed as fine vesicular structures. In the cells expressing wt Rab22a there was no change in the amount or distribution of internalised Tfn after 30 min as compared to control cells (I, Fig.4 C-E). The Alexa-Tfn staining pattern was also similar in cells expressing a dominant inhibitory mutant, S19N (I, Fig.4 I-K). Overexpression of the GTPase deficient mutant Q64L did not affect the size of Tfn-positive endosomes, however, the distribution was changed. Instead of a relatively even cytoplasmic distribution, the Tfn-positive endosomes were clustered near the cell surface at the leading edges (I, Fig.4 F-H). However, the kinetics of Tfn endocytosis or recycling was not impaired. This was studied in COS-1 cells transfected with Rab22a wt or mutant cDNAs and with biotinylated Tfn. As compared to untransfected or mock transfected cells, the cells expressing Rab22 wt, S19N or Q64L, showed no significant difference in the kinetics of either Tfn uptake or recycling (Fig.9).

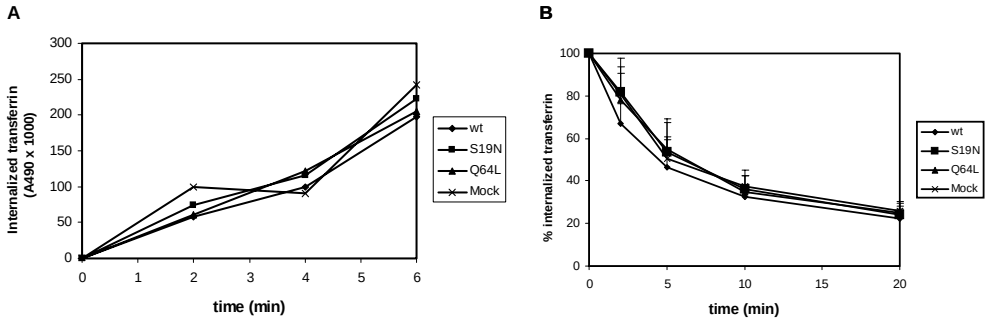

Figure 9. The effect of Rab22a overexpression on the transferrin cycle. **A**. Transferrin uptake. COS-1 cells were transfected for 24 h with Rab22a wt or mutant cDNAs in pcDNA3.1, together with a human transferrin receptor (TfR) cDNA. Biotin-transferrin was bound on cell surfaces at 0^{o}C, and the uptake was measured as described in Materials and Methods. The 0-time point background of cell surface-bound transferrin remaining on the mock-transfected cells after the holo-transferrin wash has been subtracted. The values are taken as an average of two independent experiments performed in triplicate. **B.** Transferrin recycling. COS-1 cells were transfected as above and incubated with biotin-transferrin for 2 h at 17^{o}C to accumulate the tracer in endosomes. Recycling at 37^{o}C was assessed as depicted in Materials and Methods. The symbols are the same as in A. For each curve, the value at 0-time point, the starting point after transferrin loading, has been set at 1. Mean values +/- SEM from three independent experiments, performed in duplicate, are shown.

1.3.2 Uptake of the fluid phase marker TRITC-dextran

Early endosomes also take up fluid phase cargo molecules from the plasma membrane, however, this pathway is not receptor mediated like the Tfn pathway. To test fluid phase uptake in Rab22 overexpressing cells we used fluorescently labelled (TRITC) dextran as a fluid phase marker. The transfected BHK cells were allowed to endocytose dextran for 1 h, followed by a 3 h chase without dextran. After a 1 h internalisation period, dextran remained partly in EEA1 containing early endosomal structures in the mock transfected cells. During the 3 h chase dextran reached late endosomal structures that were positive for LAMP (I, Fig.7 A-E). A majority of the cells expressing wt Rab22a or the S19N mutant form transported dextran similarly to mock-transfected cells. Even though the EEA1-containing early endosomes were enlarged in wt Rab22 transfected cells, Dextran reached late endosomal structures during the incubation (I, Fig.7 F-J, P-T.) In these cells, dextran was not seen in EEA1 positive early endosomal structures after 1 h internalisation, same was seen in control cells. Therefore, Dextran seemed to reach late endosomes directly without passing through enlarged Rab22 positive early endosomes. In the cells expressing the active mutant Q64L the situation was different. Dextran was found inside large vacuolar

structures that were positive for EEA1 and in some cases also for LAMP1. Accumulation of dextran was already observed after 1 h internalisation but not in every enlarged endosome (I, Fig.7 K-O).

1.3.3 Degradation of epidermal growth factor

The effects of Rab22a on endosomal membrane trafficking were further studied by monitoring the uptake and degradation of epidermal growth factor (EGF) in the Hep2 cell line in which the EGF binding and uptake is highly efficient. The EGF receptor level must be down-regulated rapidly after receiving the ligand. Therefore EGF is internalised with its receptor and transported through endosomal structures called multi-vesicular bodies (MVBs) to lysosomes for degradation. MVBs are endosomal structures that are negative for M6PR and LAMP (Futter et al., 1996). Hep2 cells were transfected with Rab22a cDNAs by using the Vaccinia T7 system. Fluorescently (rhodamine) labelled EGF was internalised for 1 h, followed by a 3 h chase and fixation of the cells. In Hep2 cells, the Rab22 positive endosomal structures were similar to those in HeLa cells. Even in the cells expressing wt Rab22a or the Q64L mutant form, endosomes were small and rather similar to those in non-transfected cells. After 1 h internalisation, EGF was found in Rab22a wt positive endosomal structures (I, Fig.5 A-C) and remained there even after the 3 h chase (I, Fig.5 D-F). However, in non-transfected cells, EGF was degraded during 3 h chase time and no fluorescence was observed. The same effect, albeit less pronounced, was observed in cells expressing Rab22a Q64L (I, Fig.5 G-L). Surprisingly, Q64L did not colocalise with the marker after 1 h internalisation or after 3 h chase time even though both wt and Q64L mutant protein clearly inhibited the degradation of EGF. The degradation was quantified by calculating the proportion of transfected cells that displayed EGF positive endosomal elements after the 3 h chase (I, Fig.6). The highest frequency of such cells was observed in cultures expressing wt Rab22a (84%). In the cultures expressing Q64L, the frequency was somewhat lower (70%) and the lowest percentage (44%) was in S19N cultures. Also in cells expressing the S19N mutant, the frequency was elevated as compared to mock-transfected cells (17%), but the statistical variance of the quantitation was high in S19N expressing cells. The S19N mutant protein did not colocalise with EGF either at any of the time points (I, Fig.5 M-R).

