DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
166

DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
166

PILLE SÄÄLIK

The role of endocytosis in the protein transduction by cell-penetrating peptides

TARTU UNIVERSITY
PRESS

Department of Cell Biology, Institute of Molecular and Cell Biology, University of Tartu, Estonia

Dissertation is accepted for the commencement of the degree of Doctor of Philosophy in cell biology on May 13th, 2009 by the council of the Institute of Molecular and Cell Biology, University of Tartu.

Opponent: Dr. Arwyn Tomos Jones
Welsh School of Pharmacy,
Cardiff University, Cardiff, United Kingdom.

Commencement: Room 217, Riia St. 23, Tartu, at 10:00 on June 30th, 2009

Publication of this dissertation is granted by the University of Tartu

ISSN 1024–6479
ISBN 978–9949–19–146–8 (trükis)
ISBN 978–9949–19–147–5 (PDF)

Tartu Ülikooli Kirjastus
www.tyk.ee
Tellimus nr. 205

TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The current thesis is based on the following original publications, which will be referred to by their Roman numerals in the text.

I. **Säälik, P.,** Elmquist, A., Hansen, M., Padari, K., Saar, K., Viht, K., Langel, Ü., Pooga, M. (2004) Protein cargo delivery properties of cell-penetrating peptides. A comparative study. *Bioconjug Chem.* 15(6): 1246–53

II. Padari, K., **Säälik, P.,** Hansen, M., Koppel, K., Langel, Ü., Pooga, M. (2005) Cell transduction pathways of transportans. *Bioconjug Chem.* 16(6):1399–410

III. **Säälik, P.,** Padari, K., Lorents, A., Hansen, M., Niinep, A., Jokitalo, E., Langel, Ü., Pooga, M. (2009) Protein delivery with transportans is mediated by caveolae rather than flotillin-mediated pathways. *Bioconjug Chem.,* 20(5): 877–887

IV. Räägel, H., **Säälik, P.,** Hansen, M., Langel, Ü., Pooga, M. (2009) The cell-penetrating peptide-protein constructs induce a population of vesicles with non-acidic pH during trafficking through the endo-lysosomal pathway. *Submitted to Journal of Controlled Release.*

The articles listed above have been reprinted with the permission of the copy-right owners.
My personal contribution to the articles referred to in this thesis is as follows:

Ref. I	performed the experiments, participated in data analysis and in the writing of the paper.
Ref. II	designed and performed fluorescence microscopy experiments, participated in data analysis and in the writing of the paper.
Ref. III	designed and performed the fluorescence microscopy, Western blot and FACS experiments, participated in data analysis and in the writing of the paper.
Ref. IV	designed and performed fluorescence microscopy experiments on fixed cells, participated in data analysis and in the writing of the paper.

ABBREVIATIONS

AP	adaptor protein
Arf6	ADP ribosylation factor 6
$BclX_L$	anti-apoptotic mitochondrial membrane protein
Cdc42	cell division cycle protein 42
CLIC	clathrin- and dynamin independent carrier
CLSM	confocal laser scanning microscopy
CPP	cell-penetrating peptide
CtxB	cholera toxin B subunit
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EIPA	5-(N-ethyl-N-isopropyl) amiloride
FACS	fluorescence-activated cell sorter
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
GEEC	GPI-AP enriched early endosomal compartment
GM1	monosialotetrahexosylganglioside 1
GPI-AP	glycosylphosphatidylinositol-anchored protein
HIV-1	human immunodeficiency virus type 1
HoxB4	transcription factor from the Hox gene family
HSPG	heparan sulphate proteoglycan
HypNA	PNA-like monomer based on a *trans*-4-hydroxyl-L-proline
LAMP2	lysosome-associated membrane protein 2
LTR	long terminal repeat sequence of HIV-1, a portion of a viral genome that controls replication
MAP	model amphipathic peptide
MCD	methyl-β-cyclodextrin
mdx	gene encoding dystrophin protein
MHC I	class I major histocompatibility complex
MPG	cell-penetrating peptide designed from the fusion peptide of HIV-1 gp41 and NLS
NLS	nuclear localization signal
NPC1/NPC2	Niemann-Pick disease type C
Otx2	homeodomain-containing transcription factor from bicoid sub-family
pAntp	synonym of penetratin, a cell-penetrating peptide from the Antennapedia homeoprotein
Pax6	transcription factor from paired box gene 6, containing DNA-binding paired box and homeobox domains
Pep-1	cell-penetrating peptide designed from the dimerization domain of HIV-1 reverse transcriptase and NLS
$PI(4,5)P_2$	phosphatidylinositol-4,5-diphosphate
PI3P	phosphatidylinositol-3-phospate
PMO	phosphorodiamidate morpholino oligomer

PNA	peptide nucleic acid
PNP	purine nucleoside phosphorylase
pTat	HIV Tat protein derived cell-penetrating peptide
PTRF	polymerase I and transcript release factor
pVEC	vascular endothelial cadherin derived cell-penetrating peptide
R_9	cell-penetrating peptide consisting of nine arginines
Rac1	Ras-related C3 botulinum toxin substrate 1
RhoA	Ras homologue gene family member A, small GTPase
RISC	RNA-induced silencing complex
RXR	oligoarginine-aminohexanoic acid-arginine sequence
S4(13)-PV	cell-penetrating peptide designed from dermaseptin antimicrobial peptide S4 and NLS
siRNA	small interfering RNA
SV40	simian virus 40
TEM	transmission electron microscopy
TGFβ	transforming growth factor β
TGN	trans-Golgi network
TP10	transportan 10
VP22	herpes simplex virus-1 tegument protein VP22

INTRODUCTION

The discovery of cell-penetrating peptides (CPPs) more than a decade ago raised expectations to reach a new level of possibilities in the biological and therapeutic field. Because of the ability to translocate across the plasma membrane, a barrier that usually prevents the uptake of big hydrophilic molecules, CPPs were considered to become the vehicles that enable to reach intracellular targets instead of influencing the intracellular events through receptors at the plasma membrane, a strategy that is mainly exploited in current drug design.

However, in spite of the impressive amount of knowledge about CPPs gathered by now both *in vitro* and *in vivo* systems, more questions are raised than answers found. The initial view on the translocation of CPPs directly through the plasma membrane has been substituted by the endosomal uptake, at least for big cargo molecules. On the other hand, several studies report about the efficient targeting of CPP-coupled biomolecules independently from the endosomal pathway, showing that under certain circumstances CPPs can avoid the entrapment in endo-lysosomal organelles. The endosomal compartment is a complex and highly controlled system of vesicular carriers mediating the internalization and intracellular trafficking of various biological molecules. Various issues of endosomal trafficking are currently under intense investigation, one of which includes translocation of proteins and DNA from endosomes to the cytosol. Therefore the comprehensive knowledge about the endocytic processes in CPP-mediated delivery is of high importance.

The main purpose of this study was to characterize the protein delivery properties of transportan, transportan 10 (TP10) and the endosomal pathways used while comparing these to other CPPs like Tat peptide (pTat), penetratin (pAntp), nonaarginine (R_9) and pVEC. Transportan is a chimeric peptide obtained by linking the N-terminal fragment of neuropeptide galanin to mastoparan, a peptide from wasp venom. TP10 is a shorter version of transportan retaining high cell-penetrating activity and having less side effects. Tat peptide and nonaarginine are arginine-rich CPPs and together with penetratin derived from Antennapedia homeoprotein they represent the most studied CPPs. By using the biotinylated CPP as a carrier and avidin/streptavidin/neutravidin as protein cargo we compared the uptake and kinetics of the CPP-mediated protein cellular delivery into mammalian cells. In addition, we characterized the intracellular trafficking of CPP-protein complexes in relation to different endosomal compartments.

The knowledge obtained in the present study might help to improve the CPPs for different biotechnological applications and in developing efficient vectors in drug development.

