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Endocytosis and retrograde transport of Shiga toxin and ricin



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Abbreviations

AP	Adaptor protein
BCR	B cell receptor
CCP	Clathrin-coated pit
CCV	Clathrin-coated vesicle
CHC	Clathrin heavy chain
CI-MPR	Cation-independent mannose-6-phosphate receptor
COP	Coatomer protein
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EM	Electron microscopy
ER	Endoplasmic reticulum
Gb3	Globotriaosyl ceramide
GDI	GDP-dissociation inhibitor
GPI	Glycosyl phosphatidyl inositol
GTPase	Guanosine triphosphatase
LDL	Low density lipoprotein
MPR	Mannose-6-phosphate-receptor
NGF	Nerve growth factor
NGFR	Nerve growth factor receptor
PDI	Protein disulfide isomerase
PI	Phosphoinositide
PI(4,5)P2	Phosphatidyl inositol 4,5-bisphosphate
PI(3)P	Phosphatidyl inositol 3-phosphate
PX	Phox homology
Shiga toxin B	Shiga toxin B-subunit
siRNA	Small interfering RNA
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SNX	Sorting nexin
Tf	Transferrin
TfR	Transferrin receptor
TIRF	Total internal reflection fluorescence
TMF	TATA-element modulatory factor
TGN	<i>Trans</i> -Golgi network
Vps	Vacuolar protein sorting

List of publications

- I. Utskarpen, A*, Slagsvold, HH*, Iversen, TG, Wälchli, S, and Sandvig, K (2006) Transport of ricin from endosomes to the Golgi apparatus is regulated by Rab6A and Rab6A'. *Traffic* 7, 663-72.*Equal contribution.
- II. Skånland, SS, Wälchli, S, Utskarpen, A, and Sandvig, K (2007) Phosphoinositide-regulated retrograde transport of ricin: Crosstalk between hVps34 and sorting nexins. *Traffic* 8, 297-309.
- III. Utskarpen, A, Slagsvold, HH, Dyve, AB, Skånland, SS, and Sandvig, K (2007) SNX1 and SNX2 mediate retrograde transport of Shiga toxin. *Biochem. Biophys. Res. Commun.* 358, 566-70.
- IV. Utskarpen, A, Massol, R, van Deurs, B, Lauvrak, SU, Kirchhausen, T, and Sandvig, K. Shiga toxin increases formation of clathrin coated pits through Syk kinase. *Manuscript*.

Introduction

Protein toxins – important tools in cell biology and medicine

Protein toxins from bacteria and plants may cause life-threatening diseases and poisoning in humans. However, they are also promising as immunotoxins for targeted drug delivery, and they provide valuable information on trafficking pathways in cells important for elucidating molecular mechanisms behind disease.

The family of AB toxins consists of toxins with an enzymatically active A-subunit and a surface-binding B-subunit. AB toxins produced by bacteria include Shiga toxin and Shiga-like toxins, cholera toxin, diphtheria toxin, anthrax toxin, and *Pseudomonas* exotoxin A (1-3). Shiga toxin is produced by the bacterium *Shigella dysenteriae*, which causes dysentery in humans. The closely related Shiga-like toxins made by *Escherichia coli* and some other bacterial strains are major causes of water- and food-borne infectious diseases world-wide, resulting in severe diarrhea and hemolytic uremic syndrome (HUS), which may cause renal failure in children.

Some examples of AB toxins produced in plants are ricin, abrin, modeccin and viscumin (2,4). Ricin is found in the castor oil plant, *Ricinus communis*. The toxin is mainly present in the seeds and is purified from the aqueous phase, whereas the castor oil is

used as a laxative in medicine and as a lubricator for various industrial purposes.

The toxic and transport properties of these toxins are exploited in the construction of immunotoxins, where antibodies that recognize cancer cells are linked to the toxin for targeted therapy (5-7). Hormones or growth factors may also be used for recognition of target cells. Clinical trials show promising results in cancer treatment for conjugates made from bacterial toxins, and a drug consisting of diphtheria toxin fused to a binding subunit for the interleukin-2 receptor has been approved for lymphoma treatment (6-8). The non-toxic B-subunits of toxins may be used for delivery of therapeutic or diagnostic agents into cancer or immune cells (5). The Shiga toxin receptor Gb3 is overexpressed in certain cancers (5,9), and experiments are being performed with Shiga toxin B coupled to a drug against colon cancer (10). Shiga toxin B-conjugates targeting dendritic cells are also being investigated as vaccines against ovarian cancer (11).

Several major breakthroughs in the studies of endocytosis and intracellular transport pathways have relied on protein toxins (12-15). The toxins have thus proved to be valuable tools to increase the general knowledge of cell biology important for understanding and treatment of cancer as well as other diseases.

Ricin and Shiga toxin – enzymes exploiting the endocytic pathway

Belonging to the AB-family of toxins, ricin and Shiga toxin contain an enzymatically active A-moiety and a B-moiety responsible for binding to the cell surface. They follow the retrograde pathway to the endoplasmic reticulum (ER) before

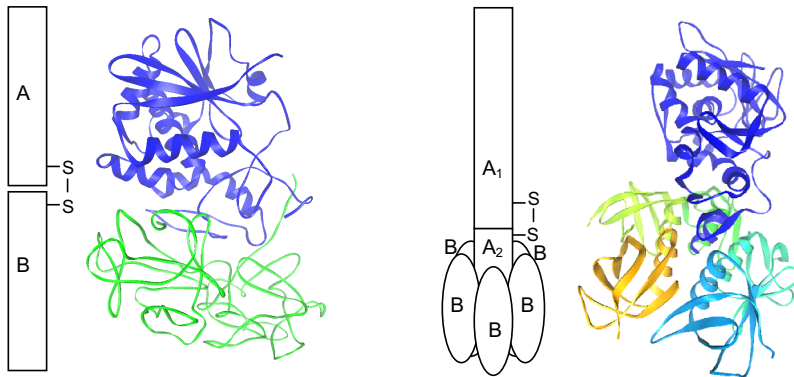


Figure 1. Schematic and crystallographic structures of ricin and Shiga toxin with A- and B-subunits (PDB protein databank). A) Ricin (PDB: 2AA1). B) Shiga toxin (PDB: 1DMO).

getting access to the cytosol and intoxicating cells (2-4,16). The A-subunit acts as a glycosidase, cleaving off an adenine from the 28S RNA on the 60S ribosomal subunit. This prevents elongation factors from binding and inhibits protein synthesis. The A-subunits are extremely efficient, and as little as one ricin molecule is sufficient to kill a cell (4). The size of the ricin A- and B-subunits is about 30 kDa, and the A-chain is linked to the B-chain by a disulfide bridge which must be cleaved for enzymatic activity (figure 1A). Ricin binds to glycolipids and glycoproteins at the cell surface with a terminal galactose-residue creating millions of binding sites (2,4).

Shiga toxin binds selectively to globotriaosylceramide (Gb3) on the cell surface, and does not bind to cells lacking this receptor (3). The Shiga toxin B-moiety consists of five B-chains of 7.5 kDa each and is non-covalently linked to the A-subunit of 32 kDa (figure 1B). Before reaching the ER, the A-subunit is cleaved into

two fragments, A₁ and A₂, by the enzyme furin (17), and at least the enzymatically active A₁-part must be translocated to the cytosol (3).

