Mechanisms in Intracellular Transport of Toxins

Sigrid S. Skånland

Centre for Cancer Biomedicine, Faculty Division Norwegian Radium Hospital, University of Oslo

Department of Biochemistry, Institute for Cancer Research, The Norwegian Radium Hospital, Rikshospitalet University Hospital

Department of Molecular Biosciences, Faculty of Mathematics and Natural Sciences, University of Oslo

RIKSHOSPITALET

Centre for Cancer Biomedicine

UNIVERSITY OF OSLO

FACULTY OF MATHEMATICS AND NATURAL SCIENCES

Series of dissertations submitted to the
Faculty of Mathematics and Natural Sciences, University of Oslo
Nr. 818

ISSN 1501-7710

Cover: Inger Sandved Anfinsen.
Printed in Norway: AiT e-dit AS, Oslo, 2008.

Produced in co-operation with Unipub AS.
The thesis is produced by Unipub AS merely in connection with the
thesis defence. Kindly direct all inquiries regarding the thesis to the copyright
holder or the unit which grants the doctorate.

Unipub AS is owned by
The University Foundation for Student Life (SiO)

“The universe is full of magical things
patiently waiting for our wits
to grow sharper”

~Eden Phillpotts, A Shadow Passes

List of publications

This thesis is based on the following original publications, included in the second part of the thesis. They will be referred to by their roman numerals I-IV.

I **Skånland SS**, Wälchli S, Utskarpen A, Wandinger-Ness A, Sandvig K (**2007**): Phosphoinositide-regulated retrograde transport of ricin: crosstalk between hVps34 and sorting nexins. *Traffic*, 8, 297-309.

II Wälchli S,* **Skånland SS**,* Gregers TF, Lauvrak SU, Torgersen ML, Ying M, Kuroda S, Maturana A, Sandvig K (**2008**): The Mitogen-activated Protein Kinase p38 Links Shiga Toxin-dependent Signaling and Trafficking. *Mol Biol Cell*, 19, 95-104.

III **Skånland SS**, Wälchli S, Brech A, Sandvig K: SNX4 in complex with clathrin and dynein: implications for endosome movement. *Submitted.*

IV **Skånland SS**, Wälchli S, Sandvig K: β–arrestins attenuate p38 mediated endosome to Golgi transport. *Submitted.*

Publications not included in this thesis:

Torgersen ML, Wälchli S, Grimmer S, **Skånland SS**, Sandvig K (**2007**): Protein kinase Cdelta is activated by Shiga toxin and regulates its transport. *J Biol Chem*, 282,16317-28.

Utskarpen A, Slagsvold HH, Dyve AB, **Skånland SS**, Sandvig K (**2007**): SNX1 and SNX2 mediate retrograde transport of Shiga toxin. *Biochem Biophys Res Commun*, 358, 566-70.

Ménétret JF, Schaletzky J, Clemons WM Jr, Osborne AR, **Skånland SS**, Denison C, Gygi SP, Kirkpatrick DS, Park E, Ludtke SJ, Rapoport TA, Akey CW (**2007**): Ribosome binding of a single copy of the SecY complex: implications for protein translocation. *Mol Cell*, 28, 1083-92.

* Equal contribution

Table of contents

Introduction

In order to understand how cells regulate endocytosis and intracellular transport routes, to gain knowledge about the proteins involved and their specific functions, it is important to define suitable model systems. Protein toxins have proven to be powerful tools to study the endocytic pathway, and important knowledge has been acquired due to these studies.

Protein toxins

Protein toxins are perhaps most known for the diseases they cause in humans, such as diphtheria, cholera and tetanus. But these molecules can paradoxically be used to our benefit (**Box 1**), and have turned out to become invaluable tools for the studies of intracellular trafficking (Schiavo and van der Goot, 2001; Sandvig and van Deurs, 2002). This is due to the long journey some of these toxins make inside cells in order to reach their final destination and eventually kill the cell.

One class of protein toxins, the AB-family, comprises both plant and bacterial toxins that share an overall structural organization. These proteins consist of an enzymatically active A-moiety, responsible for their toxic effect, and a cell binding B-moiety. The receptors for the B-moieties differ from one toxin to another. However, the toxins share the need to enter the cytosol in order to exert their toxicity. After binding to the cell surface, the toxin is endocytosed. The variety of endocytic pathways present in cells has partly been revealed through studies of protein toxins, which seem to exploit them all to their advantage (Sandvig *et al.*, 2002; Conner and Schmid, 2003). After endocytosis, the toxin can be transported by several different pathways (**Fig. 2**). In some cases, most of it is actually recycled back to the cell surface, meaning that it will not harm the cell. Another part will be degraded in the lysosomes, while only very little of the toxin is transported to the appropriate compartment from which it enters the cytosol. For some bacterial toxins, such as diphtheria toxin and anthrax toxin, translocation takes place when the toxin reaches endosomes with low pH (Sandvig and van Deurs, 2005). Shiga toxin and the plant toxin ricin, however, are transported via the Golgi apparatus all the way to the endoplasmic reticulum (ER) before translocation to the cytosol occurs (Sandvig *et al.*, 2005).

What is the purpose of this extensive voyage? There are several points to consider. To start with, some toxins are activated by proteases they meet on their way to the ER. Shiga toxin, for instance, is cleaved and thereby activated by furin, an enzyme that recycles between the plasma membrane and the Golgi apparatus (Garred *et al.*, 1995). Furthermore, the ER membrane is probably more permeable than the plasma membrane or the endosomal membrane due to its low cholesterol content. Also, protein translocons and chaperones are present in the ER, and some of these have been shown to play a role in toxin retrotranslocation (Schmitz *et al.*, 2000; Tsai *et al.*, 2001; Yu and Haslam, 2005; Slominska-Wojewodzka *et al.*, 2006).

The patient work of establishing the cellular pathways used by toxins has been fruitful. Important understanding of protein transport has been acquired (Sandvig *et al.*, 1992; Sandvig *et al.*, 2002). Furthermore, these toxins are now being used as tools

for further investigations of molecular mechanisms involved in their proper trafficking.

Ricin

Ricin is found in the seeds of the castor bean plant *Ricinus communis*. It consists of one A-moiety and one B-moiety, held together by a disulfide bridge (**Fig. 1**). The B-moiety is a lectin, and binds to the cell surface via glycoproteins and glycolipids with a terminal galactose.

The A-moiety acts by removing a single adenine residue from an exposed loop of the 28S ribosomal RNA (for review see (Olsnes and Kozlov, 2001)). This loop is involved in binding of elongation factors, and the modified ribosomes are therefore no longer able to support protein synthesis. Due to its catalytic activity, one toxin molecule is sufficient to inactivate a few thousand ribosomes per minute (Olsnes *et al.*, 1975). Thus, a single ricin molecule can be lethal to the target cell.

Shiga toxin

Shiga toxin is produced by the bacterium *Shigella dysenteriae*. It consists of one A-moiety linked to a pentamer of identical B-subunits (**Fig. 1**). The A-moiety consists of an A_1 and A_2 fragment, held together by a disulfide bond and the sequence that is cleaved by furin. Shiga toxin, like ricin, acts by inactivating ribosomes, and it is the A_1 fragment that is responsible for its toxic effect.

The B-moiety binds to the glycolipid Gb3 at the cell surface. Unlike ricin, Shiga toxin acts only on a limited number of cell types (Paton *et al.*, 1998). This is partly due to lack of receptor, but even when the toxin binds to the cell, this cell might be resistant. This can be explained by lack of transport to the Golgi apparatus and the ER (Sandvig and van Deurs, 1996).

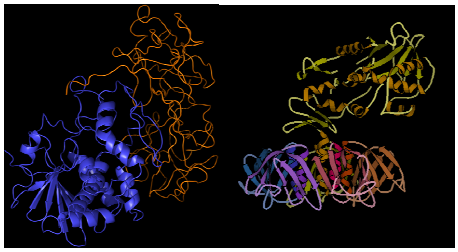

Figure 1: Structures of ricin and Shiga toxin
Ricin (left) consists of a cell-binding B-moiety (blue) coupled to a toxic A-moiety (orange) (PDB protein data bank; 2aai). Shiga toxin (right) is made up of five identical B-subunits (bottom) coupled to an A-moiety (top) (PDB protein data bank; 1dm0).

The endocytic pathway

Eukaryotic cells have developed several pathways in order to internalize material from the extracellular milieu and transport it to its proper destination inside the cell. Internalized proteins will generally be delivered to early endosomes first. From this compartment, they will either be recycled back to the cell surface, or sent to lysosomes for degradation. In other cases, the protein is sorted retrogradely to the Golgi apparatus (**Fig. 2**).

In our studies, we have mainly investigated the retrograde pathway used by protein toxins, and this will therefore be the focus of the following sections.

Endocytosis

The process by which cells internalize proteins together with patches of their plasma membrane is called endocytosis. Several forms of endocytosis exist.

Phagocytosis (literally, cell-eating) is used to internalize large particles such as bacteria, while **macropinocytosis** (literally, cell-drinking) is used to internalize large amounts of fluid (**Fig. 3**) (Cardelli, 2001). The vesicles formed by these types of endocytosis are large compared to other forms of endocytosis (>0.5-1 μm in

diameter). This is due to large scale actin-mediated remodelling of the plasma membrane (**Fig. 3**). Among the many receptor-mediated endocytic pathways, **clathrin-dependent endocytosis** constitutes a major route for selective receptor internalization in higher eukaryotes (**Fig. 3**). The vesicle formation begins with recruitment of clathrin, adaptors and endocytic accessory proteins to the plasma membrane, where they form patches ranging in diameter from 10 to more than 50 nm (Heuser, 1980). By the use of electron microscopy, stunning images of the clathrin network at various stages of invagination have emerged (Heuser, 1980). The network starts out as mainly hexagonal, with the presence of pentagons increasing with the degree of curvature (Heuser, 1980). The large GTPase dynamin is then rapidly recruited to the neck of the vesicle, probably through curvature-sensing proteins. In addition to dynamin, several other proteins mediate scission of the vesicle (Ungewickell and Hinrichsen, 2007). After vesicle internalization, the clathrin coat is released.

A second endocytic pathway that takes advantage of a coat protein is **caveolin-dependent endocytosis** (**Fig. 3**). Caveolae are commonly referred to as flask-shaped invaginations in the plasma membrane, but their morphology may vary (Richter *et al.*, 2008). In fact, they got their name because they resembled little caves when they were observed in the gall bladder epithelium (Yamada, 1955). Caveolae are 50-80 nm in diameter, and enriched in caveolins, sphingolipids and cholesterol (Thomas and Smart, 2008). However, caveolae are mostly stable structures at the plasma membrane, but can be induced to pinch off (Hommelgaard *et al.*, 2005).

In addition to these pathways, several **clathrin- and caveolin-independent pathways** exist. These can roughly be distinguished according to their dynamin dependency and the type of small GTP binding protein involved (Mayor *et al.*, 2007) (**Fig. 3**). One protein determinant of the dynamin independent pathway was recently shown to be flotillin-1 (Glebov *et al.*, 2006).

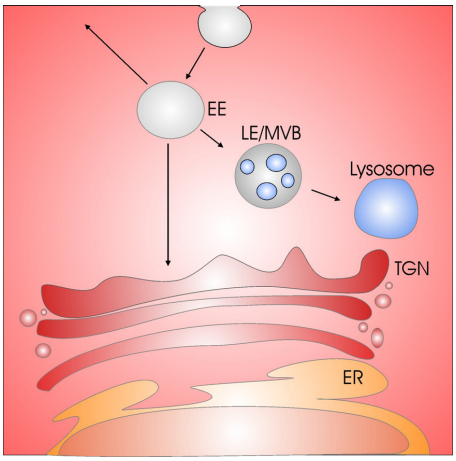

Figure 2: Intracellular pathways
After internalization, proteins can be sorted into different pathways. EE; early endosome, LE; late endosome, MVB; multivesicular body, TGN; *trans*-Golgi network, ER; endoplasmic reticulum.

Whereas Shiga toxin, at least in some cell types, is internalized mainly via clathrin-coated pits (Sandvig *et al.*, 1989; Sandvig *et al.*, 1991), ricin can use both clathrin-dependent and clathrin–independent pathways for its entry (Moya *et al.*, 1985; van Deurs *et al.*, 1989; Sandvig and van Deurs, 1991). Interestingly, though, Shiga toxin was recently shown to be able to induce clathrin independent membrane invaginations (Romer *et al.*, 2007).

Endosome to Golgi transport

From early endosomes, protein toxins can be recycled, sent to lysosomes for degradation, or sorted to the Golgi apparatus (**Fig. 2**). Although crucial for its

intoxication, less than 5% of the endocytosed ricin has been estimated to reach the *trans*-Golgi network (TGN) (van Deurs *et al.*, 1988). A well described transport route leading from early endosomes to the Golgi apparatus goes via late endosomes in a Rab9 dependent manner (Lombardi *et al.*, 1993; Riederer *et al.*, 1994). However, ricin is transported independently of Rab9, suggesting that it circumvents the late endosomes (Iversen *et al.*, 2001). Also Shiga toxin is transported directly from early endosomes to the Golgi apparatus (Mallard *et al.*, 1998).

Retromer is a protein complex involved in endosome to Golgi transport, first identified in yeast (Seaman *et al.*, 1998). It consists of five proteins, where Vps5p and Vps17p form a heterodimer which associates with a complex of Vps26p, Vps29p and Vps35p. SNX1 and SNX2 are the human orthologs of Vps5p. SNX1 is an accepted retromer component (Haft *et al.*, 2000; Gullapalli *et al.*, 2004), while the role of SNX2 is still debated (Haft *et al.*, 2000; Griffin *et al.*, 2005; Carlton *et al.*, 2005; Rojas *et al.*, 2006). The retromer subunits Vps26 and SNX1, as well as SNX2, have been shown to mediate endosome to Golgi transport of Shiga toxin (Popoff *et al.*, 2007; Bujny *et al.*, 2007; Utskarpen *et al.*, 2007). The role of retromer in ricin transport has not yet been fully investigated. But it is clear that SNX1 is not required for its normal transport, whereas SNX2 is (Paper I). SNX4, a protein first shown to mediate a pathway different from the retromer pathway in yeast (Hettema *et al.*, 2003), is also involved in endosome to Golgi transport of ricin (Paper I).

The cytoskeleton seems to be of importance for retrograde transport. Microtubules and the motor protein dynein have been shown to mediate endosome to Golgi transport of the mannose 6-phosphate receptor and of Shiga toxin (Itin *et al.*, 1999; Hehnly *et al.*, 2006). Interestingly, Shiga toxin induces microtubuli assembly (Takenouchi *et al.*, 2004; Hehnly *et al.*, 2006), and thus seems to regulate its own transport. This view is in agreement with reports of Shiga induced signalling which leads to enhanced Shiga transport (Lauvrak *et al.*, 2006)(Paper II). Microtubules and dynein appear to play a role also in ricin transport (Paper III). Dynein is found in complex with SNX4, and seems to mediate SNX4 positive vesicle movement (Traer *et al.*, 2007)(Paper III).

Translocation from ER to cytosol

From the Golgi apparatus, the transport continues all the way to the ER. Some members of the AB-family of toxins, such as cholera toxin, heat-labile enterotoxin and *Pseudomonas aeruginosa* exotoxin A (ExoA), have a C-terminal ER-retrieval signal; a KDEL or related tetrapeptide. Soluble proteins with such a signal can be transported from the Golgi to the ER by the KDEL receptor (Pelham, 1996). While the ER-retention sequence is required for

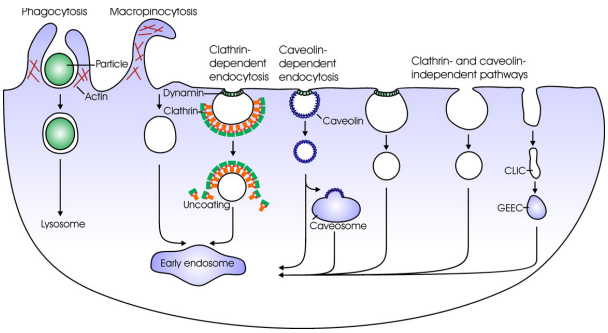

Figure 3: Endocytic pathways
Large particles are internalized by phagocytosis, whereas liquid is taken up by pinocytosis. Most internalized material is delivered to early endosomes via vesicular (clathrin- or caveolin-coated vesicles) or tubular intermediates (clathrin- and dynamin-independent carriers; CLICs). Other intermediate compartments are the caveosomes and glycosyl phosphatidylinositol-anchored protein enriched early endosomal compartments (GEEC). Adapted from (Mayor and Pagano, 2007).

ExoA toxicity (Chaudhary *et al.*, 1990), it rather enhances the transport of cholera toxin and heat-labile enterotoxin (Lencer *et al.*, 1995). It is not clear how toxins without an ER-retention sequence reach the ER. Still, Shiga toxin has been visualized in the ER using electron microscopy (Sandvig *et al.*, 1992; Sandvig *et al.*, 1994), and transport of ricin to the ER has been established (Rapak *et al.*, 1997).

When the toxins have reached the ER, they become processed into their respective A and B subunits (Ogata *et al.*, 1990; Hazes *et al.*, 1996; Tsai *et al.*, 2001; Rodighiero *et al.*, 2002; Spooner *et al.*, 2004). For both ricin and cholera toxin, the A chain is released from the holotoxin and unfolded in the lumen of the ER by the chaperone protein disulfide isomerase (PDI) (Tsai *et al.*, 2001; Spooner *et al.*, 2004). Eventually, the toxins are translocated to the cytosol where they exert their toxic effect. Accumulating evidence suggest that they take advantage of the ER-associated degradation pathway (ERAD), which normally removes misfolded or unassembled proteins from the ER (Lord *et*

Box 1 **Toxins in disease and health**

Protein toxins are the cause of several known diseases in human. Cholera toxin produced by the bacterium *Vibrio cholerae* is responsible for the massive diarrhea seen in Asiatic cholera (Lencer, 2001). Every year, 5 million people catch the disease in Asia and Africa. In its most severe forms, cholera is one of the most rapidly fatal illnesses known. A previously healthy person may die within hours of onset (Lencer, 2001).

Shiga toxin-producing *Escherichia coli* (STEC) strains have emerged as a significant cause of human gastrointestinal disease, which may result in life-threatening complications such as haemolytic-uremic syndrome. Food-borne outbreaks of STEC disease seem to be increasing, and may involve large numbers of people when food is mass-produced and mass-distributed (Paton and Paton, 1998).

Diphtheria is caused by diphtheria toxin, a third member of the AB-toxins. Symptoms include fatigue, fever, sore throat and difficulties with swallowing. The disease was previously quite common, but has largely been eradicated in developed countries due to widespread vaccination.