I. LITERATURE OVERVIEW

I.1. Endocytosis

Cells have evolved several ways to acquire compounds essential for their life from surrounding environment. Small and hydrophobic compounds such as gases reach the cells via a simple diffusion but for the transport of ions, water, amino acids and sugars, not to mention bigger macromolecules like proteins and nucleic acids, regulated uptake systems have been developed. While the availability of small biomolecules is mainly regulated by various and highly specific membrane pumps and channels, in the cellular uptake of big and hydrophilic bioactive molecules endocytosis acts as a more flexible tool. Endocytosis is a term used to describe the internalization of the extracellular material by cells using vesicular carriers, which invaginate and pinch off from the plasma membrane. Historically endocytosis is divided to **phagocytosis** and **pinocytosis**, former used for engulfment of insoluble material and latter comprising the various internalization modes of fluids (Fig. 1). Two types of pinocytosis are named **clathrin-** and **caveolin-mediated endocytosis** according to the proteins involved in the formation or the shape of the transport vesicle. The fluid phase uptake mode via engulfment of big amount of extracellular fluid by heterogeneous vesicles after extensive membrane dynamics is known as **macropinocytosis**. However, from that frontier the classification gets complicated and often all

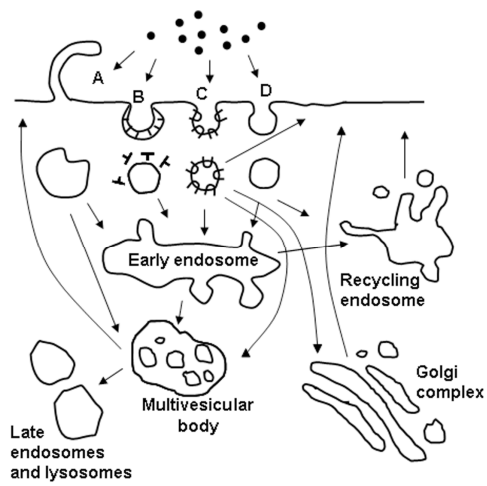

Figure 1. Schematic illustration of the main pinocytic pathways and intracellular routing of endocytosed material. A – macropinocytosis, B – clathrin-dependent, C – caveolin- and flotillin-mediated, and D – clathrin and caveolin-independent endocytosis. The endosomal trafficking routes are indicated by arrows.

the remaining vesicular internalization modes are indicated as **nonclathrin/ noncaveolar endocytosis**. Up to now, numerous biomolecules have been demonstrated to exploit the endocytic pathway without the abovementioned mediators. Vesicular entry dependent on dynamin or other small GTPases such as RhoA, Cdc42 or Arf6 has been suggested as one possibility for classification (Mayor and Pagano, 2007). On the other hand, most of the endosomal pathways – clathrin- and caveolin-dependent as well as –independent routes – are more or less interfered by the lack of membrane cholesterol, a crucial component of the membranous microdomains or so called lipid rafts. However, conflicting concepts about the nature and constituents of lipid rafts makes the discussion about lipid-raft-dependent endocytosis a topic with rather diffuse boundaries and therefore the term cholesterol-dependent endocytosis could reflect the nature of this type of endocytosis more precisely.

1.1.1 Clathrin-mediated endocytosis

The bristle coated vesicles were initially described in 1964 in insect oocytes (Roth and Porter, 1964). This internalization mode, known as clathrin-mediated endocytosis, can be counted as the best described uptake route of extracellular material. The unique structure characteristic to this type of endocytosis is formed by three molecules of clathrin heavy (190 kDa) and three molecules of clathrin light chains (25–29 kDa) (Edeling *et al.*, 2006). Together they pack into a triskelion, which is a subunit to the formation of a three-dimensional „honeycomb-like“ cage, the clathrin lattice. As the clathrin itself does not interact with membrane lipids, the contact with latter is achieved via different adaptor proteins, e.g adaptor protein (AP) compexes 1–4, AP180, epsin, arrestin, amphiphysin (Owen *et al.*, 2004). These proteins recruit other components to the forming clathrin coated vesicle and confer specificity to the otherwise ubiquitous clathrin function.

The protein responsible for the fission of clathrin-coated vesicle is dynamin, a 100 kDa GTPase, which is recruited to the membrane via its interaction with membrane lipid $PI(4,5)P_2$ leading to detachment of the clathrin-coated vesicle from the plasma membrane. Eukaryotic cells use clathrin-mediated endocytosis to obtain essential nutrients, such as iron and cholesterol, from the surrounding medium. As infers the other definition of clathrin-coated vesicle transport – receptor-mediated endocytosis – the compounds to be internalized must interact with their receptors on the plasma membrane. Concentration of the receptor-ligand complex to the clathrin-coated pit accelerates its endocytosis typically 10 to 20-fold.

1.1.2. Macropinocytosis

Macropinocytosis is the best described type of endocytosis taking place without participation of clathrin and caveolin. Macropinocytic vesicles arise from the

dynamic movement of sheet-like cell surface outgrowths called membrane ruffles, which occasionally after falling back to the plasma membrane and fusing with it capture large amounts of extracellular fluid. Macropinocytic vesicles can be very heterogeneous in size reaching from 0.2 to 10 μm in diameter (Swanson and Watts, 1995). The initiation of formation of the macropinocytic vesicle does not require recruitment of dynamin as in clathrin-mediated endocytosis but in analogy to phagocytosis is based on the dynamics of the actin cytoskeleton. Although the exact mechanisms and the interacting molecules are to date only partially known, small GTPases of Ras superfamily Rac1, RhoA, Arf6 and Cdc42 together with phosphoinositides PtdIns(4,5)P_2 and PtdIns (3,4,5)P_3 are some of the main factors shown to stimulate the actin polymerization, the recruitment of myosin and the creation of membrane curvature and fusion (Swanson and Watts, 1995). Macropinocytosis can occur constitutively as indicated for antigen-presenting dendritic cells (Sallusto *et al.*, 1995) or be induced, most often by growth factors. Macropinocytosis acts as an elimination mechanism for apoptotic cells by engulfing big amounts of extracellular fluid, but plays a role also in creation of immune response. Fast endocytosis of large membrane areas also enables operative remodelling of the cell shape in migrating cells. In addition, macropinocytosis has been shown to be used in the internalization of extracellular DNA, which first binds to certain secreted proteins and then is taken up by cells after binding to the plasma membrane heparan sulfate proteoglycans (HSPG) (Wittrup *et al.*, 2007).

Macropinocytosis is exploited also by some viruses in order to enter cells. Adenovirus type 2 uses macropinocytosis as one route to induce cellular uptake and has the ability to modulate the permeability of macropinosome, a property that is not observed in EGF-induced macropinosomes (Meier *et al.*, 2002). Moreover, Vaccinia virus has taken even a step further by simultaneously inducing macropinocytosis and apoptosis of the target cells leading to the subsequent clearance of apoptotic cellular material by immune cells and postponing the immune detection of virus by mimicking the apoptotic bodies (Mercer and Helenius, 2008).

1.1.3. Caveolin-mediated endocytosis

Caveolae are 50–80 nm flask-shaped membrane areas rich in GPI-anchored proteins, sphingolipids, cholesterol and caveolins. The cave-like structures in endothelial cells were first described by electron microscopy more than 50 years ago (Parton, 2003), but the components of these characteristic plasma membrane areas were defined much later. The main protein in caveolae is caveolin – a 21 kDa integral membrane protein with a 33-amino acid long hydrophobic segment, which is inserted as a hairpin loop into the membrane, while its amino- and carboxy-terminal regions point toward the cytosol (Dupree *et al.*, 1993). Caveolin was independently described by two research groups (Rothberg *et al.*, 1992; Dupree *et al.*, 1993). The earlier synonym VIP21, came from stud-

ies where caveolin-1 was identified as a tyrosine-phosphorylated substrate of transforming Rous sarcoma virus protein, v-Src (Glenney, 1989). Later, Simons and coworkers identified the 21 kDa protein from detergent resistant complexes derived from exosomes of epithelial cells (Dupree *et al.*, 1993). In both cases the isolated protein was demonstrated to be the main component of the filamentous structures detected in caveolae earlier by electron microscopy. Three isoforms of caveolin are known, out of which caveolin-1 and 2 have similar distribution with high expression in adipocytes, endothelial cells, fibroblasts and smooth muscle cells, while caveolin 3 is cardiac and skeletal muscle-specific (Parton, 2003). Caveolins are palmitoylated in the C-terminal segment, they can be phosphorylated on tyrosine residues, bind cholesterol and are able to form dimers and higher oligomers (Pelkmans and Helenius, 2002). Cellular internalization of caveolae is dependent on active dynamin (Nichols, 2002) and actin cytoskeleton (Thomsen *et al.*, 2002). Caveolar cargoes are diverse, ranging from lipids, proteins and lipid-anchored proteins to pathogens. In human skin fibroblasts and endothelial cells albumin is internalized by caveolae (Mayor and Pagano, 2007). Until quite recently caveolae were thought to be the only cellular entry point of simian virus 40 (SV40) (Pelkmans *et al.*, 2001). However, recently the ability of SV40 to enter the caveolin-1 knock-out fibroblast was reported, demonstrating the ability of the virus to switch between different endocytic pathways (Damm *et al.*, 2005).