The endocytic pathway and its key players

Nutrients, growth factors, signaling molecules and other macromolecules in the extracellular matrix are taken up into cells by various types of endocytosis (18-21). Transport vesicles fuse with each other to form early endosomes, which constitute important sorting stations within the cell. The destiny of the vesicles is partly controlled by different surface markers dependent on origin and cargo. From the early endosomes cargo is recycled back to the plasma membrane, transported to lysosomes for degradation or delivered to the Golgi apparatus or the ER (figure 2). Newly synthesized proteins, lipids and carbohydrates follow the opposite trafficking pattern, the biosynthetic-secretory pathway, and there is a continuous, large-scale flow of transport vesicles in both directions.

Rab GTPases, phosphoinositides (PIs) and coat proteins such as clathrin and sorting nexins (SNXs) are all key components in intracellular trafficking which have been studied in this thesis. The different Rabs and PIs are specific for certain organelles and thus maintain the compartmentalization of the cell (22-24). PIs recruit proteins that promote vesicular traffic to membranes, where some of the proteins form complexes such as clathrin coats, COP coats or SNX complexes (22,25,26). Rab GTPases can regulate PI metabolism by affecting downstream kinases. Furthermore, they interact with tethering factors and mediate docking of transport vesicles to the correct membrane (27-30). The SNARE proteins constitute another group of key factors in vesicle traffic. V-SNAREs pack with different cargo on the coat

of a budding vesicle, and bind to the complementary t-SNAREs on the target membrane to mediate fusion (25,31). Motor proteins such as dynein and kinesin connect vesicles to the actin filaments and microtubules of the cytoskeleton and promote transport between compartments (32).

Rab GTPases

Rab GTPases are molecular switches cycling between a cytosolic, inactive GDP-bound form and an active, membrane-attached GTP-bound form (22,28-31). Guanine nucleotide exchange factors (GEFs) replace GDP by GTP whereas GTPase-activating proteins (GAPs) catalyze the reverse reaction. Rab GTPases regulate cargo sorting, budding, transport, tethering, and fusion of transport vesicles. In the cytosol they form a complex with a GDP-dissociation inhibitor (GDI) which prevents insertion into membranes. GDI displacement factors (GDF)s are proposed to dissociate GDIs from Rabs at certain membrane surfaces, allowing nucleotide exchange and subsequent binding of effector proteins to the now active form (28).

More than 60 human Rabs have been identified, and their cellular distribution is compartment specific (27). Rab4 and Rab5 are associated with different subdomains of early endosomes (33), and Rab5 has been shown to mediate early endosomal fusion (34). Rab7 is thought to replace Rab5 as the early endosomes mature into late endosomes (35), whereas Rab9 is a late endosomal marker found to mediate transport from late endosomes to the TGN (36-38). Rab11 is associated with early/recycling endosomes and the Golgi (39-41), and yet another Rab, Rab1, is mainly localized to the ER and controls vesicular traffic between the ER and the Golgi apparatus (42-44).

The two Golgi-localized Rab6 isoforms Rab6A and Rab6A' are created by alternative splicing of a duplicated exon and differ in only three amino acids near the GTP-binding domain (45). They bind GTP to similar extents and are ubiquitously expressed at

similar levels, whereas the third Rab6 isoform, Rab6B, is only expressed in a subset of neurons (45,46).

Phospholipids

PIs as well as Rab proteins are important markers of organelle identity. They serve as recruitment sites for coat proteins, motor proteins, tethering factors etc., thereby forming membrane sub-domains and regulating signaling and transport events in space and time (22,24,47). Their metabolism is controlled by organelle-specific kinases and phosphatases. PI(4,5)P₂ is abundant at the plasma membrane and interacts with proteins important for endocytosis; together with PI(4)P and PI(3)P it is also present at the Golgi apparatus (24,48). PI(3)P is most abundant on early endosomes, internal vesicles of multivesicular bodies, and in yeast vacuoles (49).

Many proteins important for transport are peripheral-membrane proteins that possess specific PI-binding modules such as the FYVE, PHOX homology (PX), pleckstrin homology (PH), ENTH, and ANTH domains (23). PI(3)P is recognized by FYVE domains in proteins like EEA1, which regulate endosomal fusion, and by the PX domains of SNXs (50). PI(3)P can be generated either by a PI3-kinase phosphorylating PI or a phosphatase converting PI(3,5)P₂ or PI(3,4,5)P₃ into PI(3)P (51). The class III PI3-kinase Vps34 is conserved from yeast to mammals and has phosphatidylinositol as its only substrate. Being localized to early endosomes and forming different sub-complexes with other kinases, Vps34 has been shown to create PI(3)P for the autophagic pathway, for the formation of multivesicular bodies and transport to late endosomes, as well as endosome-to-Golgi trafficking (52-55).

Sorting nexins

So far 33 mammalian sorting nexins (SNXs) and 10 yeast SNXs have been identified (26,56). Members of the SNX protein family are recruited to membranes by their phosphoinositide-binding PX domain. Several SNXs, including SNX1, SNX2, and SNX4 possess a BAR domain, which binds to curved membranes and in some cases induces membrane curvature (57). SNXs bind preferably to PI(3)P but can also bind to other PIs with different affinities and thereby localize to specific compartments in the early endocytic pathway. Many SNXs are conserved, and the retromer protein sorting complex on early endosomes was first identified in yeast (58). In mammals the retromer consists of a cargo binding sub-complex containing hVps26, hVps29 and hVps35 and a sub-complex thought to mediate membrane interactions consisting of SNX1 and SNX2. SNX5 and SNX6 have also been suggested as retromer components (59). SNX4 regulates a retromer-independent pathway to the Golgi in yeast (60) as well as recycling of transferrin (Tf) in human cells (61). SNX9 is involved in the activation of dynamin and N-WASP regulating endocytosis (62-64).

Retrograde transport of ricin and Shiga toxin

When endocytosed, ricin and Shiga toxin both follow the retrograde transport pathway from early endosomes through the Golgi apparatus before they enter the ER and are translocated to the cytosol (figure 2) (2-4,16). Notably, Shiga toxin was the first lipid-binding ligand found to be endocytosed by coated pits (14) and the first compound shown to exploit the retrograde pathway for trafficking all the way to the ER (15). In that way, the toxin may escape lysosomal degradation on its way to the cytosol. The

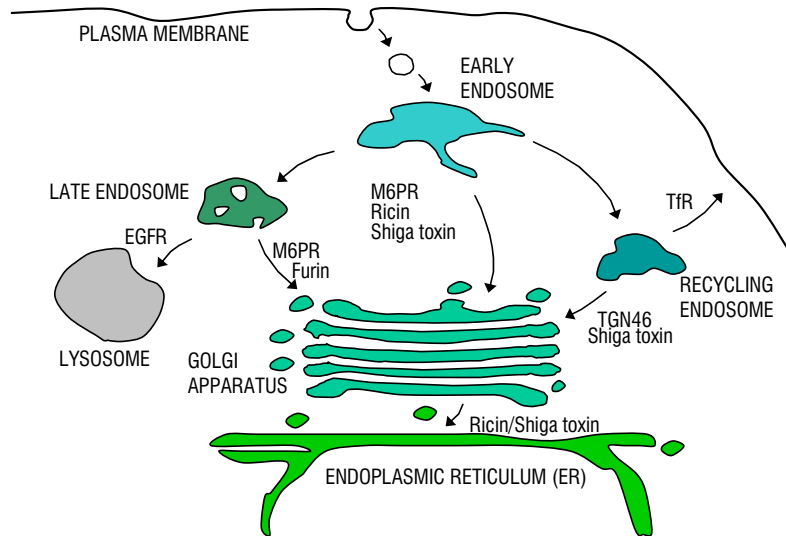


Figure 2. Intracellular transport routes in the endocytic pathway followed by different proteins. Ricin and Shiga toxin follow the retrograde pathway from early endosomes via the Golgi apparatus to the ER. Transport of Shiga toxin as well as recycling of TGN46 to the TGN is dependent on Rab11, which is associated with recycling endosomes. EGFR is transported via early and late endosomes to lysosomes, whereas TfR is recycled back to the plasma membrane. M6PR and furin are retrieved to the TGN from late endosomes via a Rab9-dependent route; however, M6PR can also be retrieved from earlier compartments.

toxins may also be recycled back to the plasma membrane from early endosomes or transported to lysosomes for degradation. In fact, only 5 % of internalized ricin enters the retrograde pathway (65).