When it was discovered that some toxins consist of two distinct parts with different functions, the idea of using them for targeted cell killing arose. Immunotoxins were originally generated by coupling a protein toxin, or only its enzymatic moiety, to an antibody. Other immunologic proteins such as growth factors and cytokines have later been conjugated to toxins, and therefore a wider definition of immunotoxins has been proposed to include protein toxins connected to a cell binding ligand of immunologic interest (Kreitman, 2006). The goal is clear - targeted cancer therapy (Pastan *et al.*, 2007). Denileukin diftitox is an example of a promising immunotoxin. It is composed of the enzymatically active subunit of diphtheria toxin coupled to interleukin-2 (IL-2), and has now been approved for the treatment of patients with persistent or recurrent cutaneous T-cell lymphoma (Pastan *et al.*, 2006; Wong *et al.*, 2007).

Due to their ability to reach the cytosol, toxins can be used as vectors to deliver antigens for proteasomal processing. The resulting peptides will then be presented by major histocompatibility complex (MHC) class I molecules at the cell surface. By using selected antigens, the aim is to provoke a specific immune response (Smith *et al.*, 2002).

Toxins are still a serious threat to human health. However, knowledge of how they target cells has made it possible to use them to our advantage in medicine. This serves as a great example of how important it is to understand the molecular mechanisms underlying intracellular trafficking.

al., 2005; Slominska-Wojewodzka *et al.*, 2006). The toxins are recognised by the ERAD pathway due to their now unfolded state. ExoA, cholera toxin, Shiga toxin and ricin have all been found to interact with the Sec61 channel (Wesche *et al.*, 1999; Schmitz *et al.*, 2000; Koopmann *et al.*, 2000; Yu *et al.*, 2005), and ricin has been shown to be transported through it (Simpson *et al.*, 1999).

ERAD substrates are normally targeted for degradation by proteasomes in the cytosol. How do toxins avoid this destiny? Sequence analysis of AB toxins that enter the cytosol via the ER revealed that lysine residues were rare, if not absent, in their A subunits (London and Luongo, 1989). In contrast, toxins that do not travel to the ER have lysine residues distributed throughout their sequence (Hazes and Read, 1997). This feature was early proposed to be the mechanism for escaping ubiquitin-mediated protein degradation (Stein *et al.*, 1996), and has later been shown to be true for ricin (Deeks *et al.*, 2002).

Aims of the thesis

The overall goal of this work has been to increase our knowledge of mechanisms in intracellular transport, using toxins as model proteins. The specific aims of the different studies have been as follows:

I **Phosphoinositide-regulated retrograde transport of ricin: crosstalk between hVps34 and sorting nexins**
To investigate the role of phosphatidylinositol 3-phosphate (PI(3)P), produced by hVps34, in endosome to Golgi transport of ricin. And to identify effectors of PI(3)P in this pathway.

II **The Mitogen-activated Protein Kinase p38 Links Shiga Toxin-dependent Signaling and Trafficking**
To study the role of the signaling protein MAPK p38 in intracellular transport of Shiga.

III **SNX4 in complex with clathrin and dynein: implications for endosome movement**
To study mechanisms in endosome to Golgi transport mediated by the hVps34 effector protein SNX4.

IV **β–arrestins attenuate p38 mediated endosome to Golgi transport**
To identify effectors of the p38 regulated endosome to Golgi pathway used by Shiga toxin.

Summary of the included papers

Papers I and III, as well as II and IV, are more related to each other. For clarity, they are mentioned in this order below.

I: Phosphoinositide-regulated retrograde transport of ricin: crosstalk between hVps34 and sorting nexins

Different species of phophoinositides are known to localize to distinct membranes in the cell. PI(3)P is localized to early endosomes, multivesicular bodies (MVBs) and vacuoles (Gillooly *et al.*, 2000), where it can serve as an anchor for PI(3)P binding proteins. We were interested in the role of PI(3)P and its effectors in endosome to Golgi transport of ricin. The PI3-kinase hVps34 is responsible for the major pool of PI(3)P on endosomes (Shin *et al.*, 2005). By targeting this kinase, the endosomal level of PI(3)P will consequently be reduced. When the activity of hVps34 was inhibited, either by PI3-kinase inhibitors, by expressing dominant negative mutants of the kinase or by targeting small interfering RNA (siRNA) against its mRNA, we observed a reduced endosome to Golgi transport of ricin in HEK293 cells. The endocytosis of ricin remained unaffected by all these treatments.

In cells with reduced hVps34 activity, we observed ricin in endosomes positive for Rab7, a protein found on late endosomes. This was in contrast to the Golgi localization seen in control cells. The reduced Golgi transport observed in cells with inactive hVps34 could therefore possibly be due to retention of ricin in early endosomes that mature into late endosomes (Rink *et al.*, 2005).

We were next interested in identifying effectors of this PI(3)P dependent pathway. Retromer is a protein complex involved in endosome to Golgi transport, and Vps34 has been shown to regulate retromer function in yeast (Burda *et al.*, 2002). Two human orthologs of the retromer subunit Vps5, SNX1 and SNX2, have a PI(3)P binding PX domain, and were therefore of interest to us. Furthermore, the PI(3)P binding protein SNX4 has been shown to mediate an endosome to Golgi pathway different from the retromer in yeast (Hettema *et al.*, 2003), and so we chose to study this protein as well. We observed that retrograde transport of ricin could occur normally in cells where SNX1 was knocked down. However, knockdown of either SNX2 or SNX4 resulted in a similar reduction in ricin transport as when hVps34 was targeted. Furthermore, double knockdown of SNX2 and SNX4 resulted in an additional reduction in ricin transport, suggesting that these proteins may mediate different endosome to Golgi pathways.

III: SNX4 in complex with clathrin and dynein: implications for endosome movement

Having established that SNX4 plays a role in retrograde transport of ricin (Paper I), we aimed at understanding the mechanisms underlying this event better. We started by screening for SNX4 binding partners, and identified clathrin, tubulin and dynein. These findings suggested that SNX4 positive vesicles move on microtubuli guided by the minus-end directed motor protein dynein. This was confirmed by another report during our studies (Traer *et al.*, 2007). The role of clathrin, however, was not obvious. To investigate its

function in ricin transport, we took advantage of siRNA. When clathrin was knocked down, an increased ricin transport to the Golgi was observed both by biochemical and imaging assays. Furthermore, also other endosomal proteins including EEA1, SNX4 and the transferrin receptor (TfR) were localized to the Golgi region in clathrin knockdown cells. Perinuclear localization of TfR and EEA1 has previously been demonstrated using a clathrin mutant (Bennett *et al.*, 2001). These data suggested that clathrin serves as a negative regulator of endosome transport.

We characterized the clathrin interaction site on SNX4 to be a short sequence resembling the clathrin box motif (Dell'Angelica, 2001). Deletion of this motif reduced the SNX4-clathrin interaction with ~80%, and a small peptide displaying this sequence was sufficient to bind clathrin *in vitro*. Interestingly, the corresponding sequences in SNX1, SNX2 and SNX3 were also able to bind clathrin *in vitro*. Like other clathrin boxes, we found that the SNX4 sequence bound the terminal domain of clathrin.

We hypothesised that clathrin serves as a physical brake of SNX4 vesicle movement. Upon clathrin release, dynein may bind and thus mediate vesicle movement. To test this, we needed to characterize how the three proteins bind to each other. When clathrin was knocked down, dynein could still bind to SNX4, showing that clathrin is not required for the SNX4-dynein interaction. We further showed, by using the tubulin depolymerising agent nocodazole, that tubulin is not needed for the binding of either clathrin or dynein to SNX4. Rather, there was an increased binding of clathrin to SNX4 in the presence of nocodazole. This suggested that when the vesicle motility is affected due to altered tubulin network, SNX4 will remain bound to its "brake". Finally, we observed that dynein bound to the same region of SNX4 as clathrin. This indicated that they do not bind to SNX4 simultaneously.

We propose a model in which clathrin serves as a negative regulator of SNX4 vesicle motility through its interaction with SNX4. When clathrin is released, dynein may bind to SNX4 and mediate retrograde transport.

II: The Mitogen-activated Protein Kinase p38 Links Shiga Toxin-dependent Signaling and Trafficking

Shiga toxin is known to induce signaling events upon cell binding or following its internalization (Katagiri *et al.*, 1999; Ikeda *et al.*, 2000; Mori *et al.*, 2000; Cameron *et al.*, 2003; Takenouchi *et al.*, 2004; Lauvrak *et al.*, 2006). Interestingly, some of these studies demonstrate that Shiga plays an active role in its own transport (Lauvrak *et al.*, 2006; Hehnly *et al.*, 2006). Our lab had recently shown that Shiga binding activates the tyrosine kinase Syk in HeLa cells, and several proteins, including clathrin, become phosphorylated (Lauvrak *et al.*, 2006). This results in an increased uptake of Shiga into cells. We were therefore interested in finding out whether Shiga can regulate other steps of its transport.

We observed that Shiga rapidly activated the mitogen-activated protein kinase (MAPK) p38. When p38 was targeted, either with chemical inhibitors or siRNA, endosome to Golgi transport of Shiga was reduced in both HeLa and HEp2 cells. Interestingly, ricin was not affected by such treatments, suggesting that the requirement for p38 is specific to Shiga.

It has been known for a long time that divalent ions are able to modulate Shiga transport (Sandvig and Brown, 1987). Interference with calcium homeostasis affects both ricin and Shiga trafficking (Lauvrak *et al.*, 2002). Furthermore, a link between Ca^{2+} and p38 activation has been proposed (Chao *et al.*, 1992; Ikeda *et al.*, 2000; Takeda *et al.*, 2004; Fazal *et al.*, 2005). We therefore tested the involvement of Ca^{2+} in the p38

dependent pathway used by Shiga. We observed that Shiga strongly attenuated Ca^{2+} oscillations induced by histamine or ATP. The effect was seen after 15 minutes incubation with Shiga, suggesting that the toxin must be internalized in order to act. The effect was not dependent on p38, as p38 inhibition did not affect the outcome. We next demonstrated that Shiga transport is regulated by Ca^{2+}, and that its requirement is part of the p38 pathway.

That p38 plays a role in endosomal trafficking suggests that it is localized to endosomes. We observed that part of it localized to endosomes positive for Shiga, and confirmed its endosomal localization by a biochemical endosome purification assay. Interestingly, p38 was recruited to the early endosome fraction upon Shiga stimulation in a Ca^{2+} dependent manner.

The observations that Shiga stimulates activation and endosomal recruitment of p38 required for its transport, clearly shows that Shiga has neat ways of promoting its own transport.

IV: β–arrestins attenuate p38 mediated endosome to Golgi transport

What are the p38 effectors required for Shiga transport? This was the question we wished to answer for our next study. β-arrestin 1 has been shown to bind to and co-localize with phosphorylated p38 (McLaughlin *et al.*, 2006; McLaughlin *et al.*, 2008), while suppression of β-arrestin 2 blocks G-protein coupled receptor (GPCR) CXCR4-mediated activation of the MAPKs ERK (extracellular signal-regulated kinase) and p38 (Sun *et al.*, 2002). We therefore chose to investigate whether β-arrestins are effectors of the p38 pathway used by Shiga in HEp2 cells.

When either β-arrestin 1 or β-arrestin 2 was knocked down by specific siRNAs, an increased endosome to Golgi transport of the toxin was observed. A number of studies have shown that β-arrestins play important roles in endocytosis of receptors (Lefkowitz and Whalen, 2004). It was therefore important to test whether knockdown of these proteins affected the uptake of Shiga. It did not, allowing us to conclude that it is the endosome to Golgi transport step that is regulated by β-arrestins.

The increased Shiga transport upon β-arrestin knockdown was in contrast to what was previously observed upon reduced p38 activity (Paper II). However, when we treated cells with the p38 activator anisomycin, an equally increased Golgi transport was seen. To test whether β-arrestin and p38 mediate the same pathway, we treated cells with both β-arrestin siRNA and anisomycin. No additional increase in Shiga sulfation was detected, suggesting that β-arrestin and p38 act on the same transport route. Furthermore, combined knockdown of p38 and β-arrestin 1 resulted in the same phenotype as p38 knockdown alone. Cells with combined p38 and β-arrestin 2 knockdown were unhealthy.

We next studied the cellular localization of β-arrestins. By confocal microscopy, we observed that they were localized to Shiga positive structures. Not only so, they even seemed to change their distribution in the presence of Shiga. To investigate this further, we performed cellular fractionation studies. As for p38, we found that the β-arrestins were recruited to the early endosomal fraction upon stimulation with Shiga toxin. The same phenotype was observed upon anisomycin treatment. Since p38 is activated both by Shiga (paper II) and anisomycin, this suggested that β-arrestin is recruited to endosomes in a p38P dependent manner. In order to confirm this, we performed fractionation experiments on p38 knockdown cells. At this condition, a strong reduction in the β-arrestin recruitment was observed.

We next investigated whether an interaction between p38 and β-arrestin could be detected in our system. Indeed, p38 and β-arrestin co-immunoprecipitated

after Shiga treatment. Although we were unable to detect p38P in the precipitates, the time-point of the interaction correlated with activation of p38 by Shiga.

We suggest that β-arrestin binds activated p38 and attenuates its signaling.

Discussion

The work presented in this thesis has contributed to elucidate mechanisms in intracellular transport of ricin and Shiga toxin. We have shown that Shiga is able to regulate its own intracellular transport through signaling events, and identified effectors of this pathway. Our studies have further revealed mechanisms in endosome to Golgi transport of ricin. We have shown that SNX4 positive vesicles loaded with ricin are transported in a clathrin and dynein dependent manner, and that clathrin and dynein serve contrary roles in this regulated transport.

hVps34 and effectors in transport

hVps34 is the human homologue of yeast Vps34, and the only identified member of the class III PI3-kinases. This kinase phosphorylates phosphatidylinositol in its 3-position to produce PI(3)P. This lipid is highly enriched on early endosomes and in the internal vesicles of MVBs, and in yeast vacuoles (Gillooly *et al.*, 2000). As its name implies, Vps34 is required for vacuolar protein sorting in yeast (Schu *et al.*, 1993). It has further been shown that hVps34 is needed for normal functioning of MVBs (Futter *et al.*, 2001) as well as early to late endosomal transport (Stein *et al.*, 2003).

Studies to better understand how PI3-kinases regulate membrane trafficking in mammalian cells have largely been based on the use of chemical inhibitors. These investigations in combination with biochemical approaches lead to the identification of the early endosomal antigen 1 (EEA1), which directly binds to PI(3)P (Patki *et al.*, 1997). Further analysis revealed that a FYVE domain (named after four proteins initially found to contain this domain: Fab1, YGLO23, Vps27 and EEA1) at the C-terminal end of EEA1 was responsible for the specific binding to PI(3)P both *in vitro* and *in vivo* (Stenmark *et al.*, 1996; Burd and Emr, 1998; Gaullier *et al.*, 1998; Patki *et al.*, 1998). EEA1 is an important regulator of endosome fusion (Simonsen *et al.*, 1998; Christoforidis *et al.*, 1999).

Also other domains can bind phosphoinositides (Lemmon, 2003). A second PI(3)P binding domain is the Phox homology (PX) domain (Simonsen and Stenmark, 2001). A subgroup of this domain is found in the sorting nexin protein family (Teasdale *et al.*, 2001). Members of this family are regulators of membrane trafficking.

hVps34 and sorting nexins in ricin transport

With this knowledge as background, we chose to study the role of hVps34 in intracellular transport of ricin. A reduced endosome to Golgi transport was observed upon hVps34 inhibition or knockdown (Paper I). We next wished to identify effectors of this pathway. As mentioned above, sorting nexins have been implicated in membrane trafficking. We selectively studied SNX1, SNX2 and SNX4, which all bind to PI(3)P. SNX1 and SNX2 have been suggested to be part of the retromer, while SNX4 has been shown to mediate an endosome to Golgi retrieval pathway different from the retromer in yeast (Hettema *et al.*, 2003). We found that ricin transport was unaffected by SNX1 knockdown, while both SNX2 and SNX4

were required for normal transport to the Golgi (Paper I).

SNX1 and SNX2 are human orthologs of the Vps5 subunit in the yeast retromer (Horazdovsky *et al.*, 1997; Haft *et al.*, 1998). While SNX1 has been shown to interact with the core retromer complex (Haft *et al.*, 2000; Gullapalli *et al.*, 2004), the role of SNX2 is still debated (Haft *et al.*, 2000; Griffin *et al.*, 2005; Carlton *et al.*, 2005; Rojas *et al.*, 2006). Our results showing that SNX1 and SNX2 play different roles in ricin transport may suggest that they are not both part of the retromer, alternatively that they can also function without this complex. The latter is likely, as several proteins are known to serve different roles in cells. In either case, however, our findings suggest that the retromer is not critical for proper transport of ricin to the Golgi apparatus since knockdown of the established retromer component SNX1 did not affect ricin transport.

We further observed that simultaneous knockdown of SNX2 and SNX4 resulted in an additional reduction in ricin transport as compared to knockdown of either of the proteins alone. This indicates that SNX2 and SNX4 act on separate pathways. If we assume that the role of SNX2 in ricin transport is unrelated to retromer function, it is possible that at least three different endosome to Golgi pathways exist in mammalian cells.

Partners of SNX4 at the endosome

When we initiated our study on sorting nexins, very little was known about SNX4 function. Since we found that it is needed for normal transport of ricin (Paper I), we were interested in investigating further the mechanisms underlying SNX4 mediated transport. As a start, we identified SNX4 binding partners. Tubulin and the minus end-directed motor protein dynein were found in this screen, suggesting that SNX4 vesicle movement is mediated by dynein on microtubuli. During the progress of our work, a publication confirmed that SNX4 and dynein interact (Traer *et al.*, 2007). In this study, SNX4 was found to play a role in sorting of the transferrin receptor between early and recycling endosomes (Traer *et al.*, 2007).

It has previously been reported that Rab5 can mediate endosome movement on microtubuli in an hVps34-dependent manner (Nielsen *et al.*, 1999). In agreement with our studies, this report showed that treatment with the PI3-kinase inhibitor wortmannin, or inhibitory antibodies against hVps34, led to reduced minus-end directed motility (Nielsen *et al.*, 1999). The motor protein responsible for this movement was not identified (Nielsen *et al.*, 1999). In a later study by the same group, however, the kinesin-3 KIF16B was found to transport early endosomes to the plus end of microtubules in a process regulated by Rab5 and hVps34 (Hoepfner *et al.*, 2005).

In our screen for SNX4 interacting proteins, we also identified clathrin (Paper III). Clathrin has mainly been studied for its role in endocytosis. However, in addition to its localization at the plasma membrane, clathrin can also be found on intracellular membranes. The interaction between SNX4 and clathrin clearly required endosomal localization of SNX4, as no interaction was observed in the presence of wortmannin (Paper III). Treatment with this PI3-kinase inhibitor leads to endosomal dissociation of SNX4 (Paper I)(Traer *et al.*, 2007). In screens for clathrin interacting proteins, several sorting nexins have been reported, but not SNX4 (Borner *et al.*, 2006). The reason for this is not clear, but it could be due to a strict cut-off in the analysis of the data, as neither the clathrin recruiting protein Hrs (Raiborg *et al.*, 2001) was identified (Borner *et al.*, 2006).

Why the attraction to SNX4?

Having identified dynein and clathrin as SNX4 interacting partners, we focused our

further study on understanding how these proteins interact, and the role they serve in complex with SNX4.