1.3.4 Analysis of the intracellular trafficking of AGA

To elucidate the possible effects of Rab22a on protein trafficking from the biosynthetic pathway to endosomes, we followed the transport of human aspartylglucosaminidase (AGA). AGA is a lysosomal hydrolase that is transported through endosomes to lysosomes via the M6PR-mediated pathway (Enomaa et al., 1995; Tikkanen et al., 1997). AGA and Rab22 wt or mutant forms were co-expressed in BHK cells by using a transient transfection. The proteins were expressed for 24 h, followed by 3 h incubation in the presence of cycloheximide (CH). CH prevents protein translation and therefore allows AGA to chase through the biosynthetic pathway to the lysosomes. The cells were triple immunostained for AGA, EEA1 (early endosomes) and LBPA (late endosomes, inner membrane), or in some experiments Rab22 was visualised instead of LBPA. In mock transfected cells AGA was chased to LBPA-containing structures which were negative for EEA1 (I, Fig.7 A-E). In cells expressing wt Rab22a, the transport of AGA into late endosomes/lysosomes proceeded in a normal fashion despite the presence of large EEA1 positive early endosomes (I, Fig.8 F-J). In most of the cells (>85%) expressing Rab22a Q64L, the AGA accumulated in large endocytic compartments that were positive for both EEA1 and LBPA (I, Fig.8 K-O). However, similar to the fluid phase marker TRITC-Dextran, AGA did not accumulate in every large endosome. Expression of Rab22a S19N had no apparent effect on the trafficking of AGA (I, Fig.8 P-T).

Several experiments reported in this thesis on known early endosomal trafficking routes provided evidence for a role of Rab22a in early endosomal sorting. Rab22a did not affect transferrin endocytosis or recycling but the distribution of transferrin was changed in Q64L overexpressing cells. This is most likely due to influence of Rab22a on motor proteins involved in motility of early endosomes (Pfeffer, 1999). However, trafficking steps on the endocytic pathways were partly impaired; the active mutant Q64L caused accumulation of both dextran and EGF. In addition, overexpression of Rab22a wt inhibited the degradation of EGF and the protein was collected to the same structures with it. The EGF experiments indicate that Rab22a-positive endosomes/MVBs are unable to communicate with lysosomes. Also, dextran was unable to move to late endocytic compartments, and the lysosomal enzyme AGA accumulated inside Rab22a Q64L-positive vesicles containing partly

late endosomal/ lysosomal markers. One common feature was that only a few of the enlarged Rab22a Q64L-endosomes accumulated the studied marker proteins, most of the structures being devoid of the marker. This data indicates that upon overexpression of Rab22a Q64L the enlarged early endosomes appear first and then start to acquire late endosomal markers. It is possible that the Q64L mutant causes fast retrograde transport from late to early endosomes and therefore blocks some cargo to early endosomes or pushes it there from later compartments.

1.4 Effects of Rab22a overexpression on the Golgi apparatus

Rab22 wt and Q64L overexpression caused a trafficking defect from the biosynthetic pathway to endosomes. This observation raised the question, is trafficking abnormal already in the Golgi complex? A simple Golgi immunofluorescence triple-staining experiment was done with two Golgi markers, mannosidase II (a Golgi resident enzyme, *cis*-/medial-Golgi marker) and GM-130 (a Golgi matrix protein, *cis*-Golgi marker, respectively). Simultaneously with the Golgi marker, antibodies against Rab22a and EEA1 were used. BHK-21 and HeLa cells were transfected with Rab22 wt or mutant cDNA constructs for 24 h. Overexpression of Rab22 wt or Q64L mutant caused complete fragmentation of the Golgi in HeLa cells (I, Fig.9 A-J). Both of the Golgi markers were redistributed into small vesicular structures all over the cell periphery but did not mix with EEA1 containing early endosomal structures. The phenomenon was also seen in BHK cells but it was not prominent in this cell line. The Golgi had a normal compact structure in S19N overexpressing cells (I, Fig.9 K-O). The effects of Rab22a on the Golgi apparatus were unique among the early endosomal Rab proteins studied: Rab4, Rab5, Rab7 and Rab11 did not cause this effect.

The finding that EEA1 binds syntaxin 6 with its C-terminal part (Simonsen et al., 1999) provides a possible mechanistic link between Rab22a function and early endosomal trafficking. Syntaxin 6 is a t-SNARE protein in the TGN. Interestingly, a yeast EEA1-like protein Vac1p regulates the TGN to endosome trafficking. Even though Rab22a Q64L causes the partial mixing of early and late endosomal marker proteins, the Golgi markers remain separately distributed. Studies of Rab1 suggest that one Rab protein can bind its effector proteins/vesicle tethering factors in both donor and acceptor membranes (Moyer et al., 2001). It is possible that Rab22a interacts with a yet unknown Golgi tethering factor and causes Golgi fragmentation

when it is present in excess amounts. Also, possible interactions with microtubular elements can cause fragmentation of the Golgi complex. Studies of the endocytic route indicate a function of Rab22a Q64L in retrograde transport (from late to early endosomes) but the Golgi fragmentation data suggest that Rab22a may function already in the TGN, maybe mediating trafficking between the TGN and early endosomes.

2. Characterisation of a mammalian Sec1/Munc18 (SM) protein Munc18b

2.1 Design of Munc18b mutants

Three mammalian SM proteins control trafficking at the plasma membrane of different cell types. Munc18a (also know as Munc18-1/rbSec1/n-Sec1) is a neuronal protein, which binds neuronal syntaxin1A and B as well as syntaxins 2 and 3 (Hata and Südhof, 1995; Katagiri et al., 1995; Tellam et al., 1995). Munc18b (also known as Munc18-2) is mainly expressed in epithelial cells and interacts with plasma membrane syntaxins 2 and 3 (Hata and Südhof, 1995; Katagiri et al., 1995; Riento et al., 1998; Riento et al., 1996; Tellam et al., 1995). The ubiquitously expressed Munc18c (also know as PSP in platelets) binds to syntaxins 2 and 4, and has been shown to regulate glucose transporter trafficking in adipocytes and platelet granule exocytosis (Reed et al., 1999; Tamori et al., 1998; Tellam et al., 1997; Tellam et al., 1995; Thurmond et al., 1998). In order to gain further insight into the function of Munc18b and the roles of different plasma membrane syntaxins in epithelial cells, we generated a set of Munc18b point mutations. The mutants were designed according to three different criteria. The first set of mutations was generated based on mutations discovered in genetic studies on other known SM proteins. Identification of the correct amino acid to be mutated is, however, not certain in all cases, since the sequences of SM family members are markedly divergent in some regions. The second set was based on highly conserved sequence regions among different SM species, which were then mutated in Munc18b. The last group was based on a molecular model of the Munc18b-syntaxin 3 complex. During the generation of the first two sets of mutants there was no 3D-structural information available from SM proteins. When the last set of mutations was generated the neuronal Munc18a-syntaxin1A crystal structure was solved and based