Internalization of caveolae can lead to the formation of caveosomes, which can later fuse with early endosomes. In this case the fused vesicle is able to retain its mosaic nature despite the release of the cargo into the lumen of the early endosome (Pelkmans *et al.*, 2004). Caveolae were considered as rather immobile plasma membrane areas (Thomsen *et al.*, 2002), but recently the existence of a quickly moving caveosomal population, which is in frequent contact with the plasma membrane by a so-called „kiss and run“ mode was demonstrated (Pelkmans and Zerial, 2005). A recent study suggests polymerase I and transcript release factor (PTRF) to be required for caveolae formation in cultured mammalian cells and zebrafish embryo (Hill *et al.*, 2008). However, the exact mechanisms governing the formation of caveolae are still largely unknown.

1.1.4. Non-clathrin and non-caveolar endocytosis

It has been repeatedly shown that several molecules and receptors are internalized into cells via vesicular carriers, which are not pinched off from clathrin-coated pits or caveolae. These intermediates are internalized rapidly and often by forming tubular structures that are sensitive to fixation. However, all these endosomal structures, which are named as clathrin-independent carriers (CLIC), or GPI-enriched endocytic compartments (GEEC) still summarize a set of poorly described mechanisms. Actin and small GTPases like dynamin, RhoA, Arf1, Arf6 and Cdc42 have been shown to mediate the formation of CLICs. Among the plasma membrane molecules entering cells via clathrin- and caveo-

lin independent endocytosis, interleukin receptor 2, major histocompatibility complex class I (MHC I) and GPI-anchored proteins are the most well known examples. In addition, SV40, cholera toxin B subunit and dextran are shown to exploit this non-conventional uptake route. However, the mentioned cellular mediators are involved in the CLIC pathway to a different extent. Interleukin-2 receptor was one of the first plasma membrane receptors demonstrated to use cellular entry via CLIC pathway and to be dependent on RhoA (Lamaze *et al.*, 2001; Sabharanjak *et al.*, 2002; Cheng *et al.*, 2006). Activity of Rho A is necessary also in the uptake of fluid phase marker dextran (Sabharanjak *et al.*, 2002). On the other hand, the internalization of folate receptor, which is a GPI-anchored protein, takes place via a Cdc42-dependent pathway (Sabharanjak *et al.*, 2002). To further complicate the picture, Arf1 has also been shown to regulate the uptake of folate receptor (Kumari and Mayor, 2008), suggesting that Arf1 might act upstream of Cdc42. Arf6 has been reported to regulate the dynamin-independent endocytosis of several proteins such as MHC I, β1 integrin, E-cadherin and GPI-APs (Mayor and Pagano, 2007). On the contrary, Arf6-independent but cholesterol-sensitive pathway has been described for the cellular internalization of CtxB (Kirkham *et al.*, 2005) and SV40 (Damm *et al.*, 2005). Recently an unexpected role of Arf6 in AP-2 regulated post-endocytic trafficking was demonstrated, where AP-2 depletion modestly delayed the internalization and enhanced the lysosomal targeting of Arf6-dependent trafficking of β1 integrin and MHC I (Lau and Chou, 2008), demonstrating the function of well-described clathrin-binding AP-2 in clathrin-independent endocytosis.

In addition to the abovementioned proteins as mediators for clathrin- and caveolin-independent endocytic pathways, some more factors are shown to have an important role in these processes. Flotillin-1 and 2, named also Reggie-2 and 1, respectively, are widely expressed integral membrane proteins with a propensity to form oligomers (Solis *et al.*, 2007). They have been reported to reside in cholesterol rich membrane microdomains and in invaginations that do not contain caveolins (Stuermer *et al.*, 2001; Frick *et al.*, 2007). Recently, the flotillin-mediated endocytosis was discovered and shown to be a new clathrin- and caveolin-independent endocytic pathway (Glebov *et al.*, 2006). Although it has been demonstrated that the flotillin-1-mediated endocytosis is dynamin-independent, at least for the internalization of CtxB (Glebov *et al.*, 2006), the actual relevance of this pathway in cell physiology has remained largely elusive due to the lack of specific markers of uptake. Flotillins have been suggested to participate in cellular signalling as they associate with src family tyrosine kinase fyn and GPI anchored proteins Thy-1 and F3 (Stuermer *et al.*, 2001). The absence of flotillin-1 is also indicated to accelerate the lysosomal degradation of caveolin-1 in human intestinal epithelial cells (Vassilieva *et al.*, 2009), implying a crosstalk between these proteins.

When excluding flotillins as markers of a specific type of clathrin-and caveolin-independent endocytosis, the attempts to define a noncargo marker for CLIC/GEEC pathway have not been successful. However, a recent report by a

group of McMahon postulated a Rho-GAP-domain-containing protein GRAF1 to be the first noncargo marker for the CLIC/GEEC endocytic membranes (Lundmark *et al.*, 2008). This protein was shown to be present in dynamic tubular and vesicular lipidic structures together with PI(4,5)P2 and Cdc42.

Factors shown to act in CLIC-mediated endocytosis are also plasma membrane proteoglycans, especially heparan sulfate proteoglycans (HSPGs). HSPGs have various roles in animal development and for several biological molecules they act as co-receptors or bridging agents between the ligand and the plasma membrane receptor (Bishop *et al.*, 2007). HSPGs mediate the uptake of cationic transfection agents like polyethyleneimine, cationic lipid mixture lipofectamine and polypeptide polyarginine in a dynamin- and flotillin-1-dependent manner (Payne *et al.*, 2007).

1.1.5. Endocytic routing of internalized cargo

Analogously to the clathrin-mediated endocytosis the intracellular fate of the clathrin-coated vesicles and the endosomal pathway downstream are more thoroughly studied. Historically, four different classes of endosomes are distinguished along the route of clathrin-mediated endocytosis pathway. The internalized proteins are first delivered to a tubulo-vesicular intermediate called an early endosome. It represents a highly dynamic structure, being responsible for the rapid recycling of internalized proteins, lipids and intracellular fluid. The fusion events between early endosomal structures are mediated by early endosomal antigen 1 (EEA1) (Christoforidis *et al.*, 1999), which is recruited to the vesicles by lipid phosphatidylinositol-3-phosphate (PI3P) (Gaullier *et al.*, 1998) and a small GTPase Rab5 (Simonsen *et al.*, 1998). Recent studies suggest that the clathrin-derived endosomes are able to sort the internalized cargo immediately before fusing with the early endosomes. Lakadamyali and collaborators presented the existence of two populations of clathrin-coated pits with different maturation kinetics and mobility (Lakadamyali *et al.*, 2006). By their observations, the transport of cargo towards degradation was mediated by high mobility population, while the contents meant for recycling were processed by slow-type endosomes (Lakadamyali *et al.*, 2006). In addition, the group of De Camilli defined a subset of early endosomal vesicles of clathrin- as well as of fluid phase origin, which were formed on the cell edge and marked by membrane adaptor protein APPL before the recruitment of Rab5 and PI3P (Zoncu *et al.*, 2009). Although in this study the clathrin-derived vesicles shared the APPL marker with macropinocytic vesicles, the intracellular fate of macropinosomes is probably dependent on cell-type, as both fusion of the macropinosomes with the early endosomal pathways and avoidance of this route has been reported (Jones, 2007).

The next organelle of the endosomal pathway, a multivesicular body, receives vesicles from early endosomes. In parallel, the gradual loss of membrane markers and the recycling of receptors takes place in its several internal mem-

branous vesicles. By sequential pH decrease due to the activity of Na^+/H^+ATPase in the endosomal membrane and by recruitment of acidic hydrolases from *trans*-Golgi network (TGN) multivesicular bodies step by step mature to late endosomes, which ultimately fuse with lysosomes, the organelles specialized in degradation of received molecules.