A well established clathrin interacting motif is the so-called clathrin box (Dell'Angelica, 2001). This motif is only five amino acids long, and the consensus sequence is LΦpΦ(−), where L is leucine, Φ denotes a bulky hydrophobic residue, p a polar residue, and (−) is a negatively charged residue (Dell'Angelica, 2001). However, it is becoming clear that several versions of the clathrin box exist. Having found that SNX4 is a clathrin interacting protein (Paper III), we searched the SNX4 sequence for the presence of a clathrin box. The consensus sequence was not present. However, an "inverted" clathrin box was found in the PX domain, $E_{109}FELL_{113}$. When we deleted this sequence from SNX4, an ~80% reduction in clathrin binding was observed (Paper III). Furthermore, a small peptide displaying this motif was sufficient to pull down clathrin (Paper III). Like other clathrin boxes, the SNX4 sequence interacted with the terminal domain of clathrin. These data demonstrate that EFELL serves as a clathrin box in SNX4. Interestingly, the corresponding sequences in SNX1, SNX2 and SNX3 were also able to bind clathrin. We may thus have identified a new member of the clathrin boxes. We next performed an alanine scan of the SNX4 motif, meaning that each amino acid was replaced by an alanine, one at the time, to study their individual role in clathrin binding. The E109A mutant was still able to bind clathrin, perhaps even stronger than the wild type. While the four following mutants only showed background binding to clathrin (Paper III). This showed that especially the amino acids $F_{110}ELL_{113}$ were of importance for proper clathrin binding.

The clathrin motif in SNX4 is part of its PX domain. The sequence is located to the α1 helix, conserved among PX domain containing proteins (Seet and Hong, 2006). The crystal structure of the PX domain from $p40^{phox}$ suggests that this alpha helix is available at the protein surface (Bravo *et al.*, 2001). Interestingly, amino acids directly upstream of the clathrin motif in SNX4 are involved in the PI(3)P binding. Importantly, we cannot exclude that mutation analysis of this sequence might destabilize the alpha helix. However, the deletion mutant SNX4 109-113Δ showed a similar cellular localization as the wild type (Paper III).

What is the purpose of the SNX4-clathrin interaction? When SNX4 is knocked down, a reduction is seen in endosome to Golgi transport of ricin (Paper I). When clathrin was knocked down, however, the opposite effect was observed (Paper III). This suggested that clathrin serves as a negative regulator of ricin transport. We next studied clathrin knockdown cells by confocal microscopy, and saw that ricin was localized in large perinuclear structures together with SNX4. These structures were positive for the Golgi protein TGN46. This is in agreement with the role of SNX4 in ricin transport, and supports the biochemical data showing increased retrograde transport upon clathrin knockdown.

We hypothesised that clathrin serves as a physical brake of SNX4 vesicle movement. When clathrin is released from the complex, dynein can bind and mediate vesicle motility. Our data showing that dynein binds to SNX4 in a clathrin-independent manner, together with the finding that clathrin and dynein bind to the same region of SNX4, support such an hypothesis.

Conclusions from Papers I and III

Our studies of ricin transport presented in this thesis have shown that endosomal PI(3)P produced by hVps34 is required for normal endosome to Golgi transport (Paper I). We found that SNX2 and SNX4, but not SNX1, are effectors of this pathway (Paper I). Interestingly, the three sorting nexins seem to mediate three different endosome

to Golgi pathways in cells. We have further shown that clathrin and dynein bind to SNX4 at the endosome (Paper III). From our data we propose a model in which clathrin serves as a brake of vesicle motility when bound to SNX4. When clathrin is released, dynein can bind and mediate vesicle movement (**Fig. 4**).

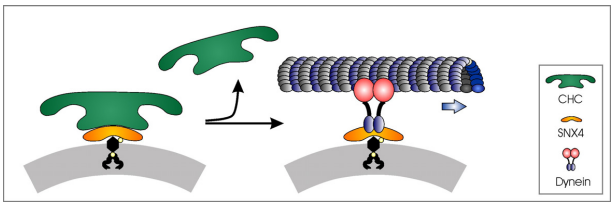

Figure 4: Simplified model of SNX4 mediated vesicle movement
When SNX4 is bound to PI(3)P on endosomes, it can interact with clathrin, probably upstream of its interaction with dynein. When clathrin is released, SNX4 may gain access to dynein and the vesicle move on microtubuli. Additional proteins may be involved.

Signaling

When the human genome was sequenced in 2001, one of the biggest surprises was the small number of genes it encoded. At that time, it was reported to be around 26000 protein coding genes (Venter *et al.*, 2001). Since then, the number has decreased, and it is now likely that a realistic number is closer to 18000, not much more than for the fruit fly *Drosophila melanogaster*. How is it possible that such a complex organism as the human does not have more genes? One of the explanations is that one protein can serve several roles in the cell, and its activity can be precisely tuned by small posttranslational modifications.

Phosphorylation is a posttranslational modification mediated by protein kinases, and the amino acids serine, threonine and tyrosine are the common targets. Protein phosphorylation may induce protein interactions, or lead to a change in the activity status of a protein. Depending on the protein, it can either be turned on or off by this modification. Signaling events are important for normal functioning of the cell, including its intracellular transport. When a ligand binds to its receptor at the plasma membrane, this may lead to autophosphorylation of the receptor and serve as signal for endocytosis, and thus silencing, of the ligand-receptor complex.

Signaling mediated by Shiga

As is the case when other ligands bind their receptors, Shiga triggers signaling events when binding to its cell surface receptor. A transient increase in microtubule levels is induced by Shiga binding (Takenouchi *et al.*, 2004; Hehnly *et al.*, 2006). Possibly, Shiga modifies the cytoskeleton in a manner that facilitates its retrograde transport (Hehnly *et al.*, 2006). The tyrosine kinase Syk regulates both the clathrin-mediated endocytosis of Shiga (Lauvrak *et al.*, 2006) as well as microtubule formation (Sulimenko *et al.*, 2006). It was therefore tested whether Syk mediates signaling between Shiga and the microtubuli, but this turned out negative (Hehnly *et al.*, 2006). Binding of Shiga induces activation of Syk, which again phosphorylates several proteins, among them clathrin (Lauvrak *et al.*, 2006). Syk activity is required for normal endocytosis of the toxin (Lauvrak *et al.*, 2006), and thus this represents another example of how Shiga can induce signaling to promote its own transport. Also Yes and Lyn, two additional members of the Src family kinases, are activated by Shiga (Katagiri *et al.*, 1999; Mori *et al.*, 2000). Not only tyrosine kinases are activated by Shiga. Members of the serine/threonine MAP kinases, including ERK1/2, JNK and p38, are also shown to be activated by Shiga (Ikeda *et al.*, 2000; Foster and Tesh, 2002; Cameron *et al.*, 2003; Smith *et al.*, 2003). However, these are often late events

related to ribotoxic stress, a consequence of ribosomal inactivation by the toxin.

p38 in Shiga transport

Since it was known that Shiga is able to induce signaling events to enhance its transport, we set out to investigate whether the MAP kinase p38 was involved in Shiga transport. In agreement with previous reports, we found that Shiga activates p38, with a peak around 15 minutes (Paper II). When p38 was knocked down or inhibited with chemical inhibitors, we observed a reduced endosome to Golgi transport of Shiga (Paper II). Interestingly, the transport of ricin was not affected by these treatments. A link between p38 activity and Ca^{2+} has previously been proposed (Chao *et al.*, 1992; Ikeda *et al.*, 2000; Takeda *et al.*, 2004; Fazal *et al.*, 2005), and cytosolic Ca^{2+} levels are known to affect Shiga transport (Sandvig *et al.*, 1987; Lauvrak *et al.*, 2002; Chen *et al.*, 2002). We therefore studied the effect of Shiga on Ca^{2+} homeostasis, and observed that Shiga strongly attenuated Ca^{2+} oscillations induced by ATP or histamine after 15 minutes incubation (Paper II). This phenotype was not sensitive to p38 inhibition. We next showed that the Ca^{2+} chelator BAPTA-AM, as well as TMB-8, an inhibitor of intracellular Ca^{2+} release, reduced endosome to Golgi transport of Shiga (Paper II). Furthermore, BAPTA-AM efficiently inhibited p38 activation, suggesting that free intracellular Ca^{2+} is required for this process. Most interestingly, we discovered that p38 is recruited to early endosomes in a Ca^{2+} dependent manner upon Shiga stimulation (Paper II). These data clearly support a link between Ca^{2+} and active p38. Since the effect of Shiga on Ca^{2+} homeostasis was independent of p38 activity, while Shiga induced p38 activation required cytosolic Ca^{2+}, we suggest that a Ca^{2+} signal is required for p38 activation. In agreement with this, our data show that p38 and Ca^{2+} mediate the same endosome to Golgi pathway, since there was no additional reduction in Shiga transport when treatments with TMB-8 and a p38 inhibitor were combined (Paper II). The Ca^{2+} requirement in endosome recruitment of p38 further confirm their crosstalk.

Although these findings are interesting in them selves, the role of Shiga adds another dimension to it. Without Shiga, we observed no activation of p38. And without Shiga, we observed no change in Ca^{2+} oscillations. In other words, Shiga induces signaling events which interplay and serve to enhance its transport.

β-arrestins in the p38 pathway

Having established that p38 plays an important role in retrograde transport of Shiga, we next wanted to determine effectors of this pathway. We were interested in finding binding partners of p38 involved in Shiga transport. Recent reports have shown that β-arrestin can scaffold GPCRs to MAP kinase cascades (McDonald *et al.*, 2000), and a specific link to p38 has been reported (Sun *et al.*, 2002; McLaughlin *et al.*, 2006). We therefore chose to investigate the role of β-arrestin in Shiga transport.

When either β-arrestin 1 or β-arrestin 2 was knocked down with siRNA, a strong increase in endosome to Golgi transport of Shiga was observed (Paper IV), opposite of what was seen upon p38 knockdown (Paper II). When cells were incubated with Shiga for 20 minutes, we detected an altered localization of β-arrestin by confocal microscopy. β-arrestin and the toxin were observed in the same endosomal structures (Paper IV). We confirmed the protein redistribution by a biochemical assay where we purify endosomes. Upon Shiga incubation, β-arrestin was recruited to the early endosome fraction together with p38 (Paper IV). This recruitment was possibly dependent on active p38, since we observed it also after treatment with

anisomycin, a p38 activator. And, importantly, p38 knockdown reversed this phenotype (Paper IV). β-arrestin 1 has previously been shown to bind phosphorylated p38 (McLaughlin *et al.*, 2006), and β-arrestins seem to have a preference for phospho proteins (Xiao *et al.*, 2007).

When cells were treated with anisomycin, a strong increase was seen in endosome to Golgi transport of Shiga (Paper IV), similarly to what was observed upon β-arrestin knockdown. To test whether p38 and β-arrestin regulate the same pathway, we treated cells with both anisomycin and β-arrestin siRNA. No additional effect should be observed if they mediate the same pathway, as was the case (Paper IV). Furthermore, combined knockdown of p38 and β-arrestin 1 resulted in the same phenotype as p38 knockdown alone. From these data, we suggest that β-arrestin is recruited to p38 positive endosomes in a p38P dependent manner. We suggest that β-arrestin binds to p38P and serves to attenuate its signal. This leads to an inhibition of Shiga transport. This view is in agreement with β-arrestin's role as silencer of receptor signaling at the plasma membrane (Lefkowitz *et al.*, 2004).

Conclusions from Papers II and IV

Our studies show that Shiga is an active player in its own transport. Upon Shiga binding, p38 is activated and recruited to endosomes in a Ca^{2+} dependent manner. These events are required for proper endosome to Golgi transport of the toxin (Paper II). We have further identified β-arrestin 1 and β-arrestin 2 as effectors of this pathway. β-arrestins negatively regulate endosome to Golgi transport of Shiga, and are recruited to endosomes in what seems to be a p38P dependent manner. We suggest that β-arrestins bind active p38 and attenuate its signaling (**Fig. 5**).

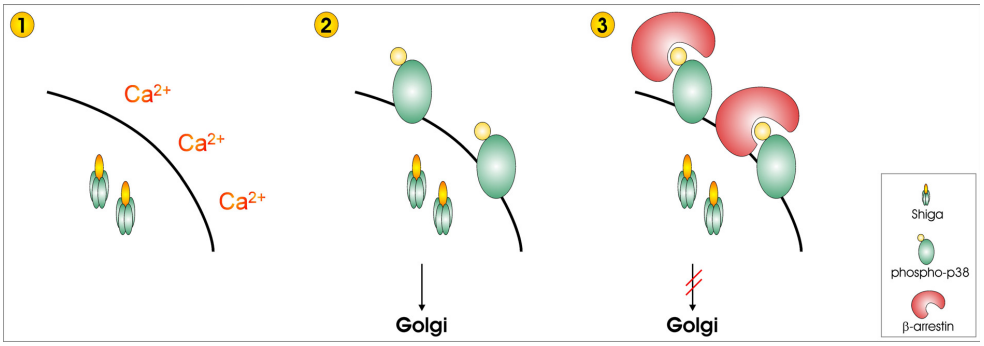

Figure 5: Tentative model of how p38 and β-arrestin regulate Shiga transport
When Shiga toxin reaches the endosome, a change in ATP/histamine-induced Ca^{2+} oscillations is detected (1). p38 is recruited to early endosomes in a Shiga and Ca^{2+} dependent manner, and active p38 is required for proper endosome to Golgi transport of Shiga (2). β-arrestin is recruited to early endosomes upon p38 activation, and is a negative regulator of Shiga transport (3).

Experimental systems

A variety of methods have been employed in order to achieve the results presented in this thesis. Detailed descriptions of all these methods are included in the respective publications, while a brief overview of some important techniques is presented below.

Cell lines

The studies presented in this thesis have been performed on cultured cell lines, the minimal model system for the human organism. We have taken advantage of three different cell lines for our purposes. The human embryonic kidney cell line HEK293 was chosen for our studies of PI(3)P and SNX mediated ricin transport. These cells are easily transfected, an important determinant for our selection. When the p38 study was initiated, we chose to use the HeLa cell line. These cells are derived from cervical cancer cells in the patient Henrietta Lacks (Jones, 1997). They are commonly used worldwide, and unlike the HEK293 cells, they are sensitive to Shiga toxin. In the progress of our study, however, we discovered that it was difficult to isolate endosomes from these cells with the protocol available, and chose to do those experiments in HEp2 cells. Since there are variabilities between cell lines, we needed to confirm that the requirement for p38 in Shiga transport was the same as in HeLa cells. In the follow-up story with β-arrestin, we used only HEp2 cells for simplicity. The HEp2 cells originate from larynx carcinoma (Moore *et al.*, 1955).

Transient transfection and knockdown

Common for the four papers included in this thesis is the study of which role selected proteins play in intracellular transport. To be able to study this, we have taken advantage of both transient expression of wildtype and mutant proteins, as well as knockdown of the protein of interest. The phenotype observed under such conditions gives a hint to the protein's role under normal conditions.

RNA interference (RNAi) is a method where small interfering RNA (siRNA) is used to target specific genes for silencing (Tuschl, 2001). There are two major steps to silencing (**Fig. 6**). First, the

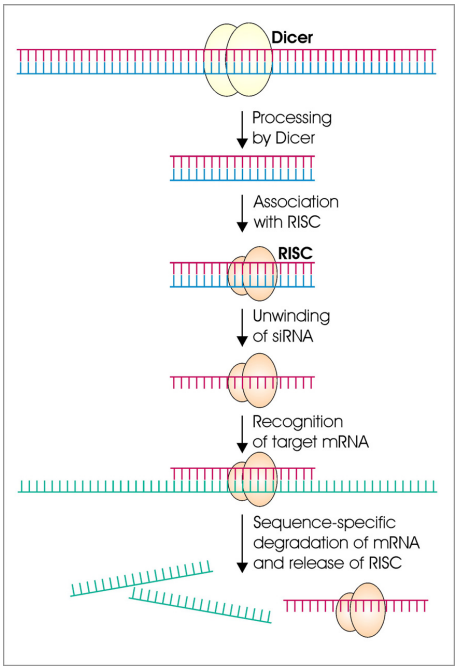

Figure 6: The RNAi pathway
Double-stranded RNA is processed into siRNA by Dicer. RISC binds to the siRNA and guides the antisense strand to the target mRNA for destruction.

introduced double-stranded RNA (dsRNA) is recognized by Dicer, an enzyme of the RNase III family of nucleases, and the dsRNA is cut into small double-stranded molecules termed siRNA. Next, an RNA-induced silencing complex (RISC) binds the siRNA and guides the antisense strand to the target mRNA. This causes cleavage of the target mRNA and further degradation.

In this work, both vector-based and oligo-based RNAi were applied. The oligo-based approach is now the most common, as one can easily order synthetic RNA ready to use from several companies. However, advantages with the vector-based approach are that it is cost-effective, and one can easily produce more of the construct when needed. It may also be more efficient than oligo-based siRNA since the product of the vector is continuously produced in the cell. On the other hand, it is more difficult to regulate the amount of siRNA in the cells by this approach.

Measurement of transport to the Golgi apparatus

In order to measure the amount of toxin that reaches the TGN, we have taken advantage of the fact that sulfation is a protein modification that takes place only in that compartment. Modified Shiga and ricin molecules containing sulfation sites have been constructed (Rapak *et al.*, 1997). By performing toxin incubations in medium where the only sulfate source is radioactive sulfate, one can easily measure the amount of toxin that has reached the TGN by quantifying their radioactive labelling.

Confocal microscopy

To visualize where proteins are located in cells, we have performed immunofluorescence experiments using confocal microscopy. When we study fixed cells, we use fluorescent antibodies to label the proteins of interest, making it possible to detect them with the microscope. It is also possible to directly fuse a fluorophore to the protein of interest, such as the green fluorescent protein GFP. In this way, one can avoid problems with poor antibodies or limited antibody availability. However, it is important to be critical to results obtained with overexpressed proteins as their effect may deviate from that of the endogenous protein.

Acknowledgements

This work was carried out in Prof. Kirsten Sandvig's laboratory at the Department of Biochemistry, The Norwegian Radium Hospital, Oslo from 2005-2008. Financial support from The Norwegian Radium Hospital made this work possible.

I am thankful to Prof. Kirsten Sandvig for welcoming me in her lab. I have appreciated her excellent supervision, as well as the freedom she has allowed me to follow my own ideas.

I wish to thank Prof. Sjur Olsnes and Prof. Harald Stenmark, as well as all other members of the Department of Biochemistry through these years, for contributing to a good scientific environment. A special thanks to Dr. Sébastien Wälchli for exceptional supervision.

Thanks to colleagues abroad,
to family and friends.

References

1. Bennett EM, Lin SX, Towler MC, Maxfield FR, and Brodsky FM (2001) Clathrin hub expression affects early endosome distribution with minimal impact on receptor sorting and recycling. *Mol Biol Cell,* **12,** 2790-2799.

2. Borner GH, Harbour M, Hester S, Lilley KS, and Robinson MS (2006) Comparative proteomics of clathrin-coated vesicles. *J Cell Biol,* **175,** 571-578.