on that, together with sequence alignments of different syntaxins and SM proteins, we created a homology model for Munc18b and syntaxin 3 (Fig.10; III, Fig .1 and Fig.2).

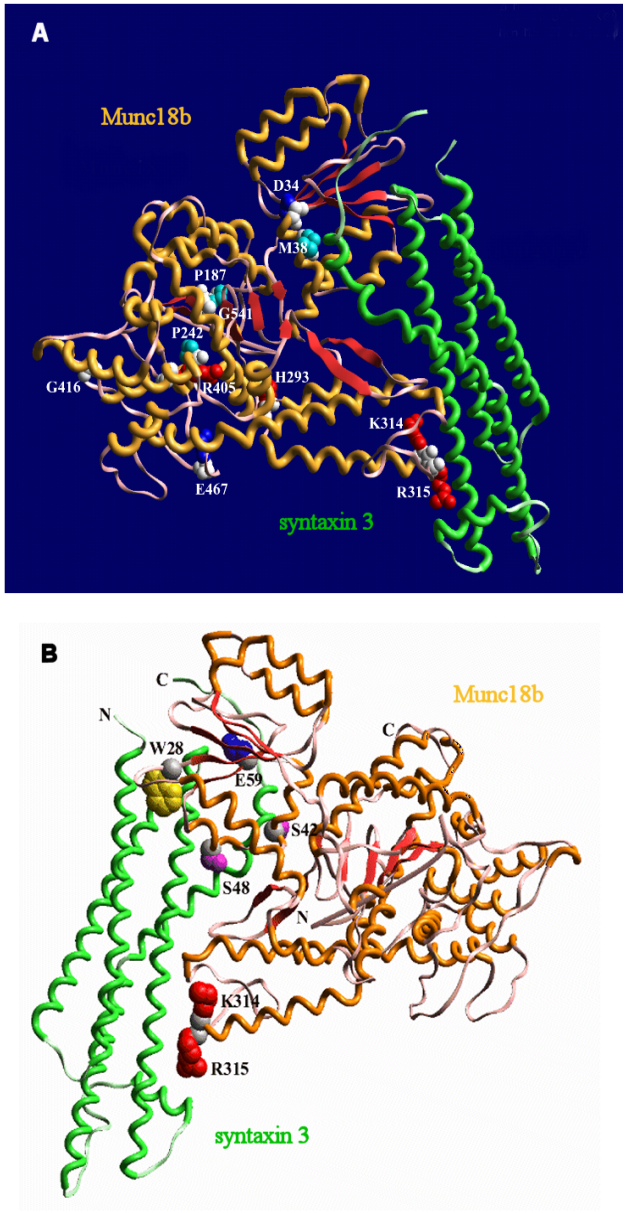

Figure 10. Two ribbon representations of the structural model of the Munc18b-syntaxin 3 complex. The the side chains of residues subjected to mutagenesis in the present study are shown in red (positively charged), blue (negatively charged), violet (polar) and yellow (aromatic). In panel **A,** group I and group II mutations are shown. These mutations were generated according to other known mutations in SM proteins or from conserved sequence regions among different species. Panel **B** represents group III mutations, that were generated to alter the binding specificity of Munc18b to syntaxins.

Munc18b is the closest relative of Munc18a showing overall sequence identity of 63%. The third set of mutants was designed to alter the binding specificity of Munc18b to syntaxin 2 and 3. All Munc18b mutant forms are presented in Table V, where the three groups described above are identified with roman numerals I, II and III. Predictions in group III are based on the analysis of Munc18b-syntaxin3 binding interface in the homology model, and on comparison of differences in syntaxin1A, 2 and 3 sequences.

Table V. Munc18b mutations used in this study

GROUP	MUTATION	CORRESPONDS TO
I	D34N/M38V	Munc18a D34N/M38V, abolishes syntaxin 1A binding [1]
	P242S	*Drosophila* ROP P254S, increase of evoked neurotransmission [2]
	H293Y	*Drosophila* ROP H302Y, reduction in evoked and spontaneous neurotransmission [2]
	R405P	*S.c. sec1-11* temperature sensitive (ts) mutation R432P [3]
	G416E	*S.c. sec1-1* ts mutation G443E, post-Golgi secretion arrest at the restrictive temp. [4]
	G416W	a bulky hydrophobic residue at the position G416
	E467K	*S.c. SLY1-20* E532K, a dominant single copy suppressor of deletion of *YPT1* [5]
II	P187A	highly conserved proline which can affect the neighboring secondary structures
	K314L/R315L	conserved residues
	G541E	highly conserved glycine
III	W28S	predicted to be a non-binder for syntaxin 1A, 2 and 3
	S42K	predicted to bind only syntaxin 2 and 3
	S48D	predicted to bind only syntaxin 2
	E59K	predicted to be a non-binder for syntaxin 1A, 2 and 3

[1] Naren *et al.* 1997; [2] Wu *et al.* 1998; [3] Aalto *et al.* 1991; [4] Novick *et al.* 1980; [5] Dascher *et al.* 1991