However, different endosomal pathways interact also horizontally, either already on the plasma membrane by sorting the membrane-interacted molecules, or between compartments taken up by cells via different endosomal routes. The flexible switching between entrance routes has been demonstrated to take place for TGF-β receptor, which can use both clathrin and caveolin-dependent pathway depending on the need of signalling or rapid receptor turnover, respectively (Di Guglielmo *et al.*, 2003).

Multivesicular bodies are able to fuse back to the plasma membrane and release their small internal vesicles (30–100 nm), exosomes. The majority of knowledge about these vesicular structures is obtained from studies with dendritic cells and cells from hematopoietic lineages (Théry *et al.*, 2002). The protein composition of exosomes assures their endosomal heritage, showing the presence of tetraspanins, Rab proteins and MHC class molecules. The functions of exosomes are poorly understood, although their role in the antigen presentation, secretion of soluble molecules like mRNA, micro RNA, as well as infectious particles like HIV and prion protein has been demonstrated (Schorey and Bhatnagar, 2008).

1.1.6. Cholesterol and the involvement of lipid rafts in endosomal pathways

Cholesterol constitutes one fifth of the lipid composition of the plasma membrane, but it can also be found in early endosomal vesicles and multivesicular bodies (Ikonen, 2008). However, its concentration in late endosomes and lysosomes is low (Möbius *et al.*, 2003). This phenomenon has been associated with the degradation of cholesterol-binding sphingomyelin by acid sphingomyelinase to cholesterol-non-binding ceramide in acidic endosomes, facilitating the transport of cholesterol to other cellular membranes by NPC1/NPC2 (Niemann-Pick disease Type C) system (Schulze *et al.*, 2009).

The ability of cholesterol to interact with specific proteins and with the more saturated membrane lipids was the premise to the hypothesis of plasma membrane microdomains as pre-existing and more densely packed raft-like entities (Simons and Ikonen, 1997). Now, during a decade the lipid raft concept has developed in parallel with improvements in imaging technology and the "modern" rafts are depicted as short-living nanoscale integrators in the signal transduction process (Harding and Hancock, 2008). The initial hypothesis was also based on the methodological considerations – the cell lysis in detergent-containing solution yielded a detergent-insoluble fraction, which floated in low sucrose gradient and contained cholesterol, sphingolipids and several proteins.

Caveolin was one of the characteristic proteins that sequestered with cholesterol and that is the reason why even currently some investigators consider lipid raft microdomains to be identical with caveolae. Another reason for classifying the lipid rafts and caveolin-containing membrane invaginations in one group also lies in methodology. In cellular trafficking studies the removal of cholesterol is often used, either by applying inhibitors of cholesterol synthesis, or pharmacological agents like methyl-β-cyclodextrin, which deplete cholesterol from caveolae and from lipid rafts as well. Lack of cholesterol causes malfunctioning of most endosomal pathways and this has been the ground for discussion about lipid raft-dependent endocytosis (Lajoie and Nabi, 2007). This classification, however, is a rather disputable way to distinguish between the endosomal processes where the role of cholesterol is poorly understood. Still, several contradictory results can be re-evaluated as a recent study demonstrated efficient sequestration of caveolae and non-caveolar lipid microdomains on the modified sucrose density gradient (Yao *et al.*, 2009). The authors also emphasize the different lipid composition of these particular fractions (Yao *et al.*, 2009), which strongly supports the hypothesis of caveolae and lipid rafts as separate membrane areas. Moreover, the facilitated differentiation between caveolae and other cholesterol-rich membrane areas might help to elucidate the involvement of cholesterol in endosomal processes as a possible modulator of intracellular membrane trafficking.

1.2. Cell-penetrating peptides

The ability of histones and polyamino acids to enhance the cellular uptake of serum albumin was noticed already decades ago (Ryser and Hancock, 1965). However, the recognition of cell-penetrating peptides (CPPs) as a specific class of transporters can be dated back to works on transforming *trans*-activator protein of human immunodeficiency virus (Frankel and Pabo, 1988; Green and Loewenstein, 1988). During those 20 years more than 100 peptidic sequences have been described to possess the ability to internalize into mammalian, plant and bacterial cells and mediate the transport of otherwise impermeable biologically active molecules. The length of these peptide sequences is usually from 5 to 40 amino acids and often they share a high content of basic amino acids, which gives the peptide a positive charge at physiological pH. Still, besides doubtlessly remarkable improvement in the cellular delivery of various biologically active cargos, the application of the CPPs as powerful drug delivery agents faces several obstacles. By now it is clear that CPPs use different mechanisms for cell entry, which is caused by the high variability of chemical and physical properties of the transducing sequences, and by the complex and cell-type-dependent composition of the plasma membrane, a gate and a barrier for every kind of drug delivery. The role of endocytosis in the entry of CPPs is demonstrated to be significant but its details are incompletely understood so far. However, in parallel the studies on the transducing peptide sequences have helped to

understand other biological processes like the action mechanisms of proteins containing nuclear localization signal (NLS) and the cell entry of viruses, which also often utilize the existing cellular endosomal pathways. In addition, the studies on so-called messenger proteins (Prochiantz, 2000) have demonstrated intercellular movement of homeodomain-containing transcription factors Engrailed-2, HoxB4 and Pax6 in *in vitro* cell culture systems (Amsellem *et al.*, 2003; Brunet *et al.*, 2005; Lesaffre *et al.*, 2007). Moreover, very recently the non-cell autonomous accumulation of homeprotein Otx2 in parvalbumin cells was demonstrated *in vivo,* an event switching on the period of plasticity in the eye development (Sugiyama *et al.*, 2008). These properties of homeoproteins suggest that the trafficking of proteins from cell to cell is a physiological phenomenon at least in neural tissue, although the broader significance of multifunctional non-cell-autonomous movement of proteins needs further elucidation.

1.2.1. Variety of CPPs

There are several possibilities to classify cell-penetrating peptides. According to their primary sources, CPPs can be grouped as of natural, chimeric and artificial origin. Although this classification reflects the broad diversity of sources from which peptide sequences with transduction ability have been found or designed, it does not give information about the physical or chemical properties of these peptides. A classification of CPPs, which is based on studies performed on model membranes was recently proposed by André Ziegler. According to the physical properties of the peptides in the presence of membranes of different compositions, cell-penetrating peptides were divided into subclasses as primary, secondary and non-amphipathic CPPs (Ziegler, 2008).

1.2.1.1. Primary amphipathic CPPs

The most well known peptides belonging to the group of primary amphipathic CPPs include transportan, transportan 10 (TP10), MPG and Pep-1 (Table 1). They all comprise of hydrophobic and cationic moieties sequentially in their primary structure and their common characteristic is to interact with anionic as well as neutral lipid membranes (Ziegler, 2008).

Transportan is a chimeric peptide consisting of 27 amino acids. It was obtained by linking an N-terminal fragment of neuropeptide galanin (1–13) and a wasp venom peptide mastoparan, i.e. using a strategy exploited earlier to obtain efficient ligands for galanin receptor (Langel *et al.*, 1996). However, the new ligand called galparan was able to internalize into cells in a receptor-independent manner and activate G-proteins (Zorko *et al.*, 1998). Later the last amino acid from the galanin part (^{13} Pro) was substituted by lysine to enable attachment of reporter groups for visualization and cargo attachment. First,

biotin was used to tag the peptide, which exhibited seemingly non-endocytic cellular internalization mode and the peptide was named transportan (Pooga *et al.*, 1998a). The search for transportan derivatives with lower G-protein activation potential yielded an N-terminally truncated analogue, which exhibited equal cellular uptake and lower toxicity than the parent peptide (Soomets *et al.*, 2000). In the presence of different artificial membranes the mastoparan part of transportan acquires an α-helical conformation independently of the membrane model system, while the galanin part is more unstructured as shown by NMR studies (Barany-Wallje *et al.*, 2004).