3. Bravo J *et al.* (2001) The crystal structure of the PX domain from p40(phox) bound to phosphatidylinositol 3-phosphate. *Mol Cell,* **8,** 829-839.

4. Bujny MV, Popoff V, Johannes L, and Cullen PJ (2007) The retromer component sorting nexin-1 is required for efficient retrograde transport of Shiga toxin from early endosome to the trans Golgi network. *J Cell Sci,* **120,** 2010-2021.

5. Burd CG and Emr SD (1998) Phosphatidylinositol(3)-phosphate signaling mediated by specific binding to RING FYVE domains. *Mol Cell,* **2,** 157-162.

6. Burda P, Padilla SM, Sarkar S, and Emr SD (2002) Retromer function in endosome-to-Golgi retrograde transport is regulated by the yeast Vps34 PtdIns 3-kinase. *J Cell Sci,* **115,** 3889-3900.

7. Cameron P, Smith SJ, Giembycz MA, Rotondo D, and Plevin R (2003) Verotoxin activates mitogen-activated protein kinase in human peripheral blood monocytes: role in apoptosis and proinflammatory cytokine release. *Br J Pharmacol,* **140,** 1320-1330.

8. Cardelli J (2001) Phagocytosis and macropinocytosis in Dictyostelium: phosphoinositide-based processes, biochemically distinct. *Traffic,* **2,** 311-320.

9. Carlton JG, Bujny MV, Peter BJ, Oorschot VM, Rutherford A, Arkell RS, Klumperman J, McMahon HT, and Cullen PJ (2005) Sorting nexin-2 is associated with tubular elements of the early endosome, but is not essential for retromer-mediated endosome-to-TGN transport. *J Cell Sci,* **118,** 4527-4539.

10. Chao TS, Byron KL, Lee KM, Villereal M, and Rosner MR (1992) Activation of MAP kinases by calcium-dependent and calcium-independent pathways. Stimulation by thapsigargin and epidermal growth factor. *J Biol Chem,* **267,** 19876-19883.

11. Chaudhary VK, Jinno Y, FitzGerald D, and Pastan I (1990) Pseudomonas exotoxin contains a specific sequence at the carboxyl terminus that is required for cytotoxicity. *Proc Natl Acad Sci U S A,* **87,** 308-312.

12. Chen JL, Ahluwalia JP, and Stamnes M (2002) Selective effects of calcium chelators on anterograde and retrograde protein transport in the cell. *J Biol Chem,* **277,** 35682-35687.

13. Christoforidis S, McBride HM, Burgoyne RD, and Zerial M (1999) The Rab5 effector EEA1 is a core component of endosome docking. *Nature,* **397,** 621-625.

14. Conner SD and Schmid SL (2003) Regulated portals of entry into the cell. *Nature,* **422,** 37-44.

15. Deeks ED, Cook JP, Day PJ, Smith DC, Roberts LM, and Lord JM (2002)

The low lysine content of ricin A chain reduces the risk of proteolytic degradation after translocation from the endoplasmic reticulum to the cytosol. *Biochemistry (Mosc),* **41,** 3405-3413.

16. Dell'Angelica EC (2001) Clathrin-binding proteins: got a motif? Join the network! *Trends Cell Biol,* **11,** 315-318.

17. Fazal N, Choudhry MA, and Sayeed MM (2005) Inhibition of T cell MAPKs (Erk 1/2, p38) with thermal injury is related to down-regulation of Ca2+ signaling. *Biochim Biophys Acta,* **1741,** 113-119.

18. Foster GH and Tesh VL (2002) Shiga toxin 1-induced activation of c-Jun NH(2)-terminal kinase and p38 in the human monocytic cell line THP-1: possible involvement in the production of TNF-alpha. *J Leukoc Biol,* **71,** 107-114.

19. Futter CE, Collinson LM, Backer JM, and Hopkins CR (2001) Human VPS34 is required for internal vesicle formation within multivesicular endosomes. *J Cell Biol,* **155,** 1251-1264.

20. Garred O, van DB, and Sandvig K (1995) Furin-induced cleavage and activation of Shiga toxin. *J Biol Chem,* **270,** 10817-10821.

21. Gaullier JM, Simonsen A, D'Arrigo A, Bremnes B, Stenmark H, and Aasland R (1998) FYVE fingers bind PtdIns(3)P. *Nature,* **394,** 432-433.

22. Gillooly DJ, Morrow IC, Lindsay M, Gould R, Bryant NJ, Gaullier JM, Parton RG, and Stenmark H (2000) Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells. *EMBO J,* **19,** 4577-4588.

23. Glebov OO, Bright NA, and Nichols BJ (2006) Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells. *Nat Cell Biol,* **8,** 46-54.

24. Griffin CT, Trejo J, and Magnuson T (2005) Genetic evidence for a mammalian retromer complex containing sorting nexins 1 and 2. *Proc Natl Acad Sci U S A,* **102,** 15173-15177.

25. Gullapalli A, Garrett TA, Paing MM, Griffin CT, Yang Y, and Trejo J (2004) A role for sorting nexin 2 in epidermal growth factor receptor down-regulation: evidence for distinct functions of sorting nexin 1 and 2 in protein trafficking. *Mol Biol Cell,* **15,** 2143-2155.

26. Haft CR, de la Luz SM, Bafford R, Lesniak MA, Barr VA, and Taylor SI (2000) Human orthologs of yeast vacuolar protein sorting proteins Vps26, 29, and 35: assembly into multimeric complexes. *Mol Biol Cell,* **11,** 4105-4116.

27. Haft CR, de la Luz SM, Barr VA, Haft DH, and Taylor SI (1998) Identification of a family of sorting nexin molecules and characterization of their association with receptors. *Mol Cell Biol,* **18,** 7278-7287.

28. Hazes B, Boodhoo A, Cockle SA, and Read RJ (1996) Crystal structure of the pertussis toxin-ATP complex: a molecular sensor. *J Mol Biol,* **258,** 661-671.

29. Hazes B and Read RJ (1997) Accumulating evidence suggests that several AB-toxins subvert the endoplasmic reticulum-associated protein degradation pathway to enter target cells. *Biochemistry (Mosc),* **36,** 11051-11054.

30. Hehnly H, Sheff D, and Stamnes M (2006) Shiga toxin facilitates its retrograde transport by modifying microtubule dynamics. *Mol Biol Cell,* **17,** 4379-4389.

31. Hettema EH, Lewis MJ, Black MW, and Pelham HR (2003) Retromer and the sorting nexins Snx4/41/42 mediate distinct retrieval pathways from yeast endosomes. *EMBO J,* **22,** 548-557.

32. Heuser J (1980) Three-dimensional visualization of coated vesicle formation in fibroblasts. *J Cell Biol,* **84,** 560-583.

33. Hoepfner S, Severin F, Cabezas A, Habermann B, Runge A, Gillooly D, Stenmark H, and Zerial M (2005) Modulation of receptor recycling and degradation by the endosomal kinesin KIF16B. *Cell,* **121,** 437-450.

34. Hommelgaard AM, Roepstorff K, Vilhardt F, Torgersen ML, Sandvig K, and van DB (2005) Caveolae: stable membrane domains with a potential for internalization. *Traffic,* **6,** 720-724.

35. Horazdovsky BF, Davies BA, Seaman MN, McLaughlin SA, Yoon S, and Emr SD (1997) A sorting nexin-1 homologue, Vps5p, forms a complex with Vps17p and is required for recycling the vacuolar protein-sorting receptor. *Mol Biol Cell,* **8,** 1529-1541.

36. Ikeda M, Gunji Y, Yamasaki S, and Takeda Y (2000) Shiga toxin activates p38 MAP kinase through cellular Ca(2+) increase in Vero cells. *FEBS Lett,* **485,** 94-98.

37. Itin C, Ulitzur N, Muhlbauer B, and Pfeffer SR (1999) Mapmodulin, cytoplasmic dynein, and microtubules enhance the transport of mannose 6-phosphate receptors from endosomes to the trans-golgi network. *Mol Biol Cell,* **10,** 2191-2197.

38. Iversen TG, Skretting G, Llorente A, Nicoziani P, van Deurs B, and Sandvig K (2001) Endosome to Golgi transport of ricin is independent of clathrin and of the Rab9- and Rab11-GTPases. *Mol Biol Cell,* **12,** 2099-2107.

39. Jones HWJr (1997) Record of the first physician to see Henrietta Lacks at the Johns Hopkins Hospital: history of the beginning of the HeLa cell line. *Am J Obstet Gynecol,* **176,** S227-S228.

40. Katagiri YU, Mori T, Nakajima H, Katagiri C, Taguchi T, Takeda T, Kiyokawa N, and Fujimoto J (1999) Activation of Src family kinase yes induced by Shiga toxin binding to globotriaosyl ceramide (Gb3/CD77) in low density, detergent-insoluble microdomains. *J Biol Chem,* **274,** 35278-35282.

41. Koopmann JO, Albring J, Huter E, Bulbuc N, Spee P, Neefjes J, Hammerling GJ, and Momburg F (2000) Export of antigenic peptides from the endoplasmic reticulum intersects with retrograde protein translocation through the Sec61p channel. *Immunity,* **13,** 117-127.

42. Kreitman RJ (2006) Immunotoxins for targeted cancer therapy. *AAPS J,* **8,** E532-E551.

43. Lauvrak SU, Llorente A, Iversen TG, and Sandvig K (2002) Selective regulation of the Rab9-independent transport of ricin to the Golgi apparatus by calcium. *J Cell Sci,* **115,** 3449-3456.

44. Lauvrak SU, Walchli S, Iversen TG, Slagsvold HH, Torgersen ML, Spilsberg B, and Sandvig K (2006) Shiga Toxin Regulates Its Entry in a Syk-dependent Manner. *Mol Biol Cell.*

45. Lefkowitz RJ and Whalen EJ (2004) beta-arrestins: traffic cops of cell signaling. *Curr Opin Cell Biol,* **16,** 162-168.

46. Lemmon MA (2003) Phosphoinositide recognition domains. *Traffic,* **4,** 201-213.

47. Lencer WI (2001) Microbes and microbial Toxins: paradigms for microbial-mucosal toxins. V. Cholera: invasion of the intestinal epithelial barrier by a stably folded protein toxin.

Am J Physiol Gastrointest Liver Physiol, **280,** G781-G786.

48. Lencer WI, Constable C, Moe S, Jobling MG, Webb HM, Ruston S, Madara JL, Hirst TR, and Holmes RK (1995) Targeting of cholera toxin and Escherichia coli heat labile toxin in polarized epithelia: role of COOH-terminal KDEL. *J Cell Biol,* **131,** 951-962.

49. Lombardi D, Soldati T, Riederer MA, Goda Y, Zerial M, and Pfeffer SR (1993) Rab9 functions in transport between late endosomes and the trans Golgi network. *EMBO J,* **12,** 677-682.

50. London E and Luongo CL (1989) Domain-specific bias in arginine/lysine usage by protein toxins. *Biochem Biophys Res Commun,* **160,** 333-339.

51. Lord JM, Roberts LM, and Lencer WI (2005) Entry of protein toxins into mammalian cells by crossing the endoplasmic reticulum membrane: co-opting basic mechanisms of endoplasmic reticulum-associated degradation. *Curr Top Microbiol Immunol,* **300,** 149-168.

52. Mallard F, Antony C, Tenza D, Salamero J, Goud B, and Johannes L (1998) Direct pathway from early/recycling endosomes to the Golgi apparatus revealed through the study of shiga toxin B-fragment transport. *J Cell Biol,* **143,** 973-990.

53. Mayor S and Pagano RE (2007) Pathways of clathrin-independent endocytosis. *Nat Rev Mol Cell Biol,* **8,** 603-612.

54. McDonald PH, Chow CW, Miller WE, Laporte SA, Field ME, Lin FT, Davis RJ, and Lefkowitz RJ (2000) Beta-arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3. *Science,* **290,** 1574-1577.

55. McLaughlin NJ, Banerjee A, Kelher MR, Gamboni-Robertson F, Hamiel C, Sheppard FR, Moore EE, and Silliman CC (2006) Platelet-activating factor-induced clathrin-mediated endocytosis requires beta-arrestin-1 recruitment and activation of the p38 MAPK signalosome at the plasma membrane for actin bundle formation. *J Immunol,* **176,** 7039-7050.

56. McLaughlin NJ *et al.* (2008) Platelet-Activating Factor-Mediated Endosome Formation Causes Membrane Translocation of p67phox and p40phox That Requires Recruitment and Activation of p38 MAPK, Rab5a, and Phosphatidylinositol 3-Kinase in Human Neutrophils. *J Immunol,* **180,** 8192-8203.

57. Moore AE, Sabachewsky L, and Toolan HW (1955) Culture characteristics of four permanent lines of human cancer cells. *Cancer Res,* **15,** 598-602.

58. Mori T *et al.* (2000) Globotriaosyl ceramide (CD77/Gb3) in the glycolipid-enriched membrane domain participates in B-cell receptor-mediated apoptosis by regulating lyn kinase activity in human B cells. *Exp Hematol,* **28,** 1260-1268.

59. Moya M, Dautry-Varsat A, Goud B, Louvard D, and Boquet P (1985) Inhibition of coated pit formation in Hep2 cells blocks the cytotoxicity of diphtheria toxin but not that of ricin toxin. *J Cell Biol,* **101,** 548-559.

60. Nielsen E, Severin F, Backer JM, Hyman AA, and Zerial M (1999) Rab5 regulates motility of early endosomes on microtubules. *Nat Cell Biol,* **1,** 376-382.

61. Ogata M, Chaudhary VK, Pastan I, and FitzGerald DJ (1990) Processing of Pseudomonas exotoxin by a cellular protease results in the generation of a 37,000-Da toxin fragment that is translocated to the cytosol. *J Biol Chem,* **265,** 20678-20685.

62. Olsnes S, Fernandez-Puentes C, Carrasco L, and Vazquez D (1975) Ribosome inactivation by the toxic lectins abrin and ricin. Kinetics of the enzymic activity of the toxin A-chains. *Eur J Biochem,* **60,** 281-288.

63. Olsnes S and Kozlov JV (2001) Ricin. *Toxicon,* **39,** 1723-1728.

64. Pastan I, Hassan R, FitzGerald DJ, and Kreitman RJ (2006) Immunotoxin therapy of cancer. *Nat Rev Cancer,* **6,** 559-565.

65. Pastan I, Hassan R, FitzGerald DJ, and Kreitman RJ (2007) Immunotoxin treatment of cancer. *Annu Rev Med,* **58,** 221-237.

66. Patki V, Lawe DC, Corvera S, Virbasius JV, and Chawla A (1998) A functional PtdIns(3)P-binding motif. *Nature,* **394,** 433-434.

67. Patki V, Virbasius J, Lane WS, Toh BH, Shpetner HS, and Corvera S (1997) Identification of an early endosomal protein regulated by phosphatidylinositol 3-kinase. *Proc Natl Acad Sci U S A,* **94,** 7326-7330.

68. Paton JC and Paton AW (1998) Pathogenesis and diagnosis of Shiga toxin-producing Escherichia coli infections. *Clin Microbiol Rev,* **11,** 450-479.

69. Pelham HR (1996) The dynamic organisation of the secretory pathway. *Cell Struct Funct,* **21,** 413-419.

70. Popoff V, Mardones GA, Tenza D, Rojas R, Lamaze C, Bonifacino JS, Raposo G, and Johannes L (2007) The retromer complex and clathrin define an early endosomal retrograde exit site. *J Cell Sci,* **120,** 2022-2031.

71. Raiborg C, Bache KG, Mehlum A, Stang E, and Stenmark H (2001) Hrs recruits clathrin to early endosomes. *EMBO J,* **20,** 5008-5021.

72. Rapak A, Falnes PO, and Olsnes S (1997) Retrograde transport of mutant ricin to the endoplasmic reticulum with subsequent translocation to cytosol. *Proc Natl Acad Sci U S A,* **94,** 3783-3788.

73. Richter T, Floetenmeyer M, Ferguson C, Galea J, Goh J, Lindsay MR, Morgan GP, Marsh BJ, and Parton RG (2008) High-resolution 3D quantitative analysis of caveolar ultrastructure and caveola-cytoskeleton interactions. *Traffic,* **9,** 893-909.

74. Riederer MA, Soldati T, Shapiro AD, Lin J, and Pfeffer SR (1994) Lysosome biogenesis requires Rab9 function and receptor recycling from endosomes to the trans-Golgi network. *J Cell Biol,* **125,** 573-582.

75. Rink J, Ghigo E, Kalaidzidis Y, and Zerial M (2005) Rab conversion as a mechanism of progression from early to late endosomes. *Cell,* **122,** 735-749.

76. Rodighiero C, Tsai B, Rapoport TA, and Lencer WI (2002) Role of ubiquitination in retro-translocation of cholera toxin and escape of cytosolic degradation. *EMBO Rep,* **3,** 1222-1227.

77. Rojas R, Kametaka S, Haft CR, and Bonifacino JS (2006) Interchangeable but Essential Functions of SNX1 and SNX2 In the Association of Retromer with Endosomes and the Trafficking of Mannose 6-phosphate Receptors. *Mol Cell Biol,* **27,** 1112-1124.

78. Romer W *et al.* (2007) Shiga toxin induces tubular membrane invaginations for its uptake into cells. *Nature,* **450,** 670-675.

79. Sandvig K and Brown JE (1987) Ionic requirements for entry of Shiga toxin from Shigella dysenteriae 1 into cells. *Infect Immun,* **55,** 298-303.

80. Sandvig K, Garred O, Prydz K, Kozlov JV, Hansen SH, and van Deurs B (1992) Retrograde transport of endocytosed Shiga toxin to the

endoplasmic reticulum. *Nature,* **358,** 510-512.

81. Sandvig K, Olsnes S, Brown JE, Petersen OW, and van Deurs B (1989) Endocytosis from coated pits of Shiga toxin: a glycolipid-binding protein from Shigella dysenteriae 1. *J Cell Biol,* **108,** 1331-1343.

82. Sandvig K, Prydz K, Ryd M, and van Deurs B (1991) Endocytosis and intracellular transport of the glycolipid-binding ligand Shiga toxin in polarized MDCK cells. *J Cell Biol,* **113,** 553-562.

83. Sandvig K, Ryd M, Garred O, Schweda E, Holm PK, and van Deurs B (1994) Retrograde transport from the Golgi complex to the ER of both Shiga toxin and the nontoxic Shiga B-fragment is regulated by butyric acid and cAMP. *J Cell Biol,* **126,** 53-64.

84. Sandvig K and van Deurs B (1996) Endocytosis, intracellular transport, and cytotoxic action of Shiga toxin and ricin. *Physiol Rev,* **76,** 949-966.

85. Sandvig K and van Deurs B (2002) Membrane traffic exploited by protein toxins. *Annu Rev Cell Dev Biol,* **18,** 1-24.

86. Sandvig K and van Deurs B (1991) Endocytosis without clathrin (a minireview). *Cell Biol Int Rep,* **15,** 3-8.

87. Sandvig K and van Deurs B (2005) Delivery into cells: lessons learned from plant and bacterial toxins. *Gene Ther,* **12,** 865-872.