2.2 Basic characterisation of the mutants

2.2.1 Stability test

The stability of all the Munc18b variants constructed was tested. The mutant proteins were all N-terminally *myc*-tagged and contained one (or two in some constructs) amino acid point mutation, which may (in principle) affect the stability of the protein. The proteins were overexpressed in Caco-2 cell by using the recombinant SFV expression system. The cells were infected for 4 h at which time the expression was approximately 5-fold as compared to the endogenous protein. The cells were

metabolically labelled with [^{35}S] Met/Cys for 0.5 h followed by 2 h chase. The cells were lysed right after pulse or after chase, immunoprecipitated with myc-antibody, and further analysed by SDS-page and fluorography. The mutants fell into three stability categories. K314L/R315L, E467K, and all mutants in Group III, were as stable as wt Munc18b. Almost all proteins in Group I (G416E, G416W, R405P, P242S, H293Y) showed a mild decrease after a 2h chase (50-80% of the amount of labelled protein remaining after chase). The rest of the mutants (D34N/M38V, P187A, G541E) were remarkably unstable (20-25% remaining after chase). These results confirm the important structural role of the conserved amino acids Pro^{187} and Gly^{541} in SM proteins. However, in the overexpression situation, the amount of even the unstable protein variants at the expression rate reached with the SFV vector were high enough for biochemical studies to be carried out (II, Fig.3, Table I; III, Fig.4). Several of the mutated residues in groups I and II were found to be located in the model far from the syntaxin binding interface (Fig. 10A), suggesting that the effects of these mutations are due to distortion of the SM protein conformation or a defective interaction with potential non-syntaxin binding partners (mutations at the surface of the predicted Munc18b structure).

2.2.2 In vitro binding assays

All mutant proteins were tested for their syntaxin 3 binding ability *in vitro* and *in vivo*. *In vitro* binding was performed by using a GST-tagged syntaxin bound on a 96-well plate. *In vitro* translated and [^{35}S] Met/Cys labelled Munc18b variants were incubated on the plate at 37°C, and the bound Munc18b was measured by scintillation counting. Also, unlabelled Munc18b was used to compete specific binding. Optimisation with different amounts of labelled Munc18b and the competition showed that the binding assay was highly specific and gave a linear response in the entire radioactivity range used (II, Fig.2). In Group I, the counterpart of yeast *sec1*-temperature sensitive mutation G416E bound syntaxin 3 at roughly 50% efficiency compared with the wt protein. The other mutant protein related to yeast *sec1*-temperature sensitive mutation, R405P, showed no detectable binding to syntaxin 3. The binding did not change when the incubation was done at 24°C. When amino acid Gly^{416} was replaced with a bulky hydrophobic tryptophan (G416W) the binding was more reduced (~90%) than for G416E, indicating more extensive structural distortion than with the smaller charged

residue. Both ROP related mutations, H293Y and P242S, showed only negligible association with syntaxin 3 similar to the neuronal Munc18a based mutation D34N/M38V, which abolishes syntaxin 1A binding of Munc18a as well. Interestingly, the counterpart of yeast *sly1-20* mutation, E467K, displayed enhanced binding to syntaxin 3. The novel mutations in group II, K314L/R315L, P187A and G514E, showed no detectable or extremely weak binding to syntaxin 3 (II, Fig.2, Table I). The fact that the counterparts of the loss-of-function mutations in other SM proteins all show defects in syntaxin binding supports the idea that syntaxin interaction is a major functional feature of SM proteins.

The binding of Group III variants to other plasma membrane syntaxins (1A, 2, 3 and 4) was tested with a similar plate assay. In this assay, wt Munc18b shows the highest binding to syntaxin 2 (100%) and the apparent binding efficiency for syntaxin 3 is about 50% of syntaxin 2. The wt protein binds syntaxin 1A with roughly 25% of the syntaxin 2 binding efficiency. There was no detectable binding to syntaxin 4. W28S, S42K and E59K did not show any binding to the syntaxins tested. S48D showed highly reduced binding to syntaxin 3 whereas its binding efficiency for syntaxin 1A and 2 remained close to wt levels. This mutant and the non-binder mutants, W28S and E59K, behaved according to the created model and could be used in assays in which selective syntaxin binding efficiency or totally abolished syntaxin binding is needed. K314L/R315L was tested in this assay as well. Compared to the wt protein, K314L/R315L showed decreased binding to syntaxin 1A and 2 and the binding to syntaxin 3 was significantly reduced but detectable as compared to the wt protein (III, Fig.3).

2.2.3 In vivo binding assays

In vivo binding assays were performed using SFV-mediated overexpression and myc-immunoprecipitation. The precipitated proteins were analysed by SDS-PAGE and by Western blotting with anti-syntaxin 3 antibody (and with anti-syntaxin 2 antibody for Group III mutants). For quantification, the results from Group I and II mutants were analysed by using [^{35}S]-protein A and the phosphorimager technique.

In vivo results coincided well with the *in vitro* results. The *in vitro* analysis was, however, more sensitive and some mutants that showed a weak but detectable signal in the *in vitro* binding assay, showed no binding *in vivo* (D34N/M38V,

K314L/R315L, P187A, P242S and H293Y). This could also indicate the presence of some other factors that can regulate the binding *in vivo* or in some cases the result could be due to reduced stability of the mutant (D34N/M38V, P187A, G541E) in the living cells. Also, an increase of E467K syntaxin 3 binding was seen *in vivo* (II, Fig.4, Table I; III, Fig.5), as suggested by the *in vitro* results.

2.2.4 SNAP-23 displacement

All Group I and II mutant proteins were tested for their ability to displace SNAP-23 from syntaxin 3-based SNARE complexes. Overexpressed wt Munc18b disrupts apical SNARE complexes containing syntaxin 3, SNAP-23 and cellubrevin (Riento et al., 1998). The displacement studies were carried out in Caco-2 cells using a similar SFV infection as above and immunoprecipitation with syntaxin 3 antibody. The precipitates were analysed by Western blotting with anti-SNAP-23 antibody and quantified with ^{35}S-protein A and the phosphorimager technique. Overexpression of wt Munc18b decreased the amount of SNAP-23 bound to syntaxin 3 by 50% compared with the control-infected cells. The ability of the mutant proteins to displace SNAP-23 from the complex correlated reasonably well with their ability to bind syntaxin 3. The strongest syntaxin 3 binder *in vitro* and *vivo*, E467K, showed similar displacement ability as the wt Munc18b. The only exception was H293Y (counterpart of *Drosophila* ROP H302Y mutation), which did not bind syntaxin 3 *in vivo* but was still able to reduce the amount of SNAP-23 in the immunoprecipitates. However, this mutant interacted with syntaxin 3 *in vitro*, so it is possible that some interaction occurs also in the overexpression situation *in vivo*.