The MPG and Pep-1 peptides are also two-domained, consisting of a less structured basic domain and a helix-prone hydrophobic part connected with a three-amino acid long (WSQ) linker fragment. The basic C-terminal domain of both peptides is derived from the nuclear localization sequence of SV40 large T antigen. The hydrophobic part of MPG is taken from the HIV-1 fusion protein gp41 (Chaloin *et al.*, 1998), and the hydrophobic sequence of Pep-1 is derived from the dimerization motif of the interface of HIV-1 reverse transcriptase (Morris *et al.*, 2001). In spite of bearing structural similarities, MPG is reported to form a β-barrel *in vitro* in the presence of SDS micelles (Deshayes *et al.*, 2004a) whereas Pep-1 is prone to form an α-helix (Deshayes *et al.*, 2004b)

1.2.1.2. Secondary amphipathic CPPs

Experiments with artificial membranes suggest that secondary amphipathic CPPs have a poor affinity to electrically neutral membranes and the affinity is increased by increasing the content of anionic lipids in the membrane. Upon membrane binding these peptides usually change the secondary structure and adopt a helical or β-strand conformation separating charged and non-charged residues (Ziegler, 2008). More thoroughly studied CPPs grouped as of secondary amphipathic nature are penetratin, KLAL (known also as model amphipathic peptide – MAP), S4(13)-PV and pVEC (Table 1). By far the most investigated peptide out of these CPPs is penetratin. This 16 amino acid peptide is derived from the third helix of homeodomain of the homeoprotein Antennapedia and is also called pAntp in parallel (amino acids 43–58 from the 60 amino acid-long homeodomain) (Derossi *et al.*, 1994). To this group belong also pVEC, a cytosolic fragment of transmembrane adhesion protein E-cadherin (Elmquist *et al.*, 2001), and S4(13)-PV, a chimeric peptide obtained by combining a nuclear localization signal from SV40 with the first 13 amino acids of S4 antimicrobial peptide from dermaseptin family (Hariton-Gazal *et al.*, 2002). The 18 aa long KLAL, on the other hand, was designed to be amphipathic containing only lysine, leucine and alanine residues (Steiner *et al.*, 1991) and its ability to internalize into cells was demonstrated later (Oehlke *et al.*, 1998). KLAL has repeatedly shown to be one of the most membrane-active CPPs, causing membrane leakage and efflux of molecules already at 1 μM concentrations (Hällbrink *et al.*, 2001).

1.2.1.3. Non-amphipathic CPPs

Tat peptide (pTat) and polyarginines are the shortest CPPs known so far and these contain mostly (pTat) or exclusively (polyarginines) cationic amino acids, preferably arginines (Table 1). As they do not have a stable secondary structure in solution (Futaki *et al.*, 2001), they are less prone to associate with lipid membranes unless these contain a high fraction of monovalent anionic lipids (Ziegler, 2008). In spite of that, non-amphipathic CPPs are reported to be very efficient cargo transporters in several systems. After the first demonstration of cellular internalization of HIV-1 transactivator protein (Frankel and Pabo, 1988; Green and Loewenstein, 1988) the property of its shorter fragments to deliver various cargo molecules was reported by Fawell and colleagues (Fawell *et al.*, 1994). Later the minimal sequence necessary for cellular internalization was demonstrated to be confined within residues 48–60 (Vivès *et al.*, 1997). Encouraged to test the contribution of basic amino acids to the internalization ability, several peptides consisting of arginines only were also found to possess similar characteristics (Mitchell *et al.*, 2000). Peptides of 6–9 Arg residues were the most efficient in entering Jurkat cells (Mitchell *et al.*, 2000). This length seems to be optimal for oligoarginines' cellular uptake as shorter sequences are poor delivery agents and the longer oligomers tend to be cytotoxic (Nakase *et al.*, 2004).

Table 1. The origin and amino acid sequence of CPPs discussed in this thesis.

CPP	Origin	Sequence	Reference
Primary amphipathic			
*Transportan	Galanin-Lys-mastoparan	GWTLNSAGYLLGKI NLKALAALAKKIL	(Pooga *et al.*, 1998a)
*TP10	truncated analogue of transportan	AGYLLGKINLKALA ALAKKIL	(Soomets *et al.*, 2000)
MPG	fusion peptide of HIV-1 gp41 + NLS	GALFLGFLGAAGST MGAWSQPKSKRKVC	(Morris *et al.*, 1997)
Pep-1	dimerization domain of HIV-1 reverse transcriptase + NLS	KETWWETWWTEWS QPKKKRKV	(Morris *et al.*, 2001)
Secondary amphipathic			
*Penetratin	*Drosophila* Antennapedia homeodomain	RQIKIWFQNRRMKW KK	(Derossi *et al.*, 1994)
*pVEC	Murine vascular endothelial cadherin	LLIILRRRIRKQAHAH SK	(Elmquist *et al.*, 2001)
S4(13)-PV	Dermaseptin S4 + NLS	ALWKTLLKKVLKAPK KKRKV	(Hariton-Gazal *et al.*, 2002)
MAP (KLAL)	Model amphipathic peptide	KLALKLALKALKAAL KLA	(Oehlke *et al.*, 1998)
Non-amphipathic			
*Tat (48–60)	HIV-1 Tat protein	GRKKRRQRRRPPQ	(Vivès *et al.*, 1997)
*oligoarginine	Designed	$(R)_n$	(Mitchell *et al.*, 2000; Futaki *et al.*, 2001)

*CPPs used in this study.

1.2.2. Studying the internalization of CPPs and CPP-cargo constructs

During the first ten years the routine CPP cellular uptake protocol included fixation of cells, as the imaging of internalized peptides was usually accomplished by incubating cells with biotinylated peptide and a subsequent treatment of cells with fluorescent avidin or streptavidin. As these reporter molecules do not enter cells by themselves, the fixation and permeabilization step is required. After fixation with methanol or aldehydes, CPPs were often detected diffusely in cells' cytoplasm and nucleus. Therefore, different models of non-endocytic internalization of CPPs were proposed (Derossi *et al.*, 1996; Pokorny *et al.*, 2002). Then, after the demonstration that pTat, VP22-GFP and histone H1 changed their vesicular and plasma membranous localization in living cells into the artefactual diffuse staining when cells were fixed (Lundberg and Johansson, 2002; Richard *et al.*, 2003), the results obtained in experiments with fixed cells were re-evaluated and visualization of CPPs in live cells became a standard. Paradoxically the visualization of fluorescently labelled CPPs in live cells has enabled to uncover the multitude of uptake mechanisms and seemingly non-endocytic rapid internalization mode has been observed in several cases (Ziegler *et al.*, 2005; Duchardt *et al.*, 2007; Fretz *et al.*, 2007).

To shed light on the transmembrane passage of CPPs, many experiments have been performed with model membranes, which have contributed a lot to the better understanding of interaction of peptides with the lipid membranes. The model membranes are an exactly defined system that makes the interpretation of data easier compared to the very complex and dynamic cellular organelle – plasma membrane. On the other hand, the application of data obtained in model systems in description of cellular events has to be taken with great circumspection. In the current thesis, only data from experiments with CPPs in cell culture or on the organism level will be discussed.

Determinants reported to act in cellular systems as mediators of CPP and CPP-cargo uptake most often include endosomal pathways and membranous heparan sulphate proteoglycans (HSPGs). Therefore, methods applied in studies of endocytosis are also used in elucidating the cellular internalization of CPPs. For assessing the involvement of endocytosis and energy-consuming processes in the cellular uptake of CPPs, the experiments are often carried out at low temperature (0–4 ºC) or in conditions of energy deficiency. In order to distinguish the endosomal pathways used, several molecular approaches can be exploited. First, the cellular uptake of CPPs can be compared to the agents that utilize defined pathways for cellular uptake, like transferrin as a molecule entering cells via clathrin-coated vesicles. Secondly, different natural or synthetic pharmacological agents are exploited to disrupt or inhibit the cellular processes involved in the formation or transport of vesicles. As a few examples – fungal metabolite cytochalasin D disrupts cellular actin filaments, and synthetic dynasore inhibits GTPase activity of dynamin (Macia *et al.*, 2006). The role of HSPGs in the uptake of CPPs can be detailed by enzymatic digestion to eliminate specifically

the polysaccharides of interest from the plasma membrane. Third, the function of a particular molecule in the uptake processes can be assessed by using cell lines in which its synthesis is impaired or the respective gene is knocked out. Cell lines with defective HSPG synthesis and with caveolin-1 knock-out mutation have been often used in CPP studies. All these methods have pros and cons as for example most pharmacologicals have low selectivity and inhibition of one endosomal route leads to up-regulation of other pathways (Lamaze and Schmid, 1995).