Endocytosis

Both ricin and Shiga toxin are endocytosed by clathrin-dependent as well as clathrin-independent mechanisms (2,3,21). Different

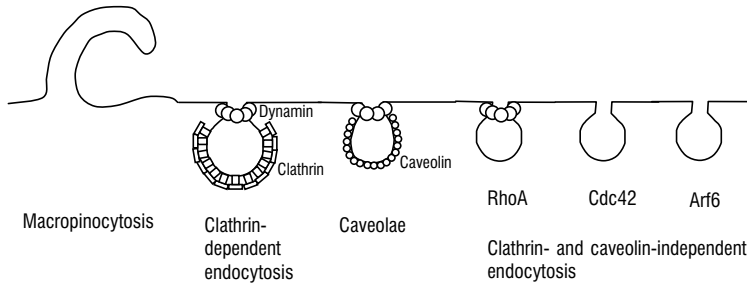


Figure 3. Endocytic mechanisms

types of endocytosis are summarized in figure 3. The endocytic pathways differ in regards to the size of the vesicle, the type and destination of cargo, and the mechanisms of vesicle formation. The two main categories of endocytosis are phagocytosis (cell-eating), the uptake of large particles such as bacteria into specialized cells, and pinocytosis (cell-drinking), the uptake of fluids and solutes which occur in all cell types (66). Macropinocytotic membrane protrusions are induced by signaling through Rho-GTPases and actin remodeling. They fuse with the plasma membrane and form endocytic vesicles greater than $1\mu\text{m}$ in diameter (66).

Caveolae are membrane invaginations $\sim 60\text{ nm}$ in size that are defined by the presence of the protein caveolin. They form on microdomains rich in cholesterol and sphingolipids, and dynamin-mediated remodeling of the actin cytoskeleton seems to be important for invagination. (18,21)

Several endocytic processes require neither clathrin nor caveolin and may be divided into groups based on their requirements for dynamin, Cdc42, RhoA, or ARF6 (18,21,67).

Clathrin-mediated internalization, where coated pits of $\sim 120\text{ nm}$ in diameter pinch off to fuse with early endosomes, is the type of endocytosis best characterized (19,20,66,68,69). In the classical

view of clathrin-mediated endocytosis ligand-induced uptake of receptors upon cargo-binding, such as growth factors or G-protein-coupled receptors, can be distinguished from constitutive endocytosis of non-signaling receptors like LDL receptor and transferrin receptor (TfR) occurring independently of ligand binding. So far four main types of internalization signals, which are recognized by adaptor or accessory proteins, have been described in transmembrane proteins. An increasing number of accessory proteins have been identified, some of them being specific for certain types of cargo. Thus, the existence of cargo-specific clathrin-coated pits (CCPs) has been discussed (19).

Displaying by far the largest number of interactions with other proteins, clathrin and AP-2 may be considered as hubs in the network of clathrin-coated vesicle (CCV) formation (68). The AP-2 complex, consisting of α , β 2, μ 2 and σ 2 subunits, binds PI(4,5)P₂ and cargo receptors and links cargo into the forming CCP. Accessory proteins such as AP180/CALM and epsin recruit clathrin and promote membrane curving. Clathrin triskelions form polygonal networks thought to stabilize the coated vesicle (70). Dynamin is recruited by the accessory proteins amphiphysin, SNX9 and/or intersectin and hydrolyses GTP for scission of the CCV. The actin cytoskeleton is also important in the scission. Auxilin and HSC70 ATP-ase are involved in un-coating of the CCV, leaving clathrin and AP-2 accessible for the formation of new CCPs (68,71).

Signaling and endocytosis

Several studies have revealed that signaling and endocytic events are mechanistically integrated in clathrin- as well as clathrin-independent internalization (72,73). Binding of epidermal growth factor (EGF) and neuronal growth factor (NGF) to their tyrosine kinase receptors is known to induce auto-phosphorylation and subsequent signal transduction promoting clathrin-mediated endocytosis and degradation of the receptors. Moreover, the

induced activation of Src-kinases seems to affect clathrin phosphorylation and recruitment (74-76). Also endocytosis of the Fc receptors in macrophages and the B and T cell antigen receptors involve activation of Syk and Src kinases; in the case of BCR and TCR the activation is followed by clathrin phosphorylation (77-79).

Although binding to a glycolipid, Shiga toxin can induce apoptotic cascades as well as more rapid signaling in cells. Shiga toxin binding has been shown to activate the Src-kinases Yes and Lyn (80,81), the MAP-kinase p38 (82), the serine/threonine kinase PKC δ (83), and the tyrosine kinase Syk (84,85), as well as increasing microtubule formation (86,87). Syk-mediated phosphorylation of clathrin heavy chain seems to be important for uptake of Shiga toxin (84). Shiga toxin can promote its own recruitment to CCPs and induce its own uptake (14,88). Whereas Shiga toxin B seems to be sufficient for activating signaling cascades, the A-subunit increases the clathrin-dependent endocytosis of the toxin (88). Shiga toxin B has also been reported to induce reorganization of membrane lipids causing tubular invaginations of the plasma membrane independent of CCPs (89).

Endosome-to-Golgi transport

Retrograde transport from endosomes to the Golgi apparatus has not been as well characterized as endocytic mechanisms and the lysosomal pathway. However, an increasing number of coat proteins, lipids, and recruitment, sorting, tethering and fusion factors in endosome-to-Golgi trafficking have now been identified, see figure 4 (90,91). Endogenous proteins utilizing this pathway include acid hydrolase sorting receptors like the mammalian mannose-6-phosphate receptors (MPRs) and the yeast Vps10p (90,91). These receptors deliver acid hydrolase precursors destined for lysosomes to early endosomes, and are recycled back to the TGN from early or late endosomes. Multiligand receptors such as sortilin and SorLa (92,93), the

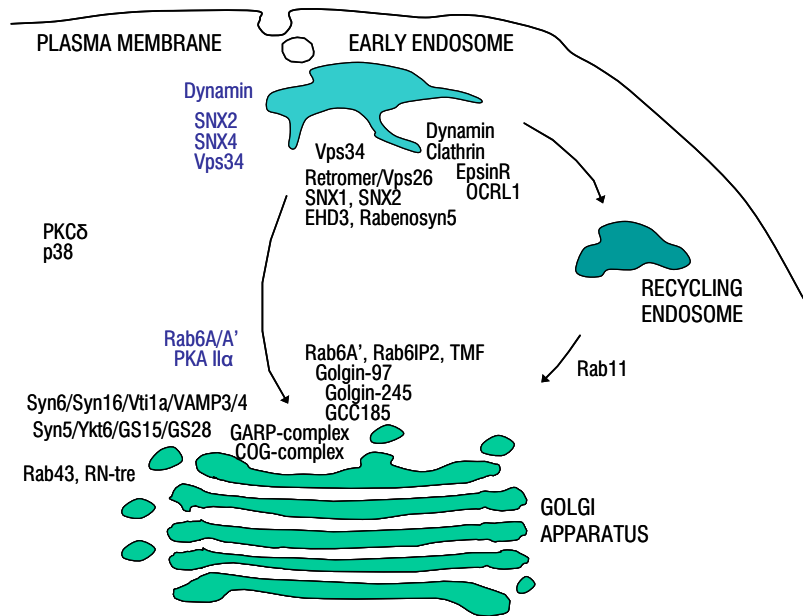


Figure 4. Proteins associated with endosome-to-Golgi transport of Shiga toxin (black color) and ricin (blue color). Proteins thought to be involved in budding of vesicles are shown around early endosomes, whereas proteins thought to mediate tethering or fusion are shown near the Golgi apparatus. The sites of action of PKC and p38 are not known. Coat proteins and their adaptors contribute to vesicle formation at early endosomes. Rab proteins mediate correct docking and interact with tethering factors (Golgins and GARP/COG-complexes) at the TGN, whereas SNARE complexes mediate fusion.