2.2.5 Membrane association

The Group I and II mutants were tested for their ability to associate with cellular membranes. These mutants were non-binders or weak binders for syntaxin 3. Caco-2 cells were infected with SFVs expressing wt Munc18b or the mutant forms for 4h. The cells were collected and the membranes were separated by sucrose gradient centrifugation from the post-nuclear supernatant. Fractions were precipitated and analysed by Western blotting. Under these conditions, syntaxin 3 was only in the membrane fraction and a cytosolic control protein gelsolin in the soluble bottom

fraction. It is known that overexpressed wt Munc18b localises to the apical plasma membrane of Caco-2 cells with simultaneously expressed syntaxin 3 (Riento, 1999). When overexpressed alone, Munc18b seems to be mainly cytosolic in immunofluorescense microscopy studies but its membrane association has not been addressed. Now in sucrose gradient centrifugation, wt Munc18b showed clear membrane association although a small part of it remained cytosolic. In control infected cells, the distribution of endogenous Munc18b was similar. Interestingly, mutant proteins D34N/M38V, K314L/R315L and P242S also showed membrane association in the absence of syntaxin 3 binding. A major part of these proteins was found in the membrane fraction (II, Fig.6). These results indicate that Munc18b can be membrane associated independently of syntaxin 3. It is possible that the interaction occours via other plasma membrane syntaxins even though the other Munc18b binding plasma membrane syntaxins, syntaxin 1A and syntaxin 2, are not abundant in Caco-2 cells.

2.3 Functional studies

2.3.1 Assay of HA trafficking by surface immunoprecipitation

Munc18b is mainly expressed in epithelial cells but its function in polarised trafficking is unknown. To elucidate this question we did two types of trafficking assays with the marker protein influenza virus hemagglutinin HA. HA is a well-characterised apically transported protein (Matlin and Simons, 1984; Rodriguez-Boulan et al., 1984). We generated double-SFVs, where HA and Munc18b were both expressed separately under two independent promoters. This approach guaranteed that both proteins were expressed in the same cells. SFV infection causes host protein synthesis shut-off. Therefore it was not possible to monitor transport of endogenous proteins. Filter-grown polarised Caco-2 cells were infected with double-SFVs encoding HA and wt Munc18b or variants (D34N/M38V, K314L/R315L, P242S). The cells were pulse-labelled with [^{35}S] Met/Cys followed by a 2h chase. Antibody against HA was added to the cells and allowed to bind to surface exposed HA protein on either apical or basolateral membranes. The cells were lysed and immunocomplexes were precipitated with protein A-sepharose. The total amount of HA was also analysed by adding the antibody to the lysed cells. Precipitates were

analysed by Western blotting. HA appears in two different forms, an 80 kDa fully glycosylated form and a smaller 70 kDa high mannose form likely to represent protein in the ER or Golgi. This latter form was predominant in the Caco-2 cells (II, Fig.7A). In control-infected cells HA was transported to both membrane domains. Even though transport of HA to the apical domain of Caco-2 cells is well characterised (Jacob et al., 1999; Zurzolo et al., 1992), it is possible that some influenza strains behave differently. Alternatively, under the growth conditions used, the specific Caco-2 cell clone may not transport HA faithfully to only the apical membrane. This unpolarised trafficking made it possible for us to monitor trafficking to both membrane domains. In wt Munc18b infected cells the amount of HA on apical membranes was 30% lower than in control cells. The HA amount in basolateral membranes remained the same indicating that wt Munc18b inhibits selectively apical transport, not basolateral transport or trancytosis, in which the cargo molecules are transported via the basolateral membrane to the apical surface. All mutant forms of Munc18b selected for this study were unable to bind syntaxin 3 or were weak binders. Interestingly, all three behaved differently. D34N/M38V showed a similar 30% inhibition in apical transport as the wt protein, whereas K314L/R315L appeared to enhance HA apical delivery by approximately 50%. However, the results from K314L/R315L varied within a large range. P242S did not show any effect on HA transport. It is likely that the inhibition caused by the wt protein is due to syntaxin 3 binding of the overexpressed protein. Wt Munc18b most likely sequesters free monomeric syntaxin 3 and therefore prevents SNARE complex formation and exocytosis. However, D34N/M38V caused inhibition without capacity for syntaxin 3 binding and K314L/R315L enhanced apical transport of HA (Fig.11). These effects could be due to yet unknown factors which the mutant forms can bind and sequester or bring to the SNARE complex assembly site and thereby cause changes in regulation of the SNARE machinery.

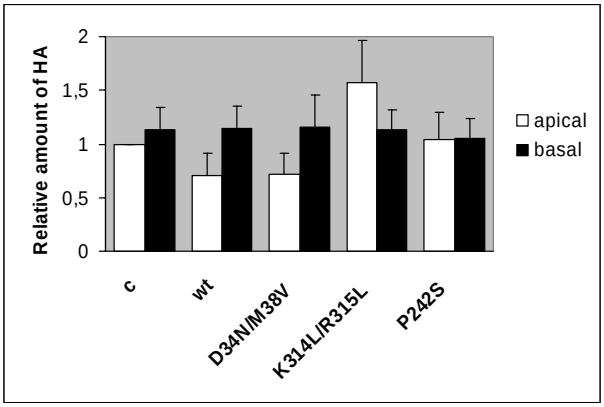

Figure 11. Quantitation of HA transported to the distinct plasma membrane domains in Munc18b overexpressing Caco-2 cells. Polarised Caco-2 cells were infected with double-SVF viruses expressing both HA and Munc18b, or one of its variants. The cells were labeled for 2h with [^{35}S] Met/Cys followed by a 2h chase. HA was surface-immunoprecipitated from apical and basolateral domains, respectively. The amount of HA is divided by the total immunoprecipitated HA. The values obtained (±S.E., n=4) are presended relative to that of apically delivered HA in the control-infected (c) cells, which was set at 1.