1.2.2.1. Cellular uptake of CPPs

Most extensive studies on the internalization mechanism of CPPs are performed with non-amphipathic peptides Tat (48–60) and oligoarginine. Richard and colleagues were the first to report that the uptake of pTat as well as pTat-PNA conjugates takes place via cellular energy-dependent clathrin-mediated endocytosis (Richard *et al.*, 2003). Endocytosis as the main cellular entry route for CPPs was corroborated in Namalwa lymphoma cells, which internalized pTat only at physiological temperature by using raft-dependent macropinocytosis (Kaplan *et al.*, 2005). This mechanism was proposed based on depletion of cellular cholesterol by methyl-β-cyclodextrin (MCD), and inhibition of macropinocytosis with EIPA, a pharmaceutical known to inhibit the Na^+/H^+ ATPase (Kaplan *et al.*, 2005). Analogously, the uptake of octaarginine (R_8) has been suggested to take place via macropinocytosis (Nakase *et al.*, 2004). However, in both latter works the internalization of pTat and R_8 was not completely blocked by EIPA, indicating either only a partial inhibition of macropinocytosis or a simultaneous utilization of other uptake routes. An alternative mechanism not involving endocytosis was proposed for oligoarginine peptides in three studies, which demonstrated the cytoplasmic localization and internalization of oligoarginines of different length at low and physiological temperature (Maiolo *et al.*, 2005; Zaro and Shen, 2005; Kosuge *et al.*, 2008). Moreover, the ability of pTat and oligoarginine to switch between different uptake modes was confirmed by three recent works, which demonstrated that at low concentrations (2 μM) the peptide was trapped in endosomal vesicles. However, when peptide concentration was increased by more than ten-fold or when the incubation temperature was lowered from 37 ˚C to 4 ˚C, the localization of the peptide turned uniform and it was detected also in the nucleus (Potocky *et al.*, 2003; Duchardt *et al.*, 2007; Fretz *et al.*, 2007). In addition, Ziegler and coworkers described the formation of round-shaped aggregates on the plasma membrane upon incubation with pTat shortly before the appearance of diffuse fluorescence staining (Ziegler *et al.*, 2005). The structures were proposed to contain CPP and plasma membrane proteoglycans. Similarly, Duchardt and colleagues observed that the fluorescent peptide accumulated to certain plasma membrane areas (nucleation zones), followed by a rapid flash-like staining of the whole cell (Duchardt *et al.*, 2007). Intriguingly, the diffuse cytoplasmic staining observed at higher peptide

concentration was dependent on endocytic machinery, as the uniform diffuse localization was abolished in cells pretreated with chlorpromazine, an inhibitor of clathrin-dependent endocytosis, and by the expression of a dominant negative form of dynamin. In parallel, the switching of pTat and R_9 from the vesicular fluorescence to diffuse was facilitated by MCD and EIPA treatment (Duchardt *et al.*, 2007). The authors suggest that a critical concentration of peptide on the membrane is required to induce the rapid non-endosomal internalization, and that the depletion of cellular cholesterol changes plasma membrane properties to the extent, which enables its rapid crossing by CPPs. Negatively charged HSPGs probably facilitate the ionic interactions and membrane accumulation of the non-amphipathic and highly positively charged pTat and R_9, as it has been shown that R_9 has strong affinity to heparan sulfates (Gonçalves *et al.*, 2005). Moreover, Tat peptide was reported to efficiently inhibit polyamine-induced cell growth (Mani *et al.*, 2007), suggesting that the peptide blocked the polyamine binding sites on HSPGs.

Secondary and primary amphipathic CPPs are reported to have cellular activities different from non-amphipathic peptides. At low concentrations (0.2 μM), S4(13)-PV internalization is reported to partially involve clathrin-mediated endocytosis and cellular HSPGs, but at a higher peptide concentration (2 μM) the uptake process is less sensitive to the lack of proteoglycans and clathrin-dependent endocytosis (Mano *et al.*, 2005). Electron microscopy studies have shown that S4(13)-PV peptide assembles in nanoparticle-like structures to gain entry to cells (Koppel *et al*, submitted).

The uptake of fluorescently labelled penetratin in live cells has yielded a cytoplasmic staining in some cases analogously to pTat and oligoarginine (Thorén *et al.*, 2003; Christiaens *et al.*, 2004; Fischer *et al.*, 2004; Maiolo *et al.*, 2005), whereas exclusively endosomal uptake even at 100 μM peptide concentration has been suggested by others (Duchardt *et al.*, 2007). The discrepancy could be caused by differences between the used cell lines, as Fischer and coworkers presented diffuse penetratin fluorescence in MC57 fibrosarcoma cells, while in HeLa cells the localization of the peptide at the same concentration was vesicular (Fischer *et al.*, 2004).

1.2.2.2. Cellular uptake of CPP-cargo constructs

In parallel with the development in the elucidation of cellular uptake modes of CPPs, which gives fundamental information about peptide/protein-lipid interactions, the pragmatic side of CPP studies lies in their ability to facilitate the cellular uptake of various bioactive molecules. Therefore, studies on the internalization mechanisms of CPP-cargo constructs, the activity of the delivered cargo in cells and possible side effects are extensively studied. The payloads delivered into eukaryotic cells using CPPs have reported to be of different size (peptides, nanoparticles), functionality (siRNA, enzymes) and character (nucleic acids, proteins).

Analogously to the conflicting ideas about the internalization mode of naked or only fluorescent tag-carrying CPP, the reports about the cellular delivery of cargo with peptides are also contradictory. However, the differences between CPPs have turned out to be beneficial in cases where some peptides have been found to be efficient transporters for certain payloads. For example, pTat shows low cellular uptake in several systems (Richard *et al.*, 2003; Richard *et al.*, 2005; El-Andaloussi *et al.*, 2007b). On the other hand pTat has been demonstrated to be very efficient in protein cellular transduction. In 1994, Fawell and colleagues reported the delivery of β-galactosidase into mouse liver, spleen and heart using Tat-(37–72) peptide as transporter (Fawell *et al.*, 1994). Five years later the ability of pTat to carry β-galactosidase into most tissues of mouse and to even cross the blood-brain-barrier was demonstrated (Schwarze *et al.*, 1999). Since then, the activity of cargo delivered into cells by pTat has been shown repeatedly in different disease model systems (Langel, 2006). The fusion with the anti-apoptotic protein Bcl-X_L increased the survival of retinal ganglion cells and rescued them from apoptosis after induced lesion (Dietz *et al.*, 2002). Analogously, pTat has a potential in enzyme replacement therapy, since the systemic delivery of purine nucleoside phosphorylase (PNP) was facilitated by fusion to pTat. The prolonged administration of pTat-PNP led to the correction of metabolic abnormalities and immunodeficiency and prevented the early lethality of $PNP^{-/-}$ mice (Toro and Grunebaum, 2006).

Cargo delivery efficiency with oligoarginines is also repeatedly demonstrated. Using a mouse model, Rothbard and coworkers showed a 70% reduction in contact dermatitis after topical administration of R_7-cyclosporin A conjugate (Rothbard *et al.*, 2000). Moreover, oligoarginine modified with aminohexanoic acid $(RXR)_4$ efficiently delivered antisense phosphorodiamidate morpholino oligomers (PMO) into both peripheral skeletal muscle and cardiac tissue in adult *mdx* dystrophic mice, causing exon skipping in *mdx* gene and subsequent restoration of the expression of dystrophin protein (Yin *et al.*, 2008).

pVEC as a secondary amphipathic peptide has shown potency in the delivery of a therapeutic construct containing cyclic homing peptide and an anti-cancer drug chlorambucil into tumor cells *in vitro* and *in vivo* (Myrberg *et al.*, 2008). pVEC has also exhibited efficiency in mediating the cellular uptake of peptide nucleic acid (PNA) (Lundin *et al.*, 2008). PNA is an oligonucleotide analogue in which the negatively charged sugar-phosphate skeleton is replaced by a neutral polyamide backbone. PNA oligomers bind to complementary RNA and DNA with very high affinity and can be used as antisense oligonucleotides of very high resistance to proteases and nucleases (Nielsen *et al.*, 1993). However, pVEC is not able to deliver PNA into the nucleus, as was demonstrated by the Kole's assay (Kang *et al.*, 1998). This assay is based on masking the aberrant splice site in the luciferase mRNA via binding of a complementary oligonucleotide, leading to the splicing of intact mRNA and expression of luciferase protein (Kang *et al.*, 1998). The PNA delivered to cells by pVEC, however, was not able to mask the aberrant splice site in luciferase mRNA and induce the production of reporter protein (Lundin *et al.*, 2008).