88. Schiavo G and van der Goot FG (2001) The bacterial toxin toolkit. *Nat Rev Mol Cell Biol,* **2,** 530-537.

89. Schmitz A, Herrgen H, Winkeler A, and Herzog V (2000) Cholera toxin is exported from microsomes by the Sec61p complex. *J Cell Biol,* **148,** 1203-1212.

90. Schu PV, Takegawa K, Fry MJ, Stack JH, Waterfield MD, and Emr SD (1993) Phosphatidylinositol 3-kinase encoded by yeast VPS34 gene essential for protein sorting. *Science,* **260,** 88-91.

91. Seaman MN, McCaffery JM, and Emr SD (1998) A membrane coat complex essential for endosome-to-Golgi retrograde transport in yeast. *J Cell Biol,* **142,** 665-681.

92. Seet LF and Hong W (2006) The Phox (PX) domain proteins and membrane traffic. *Biochim Biophys Acta,* **1761,** 878-896.

93. Shin HW *et al.* (2005) An enzymatic cascade of Rab5 effectors regulates phosphoinositide turnover in the endocytic pathway. *J Cell Biol,* **170,** 607-618.

94. Simonsen A *et al.* (1998) EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. *Nature,* **394,** 494-498.

95. Simonsen A and Stenmark H (2001) PX domains: attracted by phosphoinositides. *Nat Cell Biol,* **3,** E179-E182.

96. Simpson JC, Roberts LM, Romisch K, Davey J, Wolf DH, and Lord JM (1999) Ricin A chain utilises the endoplasmic reticulum-associated protein degradation pathway to enter the cytosol of yeast. *FEBS Lett,* **459,** 80-84.

97. Slominska-Wojewodzka M, Gregers TF, Walchli S, and Sandvig K (2006) EDEM is involved in retrotranslocation of ricin from the endoplasmic reticulum to the cytosol. *Mol Biol Cell,* **17,** 1664-1675.

98. Smith DC, Lord JM, Roberts LM, Tartour E, and Johannes L (2002) 1st class ticket to class I: protein toxins as pathfinders for antigen presentation. *Traffic,* **3,** 697-704.

99. Smith WE, Kane AV, Campbell ST, Acheson DW, Cochran BH, and Thorpe CM (2003) Shiga toxin 1 triggers a ribotoxic stress response leading to p38 and JNK activation and induction of apoptosis in intestinal epithelial cells. *Infect Immun,* **71,** 1497-1504.

100. Spooner RA, Watson PD, Marsden CJ, Smith DC, Moore KA, Cook JP, Lord JM, and Roberts LM (2004) Protein disulphide-isomerase reduces ricin to its A and B chains in the endoplasmic reticulum. *Biochem J,* **383,** 285-293.

101. Stein MP, Feng Y, Cooper KL, Welford AM, and Wandinger-Ness A (2003) Human VPS34 and p150 are Rab7 interacting partners. *Traffic,* **4,** 754-771.

102. Stein PE, Hazes B, and Read RJ (1996). In Parker,M.W. (Ed.), *Protein toxin structure*, . R.G. Landes Co., Austin, TX, pp. 191-216.

103. Stenmark H, Aasland R, Toh BH, and D'Arrigo A (1996) Endosomal localization of the autoantigen EEA1 is mediated by a zinc-binding FYVE finger. *J Biol Chem,* **271,** 24048-24054.

104. Sulimenko V, Draberova E, Sulimenko T, Macurek L, Richterova V, Draber P, and Draber P (2006) Regulation of microtubule formation in activated mast cells by complexes of gamma-tubulin with Fyn and Syk kinases. *J Immunol,* **176,** 7243-7253.

105. Sun Y, Cheng Z, Ma L, and Pei G (2002) Beta-arrestin2 is critically involved in CXCR4-mediated chemotaxis, and this is mediated by its enhancement of p38 MAPK activation. *J Biol Chem,* **277,** 49212-49219.

106. Takeda K, Matsuzawa A, Nishitoh H, Tobiume K, Kishida S, Ninomiya-Tsuji J, Matsumoto K, and Ichijo H (2004) Involvement of ASK1 in Ca2+-induced p38 MAP kinase activation. *EMBO Rep,* **5,** 161-166.

107. Takenouchi H, Kiyokawa N, Taguchi T, Matsui J, Katagiri YU, Okita H, Okuda K, and Fujimoto J (2004) Shiga toxin binding to globotriaosyl ceramide induces intracellular signals that mediate cytoskeleton remodeling in human renal carcinoma-derived cells. *J Cell Sci,* **117,** 3911-3922.

108. Teasdale RD, Loci D, Houghton F, Karlsson L, and Gleeson PA (2001) A large family of endosome-localized proteins related to sorting nexin 1. *Biochem J,* **358,** 7-16.

109. Thomas CM and Smart EJ (2008) Caveolae Structure and Function. *J Cell Mol Med.*

110. Traer CJ *et al.* (2007) SNX4 coordinates endosomal sorting of TfnR with dynein-mediated transport into the endocytic recycling compartment. *Nat Cell Biol,* **9,** 1370-1380.

111. Tsai B, Rodighiero C, Lencer WI, and Rapoport TA (2001) Protein disulfide isomerase acts as a redox-dependent chaperone to unfold cholera toxin. *Cell,* **104,** 937-948.

112. Tuschl T (2001) RNA interference and small interfering RNAs. *Chembiochem,* **2,** 239-245.

113. Ungewickell EJ and Hinrichsen L (2007) Endocytosis: clathrin-mediated membrane budding. *Curr Opin Cell Biol,* **19,** 417-425.

114. Utskarpen A, Slagsvold HH, Dyve AB, Skanland SS, and Sandvig K (2007) SNX1 and SNX2 mediate retrograde transport of Shiga toxin. *Biochem Biophys Res Commun,* **358,** 566-570.

115. van Deurs B, Petersen OW, Olsnes S, and Sandvig K (1989) The ways of endocytosis. *Int Rev Cytol,* **117,** 131-177.

116. van Deurs B, Sandvig K, Petersen OW, Olsnes S, Simons K, and Griffiths G (1988) Estimation of the amount of internalized ricin that reaches the

trans-Golgi network. *J Cell Biol,* **106,** 253-267.

117. Venter JC *et al.* (2001) The sequence of the human genome. *Science,* **291,** 1304-1351.

118. Wesche J, Rapak A, and Olsnes S (1999) Dependence of ricin toxicity on translocation of the toxin A-chain from the endoplasmic reticulum to the cytosol. *J Biol Chem,* **274,** 34443-34449.

119. Wong BY, Gregory SA, and Dang NH (2007) Denileukin diftitox as novel targeted therapy for lymphoid malignancies. *Cancer Invest,* **25,** 495-501.

120. Xiao K, McClatchy DB, Shukla AK, Zhao Y, Chen M, Shenoy SK, Yates JR, III, and Lefkowitz RJ (2007) Functional specialization of beta-arrestin interactions revealed by proteomic analysis. *Proc Natl Acad Sci U S A,* **104,** 12011-12016.

121. Yamada E (1955) The fine structure of the gall bladder epithelium of the mouse. *J Biophys Biochem Cytol,* **1,** 445-458.

122. Yu M and Haslam DB (2005) Shiga toxin is transported from the endoplasmic reticulum following interaction with the luminal chaperone HEDJ/ERdj3. *Infect Immun,* **73,** 2524-2532.

I

Skånland SS, Wälchli S, Utskarpen A, Wandinger-Ness A, Sandvig K (2007): Phosphoinositide-regulated retrograde transport of ricin: crosstalk between hVps34 and sorting nexins. Traffic, 8, 297-309.

II

Molecular Biology of the Cell
Vol. 19, 95–104, January 2008

The Mitogen-activated Protein Kinase p38 Links Shiga Toxin-dependent Signaling and Trafficking

Sébastien Wälchli,*† Sigrid S. Skånland,*†‡§ Tone F. Gregers,*§ Silje U. Lauvrak,*‡ Maria L. Torgersen,*‡ Ming Ying,* Shun'ichi Kuroda,‖ Andrés Maturana,‖¶ and Kirsten Sandvig*‡§

*Department of Biochemistry and ‡Centre for Cancer Biomedicine, Institute for Cancer Research, The Norwegian Radium Hospital, University of Oslo, Montebello, N-0310 Oslo, Norway; §Department of Molecular Biosciences, University of Oslo, N-0316 Oslo, Norway; and ‖Department of Structural Molecular Biology, The Institute of Scientific and Industrial Research SANKEN, Osaka University, 8-1 Mihogaoka, Ibaraki Osaka 567-0047, Japan

Submitted June 14, 2007; Revised October 5, 2007; Accepted October 15, 2007
Monitoring Editor: Jennifer Lippincott-Schwartz

Shiga toxin (Stx) binds to the cell, and it is transported via endosomes and the Golgi apparatus to the endoplasmic reticulum and cytosol, where it exerts its toxic effect. We have recently shown that Stx activates the tyrosine kinase Syk, which in turn induces clathrin phosphorylation and up-regulates Stx uptake. Here, we show that toxin-induced signaling can also regulate another step in intracellular Stx transport. We demonstrate that transport of Stx to the Golgi apparatus is dependent on the mitogen-activated protein kinase p38. Treatment of cells with chemical inhibitors or small interfering RNA targeting p38 inhibited Stx transport to the Golgi and reduced Stx toxicity. This p38 dependence is specific to Stx, because transport of the related toxin ricin was not affected by p38 inhibition. Stx rapidly activated p38, and recruited it to early endosomes in a Ca^{2+}-dependent manner. Furthermore, agonist-induced oscillations in cytosolic Ca^{2+} levels were inhibited upon Stx stimulation, possibly reflecting Stx-dependent local alterations in cytosolic Ca^{2+} levels. Intracellular transport of Stx is Ca^{2+} dependent, and we provide evidence that Stx activates a signaling cascade involving cross talk between Ca^{2+} and p38, to regulate its trafficking to the Golgi apparatus.

INTRODUCTION

Shiga toxin (Stx) is composed of a cell-binding B-moiety and an enzymatically active A-subunit. The toxin binds to the target cell, and it is subsequently taken up by endocytosis. It is then transported via early endosomes, and the Golgi apparatus to the endoplasmic reticulum (ER), from where it retrotranslocates to its final destination, the cytosol. The toxic effect of Shiga is to inactivate ribosomes and thus inhibit protein synthesis.

It is now accepted, in the case of hormone receptors, that ligand-binding induced changes in receptor structure can stimulate an intrinsic kinase activity or an associated kinase. The signaling cascade induced by receptor stimulation can also regulate endocytosis (Gonzalez-Gaitan and Stenmark, 2003; Polo and Di Fiore, 2006). The importance of kinase-mediated signaling in endocytosis and intracellular transport has been demonstrated by a genome-wide analysis (Pelkmans *et al*., 2005). Another important finding was that p38 is able to regulate formation of the Rab5–guanine nucleotide dissociation inhibitor (GDI) complex (Cavalli *et al*., 2001). Furthermore, the Rab5 effectors Rabenosyn-5 and early endosome antigen 1 (EEA1) are substrates for p38 (Mace *et al*., 2005). Interestingly, mitogen-activated protein (MAP) kinases are known to associate with endosomes (Pol *et al*., 1998), and this has been demonstrated for p38 as well. p38 has been purified with the endosome fraction in a sucrose gradient (Delcroix *et al*., 2003), and it also has been observed on endosomal structures, by confocal microscopy (Pelkmans *et al*., 2005). Recently, it was demonstrated that p38 can be recruited to a signalosome involved in the regulation of the platelet-activating factor-induced clathrin-mediated endocytosis (McLaughlin *et al*., 2006). Taken together, p38 can be localized on endosomes to phosphorylate downstream effectors from here.

Several studies have shown that Stx, upon binding or entry into cells, is able to trigger signaling cascades (Katagiri *et al*., 1999; Ikeda *et al*., 2000; Mori *et al*., 2000; Cameron *et al*., 2003; Takenouchi *et al*., 2004). However, the main focus has been on Stx-induced apoptosis and signaling related to this late event. Interestingly, not only the tyrosine kinases have been shown to be activated upon Stx intoxication (Foster and Tesh, 2002; Smith *et al*., 2003). However, such kinases are mostly activated upon ribotoxic stress, a cellular response that occurs as a consequence of toxin-induced ribosomal inactivation in the cytosol. We have previously presented data demonstrating that Stx is an active player in its own transport. We have shown that Stx binding activates the tyrosine kinase Syk, and several proteins, one of these proteins being clathrin heavy chain (CHC), are phosphorylated

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E07–06–0565) on October 24, 2007.

† These authors contributed equally to this work.

¶ Present address: Global Edge Institute, Tokyo Institute of Technology, 4259-B4 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan.

Address correspondence to: Kirsten Sandvig (ksandvig@radium.uio.no).

Abbreviations used: Stx, Shiga toxin.

(Lauvrak *et al*., 2006). For Stx, which binds to a glycolipid and not to a transmembrane protein receptor, one may ask what triggers a signaling cascade in the cytosol. So far, this is not known. However, signaling is not only caused by phosphorylation of receptors but also Ca^{2+} currents can mediate downstream cytosolic phosphorylations. It has been known for two decades that divalent ions are able to modulate Stx trafficking (Sandvig and Brown, 1987) and that in Madin Darby canine kidney cells, ricin and, to a lesser extent, Stx transport is sensitive to interference of Ca^{2+} homeostasis (Lauvrak *et al*., 2002). Furthermore, early cellular events that might precede phosphorylation cascades were detected in B-cells stimulated by Stx. Taga *et al*. (1997) have shown that exposing Burkitt's lymphoma cells to Stx triggers a Ca^{2+} influx. These events were, however, linked to apoptotic signaling rather than regulation of transport (Cherla *et al*., 2003). A link between Ca^{2+} and p38 activation has been proposed in Vero cells (Ikeda *et al*., 2000). However, in this study, p38 activation was dependent on entry of the active A subunit, thus presumably caused by ribotoxic stress.

In the present study, we have investigated the importance of the MAP kinase p38 and Ca^{2+} in the regulation of Stx transport. We show that p38 is rapidly activated by Stx and that its activity is required for transport of Stx from endosomes to the Golgi apparatus. p38-regulated trafficking seems to be specific to Stx, because transport of the related toxin ricin was insensitive to p38 inhibition. In addition, Stx is able to modulate oscillations in Ca^{2+} levels caused by the addition of ATP or histamine, suggesting a link between Stx trafficking and Ca^{2+} homeostasis. We further show that cytosolic Ca^{2+} seems to be necessary for proper targeting of p38 to the endosomes. Taken together, our data support a model in which Stx, by modifying Ca^{2+} homeostasis, recruits p38 to endosomes to regulate its intracellular transport.

MATERIALS AND METHODS

Cell Lines, Products, and Reagents

HeLa and HEp2 cells were grown under 5% CO_2 in DMEM with 10% fetal calf serum supplemented with penicillin at 100 U/ml, streptomycin at 100 U/ml, and L-glutamine at 2 mM (Invitrogen, Carlsbad, CA). SB203580 was from Calbiochem (San Diego, CA), SKF86002 was from Sigma-Aldrich (St. Louis, MO), and 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB-8) was from Alexis Biochemicals (Lausen, Switzerland). [^{3}H]Leucine was from PerkinElmer Life and Analytical Sciences (Boston, MA). All cell culture reagents were from Invitrogen (Paisley, United Kingdom). Stx1 was obtained from Nacalai Tesque (Kyoto, Japan), and Stx was a kind gift from J. V. Kozlov (Academy of Sciences of Russia, Moscow, Russia) and J. E. Brown (U.S. Army Medical Research Institute of Chemical Defense, Fort Detrick, MD). The antibodies used in this study were anti-Stx (anti-STX1-13C4; Toxin Technology, Sarasota, FL), anti-ricin (Sigma-Aldrich), anti-p38 (p38α and p38P [pT180/pY182]; BD Biosciences, Palo Alto, CA), anti-p38β2 (Zymed Laboratories, South San Francisco, CA), anti-p38 (Cell Signaling Technology, Beverly, MA), anti-α-tubulin (Sigma-Aldrich), anti-clathrin heavy chain (RDI Division of Fitzgerald Industries International, Concord, MA), and anti-Rab5, anti-EEA1, and anti-annexin (our collection). StxB-Sulf$_2$ expression construct was a kind gift from Dr. B. Goud (Institut Curie, Paris, France). StxB-Sulf$_2$, and ricin-Sulf1 were prepared as described previously (Rapak *et al*., 1997; Lauvrak *et al*., 2006).

Small Interfering RNA (siRNA) Design and Transfection

Two different siRNAs targeting different parts of p38α and p38β mRNAs were designed. They were selected according to their physicochemical profiles and their specificity to the target mRNA by BLAST analysis profile (http://www.ncbi.nlm.nih.gov/BLAST/), and they were fitted as closely as possible to the criteria depicted in Reynolds *et al*. (2004). p38 siRNA target sequences were as follows: p38α, 5-GCUGUUGACUGGAAGAACA-3 and 5-CUGCGGUUACUUAAACAUA-3 (siRNA1 and -2, respectively) and p38β, 5-AAGGACCUGAGCAGCAUCUU-3 and 5-AAGUGUACUUGGUGACCACC-3 (siRNAb1 and -b2, respectively). High-performance liquid chromatography-purified p38 siRNAs were ordered from MWG Biotech (Ebersberg, Germany), and a negative control siRNA was from Eurogentec (Seraing, Belgium). Cells were transiently transfected with the indicated siRNA by using Oligofectamine (Invitrogen) according to the manufacturer's protocol.

Calcium Analysis

Variations in cytosolic calcium concentrations were measured using the calcium probe Fura-2 as described previously (Maturana *et al*., 2002). Cells were loaded for 20 min at room temperature with the membrane permeant Fura-2 acetoxymethyl ester (AM) in medium containing 140 mM NaCl, 5 mM KCl, 1.2 mM Ca^{2+}, 1 mM $MgCl_2$, 10 mM glucose, and 20 mM HEPES, pH 7.4. Fura-2 fluorescence (excitation, 340/380 nm; emission, 510 nm) was monitored with an imaging system. Loaded cells plated on coverslips were mounted on an IX70 inverted microscope (Olympus, Tokyo, Japan), and measurements were performed at 37°C (Thermoplate from Tokai Hit, Shizuoka, Japan) unless otherwise indicated. Images were captured with a charge-coupled device Orca-ER camera (Hamamatsu, Shizuoka, Japan). The cells were illuminated with a 75-W xenon lamp through a 10% neutral density filter (Omega Optical, Tokyo, Japan), a Lambda 10-2 filter wheel (Sutter Instrument, Novato, CA), and a 60× oil immersion PlanApo objective lens (numerical aperture. 1.4; Olympus). Camera and filter wheel shutter were under the control of the MetaMorph software (Molecular Devices, Sunnyvale, CA).

Endocytosis Assays

Stx internalization was quantified following the procedure described previously (Torgersen *et al*., 2005).