2.3.2 HA trafficking assay by confocal immunofluorescence microscopy

Effects of Group III mutants, S48D and E59K, on HA trafficking were monitored by another method using a confocal microscope. Munc18b S48D binds only syntaxin 2 with high affinity whereas E59K is a non-binder for any syntaxin. Different epithelial cells express the different syntaxins in different amounts. Caco-2 cells express mainly syntaxin 3 whereas MDCK II cells express syntaxin 2 more abundantly. The porcine kidney epithelial cell line LLC-PK1 produces both of the syntaxins in significant amounts (III, Fig.7). Endogenous syntaxin 3 is localised on apical membranes in Caco-2 cells (Delgrossi et al., 1997) where syntaxin 2A is also located (Quinones et al., 1999). In stably transfected MDCK II cells, syntaxin 3 was found in the apical domain whereas syntaxin 4 was in the basolateral part, showing no overlap with syntaxin 3 staining. Syntaxin 2 was found in both membrane domains, (Low et al., 1996) which may represent different syntaxin 2 isoforms. Overexpressed syntaxin 2A localises to the apical plasma membrane in MDCK II (Quinones et al., 1999).

We selected Caco-2 and MDCK II cells and monitored the influence of Munc18b S48D mutant form on apical transport of HA. Filter-grown polarised cells were infected with double SFVs expressing both, HA and Munc18b variant, followed

by a period with cycloheximide. The cells were fixed and stained with anti-HA antibody. The distribution of HA was monitored as the mean fluorescence intensity along the apical-basal axis with a confocal microscope. Four focal planes were selected in which the first was the apical plasma membrane and the last was the bottom of the cell (Fig.12).

The wt Munc18b inhibited apical transport of HA in both cell lines compared to control infected cells. The inhibition was 33% in Caco-2 cells and 56% in MDCK II cells. Munc18b S48D caused 42% inhibition of apical transport in MDCK II cells but did not interfere with transport in Caco-2 cells. This is most likely due to selective binding of the S48D mutant to syntaxin 2, the major apical syntaxin in MDCK cells. The result suggests that in MDCK II cells, in which the function of syntaxin 3 in exocytic transport to the apical surface is well established (Lafont et al., 1999; Low et al., 1996), syntaxin 2 also plays an important role in this process. This indicates that different cell lines can use different plasma membrane syntaxins for the same task. The results obtained with the E59K mutant were unexpected. This mutant was originally assumed to have no effect on apical transport of HA since it lacks the syntaxin binding capacity but it caused clear inhibition of the apical distribution of HA in both of the cell lines used. Another mutant, K314L/R315L, with diminished syntaxin 2 binding affinity and weak syntaxin 3 affinity, did not significantly interfere with HA transport (Fig.13).

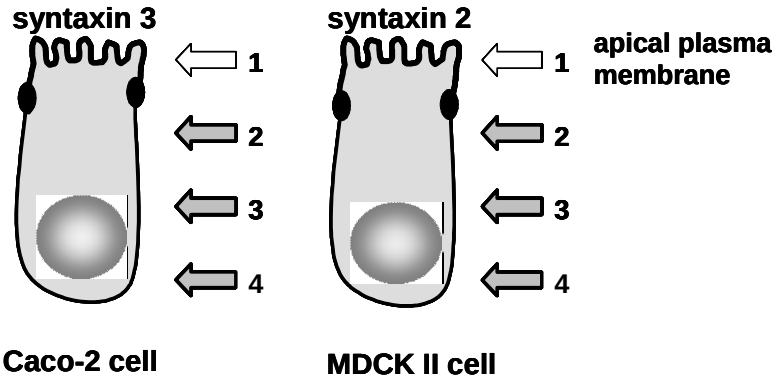

Figure 12. Four focal planes recorded from the HA trafficking assay by confocal immunofluorescence microscopy. The first plane, the apical plasma membrane, was selected above the tight junctions. The other planes were recorded at equal intervals, the fourth plane representing the basal surface. The mean fluorescence intensity was measured in each focal plane.

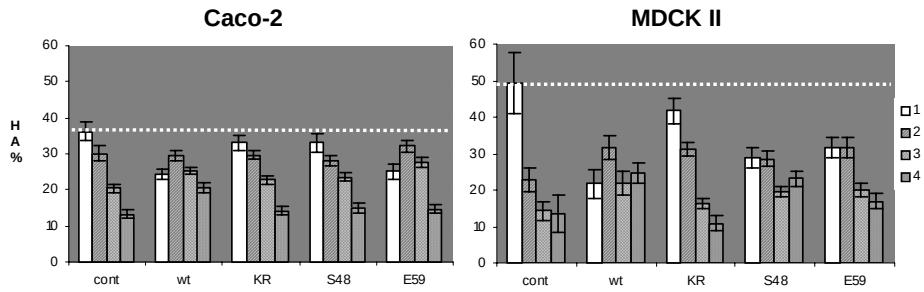

Figure 13. The effect of Munc18b variants on apical transport of HA. Polarized Caco-2 (A) or MDCK II (B) cells, grown on filters, were infected with recombinant SFVs expressing either a non-relevant control protein (ctrl), the wild-type Munc18b (wt) or mutant forms of Munc18b (K314L/R315L, S48D or E59K), together with influenza virus HA, as detailed in Methods. The cells were fixed and processed for immunofluorescence microscopy using HA antibodies. The distribution of HA along the apical-basal axis was quantified using a confocal microscope; a percentage distribution of mean fluorescence intensity at four focal planes is presented. 1, apical surface; 2, a plane at 1/3 the of cell thickness down; 3, a plane at 2/3 of the cell thickness down; 4, basal surface. The results represent the mean ± SEM from 20 cells analysed from each infection.

Some of the results obtained from Munc18b variants are summarised in Table VI.

Table VI. Summary of the results from Munc18b mutant forms

MUTATION (GROUP)	STABILITY	*In vitro* Syn2 BINDING	*In vitro* Syn3 BINDING	SNAP-23 DISPLACEMENT	HA TRANSPORT (% apical as compared to ctrl)	
					Caco-2	MDCK
wt	+++	+++	+++	+++	-30 #	-50 *
D34N/M38V(I)	+/-	ND	+/-	ND	-30 #	ND
P242S (I)	++	ND	+/-	++	+/-0 #	ND
H293Y (I)	++	ND	+/-	++	ND	ND
R405P (I)	++	ND	ND	ND	ND	ND
G416E (I)	++	ND	++	++	ND	ND
G416W (I)	++	ND	+/-	+	ND	ND
E467K (I)	+++	ND	+++	+++	ND	ND
P187A (II)	+	ND	+/-	ND	ND	ND
K314L/R315L(II)	+++	ND	+/-	+	+50 #	-10 *
G541E (II)	+	ND	+	ND	ND	ND
W28S (III)	+++	-	-	ND	ND	ND
S42K (III)	+++	-	-	ND	ND	ND
S48D (III)	+++	+++	+/-	ND	+/-0*	-40 *
E59K (III)	+++	-	-	ND	-25 *	-40 *