Golgi resident proteins TGN38/46, the SNARE membrane fusion proteins, and transmembrane enzymes/peptidases also cycle between endosomes and the TGN (90,91). Furthermore, the protein toxins ricin, Shiga toxin and cholera toxin take advantage of this pathway to reach the ER. There is evidence for more than one trafficking pathway between early endosomes and the TGN, and cargo may be transported from early, recycling or late endosomes. CI-MPR is retrieved from late endosomes to the TGN in a Rab9-dependent manner (36); however, CI-MPR can also be

retrieved from earlier compartments (94-96). The precursor protein-cleaving enzyme furin is also transported via late endosomes to the TGN (38). In contrast, Shiga toxin and ricin seem to bypass late endosomes on their way to the TGN and traffic independently of Rab9 (97,98).

Four different kinases have been reported to play a role in endosome-to-Golgi transport of toxins. The serine/threonine kinase PKC δ and the MAP kinase p38 are activated by Shiga toxin and important for efficient transport to the TGN (82,83), and β -arrestins seem to serve as negative regulators of the p38 dependent pathway (99). Protein kinase A type II α was found to regulate endosome-to-Golgi transport of ricin (100), and hVps34 is responsible for a specific pool of PI(3)P required for efficient transport of both Shiga toxin and ricin to the TGN (papers II and III).

Lipids other than PIs are also crucial for efficient endosome-to-Golgi trafficking of toxins. Cholesterol is required for targeting of ricin (101) as well as Shiga toxin (102) to the TGN. The lipid composition of the Gb3 receptor is also important for transport of Shiga toxin to the TGN and further to the ER (103-105).

Coat proteins and adaptors necessary for Shiga toxin transport include clathrin and its binding partner epsinR, as well as dynamin (106,107) and the clathrin-associated PI(4,5)P₂ phosphatase OCRL1 (108). Dynamin, but not clathrin, is also required for efficient transport of ricin (98,109). The retromer complex constitutes a coat different from clathrin, and its components hVps26, SNX1 and SNX2 have been shown to mediate endosome-to-Golgi transport of Shiga toxin (110,111) (paper III). SNX2 and SNX4, but not SNX1, are essential for transport of ricin to the TGN (paper II). Furthermore, EHD3 (eps15 homology domain-containing protein 3) and one of its interaction partners, the Rab4/5 effector rabenosyn-5, are necessary for SNX1 and SNX2 localization and thereby Shiga toxin transport from early endosomes to the Golgi (112).

The two isoforms of the small GTPase Rab6, Rab6A and Rab6A', are both involved in ricin transport (paper I), whereas only Rab6A' so far has been shown to function in transport of Shiga toxin to the TGN, along with two Rab6 effectors, the RabGAP Rab6IP2, and the Rab6-binding TATA element modulatory factor (TMF) (113-116). Rab11, being localized to early/recycling endosomes, is necessary for efficient transport of Shiga toxin, but not ricin (98,117). The Golgi-localized Rab43 and its RabGAP RNTre also seem to be needed for transport of Shiga toxin to the TGN and for maintaining Golgi morphology (118). Other RabGAPs identified as regulators of Shiga toxin transport are EV15, TBC1D10A, TBC1D10B, TBC1D10C and TBC1D17; however, possible effects on endocytosis have not been tested and their target Rabs remain unknown (118).

Tethering factors involved in Shiga toxin transport include the small GTPase Arl1 and its effector golgin-97 (119,120), golgin-245 (121), GCC185 (122), the COG (conserved oligomeric Golgi) complex (123), and the GARP (Golgi-associated retrograde protein) complex (124). Fusion of both Shiga toxin- and ricin-containing vesicles to the TGN is mediated by the SNARE complexes syntaxin5/Ykt6/GS15/GS28 and syntaxin6/syntaxin16/Vti1a/VAMP3/4 (113,125,126). The cycling *cis*-Golgi trans-membrane protein GPP130 (Golgi phosphoprotein 130) is also involved in endosome-to-Golgi trafficking of Shiga toxin, but its exact function remains unknown (127,128).

Furthermore, Shiga toxin increases the assembly of microtubules in cells (86), and both microtubules and the motor protein dynein are required for transport of this toxin (87).

Golgi-to-ER transport and translocation to the cytosol

ER-resident proteins contain a specific sequence that is recognized by the KDEL-receptor, and they are recycled back to the ER from the Golgi by a mechanism dependent on coatamer protein I (COPI). Possessing a KDEL-like sequence, *Pseudo-*

monas exotoxin A exploits this pathway to reach the ER (129). Ricin and Shiga toxin are transported to the ER despite their lack of a KDEL sequence (15,130), and they have both been shown to traffic independently of COPI (131-133). Shiga toxin follows a Rab6-regulated pathway that is also used by Golgi glycosylation enzymes (114,131,134,135). Furthermore, Cdc42, microtubule and actin have been described as regulators of Shiga toxin transport to the ER (136-138), with the movement on actin filaments being mediated by myosin motors (139).

The ER chaperone protein disulfide isomerase (PDI) cleaves the disulfide bridge between ricin A and B-chain before the A-chain is translocated to the cytosol (140). This mechanism was previously described for dissociation of the A- and B-subunits of cholera toxin (141) and *Pseudomonas* exotoxin A (142).

For translocation the toxin A-chains are thought to exploit the ERAD quality control pathway for misfolded proteins. However, they escape ubiquitin-mediated proteosomal degradation presumably due to their low lysine content as shown for ricin (143). Ricin has been reported to interact with EDEM, a protein involved in retrotranslocation (144), as well as to translocate through the Sec61-channel (145). Also Shiga toxin, cholera toxin and *Pseudomonas* exotoxin A have been shown to associate with the Sec61-complex (146-149). Moreover, Shiga toxin interacts with the ER chaperone BiP (150).

Aims of the study

The main objective of this project has been to identify new components in the retrograde trafficking of ricin and Shiga toxin, preferably in endosome-to-Golgi transport, and to study further details of the clathrin-dependent entry mechanism of Shiga toxin. Studies of toxin transport are helpful in revealing new players of these pathways, and the specific aims of the project were as follows:

- ❖ To investigate the role of the Rab6 GTPase splice variants Rab6A and Rab6A' in endosome-to-Golgi transport of ricin.
- ❖ To study whether the PI3-kinase hVps34 regulate toxin transport.
- ❖ To investigate if members of the sorting nexin protein family, namely SNX1, SNX2 and SNX4, are important for endosome-to-Golgi trafficking of Shiga toxin and ricin, and if the SNXs' requirement for membrane lipids may be sustained by hVps34.
- ❖ To study whether Shiga toxin, being able to stimulate its own uptake, is capable of increasing the formation of CCPs at the plasma membrane, and whether the observed increase is mediated by the kinase Syk.