Transportan can be considered as a remarkably efficient vector for cellular PNA transport. Pooga and coworkers have demonstrated PNA delivery with penetratin and transportan *in vivo* by efficiently down-regulating galanin receptors in the spinal cord (Pooga *et al.*, 1998b). Transportan has been also used for cellular delivery of PNA against HIV transactivation response region resulting in the inhibition of Tat-mediated HIV-1 LTR transactivation (Chaubey *et al.*, 2005).

Transportan's analogue TP10 has been an efficient vector for avidin and streptavidin proteins (El-Andaloussi *et al.*, 2007a; El-Andaloussi *et al.*, 2007b), for PNA in splicing correction system (El-Andaloussi *et al.*, 2007a) and also for peptides, which activate secretory pathways in mast cells (peptidyl secretagogues) (Howl *et al.*, 2003). TP10 also mediates the cellular uptake of siRNA, however, without retaining its biological activity (Lundberg *et al.*, 2007).

1.2.2.3. Mechanism of delivery of CPP-cargo constructs

Different endosomal pathways are postulated for the cellular uptake of CPP-cargo constructs (Console *et al.*, 2003; Ferrari *et al.*, 2003; Fittipaldi *et al.*, 2003; Khalil *et al.*, 2004; Wadia *et al.*, 2004; Tünnemann *et al.*, 2006; Rinne *et al.*, 2007). The target of the delivered bioactive molecule, however, can situate in various cellular locations outside endocytic vesicles. For that reason, the cargo has to escape from the endosomal vesicles if the target is not within the endocytic pathway. Different studies that have suggested the uptake of pTat-Cre fusion protein via macropinocytosis or actin- and energy-dependent mechanism (Wadia *et al.*, 2004; Tünnemann *et al.*, 2006), and the internalization of PNA-CPP constructs using clathrin-mediated endocytosis (El-Andaloussi *et al.*, 2007b) demonstrate the biological activity of the attached load. This indicates that the construct reaches nucleus despite being trapped in endocytic vesicles. It also suggests that fluorescence microscopy might lack sensitivity to detect the compounds in cells at low but still biologically relevant concentrations. The question to which extent the cellular uptake of PNA-CPP conjugates can be correlated with their biological activity, was addressed in a recent study using a splicing correction assay to quantify the biological effect of CPP-conjugated PNA. El-Andaloussi and colleagues reported that despite of the rather similar endosomal localization of conjugates of PNA with pTat, penetratin and transportan, the splice correction efficiency was different for every PNA-CPP conjugate, being the highest for transportan (El-Andaloussi *et al.*, 2006).

On the contrary, oligonucleotide delivery to cells with Pep family CPPs has yielded diffuse labelling of the cytoplasm and the nucleus when attached to a fluorescent cargo, revealing either a direct translocation across the plasma membrane or extensive endosomal escape (Morris *et al.*, 2004; Morris *et al.*, 2007). More precisely, in those studies a construct consisting of a phosphonate analog of PNA (pPNA) and a PNA-like monomer based on a *trans*-4-hydroxyl-L-proline (HypNA) was used to target cyclin B1 via facilitating its cellular de-

livery with Pep-2 and Pep-3. The down-regulation of cyclin B1 on the mRNA as well as on the protein level with Pep-2 was shown in different cultured cells (Morris *et al.*, 2004) and, using Pep-3, also in human prostate carcinoma xenografted mice (Morris *et al.*, 2007). It has to be mentioned that for obtaining efficient carrier with Pep family peptides, co-incubation of CPP and cargo is sufficient, although a particular size of complexes and a molar ratio of CPP/cargo has to be followed (Muñoz-Morris *et al.*, 2007).

2. AIMS OF THE STUDY

The application of cell-penetrating peptides as vector molecules in biotechnological and especially in therapeutical field requires a detailed knowledge about the cellular internalization mechanisms used by the peptide and cargo. Moreover, their post-internalizational fate is of the highest importance. The main purpose of this study was to unravel the internalization modes and intracellular trafficking of CPPs coupled to a protein cargo. Although we focused on the elucidation and characterization of the cargo delivery properties of transportan and TP10, the protein transduction abilities of other CPPs like Tat peptide, nonaarginine, pVEC and penetratin were characterized as well.

The more specific goals of this thesis can be concluded as follows

- to characterize the kinetics and efficiency of protein cellular delivery by transportan, pTat, penetratin and pVEC and to evaluate the role of energy-dependent cellular processes and cholesterol in the uptake process of CPP-protein complexes (paper I)
- to characterize the initial steps of the internalization process of protein complexes with transportans and their destination; to assess the influence of low temperature on the uptake process at an ultrastructural level (paper II)
- to evaluate the involvement of caveolin- and flotillin-dependent endocytosis on the cellular transduction of protein attached to transportans and their subsequent targeting through the early endosomal pathway (paper III)
- to map and compare the intracellular routing of avidin complexed with pTat, nonaarginine and transportan in relation to endosomal recycling compartment and acidic endosomes (paper IV)

3. METHODOLOGICAL CONSIDERATIONS

The methods used in this study are described comprehensively in the respective papers and only brief comments on the chosen methods are given here.

3.1. The cell-penetrating peptides used in this research

Due to the main focus of our research group on transportans and the efficient carrier properties of this CPP (Pooga *et al.*, 1998b; El-Andaloussi *et al.*, 2006), the protein transduction ability of transportan is discussed in all papers included in this study. In parallel, the transportan analogue TP10 was used in experiments in papers II and III as a CPP with excellent cargo delivery properties but, lower-level side effects compared to transportan at high concentration. pTat, which has been addressed in papers I, III and IV, was included in the studies for comparison as the most often used CPP and due to its high efficiency in protein cellular delivery. Penetratin and pVEC examined in paper I along with transportan and pTat were chosen as the representatives of the respective CPP families. Nonaarginine as a very efficient and structurally similar CPP to pTat was discussed in the paper IV together with pTat and transportan in order to compare the transportan-mediated protein delivery to that of arginine-rich non-amphipathic cell-penetrating peptides.

3.2. Cell cultures

Five cell lines were used for the experiments described in this thesis. **HeLa** is a cancer cell line, which was derived from the cervical carcinoma of Henrietta Lacks after her death in 1951. Since then HeLa has been the most commonly used cell line in molecular biology and in the CPP studies as well (Mueller *et al.*, 2008). Thus, application of HeLa cells in experiments described in paper I allowed us to compare the protein transduction efficiency of four CPPs in a well described system. As we also aimed to explore the intracellular routing of CPP-cargo complexes, the ratio of cytosol to nuclear volume of HeLa cells was well suited for such studies.

Bowes cells are derived from human melanoma. As this cell line expresses the galanin receptor, it was used initially in the studies of this neuropeptide and also for transportan's predecessor galparan. It has been shown that transportan displaces galanin from its receptors in Bowes cells (Pooga *et al.*, 1998a). However, it takes place in 2.7-fold higher concentration compared to galparan. In paper II we used in parallel Bowes human melanoma cells and HeLa cells cor-

roborating that in both these cell lines protein transduction takes place in an analogous manner.

In order to evaluate the cellular uptake of CPP-protein complexes in the absence of caveolin, **caveolin-1 knock-out mouse 3T3 embryonic fibroblasts** together with **wild-type mouse embryonic fibroblasts** were included in the experiments described in paper III in addition to HeLa cells.

In paper IV all the experiments were conducted in **Cos 7** cells, which is an African green monkey kidney fibroblast-like cell line immortalized with SV40 Large T antigen. Since it has been reported that in Cos 1 cells the recycling and degradative endosomes are differently localized in relation to the spherical Golgi complex (Misaki *et al.*, 2007), we exploited this property in exploring the intracellular targeting of CPP-protein complexes. After affirmation that the Golgi complex is spherical and allows differentiation of endosomal populations also in Cos 7 cells, we used this cell line due to higher homogeneity and better cultivation properties.