Sulfation Assays

Transfected or untransfected HeLa cells were washed twice with sulfate-free medium (minimal essential medium 12-126; Lonza Walkersville, Walkersville, MD) before incubation with 0.2 mCi/ml $Na_2{}^{35}SO_4$ in the same medium for 3 h at 37°C. Inhibitors were added as indicated, and then they were present in the same medium during the last 30 min of this incubation. Cells were incubated with StxB-Sulf$_2$ for 45 min or ricin-Sulf1 for 3 h. They were then washed twice with cold phosphate-buffered saline (PBS) and lysed in lysis buffer (0.1 M NaCl, 10 mM Na_2HPO_4, pH 7.4, 1 mM EDTA, 1% Triton X-100, and 60 mM *n*-octyl-β-glucopyranoside, supplemented with Complete protease inhibitors; Roche Diagnostics, Mannheim, Germany). Cleared lysate was immunoprecipitated with anti-Stx or anti-ricin antibodies. The immunoprecipitated complex was separated by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to a polyvinylidene difluoride (PVDF) membrane, and investigated by autoradiography. Band intensities were quantified using ImageQuant 5.0 software (Molecular Dynamics, Sunnyvale, CA). Total cellular protein sulfation was measured as the amount of 5% trichloroacetic acid (TCA)-precipitated $^{35}SO_4^{2-}$ in the lysates.

Subcellular Fractionation of Endosomes

HEp2 cells were starved in serum-free medium for 1 h before treatment with or without 100 μM TMB-8 for 30 min. The cells were then incubated with or without 250 ng/ml StxB for 20 min in the same medium. Endosomes were purified as described previously (Aniento *et al*., 1996). Briefly, the cells were homogenized in homogenization buffer (250 mM sucrose and 3 mM imidazole, pH 7.4), and the postnuclear supernatant (PNS) was adjusted to 40% sucrose, 3 mM imidazole, pH 7.4. The PNS was then subjected to equilibrium flotation through layers of 35%, 25%, and 250 mM sucrose solutions in 3 mM imidazole, pH 7.4. The visible bands at the PNS/35%, 35%/25%, and 25%/250 mM sucrose solution interfaces correspond to the "heavy" membrane (HM), early endosome (EE), and late endosome (LE) fractions, respectively (Aniento *et al*., 1996). The protein concentrations in the purified fractions were measured, and equal amounts of protein were separated by SDS-PAGE and analyzed by Western blotting.

Immunoprecipitation

Detection of phosphorylated proteins after Stx stimulation was performed as follows: HeLa cells were starved in HEPES medium for 2 h and treated with 250 ng/ml StxB for the indicated durations. The cells were then lysed in binding buffer (20 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, 1% NP-40, and 0.2 mM orthovanadate) supplemented with a Complete protease inhibitor cocktail (Roche Diagnostics). Tyrosine-phosphorylated proteins were immunoprecipitated overnight at 4°C by using 20 μl of the slurry of an anti-phosphotyrosine column (Upstate Biotechnology, Charlottesville, VA) in batch as described previously (Wälchli *et al*., 2004). The eluate was subjected to SDS-PAGE and transferred onto a PVDF membrane. Immunostaining was performed with the indicated antibodies.

Cytotoxicity Assays

HeLa cells were pretreated as indicated in the figure legends. The cells were washed twice with leucine-free medium, and then they were incubated with increasing concentrations of toxin (Stx or ricin) for 2.5 h. After this, cells were incubated for 30 min in the presence of 2 μCi/ml [^{3}H]leucine, and finally they were extracted twice with 5% TCA. The precipitate was dissolved in 0.1 M KOH, and the associated radioactivity was measured.

Confocal Fluorescence Microscopy

Cells grown on glass coverslips were transfected with siRNA against p38α or p38β, and they were analyzed 2 days after transfection. Alternatively, the cells

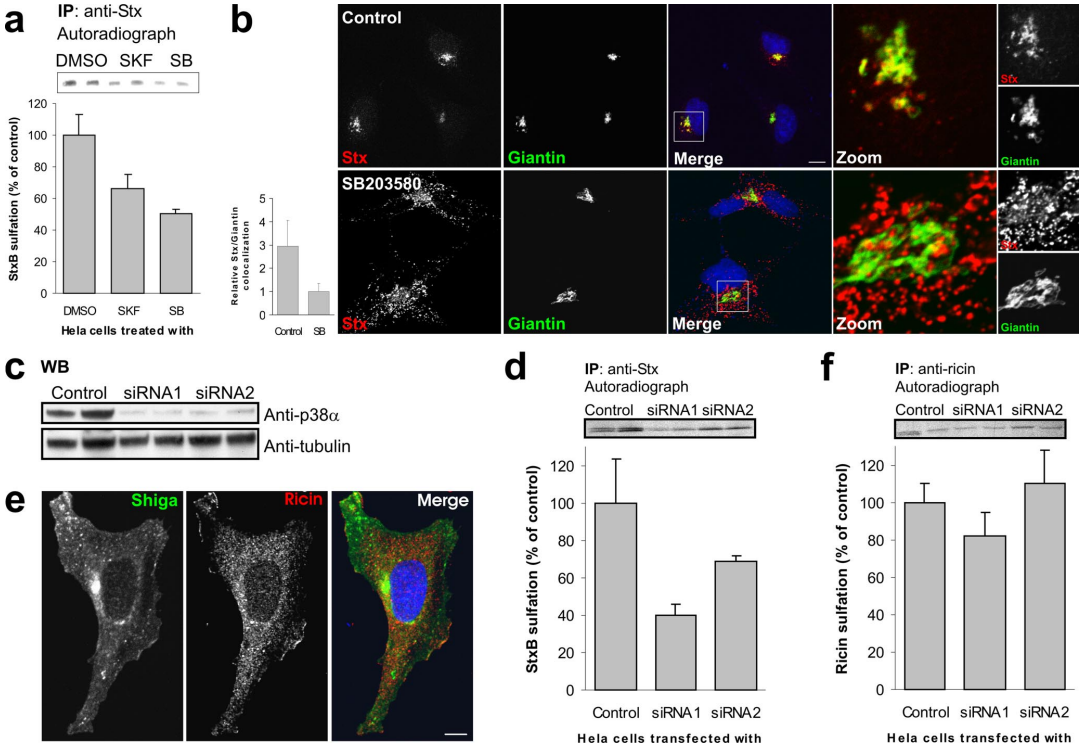

Figure 1. p38 inhibition reduces transport of StxB, but not ricin, to the TGN. (a) HeLa cells were incubated with radioactive sulfate for 3 h, and the indicated inhibitors (SKF86002 and SB203580; 30 μM) or the carrier (dimethyl sulfoxide [DMSO]; 0.1% final concentration) were present for the last 30 min. StxB was then added, and the incubation was continued for 45 min. StxB was immunoprecipitated from the lysates, and its degree of sulfation was analyzed by SDS-PAGE and autoradiography. The band intensities were quantified and the average plotted with error bars showing deviations. The experiment was performed three times with duplicates. (b) HeLa cells were incubated with or without SB203580 for 30 min, before addition of and further incubation with 2 μg/ml StxB for 20 min. The cells were then fixed and permeabilized before staining with the indicated antibodies. DRAQ5 was used for nuclear staining. Bar, 10 μm. Colocalization of StxB with the Golgi marker Giantin was quantified using Zeiss LSM Image Browser. Bars represent SD; n = 10. (c) We analyzed 1/100 of the Stx-IP supernatant (from D) for p38α by Western blot. α-Tubulin was used as loading control. (d) As in a, but in this case cells were transfected with 100 nM of the indicated siRNA and incubated for 48 h before StxB-Sulf$_2$ treatment. These experiments were repeated at least three times with duplicates. (e) Cells were incubated for 20 min with 2 μg/ml Stx and 2 μg/ml ricin before fixation and permeabilization. They were then stained with antibodies as indicated. Bar, 10 μm. (f) As in d, but with 90-min incubation of ricin sulf-1 instead of StxB-Sulf$_2$.

were treated with or without 30 μM SB203580 for 30 min, and then they were incubated with 2 μg/ml StxB for 20 min. For experiments with ricin, the concentration used was also 2 μg/ml. The cells were fixed with 10% Formalin solution (Sigma-Aldrich), permeabilized with 0.2% Triton X-100 in PBS, and immunostained with appropriate antibodies. Fluorophore-labeled secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). DRAQ5 (Alexis Biochemicals, San Diego, CA) was used to stain the nuclei. The cells were mounted in Mowiol (Calbiochem), and then they were examined by laser scanning confocal microscope LSM 510 META (Carl Zeiss, Jena, Germany). Images were prepared with the LSM Image Browser software (Carl Zeiss).

RESULTS

Retrograde Trafficking of Stx Is Dependent on p38α

Stx induces early signaling events that can regulate endocytosis, and possibly also intracellular transport, of the toxin (Lauvrak *et al*., 2006). To analyze the involvement of kinases in Stx transport, we undertook an unbiased screen of specific kinase inhibitors. In this screen, the MAP kinase p38 stood out as a candidate regulator of retrograde Stx transport. We performed a sulfation experiment (Johannes *et al*., 1997), a biochemical approach that allows quantification of the amount of Stx that reaches the *trans*-Golgi network (TGN) (see *Materials and Methods*). The p38 inhibitors SB203580 and SKF86002 reduced Stx sulfation to 50% (Figure 1a), suggesting that p38 activity is required for transport of Stx to the Golgi apparatus. This was confirmed visually by confocal immunofluorescence microscopy. Cells treated with SB203580, displayed a threefold reduction in colocalization between Stx and the Golgi marker Giantin (Figure 1b).

Next, we designed siRNAs that specifically targeted the p38α or p38β isoforms. Two days after transfection with p38α siRNA, when p38α knockdown was almost complete as determined by Western immunoblot (Figure 1c), transport of Stx to the Golgi was reduced to a similar extent as observed with the chemical inhibitors against p38 (Figure 1d). Because the available antibodies did not allow us to detect p38β by Western immunoblot, we took advantage of confocal microscopy to verify the siRNA-mediated knock-

down of p38β. As shown in Supplemental Figure S1A, p38β was knocked down to a similar extent as p38α with the respective siRNAs (compare bottom and top panels). Transfection of cells with siRNAs targeting p38β did not affect the level of Stx sulfation, that is, the transport of Stx to the TGN (Supplemental Figure S1B). These observations suggest a role for the p38α isoform only, in regulation of Stx transport. We routinely check the total levels of protein sulfation under the various experimental conditions, and no significant differences between inhibitor- or siRNA-treated cells and control cells were observed (data not shown).

To determine whether the effect of p38α-inhibition/p38α-knockdown on retrograde transport to the TGN is specific for Stx, or whether it is involved in transport to the TGN in general, we investigated the transport of the related toxin ricin. In contrast to Stx, which binds specifically to the glycosphingolipid Gb3, ricin binds to both glycolipids and glycoproteins with terminal galactose. Ricin and Stx are thus endocytosed by different mechanisms, and their nonoverlapping endosomal localization is shown in Figure 1e. Analogous to what was done for Stx, we investigated the retrograde transport of a sulfation site-containing version of ricin (Rapak *et al*., 1997). Treatment of cells with either SB203580 (data not shown) or p38α siRNA (Figure 1f) had no significant effect on ricin sulfation. Thus, p38α is specifically involved in TGN transport of Stx, and not in general transport to the Golgi apparatus.

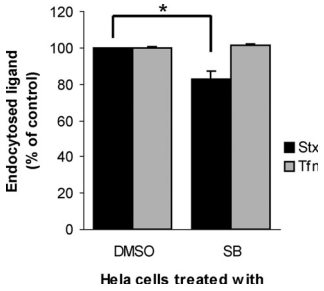

Figure 2. Effect of p38 inhibition on endocytosis. HeLa cells were incubated with 30 μM SB203580 (SB) or carrier (DMSO; 0.1% final concentration) for 30 min at 37°C before Stx-SS-Biotin (black bars) or transferrin-SS-Biotin (Tfn; gray bars) was added to the medium. The incubation was continued for 20 min. Cells were 2-mercaptoethane sulphonate sodium treated, and internalized Stx- or transferrin-SS-Biotin labeled with Ru(II)-tag either directly (transferrin) or via antibody (Stx) was fished out from cell lysates by streptavidin-coated magnetic beads and measured by electrochemiluminescence. This experiment was repeated three times with duplicates; error bars represent deviations. *$p \leq 0.005$, determined by the paired Student's *t* test.

Stx Endocytosis Is Independent of p38

The observed reduction in Stx transport to the Golgi apparatus may be due to inhibition of a specific transport step between endosomes and TGN, or to inhibition of earlier events, like binding or endocytosis. Long-term inhibition of p38 by SB203580 has been shown previously to reduce binding of Stx to human brain endothelial cells (Stricklett *et al*., 2005). We therefore tested the effect of the two p38 inhibitors, SB203580 and SKF86002, on binding and endocytosis of Stx in our cells. Neither of the inhibitors affected toxin binding (data not shown). However, a slight decrease in Stx uptake was observed in cells treated with SB203580 (Figure 2, black bars). This effect was not due to a general block in clathrin-dependent endocytosis, because the transferrin endocytosis was unaffected by the inhibitor, both after 5 and 20 min (data not shown; Figure 2, gray bars). Importantly, the siRNAs targeting p38α did not reduce binding or endocytosis of Stx (data not shown). From these data, we conclude that the main regulatory activity of p38α on Stx transport is on the step from endosomes to the Golgi apparatus.

Depletion of p38α Protects Cells against Stx Toxicity

Because knockdown of p38α resulted in a strong reduction in endosome to Golgi transport of Stx, we wanted to study the effect on Stx transport to the cytosol. To this end, we performed a toxicity assay. As shown in Figure 3, a and b, the p38 inhibitor SB203580 and siRNA against p38α were able to decrease the toxicity of Stx four- to fivefold. This is in agreement with the sulfation and immunofluorescence data, and it further shows that p38α is required for proper Stx transport. We have reported previously that toxins are sometimes able to overcome a block in their trafficking (Llorente *et al*., 2003), apparently by using compensatory transport pathways up-regulated by the cell. However, for p38, even after long-term inhibition (siRNA treatment), the cells did not seem to be able to compensate for the lack of the p38α kinase in trafficking. To further confirm that the observed effects were specific for Stx transport, we performed analogous experiments for ricin. In agreement with data from sulfation experiments, ricin toxicity was not affected by treatment with p38 inhibitor or siRNA (Figure 3, c and d). Together, these results confirm that p38 is required for proper trafficking of Stx, but not ricin.

p38 Is Activated by Stx

Previous reports have shown that the MAP kinase p38 is a regulator of endosomal transport (Cavalli *et al*., 2001; Delcroix *et al*., 2003; Fratti *et al*., 2003; Mace *et al*., 2005; Pelkmans *et al*., 2005). Therefore, we aimed at testing whether Stx can activate p38. Lysates of cells treated with Stx for different times were passed through an anti-phosphotyrosine (anti-YP) column, and eluted proteins were separated by SDS-PAGE and transferred to a PVDF membrane for immunoblot analysis. As shown, activated (Tyr- and Thr-phosphorylated) p38 (p38P) bound to the anti-YP column already after 1 min of Stx stimulation of the cells, and a peak was observed after 10–15 min (Figure 4a, top). The membrane was also probed for p38α (Figure 4a, second panel from the top). The observed binding of this isoform to the anti-YP column suggests that at least part of the p38P bands represent activated p38α. However, due to poor antibodies we were not able to detect p38β in immunoblots, and one can therefore not exclude that the p38P bands also contain activated p38β. p38P shows a peak that seems to be a bit delayed compared with p38α. The p38P-antibody recognizes the doubly phosphorylated (Thr and Tyr) protein, whereas the column binds Tyr-phosphorylated proteins. The p38α antibody does not distinguish between the different phosphorylation states of the protein, and this might therefore explain the seemingly different phosphorylation kinetics for p38P and p38α. As a control, the membrane was reprobed with anti-CHC antibody, because we have previously shown that CHC becomes phosphorylated upon Stx binding (Figure 4a, third panel from the top; Lauvrak *et al*., 2006). As shown, also the kinetics of CHC phosphorylation is rapid. As a control of equal loading to the columns, the whole cell lysate (WCL) was probed against p38α (Figure 4a, bottom). We also tested the sensitivity of the p38 response to different Stx concentrations. p38 activation (Tyr and Thr phosphory-

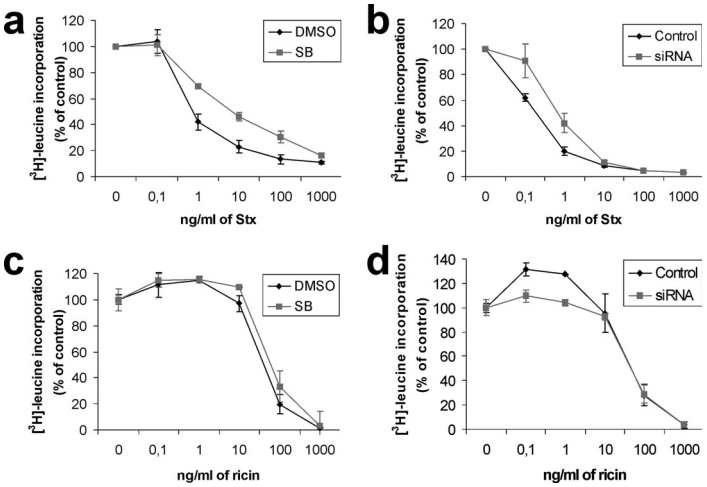

Figure 3. p38 inhibition protects against Stx, but not ricin, cytotoxicity. HeLa cells were treated with 30 μM SB or carrier (DMSO; 0.1% final concentration) for 30 min (a and c) or transfected with p38α siRNA1 or a control siRNA 48 h before the experiment (b and d). After this, the cells were incubated with increasing concentrations of Stx (a and b) or ricin (c and d) for 2.5 h. Protein synthesis was measured by [^{3}H]leucine incorporation. All experiments were repeated at least twice with duplicates. Error bars represent standard deviations.

lation) was analyzed after 5-min incubation with increasing concentrations of Stx (Figure 4b). Whole cell lysates were subjected to SDS-PAGE and analyzed by Western immunoblot. As presented, Stx concentrations as low as 10 ng/ml were able to stimulate p38 phosphorylation.