Summary of publications

Paper I. Transport of ricin from endosomes to the Golgi apparatus is regulated by Rab6A and Rab6A'.

When we initiated our study, the splice variants of the Golgi-localized small GTPase Rab6 had been proposed to take part in different steps of retrograde trafficking. Rab6A' had been shown to mediate endosome-to-Golgi transport of Shiga toxin (113), whereas Rab6A had been suggested as a mediator of Shiga toxin transport from Golgi to ER (131,135). We specifically depleted each isoform by siRNA to determine if Rab6A and/or Rab6A' played any role in endosome-to-Golgi transport of ricin. Transport of ricin to the TGN was inhibited upon Rab6A knockdown when mRNA levels were reduced by more than 40 % but less than 75 %. When Rab6A mRNA levels were reduced by more than 75 %, Rab6A' mRNA was up-regulated, and inhibition of ricin transport was no longer observed. This suggests that Rab6A' is able to compensate for the loss of Rab6A. Furthermore, an almost complete knockdown of Rab6A' inhibited ricin transport by ~40 %. However, in experiments where Rab6A at the same time was up-regulated, the inhibition of ~40 % was still observed. Combined knockdown of Rab6A and Rab6A' reduced transport to the TGN by ~70 %.

Less co-localization between ricin and TGN46 was observed by immunofluorescence microscopy when Rab6A was knocked down without any up-regulation of Rab6A', and ricin seemed to localize further away from the nucleus than in control cells.

Similar results were obtained upon combined Rab6A/A' knockdown. We concluded that both Rab6 isoforms are important in transport of ricin to the TGN and that they have some functional redundancy. Yet, only Rab6A' is able to fully compensate for loss of the other isoform. This may indicate that the two isoforms control parallel transport pathways between early endosomes and the TGN.

Paper II. Phosphoinositide-regulated retrograde transport of ricin: Crosstalk between hVps34 and sorting nexins.

Phosphoinositides are important regulators of membrane traffic whose formation is specifically regulated by kinases and phosphatases. The main supplier of PI(3)P on early endosomes is the PI3-kinase Vps34 (151), which in yeast has been shown to regulate endosome-to-Golgi transport via the retromer complex (53). We therefore investigated if hVps34 was involved in endosome-to-Golgi transport of ricin and if any effectors of hVps34 in this pathway might be identified. Perturbation of hVps34 activity by the use of PI3-kinase inhibitors, overexpression of a dominant negative mutant, and siRNA led to reduced endosome-to-Golgi transport in HEK 293 cells, thus confirming the importance of this kinase. Moreover, ricin was localized to Rab7-positive endosomes when hVps34 activity was altered.

We considered the mammalian orthologs of the yeast retromer component Vps5p, SNX1 and SNX2, as potential effector proteins for hVps34 in ricin transport. SNX4, a different PI(3)P-binding SNX, had been shown to mediate a retromer-independent retrieval pathway to the Golgi in yeast (60). We therefore used siRNA to knock down SNX1, SNX2, and SNX4 and measured endosome-to-Golgi transport of the toxin. Knockdown of SNX2 or SNX4 reduced ricin transport to the TGN by ~40 %, similar to the effect observed by hVps34 siRNA, whereas a combined knockdown of SNX2 and SNX4 led to as much as 70 % reduction. Furthermore, transport of ricin to the TGN was able to

proceed as normal even in the absence of SNX1. These results identify SNX2 and SNX4 as effectors in hVps34-dependent transport, and may suggest that SNX2 and SNX4 operate in parallel and retromer-independent pathways between endosomes and the TGN.

Paper III. SNX1 and SNX2 mediate retrograde transport of Shiga toxin.

The retromer protein sorting complex on early endosomes has previously been shown to mediate retrieval of the CI-MPR from endosomes to the TGN (95,96). SNX1 and SNX2 have been identified as mammalian orthologs of the yeast retromer component Vps5p (152,153). Thus, we considered them as candidates for mediating endosome-to-Golgi trafficking of Shiga toxin as well as ricin. We used siRNA to specifically deplete Vero cells of SNX1 and SNX2 separately or in combination, and we obtained at least 80 % knockdown in all experiments. After knockdown of SNX1 or SNX2 alone, transport from endosomes to the TGN was reduced by ≥ 40 %, whereas a combined knockdown gave an ~ 80 % reduction. The endocytosis of Shiga toxin was not affected upon knockdown of neither SNX. Treatment of the cells with the PI3-kinase inhibitor wortmannin reduced the transport of Shiga toxin to the TGN by 50-75 % in a dose-dependent manner. We observed a similar response after knockdown of hVps34 by siRNA, suggesting that hVps34 is responsible for a pool of PI(3)P important for Shiga toxin transport in the same way as for ricin. Our results demonstrate that SNX1 and SNX2 are both important for endosome-to-Golgi transport of Shiga toxin and that they are only partly redundant.

Paper IV. Shiga toxin increases formation of clathrin coated pits through Syk kinase.

Clathrin-mediated endocytosis is an important entry mechanism for Shiga toxin (14). Several reports show that binding of Shiga

toxin to the Gb3-receptor induces signaling cascades in cells (80,81,86). Our lab has previously shown, that among other proteins, the tyrosine kinase Syk is activated and found to be important for the endocytosis of Shiga toxin (84). Shiga toxin-mediated activation of Syk leads to phosphorylation of CHC and promotes the formation of a complex between Syk and clathrin. Bearing this in mind, we wished to study if Shiga toxin might have any effect on clathrin recruitment. Previous reports have suggested that binding of ligands such as EGF and NGF to their tyrosine kinase receptors may increase the recruitment of clathrin to the plasma membrane (74-76). We studied the amount and dynamics of CCPs before and after incubation with Shiga toxin/ Shiga toxin B by means of EM and live-cell confocal imaging. EM revealed that there was a 30-40 % increase in CCPs of HeLa and HEp-2 cells upon incubation with Shiga toxin. The increase was reproduced for Shiga toxin B by spinning disk confocal microscopy in HeLa cells stably expressing the AP-2 subunit $\sigma 2$ coupled to EGFP. The new CCPs were internalized in the same way as normal CCPs. Interestingly, lifetime and maximum intensity distributions were shifted towards higher values. We also studied the uptake of Tf after addition of Shiga toxin B to the cells at various time points ranging from 2 to 15 min; however, there was no effect on Tf uptake. When inhibiting Syk with piceatannol, an increase in the formation of CCPs was no longer observed, indicating that Syk is important for this process. We propose that Shiga toxin, when binding to Gb3, induces activation of Syk and formation of a complex between Syk and CHC, and subsequently increases the formation of functional clathrin coats.

Discussion

Rab GTPases, phosphoinositides and hetero-oligomeric protein complexes are crucial elements in intracellular trafficking. In the following I will discuss the importance of several such components in transport of ricin and Shiga toxin to the Golgi as well as the effect of Shiga toxin on the clathrin coated pit machinery that we established during this study.

Shiga toxin and the formation of clathrin-coated pits

Shiga toxin has been shown to play an active part in its uptake and retrograde transport in cells. Our lab has previously demonstrated that Syk, being activated by Shiga toxin, phosphorylates clathrin and is important for Shiga toxin endocytosis (84,85). In connection with this, we show in paper IV that Shiga toxin and Shiga toxin B increase the formation of CCPs at the plasma membrane in a Syk-dependent manner.