ND, Not determined; #, HA trafficking by surface immunoprecipitation; * HA trafficking assay by confocal immunofluorescence microscopy

2.3.3 Amylase secretion from pancreatic acinar cells

The pancreatic acinar cell has been a classic model to study regulated exocytosis occurring at the apical plasma membrane. The cytosol of the acinar cell is crowded with zymogen granules. Zymogens are synthesised as inactive proenzymes and are transported to the condensing vacuoles. These vacuoles undergo a series of maturation steps before ending up as large and dense mature zymogen granules, which are packed with high amount of proenzyme. When acinar cells are stimulated with cholecystokinin (CCK), the enzymes are released from the apical membrane to the pancreatic duct. This transport event is disturbed in acute pancreatitis. In this condition, the enzymes are secreted to the basal membrane where they cause severe tissue damage (Gaisano, 2000). We used pancreatic acinar cells type to study the effects of Munc18b on regulated exocytosis and measured the secretion of one enzyme, amylase, upon CCK analogue CCK-OPE stimulus. In acinar cells, the distribution of syntaxins is different compared to the previously used polarised cell lines; syntaxin 2 is located at the apical plasma membrane whereas syntaxin 4 is on the basal membrane and syntaxin 3 is on the zymogen granules (Gaisano, 2000).

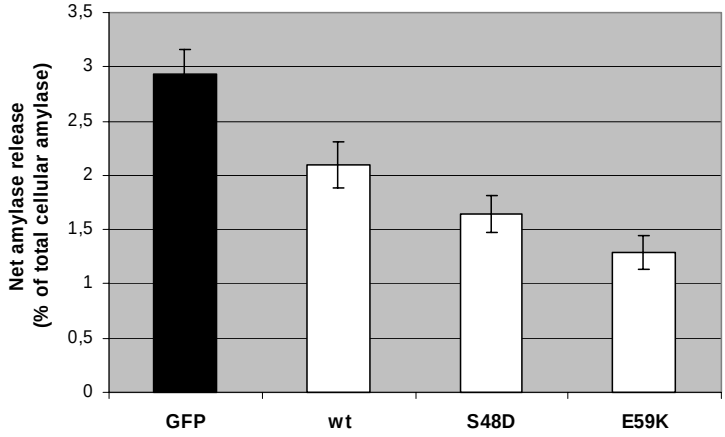

Figure 14. The effect of Munc18b variants on amylase secretion in pancreatic acinar cells. Rat acinar cells were isolated and infected with Adenoviruses expressing GFP (control), wt Munc18b, S48D or E59K. The cells were stimulated for secretion and the amount of secreted amylase was measured from the medium as detailed in the Methods section. The results represent the mean of five independent experiments ± s.e.m. For each Munc18b variant the difference from the GFP control is statistically significant (t-test; $p < 0.0001$).

Wt Munc18b expressed from an adenoviral vector caused ~30% inhibition of amylase secretion compared to control infected cells. Munc18b S48D, the mutant unable to bind syntaxin 3, inhibited amylase secretion at least as efficiently as the wt protein. Interestingly, Munc18b E59K, which is unable to bind any of the syntaxins studied, caused the most extensive, ~50%, inhibition (Fig.14). These results, together with those obtained in Caco-2 and MDCK II cells, indicate that the syntaxin interaction is not the only functionally relevant feature of Munc18b. It is evident that those mutant forms of Munc18b that are unable to bind syntaxin, are still capable of causing transport inhibition when overexpressed.

V SUMMARY AND CONCLUSIONS

Cells contain a variety of transport vesicles that carry cargo molecules to different destinations. This process is necessary for cell organelles to maintain their unique features. Although many specific transport routes exist, the molecular mechanisms involved appear to be rather similar and conserved through evolution. SNARE proteins on the vesicle membrane and the target membrane play a key role in vesicle fusion. There are several regulators of SNARE complexes that adjust the specificity as well as the correct timing of the fusion event. The two main families of SNARE regulators are the Rab protein family and the Sec1/Munc18b (SM) protein family. In the present study, we have characterised the properties of a small GTPase Rab22a and an SM family member, Munc18b.

Rab22a is a novel, early endosomal Rab protein. Early endosomes are shown to be important sorting centres within multiple endocytic pathways. Several Rab proteins are localised to these organelles where they are suggested to form specific sorting domains. Effector proteins mediate the function of Rabs through binding to the GTP bound form of the GTPase. Each early endosomal Rab protein has its unique effector proteins but they also share some binding partners (Deneka and van der Sluijs, 2002). We found that Rab22a interacts directly with the N-terminal part of the early endosomal tethering factor EEA1 thus creating a functional link with the major early endosome transport regulator Rab5. Rab5 mediates the homotypic fusion of early endosomes and binds to both ends of the long EEA1 molecule. Since Rab22a binds only the N-terminal end of EEA1, the function must differ from the function of Rab5. Overexpressed Rab22a causes several trafficking defects in the endocytic pathway. The overexpressed GTPase deficient mutant of Rab22a leads to mixing of early and late endosomal markers. This could be due to enhancement of a retrograde pathway from early to late endosomes. In addition, both forms of Rab22a, wt and the GTPase deficient mutant, cause complete disruption of the Golgi complex when overexpressed. It is therefore possible that Rab22a interacts with another tethering factor in the Golgi complex. Interestingly, EEA1 binds with its C-terminal end to syntaxin 6, a t-SNARE in the TGN. These results indicate that Rab22a may have a function in communication between early endosomes and the TGN. Further studies are needed to prove this hypothesis. It would be interesting to study the role of

Rab22a in the intoxication by Shiga toxin, which is believed to enter the TGN via early endosomes (Johannes and Goud, 2000; Sandvig and van Deurs, 2002).