Stx Modifies the Intracellular Ca^{2+} Response to Agonists

Because cytosolic Ca^{2+} levels affect Stx transport (Sandvig and Brown, 1987; Chen *et al.*, 2002; Lauvrak *et al.*, 2002), we analyzed the effect of Stx binding on Ca^{2+} homeostasis. In contrast to data obtained from studies in B cells (Taga *et al.*, 1997), binding of Stx itself (unlike addition of ionomycin) did not induce any observable Ca^{2+} response in HeLa cells (Supplemental Figure S2A). The same was observed when purified StxB was added to the cells (Supplemental Figure S2D, left). This may be due to a local effect of Stx signaling, not measurable with a cytosolic calcium probe such as Fura-2AM, or, alternatively, a negative response. To test whether Stx addition modifies Ca^{2+} fluxes, we investigated the effect of Stx on histamine- or ATP-induced oscillations in Ca^{2+} levels. Stx was added to the cells, and histamine was then added after different time points (20 s, and 4, 10, and 15 min; Figure 5, a–d, respectively). No significant effect on the cytosolic Ca^{2+} oscillations induced by histamine could be observed at the earliest time points (Figure 5, a–c). After 15 min, however, the cytosolic Ca^{2+} oscillations induced by histamine were strongly attenuated, despite the fact that an intracellular Ca^{2+} increase was still observed (Figure 5d). The same was observed after ATP stimulation (Supplemental Figure S2B) and for StxB (Supplemental Figure S2C). These results suggest that Stx must be taken up by the cell to exert its effect on cytosolic Ca^{2+} levels. We next investigated whether the observed attenuation of cytosolic Ca^{2+} oscillations is a result of Stx localization to early endosomes per se. To this end, we performed the experiment at 18°C, a temperature that leads to Stx accumulation in early endosomes (Mallard *et al.*, 1998). The histamine-induced Ca^{2+} oscillations were not affected by a 20-min Stx incubation at this temperature (Figure 5e), suggesting that the Stx-positive endosomes must reach, and perhaps fuse with, another compartment to affect the cytosolic Ca^{2+} oscillations caused by histamine. We also wanted to investigate whether the effect of Stx on oscillations in Ca^{2+} levels is p38 dependent. Cells were preincubated with SB203580 for 30 min before the experiment was performed as described. Under these experimental conditions, Stx treatment still inhibited the histamine-induced Ca^{2+} oscillations (Figure 5f), as seen without the inhibitor (Figure 5d). Interestingly, the basal level of cytosolic Ca^{2+} was increased in the presence of SB203580 (Figure 5f). In summary, these data show that Stx is able to act on Ca^{2+} homeostasis by affecting the intracellular Ca^{2+} cycling and that this is dependent on Stx internalization, but not on p38 activity. Because the effects of holotoxin and

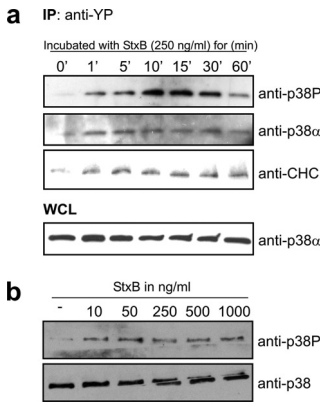

Figure 4. StxB is able to activate p38α upon binding. (a) HeLa cells were starved for 2 h in HEPES medium before incubation with 250 ng/ml StxB for the indicated times. Cells were lysed, and cell lysate was passed through an anti-YP column. The eluate was then analyzed by Western immunoblotting with the indicated antibodies. One percent of the WCL was analyzed by SDS-PAGE and Western immunoblot to serve as control of equal loading on the column. (b) HeLa cells were incubated with increasing concentrations of StxB for 5 min at 37°C. Lysates were prepared and run for Western blot analysis by using the indicated antibodies. These experiments were repeated three times.

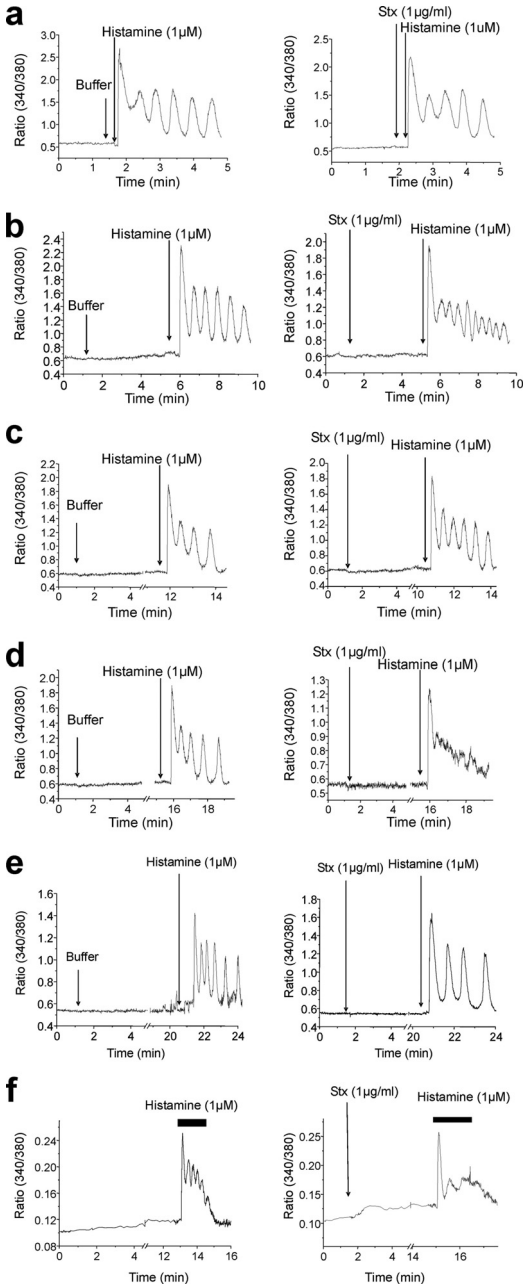

Figure 5. Stx inhibits cytosolic Ca^{2+} oscillations. Cytosolic Ca^{2+} was measured in HeLa cells, loaded with the calcium probe Fura-2AM. Stx (1 μg/ml) was added to the medium, as indicated, after initiation of Ca^{2+} measurements. Histamine (1 μM) was added 20 s (a), 4 min (b), 10 min (c), or 15 min (d) after Stx addition. For c and d, the traces are cut between 5 and 10 or 15 min, respectively. The left traces represent cells without toxin. Traces show the Fura-2AM fluorescent ratio (340 nm/380 nm). (e) After Fura-2AM loading, HeLa cells were incubated at 18°C for measurements. Stx was added as indicated. Histamine (1 μM) was added 20 min after the Stx addition. Traces presented are cut between 5 and 20 min. (f) Cells were pretreated with 2 μM SB203580 for 30 min. The cells were then loaded with Fura-2AM. Stx was added 2 min after the measurements started. Histamine (1 μM) was added 15 min after the Stx addition. Each trace is a representative Ca^{2+} response for 18–30 individual cells per experimental condition.

purified B-chain were similar, one can use the B-chain alone to investigate this phenomenon further.

Intracellular Trafficking of Stx in HeLa Cells Is Sensitive to Ca^{2+} Levels: Nonadditive Effects of TMB-8 and p38 Inhibition

Cross talk between Ca^{2+} signaling and p38 activation has been proposed previously (Chao *et al.*, 1992; Ikeda *et al.*, 2000; Takeda *et al.*, 2004; Fazal *et al.*, 2005), and we set out to investigate whether there is a link between the requirements for Ca^{2+} and p38 in Stx transport. First, endocytosis assays were performed in the presence of Ca^{2+} chelators. As reported by Chen *et al.* (2002), none of these chelators seemed to affect Stx uptake to any large extent (data not shown). However, we noticed that 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA)-AM, a membrane-permeant Ca^{2+} chelator, became cytotoxic at concentrations higher than 20 μM (data not shown). We therefore tested the effect of different concentrations on toxin sulfation. 20 μM BAPTA-AM reduced Stx sulfation by >80% compared with that of the control (Figure 6a, black bars), whereas total protein sulfation was reduced by 30% at this concentration (Figure 6a, gray bars). The effect on total protein sulfation is in agreement with the results of Chen *et al.* (2002) showing that also anterograde, ER-to-Golgi, transport is sensitive to removal of Ca^{2+}. In the further studies, we chose to work with 10 μM BAPTA-AM, a concentration that gave strong reduction in Stx sulfation, but only moderately affected total protein sulfation (Figure 6a). To confirm these data, we performed Stx toxicity experiments on cells treated with 10 μM BAPTA-AM. Under these conditions, we observed a 15-fold protection against Stx (average $\pm$ deviation, 14.8 $\pm$ 2.4; n = 2) (Figure 6b).

We also investigated the Stx-induced activation of p38 in the presence of BAPTA-AM. As shown in Figure 6c, BAPTA-AM efficiently inhibited p38 phosphorylation.

To confirm the results from experiments with BAPTA-AM–treated cells, we also tested the effect of TMB-8, an inhibitor of intracellular Ca^{2+} release (Bencherif *et al.*, 1995). This inhibitor reduced Stx sulfation with 30% compared with that of the control cells, whereas total protein sulfation remained unaffected (Figure 6d). In agreement with this, we observed a 2.5 $\pm$ 1.1 (n = 3) protection against Stx in cells treated with TMB-8 (Figure 6e). Thus, these data indicate that the cytosolic Ca^{2+} level, and perhaps even a local change in Ca^{2+} concentration, is important for proper transport of Stx to the Golgi apparatus.

Next, we wanted to study the link between Ca^{2+} and the requirement for active p38 for proper Stx transport. Because inhibitors of both p38 and Ca^{2+} release reduce Stx transport, we reasoned that if p38 and Ca^{2+} act on the same pathway, cotreatment with the two inhibitors should not cause further reduction. As shown in Figure 6f, no additive effect on Stx sulfation was observed in cells treated with both SB203580 and TMB-8 compared with cells treated with SB203580 alone. This suggests that p38 activation and the level of Ca^{2+} together regulate one step in the Stx transport.

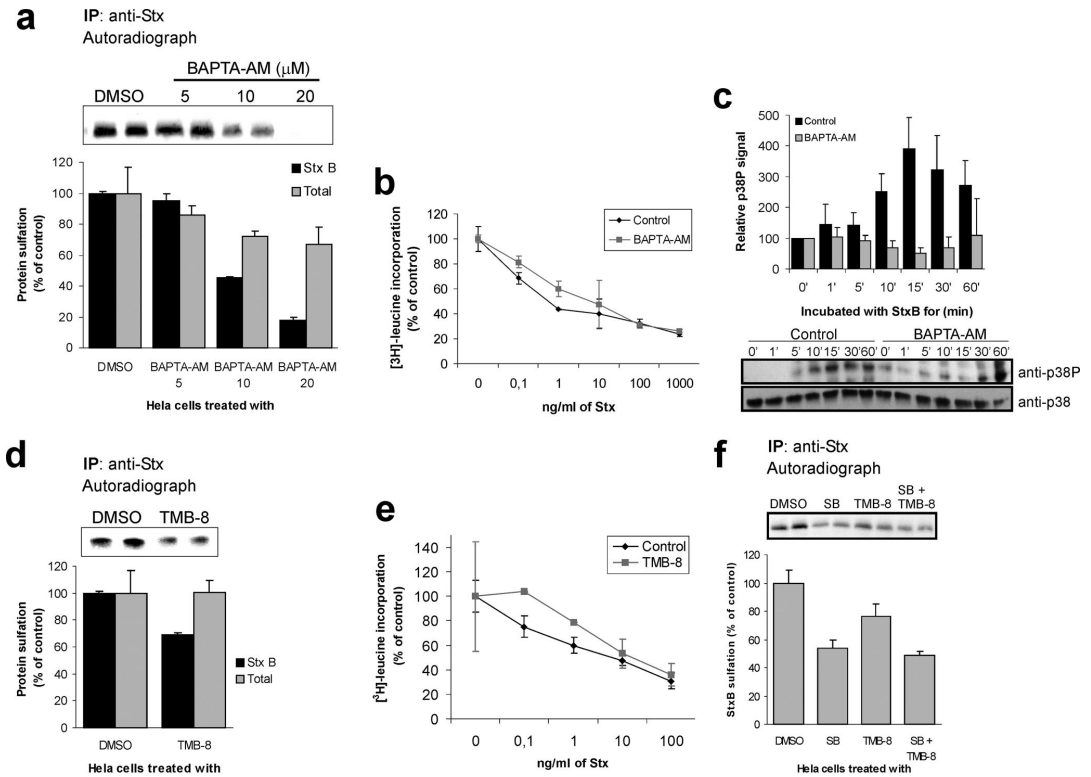

Figure 6. StxB transport to the TGN is sensitive to Ca^{2+} variations. (a) HeLa cells were incubated with BAPTA-AM at the indicated concentrations or the carrier (DMSO; 0.1% final concentration) for 30 min before incubation with StxB for 45 min and lysis of the cells. StxB was immunoprecipitated from the lysates, and its degree of sulfation analyzed by SDS-PAGE and autoradiography. The band intensities were calculated and plotted as average of parallels. Total cellular proteins sulfation was measured after TCA precipitation and plotted relative to the control. This experiment was performed twice with duplicates; error bars show deviations. (b) Cells were incubated with or without 10 μM BAPTA-AM for 30 min before addition of Stx. The experiment was then performed as described in Figure 3. This experiment was performed twice with duplicates; error bars show deviations. (c) Cells were serum starved for 1 h, and then they were treated with or without 10 μM BAPTA-AM for 30 min before StxB was added. Cell lysates were separated by SDS-PAGE and analyzed by Western blot. Quantification of three separate experiments is shown in the histogram. (d) Cells were incubated with 100 μM TMB-8 or the carrier (DMSO; 0.1% final concentration) for 30 min before incubation with StxB-$Sulf_2$ for 45 min. The experiment was then carried out as described in A. (e) Cells were incubated with or without 100 μM TMB-8 for 30 min before addition of Stx. The experiment was then performed as described in Figure 3. This experiment was repeated three times with duplicates. Error bars show deviations. (f) As in d, but in this assay cells were also treated or not with 30 μM SB203580 before addition of StxB-$Sulf_2$. This experiment was repeated twice with duplicates. Error bars show deviations.

Stx Regulates the Recruitment of p38α to Endocytic Membranes in a Ca^{2+}-dependent Manner

In neurons, MAP kinase p38 has been shown to be targeted to transport vesicles (Delcroix *et al*., 2003), and it has been suggested to be implicated in regulation of neurotransmitter transport. We therefore tested whether Stx binding was able to regulate the targeting of p38α to endosomes. To facilitate the studies, we used HEp2 cells, which, in contrast to HeLa cells, contain vesicles easily separable in a sucrose gradient. As a control, HEp2 cells were tested in Stx sulfation experiments in the presence of p38α siRNA (Supplemental Figure S3A) and SB203580 or TMB-8 (Supplemental Figure S3B), and they were shown to respond as HeLa cells. Cellular fractions were purified as described previously (Aniento *et al*., 1996). Equal amounts of the purified endosomal fractions were analyzed by SDS-PAGE and Western immunoblot. p38α was found in the early endosomal fraction, and recruitment to this fraction occurred in an Stx-dependent manner (Figure 7a). In agreement with this finding, we observed increased vesicular p38 staining pattern in HeLa cells treated with Stx, compared with control cells, as shown by immunofluorescence microscopy (Figure 7b). And, indeed, a fraction of the Stx-positive endosomes were positive for p38 (Figure 7b) and the early endosome marker EEA1 (Supplemental Figure S4). Importantly, we did not observe translocation of p38 to the nucleus upon Stx stimulation, suggesting a local activation of p38 on endosomes (see *Discussion*).

To test a possible influence of variations in Ca^{2+} levels on p38 recruitment to endosomes, we repeated the endosome purification in the presence of TMB-8. As shown in Figure 7c, this inhibitor blocked p38α recruitment to early endosomal membranes (top, compare lanes 5 and 11). In agreement

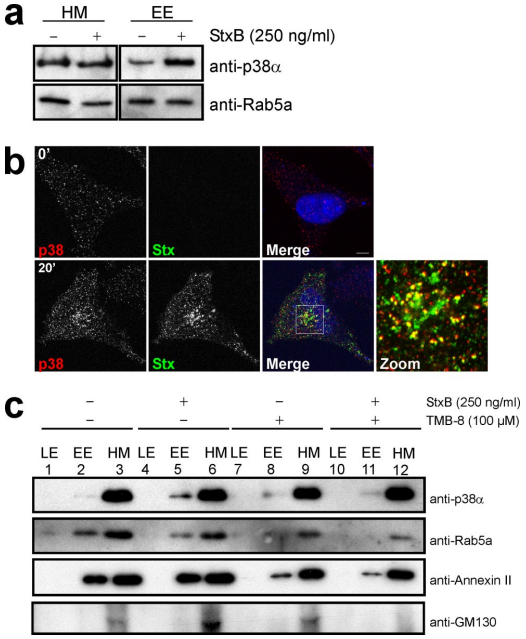

Figure 7. p38 is recruited to early endosomes by a Ca^{2+}-dependent mechanism upon Stx binding. (a) HEp2 cells were starved for 1 h before incubation with 250 ng/ml StxB for 20 min. Endosome fractions were prepared as described in *Materials and Methods*, and equal amounts of proteins were separated by SDS-PAGE and analyzed by Western immunoblotting with the indicated antibodies. (b) HeLa cells were incubated with 2 μg/ml StxB for 0 (top) or 20 min (bottom) before fixation and permeabilization. Antibodies against p38 and Stx were used. Bar, 5 μm. (c) As in a, but cells were treated with or without TMB-8 for 30 min before stimulation with 250 ng/ml StxB. Equal amounts of all fractions were separated by SDS-PAGE and analyzed by Western blot.

with previous studies (Cavalli *et al*., 2001), the early endosomal marker Rab5a was redistributed upon p38 activation (Figure 7c). Rab5a was also sensitive to Ca^{2+} depletion (Figure 7c). We therefore used annexin II as an additional internal marker (Figure 7c), because it has been shown to be only partially sensitive to Ca^{2+} depletion (Jost *et al*., 1997; Konig and Gerke, 2000). Despite the fact that a fraction of annexin II was detached from the endosomes upon TMB-8 treatment, the protein did not seem to be sensitive to Stx binding, and it could therefore be used as a marker for the stability of our system in respect to Stx. Finally, we used GM130 as a marker for Golgi membranes to show the purity of our endosome fractions (Figure 7c). These data suggest that the Ca^{2+} requirement for endosome to Golgi transport of Stx can be connected to the Ca^{2+} dependent recruitment of p38 to endosomes. Furthermore, the ability of the toxin to promote this recruitment may be related to the Stx-dependent modification of Ca^{2+} fluxes.

DISCUSSION

We have previously shown that Stx is an active player in mediating its own transport. Stx activates the tyrosine kinase Syk, which then specifically regulates cell entry of the toxin (Lauvrak *et al*., 2006). In the present article, we elucidate further on the ability of Stx to promote its own transport. We show that endosome to Golgi transport of Stx is regulated by the MAP kinase p38 and Ca^{2+}. Endocytosis of Stx induces a change in cytosolic Ca^{2+} levels, which in turn leads to activation of p38 and its recruitment to early endosomes.

The role of p38 in endosome to Golgi transport has not previously been investigated, but p38 has been found to regulate endocytosis and transport from endosomes. Fratti *et al*. (2003) have demonstrated that the activation of p38 by *Mycobacterium tuberculosis* is important for correct sorting of the pathogen (Fratti *et al*., 2003), and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor trafficking is improved by p38 (Huang *et al*., 2004). More recently, p38α has been implicated in the endocytosis of μ opioid receptor (Mace *et al*., 2005), and together with p38β, in epidermal growth factor receptor internalization (Vergarajauregui *et al*., 2006).