Signaling induced by ligand-binding to tyrosine kinase receptors has previously been found to stimulate the recruitment of CCPs. NGF increases the formation of CCPs and induces its clathrin-dependent uptake by signaling through the receptor tyrosine kinase TrkA (75). Similarly, EGF activates its receptor and induces Src-dependent phosphorylation of CHC and redistri-

bution of clathrin as well as recruitment of CCPs (74,76). The B-cell antigen receptor BCR has no kinase activity, but in spite of this has been shown to induce Src-dependent phosphorylation of CHC important for clathrin-mediated endocytosis of BCR when clathrin is associated with lipid rafts (78).

As shown in paper IV, inhibition of Syk prevents the increase in formation of CCPs, and we propose that the formation of a Syk-clathrin complex and Shiga toxin-induced phosphorylation of CHC is important for clathrin recruitment. Syk has also been shown to associate with clathrin and mediate endocytosis of the human rhinovirus via a PI(3)-kinase-dependent pathway (154). There seems to be a correlation in time between the maximum increase in the number of CCPs that we observe in paper IV and the maximum increase in Syk-induced CHC-phosphorylation (84). Yet, the mechanism of such a link needs further clarification. Accordingly, the site of Shiga toxin-induced phosphorylation on CHC was recently identified (85). This site, Y1477, is also phosphorylated during ligand-induced endocytosis of EGF (74) and lies within a domain that has been suggested to be involved in clathrin assembly (155,156). Another possibility is that the role of phosphorylated CHC is to recruit adaptor proteins necessary for the formation of CCPs.

Based on the fact that different cargo bind to different receptors and the discovery of several cargo-specialized adaptor proteins for clathrin-mediated endocytosis it has been suggested that cargo-specific subgroups of CCPs exist (19).

Because the transferrin receptor (TfR) is endocytosed independently of ligand-binding, transferrin (Tf) is frequently used as a marker of clathrin-dependent endocytosis. However, in our study we did not observe any increase in the uptake of Tf upon Shiga toxin B-incubation, a result raising the question whether Shiga toxin and Tf may be taken up partly into different types of CCPs. To address this issue, clathrin-mediated endocytosis of Shiga toxin and Tf should be followed by TIRF

microscopy. Shiga toxin and Tf, although co-localizing in immunofluorescence, are enriched in different sub-domains on early/recycling endosomes as observed by EM (107). Different requirements for endocytosis may lead to sorting at an early stage and thus result in localization to different microdomains of early endosomes (107,157).

The influenza virus has been shown to induce CCP formation at its binding sites, but whether the number of CCPs is increased in general is not clear (158). Epsin 1 has been identified as a cargo-specific adaptor for the influenza virus, and clathrin-dependent endocytosis of this virus seems to occur independently of AP-2 at 37°C (159). It has been claimed that endocytosis of EGFR may proceed independently of AP-2 (160), but the use of ligand-binding at 4°C in such studies has been criticized, and a later study performed at 37°C showed that AP-2 was essential for internalization of EGFR (161). Whether AP-2 is needed for Shiga toxin endocytosis is not known.

How signaling can be induced through binding of Shiga toxin to its glycolipid receptor has not been clarified. Receptor cross-linking by Shiga toxin may induce new interactions, and lead to binding of signaling proteins. A candidate protein may be the trans-membrane receptor Fas, whose extracellular domain interacts with Gb3, or other proteins sharing the same glycolipid-binding motif (162). Two unidentified proteins have also been found to interact with Shiga toxin (163). Moreover, Syk can be activated by mechanisms that are independent of phosphorylation, as shown when Syk binds to the integrin $\beta 3$ cytoplasmic tail (164,165). A signaling cascade involving other kinases may also be induced.

Sorting nexins and PI requirements in endosome-to-Golgi transport of toxins

In papers II and III we used siRNA to demonstrate that the PI(3)P-binding sorting nexins SNX1, SNX2, and SNX4 mediate endosome-to-Golgi trafficking of toxins. By interfering with the activity of the PI3-kinase hVps34 we also showed that a pool of PI(3)P made by hVps34 is required for this transport.

Proteins of the sorting nexin family share the phosphoinositide-binding PX-domain and have been implicated in sorting or budding events in the endocytic pathway (26,166,167). The retromer was first identified in yeast where it transports the vacuolar hydrolase receptor Vps10p from pre-vacuolar endosomes to the Golgi in a Vps34-dependent manner (53,58,168). The yeast retromer consists of two subcomplexes, a Vps26p/Vps29p/Vps35p trimer and a Vps5p/Vps17p dimer. SNX1 and SNX2 are both orthologs of yeast Vps5p, but during the course of our study, the role of SNX2 in the retromer was debated, as different studies gave conflicting results (152,153,169-173).

Genetic studies in mice indicate that SNX2 is indeed a retromer component (172), and it has been shown that SNX1 and SNX2 are essential but functionally redundant during embryonic development in mice (169,172) and in trafficking of the CI-MPR in HeLa cells (173). In paper III we concluded that SNX1 and SNX2 are both necessary for efficient endosome-to-Golgi trafficking of Shiga toxin. However, since knockdown of SNX1 or SNX2 alone also inhibits Shiga toxin transport, they are only partly redundant. The involvement of SNX1 in Shiga toxin transport to the TGN was confirmed by the work of others (110), and the importance of the retromer component hVps26 in Shiga toxin trafficking has also been demonstrated by siRNA (111). Insufficient siRNA-mediated knockdown or cell type differences may

be the reason behind the discrepancy between different studies of SNX2 in transport to the TGN.

SNX5 and SNX6 have been proposed as the mammalian orthologs of yeast Vps17p. SiRNA against these two SNXs inhibited retrieval of the CI-MPR to the Golgi, giving a phenotype similar to that of the known retromer components (59). Moreover, SNX6 was shown to form a complex with SNX1, and SNX5/6 was also required for the association of SNX1 with endosomes (59).

In paper II we reported that SNX2 and SNX4 mediate transport of ricin to the Golgi apparatus in a partly redundant manner, and we suggested that they act in parallel pathways. Furthermore, ricin transport seemed to proceed even in the absence of SNX1. With both SNX1 and SNX2 as parts of the retromer complex, such results may seem contradictory, but it is not unlikely that SNX2 can mediate a retromer-independent pathway to the Golgi. SNX1 and SNX2 may form homo-dimers as well as hetero-dimers with one another or with SNX5 and SNX6 (153). SNX1 has been shown to mediate retromer-independent sorting of PAR1 to lysosomes (174), and the other SNXs may have independent functions either alone or as part of other complexes. Consequently, we cannot exclude that SNX1 and SNX2 may act in parallel pathways when regulating transport of Shiga toxin to the TGN (paper III).

A complex consisting of SNX4, SNX41 and SNX42 has been shown to mediate a transport pathway independent of the retromer in yeast transporting cargo from an earlier endosomal compartment to the Golgi (60). The retrieval of CI-MPR, which requires functional retromer, was shown to proceed independently of SNX4 in HeLa cells (61). Thus, SNX4 and the retromer seem to function in parallel pathways also in mammalian cells, which is in agreement with our conclusion from paper II.

SNX4 contains a curvature-sensing BAR domain and may form a coat complex similar to the retromer together with yet unidenti-

fied proteins. Mammalian equivalents of SNX41 and SNX42 have not been established, but based on phylogenetic analysis SNX7 and SNX30 are possible candidates (26). There is so far no evidence for the formation of a complex between SNX2 and SNX4.