Munc18b is mainly expressed in epithelial cells and interacts with plasma membrane syntaxins 2 and 3 (Hata and Südhof, 1995; Katagiri et al., 1995; Tellam et al., 1995). We showed that Munc18b has a role in apical exocytosis in several polarised cell types. The molecular model of the Munc18b/syntaxin 3 complex was used to design mutants with altered syntaxin binding properties. The mutant S48D that binds only to syntaxin 2 provides evidence that different epithelial cells can use different syntaxins for the same task. The mutations in conserved amino acid residues (P187A and G541E) cause rapid degradation of Munc18b, indicating the important structural function of these amino acids in the correct folding of the protein. The mutants designed according to loss-of–function mutations in other SM proteins all have defects in syntaxin binding, supporting the idea that syntaxin interaction is a major functional feature of SM proteins. However, behaviour of the non-binder/weak binder mutants in the functional assay suggest that also other interaction partners must be highly important. Also, the fact that some syntaxin non-binder mutants associate with membranes supports this idea. In the present study it has been a frequent observation (with several cell lines and several different assays) that Munc18b mutants that show no or drastically reduced affinity for syntaxin behave in different ways in apical transport assays. Some of the mutants seem functionally inert while others have effects comparable to those of the wt protein.

Data obtained with non-binder or weak binder mutants allow us to suggest the following model (Fig.15). In this model Munc18b with a low affinity for syntaxin (such as K314L/R315L) is still capable of performing its essential function by associating with the syntaxin at a high turnover rate and recruiting a yet unknown essential factor to the SNARE complex assembly site. The reduced affinity would compensate for the increased protein concentration due to over-expression, resulting in no inhibitory effect such as that caused by excess wt protein. However, an over-expressed mutant with totally abolished syntaxin binding (like E59K), assuming that it is still able to bind the unknown cytosolic or membrane-associated factor, would sequester this factor and make it unavailable for the normal endogenous SM protein, thus leading to inhibition of transport. It is possible that SM proteins bridge the SNARE complex and the membrane tethering apparatus controlled by the Rab GTPases. In fact, there is evidence that several SM proteins interact with effectors that

Rab GTPases recruit to membranes. For example Rab5 effector Rabenosyn5 binds the SM family member hVPS45 and the *S. cerevisiae* tethering factor Vac1p has binding partners in both SNARE regulator families (Nielsen et al., 2000; Peterson et al., 1999; Tall et al., 1999). Creating temporal and spatial control between the tethering and docking/fusion steps is an absolutely essential but yet poorly understood process.

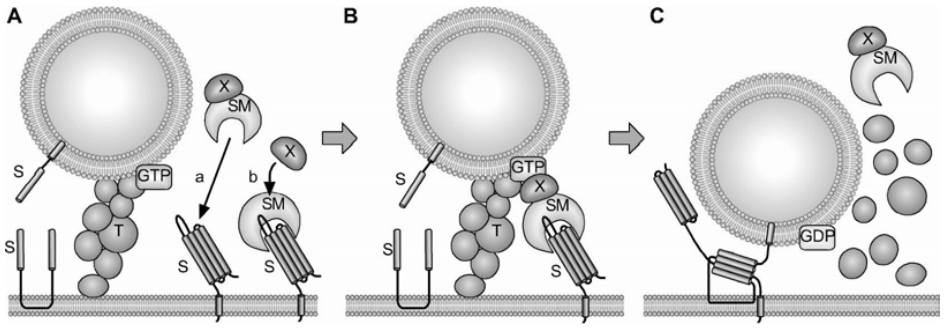

Figure 15. Model for a common function of SM proteins. **A.** A transport vesicle is tethered at the target membrane via the action of a Rab protein in its GTP-bound form (GTP) and by a tethering complex (T) recruited to membranes by the small GTPase. The SM protein binds to factor X (or possibly several factors) and then to syntaxin (alternative a), or binds to syntaxin first and then recruits factor X (alternative b). SNARE proteins are marked with S. **B.** Factor X links the SM protein and the complexed syntaxin to the Rab-based tethering apparatus. **C.** The SM protein facilitates the assembly of a trans-SNARE complex in a spatially and temporally controlled manner. The tethering complex dissociates and the Rab in GDP-bound form (GDP), the SM protein, and factor X are released. In some cases, the SM protein may remain bound to the SNARE complex and regulate events during the following fusion process.

To fully understand the role of SM proteins in vesicle transport we now need to investigate in great detail the function and the non-syntaxin interaction partners of different SM proteins with distinct modes of syntaxin binding. Furthermore, it is obvious that phosphorylation/dephosphorylation cycles of SM proteins and their binding partners play major roles in the regulation of vesicle docking and fusion (Craig et al., 2003; de Vries et al., 2000; Fujita et al., 1996; Sassa et al., 1996). Grasping the common functional principle of the SM proteins is a major task for the future and will undoubtedly represent a major leap in our comprehension of how cells control the essential but highly complex process of vesicle transport.

VI ACKNOWLEDGEMENTS

This work was carried out at the National Public Health Institute at the Department of Biochemistry and Molecular Medicine during the years 1999-2004. I wish to express my gratitude to the following people:

Former and present directors of the Institute, Professor Jussi Huttunen and Professor Pekka Puska, respectively, for providing the excellent research facilities

Former and present heads of the department, Professor Christian Ehnholm and Professor Leena Palotie, respectively, for allowing me the opportunity to carry out my research in such a well-equipped and dynamic laboratory

Professor Carl G. Gahmberg, from the Division of Biochemistry, University of Helsinki, for his assistance and flexibility in arranging the coursework component of my studies

My supervisor Docent Vesa Olkkonen for his enthusiasm in my project, active collaboration and overall guidance throughout my studies

My collaborators Dr. Sirkka Keränen, Dr. Kirsi Riento, Dr. Gerd Wohlfahrt and the group of Professor Harald Stenmark (Oslo, Norway), as well as the group of Dr. Herbert Gaisano (Toronto, Canada) for welcoming me into their laboratories and for providing me an inspiring environment that was enjoyable to work in

Professor Elina Ikonen and Docent Matti Jauhiainen for their sound advice and enthusiastic discussions

Docent Markku Saloheimo (VTT, Espoo) and Dr. Ulrich Blank (CNRS, Paris, France) for reviewing this thesis and providing useful comments

Sarah Siggins for revising the language of the thesis

Seija Puomilahti and Pirjo Ranta for their technical assistance during the project

Everyone in the lab for the enjoyable atmosphere and scientific collaboration

Family and friends for their unfailing help and support

Helsinki Graduate School in Biotechnology and Molecular Biology for offering travel grants as well as many interesting courses and events

The Finnish Cultural foundation, Instrumentariumin Tiedesäätiö, Farmoksen Tutkimus- ja Tiedesäätiö for financial support

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