In the present study, inhibition of p38 activity, or knockdown of p38α, impaired transport of Stx from endosomes to the Golgi apparatus and protected the cells against the toxin. Importantly, the phenotypes observed by the two different methods used to validate the implication of p38α in intracellular trafficking of Stx, were similar, even though the incubation times differed significantly for the chemical inhibitors and siRNA. The requirement for p38 in retrograde transport seems to be specific for Stx, because there was no effect on transport of the related toxin ricin upon inhibition of p38 activity. These data are in agreement with the findings that p38 regulates sorting from early endosomes by phosphorylation of Rab5-GDI, EEA1, and Rabenosyn-5 (Cavalli *et al*., 2001; Fratti *et al*., 2003; Huang *et al*., 2004; Mace *et al*., 2005). It was recently suggested that Stx retrograde transport is independent of Rab5 (Fuchs *et al*., 2007). Future studies are needed to clarify these questions.

Previous reports show that p38 inhibitors protect different cell lines against Stx (Ikeda *et al*., 2000; Smith *et al*., 2003; Stricklett *et al*., 2005). However, these were studies of apoptotic cell death after long-term incubation with the toxin (24 h), and p38 activation was in these cells (Vero and brain endothelial cells) a late event induced by entry of active A-chain into cytosol. In contrast, we show that Stx rapidly activates p38 in HeLa cells. Also, as shown, after only 2.5 h of incubation with the toxin, there is a strong decrease in Stx-induced toxicity in cells treated with p38 inhibitor. A similar protection could be observed even after 90 min (data not shown). The strong protection might be due to a combination of two effects, the p38-dependent trafficking described in the present article, and ribotoxic stress attenuation described in previous reports (Foster and Tesh, 2002; Smith *et al*., 2003). Nevertheless, ricin, which also triggers ribotoxic stress (Iordanov *et al*., 1997; Foster and Tesh, 2002), was not affected by p38 inhibition, suggesting that Stx-related p38 activity is mainly dedicated to Stx trafficking in HeLa cells. The strong effect on Stx toxicity after p38 inhibition may indicate that p38 regulates transport from the Golgi apparatus to the ER as well. It would, therefore, be interesting to test whether p38α is also recruited to the Golgi membrane upon Stx binding.

The importance of Ca^{2+} homeostasis for Stx transport has been demonstrated, but signaling proteins linked to this Ca^{2+} requirement have not previously been characterized (Sandvig and Brown, 1987; Ikeda *et al*., 2000; Chen *et al*., 2002). Here, we have shown that Stx is able to trigger changes in Ca^{2+} levels induced by ATP or histamine. This effect was not observed under conditions where Stx was retained in the early endosomes, suggesting that endosome

fusion with a later compartment might be required for the Stx-induced change in histamine-induced cytosolic Ca^{2+} oscillations. However, this finding does not exclude that Stx is able to induce Ca^{2+} changes in proximity to endosomes without fusion to another compartment, and with different kinetics. We further show that modification in Ca^{2+} levels is necessary for activation of p38 and its recruitment to early endosomes after Stx stimulation. p38 inhibition did not affect the Stx-induced change in Ca^{2+} oscillations, supporting that p38 activation and recruitment to endosomes may occur after Ca^{2+} signaling.

We have presented data showing that there is a link between Ca^{2+}, p38 and Stx trafficking on the endosomal level. MAP kinases were reported to be found on endosomes even before any connection to trafficking was demonstrated (Pol *et al*., 1998). In our assay, we observed that a small fraction of p38 is activated upon Stx binding, suggesting that a small pool of p38 is sufficient to regulate toxin transport. One can speculate that this pool has to be targeted to the endosomal membrane in order to regulate Stx trafficking. In other words, in contrast to EGF receptor internalization (Vergarajauregui *et al*., 2006; Zwang and Yarden, 2006), activation of p38 in itself may not be sufficient to improve Stx trafficking. The endosomal recruitment is likely to be dependent on the presence of scaffold proteins (Morrison and Davis, 2003; Kolch, 2005). Such proteins have been shown to be essential for the recruitment of extracellular signal-regulated kinase to the Golgi (Morrison and Davis, 2003; Wang *et al*., 2005) or to the late endosome/lysosome (Wunderlich *et al*., 2001; Teis *et al*., 2002). It was recently shown that p38 requires β-arrestin-1 recruitment for proper activation in platelet-activating factor-stimulated cells (McLaughlin *et al*., 2006). For the present story, it is, therefore, tempting to speculate on the existence of a Ca^{2+}-dependent scaffolding protein that is involved in recruitment of p38 to endosomes. Interestingly, it has been shown that scaffold proteins can be sufficient to activate p38α (Ge *et al*., 2002), suggesting that the proper targeting can bypass an upstream signaling cascade. Together, these results support a critical role for a yet unidentified scaffold protein that would link p38 to endosomes in a Ca^{2+}-dependent manner.

In previous studies, we have observed that compensatory trafficking events can take place upon inhibition or knockdown of proteins essential for trafficking (Llorente *et al*., 2003; Utskarpen *et al*., 2006). For p38, there were no indications of such, supporting that this kinase has a unique role in the endosome to Golgi and ER route of Stx. Thus, in contrast to the reduced Stx transport after Rab6A inhibition, which can be overcome by upregulation of Rab6A′ (Del Nery *et al*., 2006; Utskarpen *et al*., 2006), there is an absolute requirement for p38α activity for proper trafficking of Stx.

ACKNOWLEDGMENTS

We are grateful to E. Rolén and A.-G. Myrann for expert technical assistance. We acknowledge Prof. R. J. Davis (University of Massachusetts, Worcester, MA) for the p38, MKK3, and MKK6 cDNA constructs. Prof. N. Kurebayashi (Juntendo University School of Medicine, Tokyo, Japan) kindly let us use a microscope for calcium measurements. We are grateful to Dr. K. Pattni and H. Raa for critical reading of the manuscript. This study was supported by The Norwegian Radium Hospital, The University of Oslo, The Norwegian Cancer Society, The Norwegian Research Council and Humanities, The Novo Nordisk Foundation, The Jahre Foundation, and Jeanette and Søren Bothners Legacy. S.W. was in 2003 supported by Federation of European Biochemical Societies and Fond National Suisse de la Recherche Scientifique postdoctoral fellowships.

REFERENCES

Aniento, F., Gu, F., Parton, R. G., and Gruenberg, J. (1996). An endosomal beta COP is involved in the pH-dependent formation of transport vesicles destined for late endosomes. J. Cell Biol. *133*, 29–41.

Bencherif, M., Eisenhour, C. M., Prince, R. J., Lippiello, P. M., and Lukas, R. J. (1995). The "calcium antagonist" TMB-8 [3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester] is a potent, non-competitive, functional antagonist at diverse nicotinic acetylcholine receptor subtypes. J. Pharmacol. Exp. Ther. *275*, 1418–1426.

Cameron, P., Smith, S. J., Giembycz, M. A., Rotondo, D., and Plevin, R. (2003). Verotoxin activates mitogen-activated protein kinase in human peripheral blood monocytes: role in apoptosis and proinflammatory cytokine release. Br. J. Pharmacol. *140*, 1320–1330.

Cavalli, V., Vilbois, F., Corti, M., Marcote, M. J., Tamura, K., Karin, M., Arkinstall, S., and Gruenberg, J. (2001). The stress-induced MAP kinase p38 regulates endocytic trafficking via the GDI:Rab5 complex. Mol. Cell *7*, 421–432.

Chao, T. S., Byron, K. L., Lee, K. M., Villereal, M., and Rosner, M. R. (1992). Activation of MAP kinases by calcium-dependent and calcium-independent pathways. Stimulation by thapsigargin and epidermal growth factor. J. Biol. Chem. *267*, 19876–19883.

Chen, J. L., Ahluwalia, J. P., and Stamnes, M. (2002). Selective effects of calcium chelators on anterograde and retrograde protein transport in the cell. J. Biol. Chem. *277*, 35682–35687.

Cherla, R. P., Lee, S.-Y., and Tesh, V. L. (2003). Shiga toxins and apoptosis. FEMS Microbiol. Lett. *228*, 159–166.

Del Nery, E., Miserey-Lenkei, S., Falguieres, T., Nizak, C., Johannes, L., Perez, F., and Goud, B. (2006). Rab6A and Rab6A′ GTPases play non-overlapping roles in membrane trafficking. Traffic *7*, 394–407.

Delcroix, J. D., Valletta, J. S., Wu, C., Hunt, S. J., Kowal, A. S., and Mobley, W. C. (2003). NGF signaling in sensory neurons: evidence that early endosomes carry NGF retrograde signals. Neuron *39*, 69–84.

Fazal, N., Choudhry, M. A., and Sayeed, M. M. (2005). Inhibition of T cell MAPKs (Erk 1/2, p38) with thermal injury is related to down-regulation of Ca2+ signaling. Biochim. Biophys. Acta *1741*, 113–119.

Foster, G. H., and Tesh, V. L. (2002). Shiga toxin 1-induced activation of c-Jun NH(2)-terminal kinase and p38 in the human monocytic cell line THP-1, possible involvement in the production of TNF-alpha. J. Leukoc. Biol. *71*, 107–114.

Fratti, R. A., Chua, J., and Deretic, V. (2003). Induction of p38 mitogen-activated protein kinase reduces early endosome autoantigen 1 (EEA1) recruitment to phagosomal membranes. J. Biol. Chem. *278*, 46961–46967.

Fuchs, E., Haas, A. K., Spooner, R. A., Yoshimura, S., Lord, J. M., and Barr, F. A. (2007). Specific Rab GTPase-activating proteins define the Shiga toxin and epidermal growth factor uptake pathways. J. Cell Biol. *177*, 1133–1143.

Ge, B., Gram, H., Di Padova, F., Huang, B., New, L., Ulevitch, R. J., Luo, Y., and Han, J. (2002). MAPKK-independent activation of p38alpha mediated by TAB1-dependent autophosphorylation of p38alpha. Science *295*, 1291–1294.

Gonzalez-Gaitan, M., and Stenmark, H. (2003). Endocytosis and signaling: a relationship under development. Cell *115*, 513–521.

Huang, C. C., You, J. L., Wu, M. Y., and Hsu, K. S. (2004). Rap1-induced p38 mitogen-activated protein kinase activation facilitates AMPA receptor trafficking via the GDI.Rab5 complex. Potential role in (S)-3,5-dihydroxyphenylglycene-induced long term depression. J. Biol. Chem. *279*, 12286–12292.

Ikeda, M., Gunji, Y., Yamasaki, S., and Takeda, Y. (2000). Shiga toxin activates p38 MAP kinase through cellular Ca(2+) increase in Vero cells. FEBS Lett. *485*, 94–98.

Iordanov, M. S., Pribnow, D., Magun, J. L., Dinh, T. H., Pearson, J. A., Chen, S. L., and Magun, B. E. (1997). Ribotoxic stress response: activation of the stress-activated protein kinase JNK1 by inhibitors of the peptidyl transferase reaction and by sequence-specific RNA damage to the alpha-sarcin/ricin loop in the 28S rRNA. Mol. Cell. Biol. *17*, 3373–3381.

Johannes, L., Tenza, D., Antony, C., and Goud, B. (1997). Retrograde transport of KDEL-bearing B-fragment of Shiga toxin. J. Biol. Chem. *272*, 19554–19561.

Jost, M., Zeuschner, D., Seemann, J., Weber, K., and Gerke, V. (1997). Identification and characterization of a novel type of annexin-membrane interaction: Ca2+ is not required for the association of annexin II with early endosomes. J. Cell Sci. *110*, 221–228.

Katagiri, Y. U., Mori, T., Nakajima, H., Katagiri, C., Taguchi, T., Takeda, T., Kiyokawa, N., and Fujimoto, J. (1999). Activation of Src family kinase yes induced by Shiga toxin binding to globotriaosyl ceramide (Gb3/CD77) in low density, detergent-insoluble microdomains. J. Biol. Chem. *274*, 35278–35282.

Kolch, W. (2005). Coordinating ERK/MAPK signalling through scaffolds and inhibitors. Nat. Rev. Mol. Cell Biol. *6*, 827–837.

Konig, J., and Gerke, V. (2000). Modes of annexin-membrane interactions analyzed by employing chimeric annexin proteins. Biochim. Biophys. Acta *1498*, 174–180.

Lauvrak, S. U., Llorente, A., Iversen, T. G., and Sandvig, K. (2002). Selective regulation of the Rab9-independent transport of ricin to the Golgi apparatus by calcium. J. Cell Sci. *115*, 3449–3456.

Lauvrak, S. U., Walchli, S., Iversen, T. G., Slagsvold, H. H., Torgersen, M. L., Spilsberg, B., and Sandvig, K. (2006). Shiga toxin regulates its entry in a Syk-dependent manner. Mol. Biol. Cell *17*, 1096–1109.

Llorente, A., Lauvrak, S. U., van Deurs, B., and Sandvig, K. (2003). Induction of direct endosome to endoplasmic reticulum transport in Chinese hamster ovary (CHO) cells (LdlF) with a temperature-sensitive defect in epsilon-coatomer protein (epsilon-COP). J. Biol. Chem. *278*, 35850–35855.

Mace, G., Miaczynska, M., Zerial, M., and Nebreda, A. R. (2005). Phosphorylation of EEA1 by p38 MAP kinase regulates mu opioid receptor endocytosis. EMBO J. *24*, 3235–3246.

Mallard, F., Antony, C., Tenza, D., Salamero, J., Goud, B., and Johannes, L. (1998). Direct pathway from early/recycling endosomes to the Golgi apparatus revealed through the study of shiga toxin B-fragment transport. J. Cell Biol. *143*, 973–990.

Maturana, A., Van Haasteren, G., Piuz, I., Castelbou, C., Demaurex, N., and Schlegel, W. (2002). Spontaneous calcium oscillations control c-fos transcription via the serum response element in neuroendocrine cells. J. Biol. Chem. *277*, 39713–39721.

McLaughlin, N. J., Banerjee, A., Kelher, M. R., Gamboni-Robertson, F., Hamiel, C., Sheppard, F. R., Moore, E. E., and Silliman, C. C. (2006). Platelet-activating factor-induced clathrin-mediated endocytosis requires beta-arrestin-1 recruitment and activation of the p38 MAPK signalosome at the plasma membrane for actin bundle formation. J. Immunol. *176*, 7039–7050.

Mori, T. *et al.* (2000). Globotriaosyl ceramide (CD77/Gb3) in the glycolipid-enriched membrane domain participates in B-cell receptor-mediated apoptosis by regulating lyn kinase activity in human B cells. Exp. Hematol. *28*, 1260–1268.

Morrison, D. K., and Davis, R. J. (2003). Regulation of MAP kinase signaling modules by scaffold proteins in mammals. Annu. Rev. Cell Dev. Biol. *19*, 91–118.

Pelkmans, L., Fava, E., Grabner, H., Hannus, M., Habermann, B., Krausz, E., and Zerial, M. (2005). Genome-wide analysis of human kinases in clathrin- and caveolae/raft-mediated endocytosis. Nature *436*, 78–86.

Pol, A., Calvo, M., and Enrich, C. (1998). Isolated endosomes from quiescent rat liver contain the signal transduction machinery. Differential distribution of activated Raf-1 and Mek in the endocytic compartment. FEBS Lett. *441*, 34–38.

Polo, S., and Di Fiore, P. P. (2006). Endocytosis conducts the cell signaling orchestra. Cell *124*, 897–900.

Rapak, A., Falnes, P. O., and Olsnes, S. (1997). Retrograde transport of mutant ricin to the endoplasmic reticulum with subsequent translocation to cytosol. Proc. Natl. Acad. Sci. USA *94*, 3783–3788.

Reynolds, A., Leake, D., Boese, Q., Scaringe, S., Marshall, W. S., and Khvorova, A. (2004). Rational siRNA design for RNA interference. Nat. Biotechnol. *22*, 326–330.

Sandvig, K., and Brown, J. E. (1987). Ionic requirements for entry of Shiga toxin from *Shigella dysenteriae* 1 into cells. Infect. Immun. *55*, 298–303.

Smith, W. E., Kane, A. V., Campbell, S. T., Acheson, D. W., Cochran, B. H., and Thorpe, C. M. (2003). Shiga toxin 1 triggers a ribotoxic stress response leading to p38 and JNK activation and induction of apoptosis in intestinal epithelial cells. Infect. Immun. *71*, 1497–1504.

Stricklett, P. K., Hughes, A. K., and Kohan, D. E. (2005). Inhibition of p38 mitogen-activated protein kinase ameliorates cytokine up-regulated shiga-toxin-1 toxicity in human brain microvascular endothelial cells. J. Infect. Dis. *191*, 461–471.

Taga, S. *et al*. (1997). Intracellular signaling events in CD77-mediated apoptosis of Burkitt's lymphoma cells. Blood *90*, 2757–2767.

Takeda, K., Matsuzawa, A., Nishitoh, H., Tobiume, K., Kishida, S., Ninomiya-Tsuji, J., Matsumoto, K., and Ichijo, H. (2004). Involvement of ASK1 in Ca2+-induced p38 MAP kinase activation. EMBO Rep. *5*, 161–166.

Takenouchi, H., Kiyokawa, N., Taguchi, T., Matsui, J., Katagiri, Y. U., Okita, H., Okuda, K., and Fujimoto, J. (2004). Shiga toxin binding to globotriaosyl ceramide induces intracellular signals that mediate cytoskeleton remodeling in human renal carcinoma-derived cells. J. Cell Sci. *117*, 3911–3922.

Teis, D., Wunderlich, W., and Huber, L. A. (2002). Localization of the MP1-MAPK scaffold complex to endosomes is mediated by p14 and required for signal transduction. Dev. Cell *3*, 803–814.

Torgersen, M. L., Lauvrak, S. U., and Sandvig, K. (2005). The A-subunit of surface-bound Shiga toxin stimulates clathrin-dependent uptake of the toxin. FEBS J. *272*, 4103–4113.

Utskarpen, A., Slagsvold, H. H., Iversen, T. G., Walchli, S., and Sandvig, K. (2006). Transport of Ricin from endosomes to the Golgi apparatus is regulated by Rab6A and Rab6A'. Traffic *7*, 663–672.

Vergarajauregui, S., San Miguel, A., and Puertollano, R. (2006). Activation of p38 mitogen-activated protein kinase promotes epidermal growth factor receptor internalization. Traffic *7*, 686–698.

Wang, P. Y., Weng, J., and Anderson, R. G. (2005). OSBP is a cholesterol-regulated scaffolding protein in control of ERK 1/2 activation. Science *307*, 1472–1476.

Wunderlich, W., Fialka, I., Teis, D., Alpi, A., Pfeifer, A., Parton, R. G., Lottspeich, F., and Huber, L. A. (2001). A novel 14-kilodalton protein interacts with the mitogen-activated protein kinase scaffold mp1 on a late endosomal/lysosomal compartment. J. Cell Biol. *152*, 765–776.

Wälchli, S., Espanel, X., Harrenga, A., Rossi, M., Cesareni, G., and Hooft van Huijsduijnen, R. (2004). Probing protein-tyrosine phosphatase substrate specificity using a phosphotyrosine-containing phage library. J. Biol. Chem. *279*, 311–318.

Zwang, Y., and Yarden, Y. (2006). p38 MAP kinase mediates stress-induced internalization of EGFR: implications for cancer chemotherapy. EMBO J. *25*, 4195–4206.

III

This article is removed from the dissertation.

IV