Although co-localizing to some extent on endosomes, SNX1 and SNX4 are found on separate tubular profiles arising from the same compartment (61). Different affinities for different PIs may target SNXs to separate membrane domains. SNX1 has similar affinities for PI(3)P and PI(3,5)P₂ (57). It is not yet clear whether it is the direct binding of PI(3)P to SNX1 and SNX2 or rather binding of PI(3,5)P₂ that is required for transport of toxins to the TGN. The PIKFyve kinase converts PI(3)P into PI(3,5)P₂, and upon siRNA-mediated suppression of PIKFyve retrieval of CI-MPR from endosomes to the Golgi is inhibited, whereas EGFR and TfR trafficking remain undisturbed (175). Thus, it has been suggested that SNX1 may work mainly in the PI(3,5)P₂-dependent pathway, whereas SNX4 has a role in endosome-to-TGN transport as well as transport between early endosomes and the early recycling compartment (61).

PI(3)P is concentrated in Rab5-positive domains of early endosomes (49) and Rab5 has been shown to activate hVps34 (176). Thus, Rab5 may promote the hVps34-catalyzed production of PI(3)P required for recruiting SNXs to early endosomes that we report in paper II. A recent report suggests that Rab5 and Rab7 recruit retromer components to the endosome in a sequential manner, with Rab5 recruiting the SNX dimer and GTP-bound Rab 7 binding directly to the hVps trimer (177). The authors provide evidence that overexpression of inactive Rab5 and siRNA against Rab7 perturb retrieval of CI-MPR to the Golgi (177). In contrast, a screen for RabGAPs inhibiting Shiga toxin trafficking showed that neither overexpression of mutant RabGAP5 nor siRNA against Rab5 was relevant for Shiga toxin transport to the

Golgi (118). Further studies are therefore needed to clarify the possible involvement of Rab5 and Rab7 in toxin transport.

Recently identified regulators of retromer function include two proteins of the EHD family, which contain an Eps15 homology domain that interacts with PIs. EHD1 and EHD3 are required for proper localization of SNX1 and SNX2 to endosomal tubules. Moreover, EHD3 and its interaction partner, the Rab4/5 effector rabenosyn-5 have been shown to mediate trafficking of Shiga toxin as well as CI-MPR to the TGN (112,178). It would be interesting to know whether such retromer-regulating proteins or effectors are exclusive for this pathway or if they also play a role in the SNX4-mediated route to the TGN.

Rab6A and Rab6A' in retrograde transport of toxins

Being localized to the Golgi apparatus (179), Rab6 has long been considered as a candidate for regulating transport to, from, or within this compartment. In paper I we demonstrated that the two Rab6 isoforms Rab6A and Rab6A' are both involved in endosome-to-Golgi transport of ricin, and that they are partly redundant. Moreover, when Rab6A mRNA was reduced below a certain level, Rab6A' was up-regulated and seemed to take over the function of Rab6A. Rab6A, in contrast, could not substitute for Rab6A'. This may indicate that Rab6A and Rab6A' regulate parallel pathways.

Compensatory transport mechanisms have also been described in other studies. In cells expressing mutant ϵ -COP, causing vesiculation of the Golgi apparatus, ricin was still capable of reaching the ER, most likely through the induction of an alternative pathway bypassing the Golgi (133).

Different strategies, such as overexpression of mutants (see Experimental considerations), inhibitory antibodies or studies of downstream effectors, have been used to elucidate the specific functions of the Rab6 splice variants, sometimes yielding conflicting results. When we initiated our study, overexpression of a dominant negative mutant of Rab6A' had been shown to strongly inhibit endosome-to-TGN transport of Shiga toxin (113), and this finding was later strengthened by the use of siRNA (114). Overexpression of a GDP-restricted mutant of Rab6A did not inhibit the cytotoxic effect of ricin; however, this may be due to induction of an alternative pathway or the method used (180).

Results obtained with overexpression of a GDP-restricted mutant of Rab6A had suggested a role in transport of Shiga toxin to the ER via a COPI-independent route (131,135). However, such a role for Rab6A was not confirmed by the later siRNA study (114). On the contrary, Rab6A' proved to be the Rab6 isoform regulating this pathway, and a specific role for Rab6A in the transport of Shiga toxin was no longer assigned (114). Since Shiga toxin and ricin differ in their requirements for transport, it is not unlikely that Shiga toxin traffics to the Golgi independently of Rab6A. Our unpublished data nevertheless indicate that also Rab6A may play a role in Shiga toxin transport to the Golgi. Since Rab6A' can be up-regulated upon depletion of Rab6A, such up-regulation may easily mask the effects of knocking down Rab6A, making these effects very difficult to measure.

Variations in mechanisms of action between Rab6A and Rab6A' may be explained by different requirements for effectors. Several Rab6-interacting proteins have been identified, but so far the only isoform-specific effector found is the motor protein Rabkinesin6, which only interacts with Rab6A and is involved in cytokinesis (45,181-183). Furthermore, dynein light chain interacts directly with both isoforms, but binds preferentially to GTP-bound Rab6A and GDP-bound Rab6A' and Rab6B (184). Both Rab6A and Rab6A' have been shown to interact with and regulate

recruitment of the dynactin complex to Golgi membranes and thereby mediate microtubule-dependent recycling from Golgi to ER (185,186). Rab6A' has also been shown to function in cytokinesis (187).

An increasing number of components in the retrograde machinery have been identified, and new studies have shed more light on how these factors are integrated. Most likely the role of Rab6A/A' is to bind effector proteins at the TGN and thereby promote fusion. Proper function of the COG complex, a putative tethering factor that promotes Shiga toxin transport to the TGN, has been reported to depend on active Rab6 (123,188). The COG complex seems to interact with the syntaxin5/Ykt6/GS28/GS15 SNARE complex, which also has been found to play a role in trafficking of Shiga toxin (123,125). In addition, it has been described that Rab6 may regulate a pathway separate from the COG-dependent route involving the protein complex ZW10/RINT-1 (188). It would be of interest to study if Rab6A and Rab6A' might play different roles in these pathways.

Rab6 has also been shown to mediate the localization of the coil-coiled tethering protein GCC185 to the TGN in cooperation with Arl1; and GCC185 is again involved in Shiga toxin transport (122,189). TMF is yet another protein that modulates Rab6-dependent trafficking of Shiga toxin from early endosomes to the TGN and Golgi-resident enzymes to the ER (116).

Rab6 and Rab11 seem to regulate Shiga toxin transport in a sequential manner, possibly linked by Rab6-interacting protein 1 (R6IP1) (113,190). In a screen for RabGAPs inhibiting trafficking of Shiga toxin to the Golgi, RN-tre and 5 other GAPs were identified, and the target of RN-tre, the Golgi-localized Rab43, was shown to regulate transport of Shiga toxin (118). Accordingly, not yet investigated Rabs may contribute to the transport of ricin and Shiga toxin to the TGN, either acting sequentially or in parallel pathways.

Protein trafficking disorders related to Rabs and SNXs

Studies of disease may provide insight into compensatory mechanisms of the trafficking machinery and vice versa. Inherited mutations of Rab proteins and Rab-associated proteins seem to be associated with several disorders affecting the immune system and the neuronal system (191,192).

Loss of function of the Rab27a isoform can be compensated by Rab27b; however, Rab27b is not expressed in the lysosome-related organelles of melanocytes and cytotoxic T-cells (193). Mutations in Rab27a therefore specifically affect lysosomal transport in these cells, resulting in pigment dilution of the hair and uncontrolled T-lymphocyte activation, known as the Griscelli syndrome type II. Also mutations in Rab7, Rab3GAPs and GDP dissociation inhibitor 1 (GDI1) are linked to such diseases (192),

Mutations leading to disease have not yet been discovered for Rab6; however the Rab proteins may also play a more indirect role. It has been suggested that Rab6A/A' and Rab1 target the protein OCRL1 (oculocerebrorenal syndrome of Lowe protein 1) to Golgi membranes in a partly redundant manner with Rab1 being the most essential. The Rab proteins then seem to stimulate the phosphatase activity of OCRL1 (194). OCRL1 is involved in endosome-to-Golgi trafficking, and mutations in this protein cause accumulation of PI(4,5)P2 resulting in the Lowe syndrome (108).

Defects in the retrograde trafficking pathway are also associated with more common diseases such as Alzheimer and cancer.

Rab6 is up-regulated in brain tissue from patients with Alzheimer disease, and the increase in Rab6 levels does not seem to be due to activation of the unfolded protein response (195). The authors propose a role for Rab6 in post-ER quality control and that there

is an increased demand for Golgi-ER-recycling because of accumulation of proteins in the Golgi in Alzheimer disease brain.

Defects in the retromer pathway from endosomes to the Golgi apparatus may also play a role in late-onset Alzheimer disease (196,197). Vps35 expression levels on microarray are reduced in Alzheimer disease brain (196), and SNX1 and Vps35 seem to be involved in the retrieval of the amyloid precursor protein (APP) - binding receptor SorLa to the TGN (92). It has been proposed that when transport of APP from endosomes to the Golgi is delayed, there is an increase in the processing of APP into the amyloid- β peptides which are accumulated in Alzheimer disease brain (197).

Furthermore, down-regulation of SNX1 has been shown to play a role in development of colon cancer, probably due to increased signaling from endosomes (198).

Experimental considerations

siRNA

Small interfering RNA (siRNA) has become a major tool for studying protein function. RNAi was first discovered as a post-transcriptional regulatory mechanism in *Caenorhabditis elegans* (199), and it was later shown that 21 nucleotide RNA oligos delivered to mammalian cells could silence the protein of interest (200). However, there are several non-specific effects to consider. Lipid-based transfection reagents have been shown to alter the gene expression profile as opposed to using electroporation for siRNA delivery (201). Delivering siRNA to cells also triggers the interferon response and thereby activates the Jak-Stat signaling pathway (202). Yet, the greatest concern is the non-target effects arising from sequence similarities between the mRNA of interest and other mRNAs. Similarities in as few as 5 base-pairs in the 3' UTR-region may cause unwanted silencing of off-target mRNA,

an effect which is difficult to predict (203). It is therefore essential to use several siRNA oligos against a target protein to minimize the risk of false conclusions.

In our studies we used both vector-based and oligo-based siRNA. Vector-based siRNA is less suitable in cells where the transfection efficiency is low, and it is also difficult to modulate knockdown. It may be of advantage for proteins with very long half-lives, since re-transfection is not required; however we observed an efficient knockdown at the protein level from 48-96 hours after transfection in all our experiments.

Another pitfall may be the long-term nature of the siRNA experiments, which may allow for induction of compensatory mechanisms and transport pathways not operating under normal conditions. In the case of Rab6A/A' the time-course may be of importance for up-regulation of the other isoform.

Overexpression

Transient overexpression of mutant proteins was used in paper II to study the role of hVps34 in transport of ricin, and the data obtained were in agreement with the effects found by siRNA. The use of active or dominant negative mutants of Rab6A and Rab6A' seems to yield some conflicting data compared to results obtained by siRNA. Overexpression of for instance the Rab6A isoform may sequester effector proteins that are common for both isoforms. As a consequence, an observed phenotype may not be the result of activating Rab6A, but rather of inhibiting Rab6A', and the use of mutants should therefore be combined with other methods.

Early studies using overexpression of wildtype and mutant SNX1 and mutant SNX2 indicated a role for these proteins in the transport of EGFR to lysosomes for degradation (170,204,205). In contrast, siRNAs against SNX1 and SNX2 had no effect on lysosomal trafficking of EGFR (57,170). It was suggested that

SNX1 overexpression induces tubules on early endosomes promoting lysosomal transport in general (57). Thus, overexpression does not seem to be suitable for studies of SNX function.

In paper IV we relied on HeLa cells stably expressing the σ 2-subunit of the clathrin adaptor complex AP-2 fused to EGFP. In BSCI cells (monkey kidney epithelial cells) this fusion protein was shown to co-localize with endogenous AP-2 and transiently expressed clathrin light chain and did not prevent the uptake of Tf (206).

Microscopy

Studies of clathrin-mediated endocytosis have benefited from advances in live-cell imaging. The use of spinning disk confocal microscopy as in paper IV allows more rapid image collection and less photobleaching than TIRF (total internal reflection fluorescence) and ordinary confocal; however, some out-of-focus light is collected. The problem may be reduced by installing a motorized spherical aberration correction (SAC) -unit, which corrects for the differences in refractive index between the lens, oil, coverslip, media and specimen. This set-up made imaging of internalizing CCPs in the same cell area possible for up to 15 minutes. A drawback is that the cell surface attached to the coverslip, the only surface suitable for imaging, in HeLa cells displays a number of static clathrin structures in addition to internalizing CCPs. It is not known whether such plaques have a biological significance or are artifacts of cell plating.

Measurements of endosome-to-Golgi transport.

For measuring transport of ricin and Shiga toxin to the TGN we took advantage of modified toxins containing sulfation sites (130,207). Sulfation is a TGN-specific modification of newly synthesized proteins, and by supplementing media with radioactive sulfate the amount of sulfated toxin reaching the TGN

can be measured by autoradiography. The total amount of sulfated proteins should also be taken into account to make sure that the effects observed are not due to alterations in the sulfation machinery, protein synthesis or experimental conditions. This is done by counting the radioactivity in TCA-precipitates from the samples.

Conclusions and perspectives

In paper IV we show that Shiga toxin increases the formation of CCPs at the plasma membrane, most likely via signaling through the Syk kinase. Shiga toxin as well as viruses and receptor tyrosine kinases have been shown to induce their own uptake through clathrin coated pits by kinase-dependent phosphorylation of clathrin heavy chain. Yet, further details about the mechanism behind the increase in CCPs as well as the first steps of Shiga toxin-induced signaling remain to be studied.

While over the last decades endocytic pathways and lysosomal trafficking have been thoroughly studied, the components of the retrograde pathway from early endosomes to the Golgi apparatus have gained less attention. In papers I, II, and III we have studied proteins that proved to be functionally redundant in toxin trafficking between endosomes and the TGN, either acting as parts of a complex, being interchangeable in the same pathway, or operating in parallel pathways. We showed that SNX1 and SNX2 are involved in transport of Shiga toxin from endosomes to the Golgi and that they are partly redundant. Furthermore, we provide evidence that ricin depends on parallel trafficking pathways regulated by SNX2 and SNX4 on its way to the TGN. HVps34 seems to catalyze the formation of PIs important for all these transport pathways. In addition, we established a role for the two

Rab6 isoforms Rab6A and Rab6A' in endosome-to-Golgi-transport of ricin.

To fully elucidate how the increasing number of identified components in endosome-to-Golgi trafficking are connected, further studies will be necessary. Rab6A and Rab6A' may regulate parallel pathways to the TGN or be partly interchangeable in the same pathway. We assume that these Rabs are important for docking transport vesicles to the TGN membrane, but we do not know whether they interact with one or both SNARE complexes involved in Shiga toxin transport, or whether they operate in the same pathway as SNX1, SNX2 or SNX4.

Mutations in the genes encoding for the most essential proteins of the trafficking machinery will cause death of the organism before birth. In contrast, functionally redundant proteins and compensatory mechanisms render the cell less susceptible to defects in one protein or transport pathway; however, the defects may still cause illness. Thus, we may expect that future studies of compensatory mechanisms in intracellular trafficking will contribute to increased understanding of disease.

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