3.3. Constructs of CPPs with cargo proteins

Different strategies are used to couple CPPs to cargo molecules. The easiest way is the formation of CPP-cargo complexes by a simple mixing of the peptide and cargo molecules. However, this strategy necessitates high affinity of cargo molecule to a carrier and in that case usually the molar concentration of CPP must be several-fold higher than that of the molecule to be delivered. Another method to couple CPP to a cargo is the formation of a disulfide bridge between the carrier and cargo moiety. This approach is thought to have an advantage in the delivery of compounds, which have targets in the cytoplasm, as the reducing environment of the cytoplasm disrupts the disulfide linkage in the construct when it reaches the cytoplasm. For the cellular transduction of proteins, the CPP motif can be added to the protein sequence. The easiest method is the expression of the fusion protein in bacteria and the subsequent purification of the product. However, during the purification of the designed construct after bacterial expression several problems can be encountered. Another possibility to produce large proteins where only one CPP moiety is attached per protein is a covalent coupling of the carrier and the cargo moieties, which is mostly used in the delivery of peptides, oligonucleotides and oligonucleotide analogs.

Proteins used as model cargos attached to CPPs throughout this thesis are avidin, streptavidin and neutravidin. The ability of these proteins to interact non-covalently but with very high affinity – nearly irreversibly – with biotin is widely exploited in molecular biology. We took advantage of this approach and used the complexes of biotin-tagged CPPs and one of the named proteins labelled with a suitable reporter group to visualize the protein transduction mediated by CPPs (Fig. 2). All three proteins are tetrameric with one biotin binding site in each subunit. Avidin is a glycosylated egg white protein and due to its glycosylation has a very high isoelectric point (pI >10), whereas streptavidin

from *Streptomyces avidinii* is almost neutral (pI 6.8–7.5). Neutravidin is derived from avidin and lacks glycosylation, which gives the protein a slightly acidic isoelectric point, around 6.3. Probably due to the very high pI, nobody has succeeded in labelling of avidin with colloidal gold so far and therefore gold-labelled streptavidin and neutravidin were used in the electron microscopy experiments in paper II and III.

The ratio of CPPs to avidin used in the experiments was chosen considering that the biotin binding sites on the protein would be saturated and no free peptide would be in the environment to interfere with the uptake of formed CPP-protein complexes. The complexes were used in concentrations which enabled reliable detection, on one, and excluded any toxicity of used peptides, on the other hand. Higher concentration of CPPs and avidin was used in paper IV to assess the influence of concentration of complexes on their intracellular targeting.

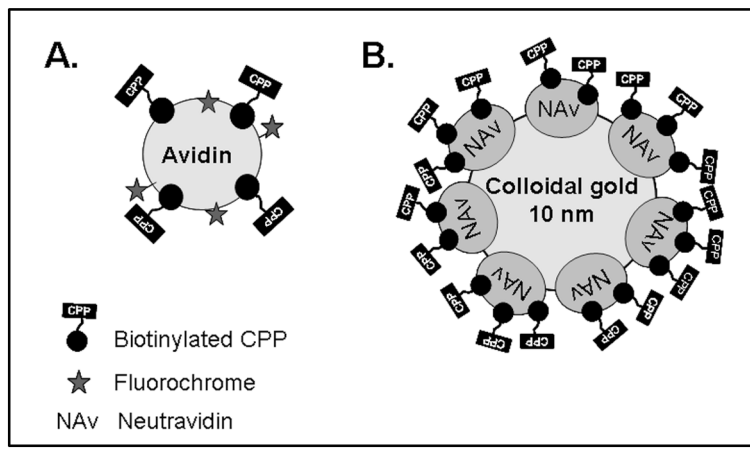

Figure 2. Schematic representation of differently labelled CPP-protein constructs used in this study. (A) In fluorescence microscopy experiments fluorochrome-labelled avidin is complexed with biotinylated CPPs. (B) For electron microscopy experiments the proteins (neutravidin or streptavidin) adsorbed to colloidal gold particle are complexed with biotinylated CPPs. (Modified from Padari, 2008).

3.4. Quantification of CPP-mediated cellular transduction of fluorescently labelled protein

In paper I, FACS and fluorescence spectrophotometry were used in parallel to evaluate the cellular uptake efficiency of avidin-FITC complexes with biotinylated CPP (CPPb). As FACS detects the fluorescence without the discrimina-

tion between intra- and extracellular signal, we applied prolonged trypsin treatment for digesting the non-internalized and merely plasma membrane-attached CPP-avidin complexes. This approach should enable the detection of internalized complexes only. In addition, in more recent FACS experiments (paper III) the cellular fluorescence was measured in the presence of low concentration of trypan blue in order to quench the extracellular fluorescence (Sahlin *et al.*, 1983), which was insensitive to trypsin treatment.

The reason to include the fluorescence spectrophotometry measurements in the experiments in paper I is the sensitivity of FITC fluorescence emission to pH especially at values below 7 (Ohkuma and Poole, 1978), which might lead to the underestimation of protein concentration in acidic organelles. In order to exclude the possibility for incorrect estimation of avidin concentration in cells, the CPP-protein complexes-treated cells were dissolved in alkaline buffer (pH 10–11) to quantify the FITC fluorescence from all cellular organelles and cell lysate by spectrofluorometrical analysis.

In addition to comparing the protein transduction efficiency of different CPPs (paper I), we used different approaches to inhibit endosomal processes in order to evaluate their role in the uptake of CPP-protein complexes. Low temperature is a commonly used method for inhibiting and blocking of endosomal processes, although some studies report that vesicular trafficking continues also at temperatures lower than 20 ˚C (Saraste *et al.*, 1986).

Hyperosmolar solution conditions generated by high-concentration of sucrose are suggested to stop the clathrin-dependent pathway (Heuser and Anderson, 1989). However, more specific treatments to inhibit clathrin-mediated endocytosis are preferred now. Expression of a dominant negative mutant of proteins crucial in clathrin-dependent endocytosis is probably the most specific among these. In addition, application of different pharmacological agents is very common but their mechanism of action could be poorly characterized and most of these have low selectivity.

Combined treatment with deoxyglucose and sodium azide leads to the energy deficiency of cells by the inhibition of glycolysis and ATP synthesis, respectively (Silverstein *et al.*, 1977), and in analogy with the low temperature conditions the cellular energy-driven endosomal processes should be arrested (Steinman *et al.*, 1974).

Cholesterol is shown to be important in endosomal pathways, which involve membrane lipid rafts and caveolae. Therefore, methyl-β-cyclodextrin (MCD) as cholesterol-depleting agent was used to study the role of rafts in the internalization process of CPP-protein complexes (paper I). The depletion of cholesterol, on the other hand, has been also reported to inhibit clathrin-mediated endocytosis (Rodal *et al.*, 1999) and therefore the application of MCD for inhibition of raft- and caveolae-mediated endocytosis can influence also clathrin-dependent pathway.

3.5. Visualization of CPP-protein complexes in relation to cellular organelles by fluorescence microscopy

Fluorescence microscopy and confocal laser scanning fluorescence microscopy, in particular, enable the studies of cellular processes on the organelle level, which is not possible by FACS or fluorescence spectroscopy. Therefore, we applied both the wide field as well as confocal fluorescence microscopy for visualization of the intracellular localization of CPP-protein complexes in relation to different cellular organelles. Besides visualizing fixed and permeabilized cells, which allows immunolabelling of various cellular proteins, we exploited several fluorescent markers applicable for live-cell imaging. Visualization of live cells avoids possible fixation-derived artefacts, a subject thoroughly addressed in CPP studies (Lundberg and Johansson, 2002; Richard *et al.*, 2003). We addressed this question in paper I analyzing the influence of fixation with 4% paraformaldehyde (used in papers II and III) on the cellular localization of CPP-protein complexes.

In papers II, III and IV we used antibodies against LAMP2, PI3P, Rab5, flotillin-1, caveolin and TGN46 to visualize different endosomal organelles in relation to CPP-protein complexes. In immunofluorescence studies care must be taken in optimizing fixation and permeabilization processes to retain the specific binding sites and to reduce the possibility of non-specific binding of antibodies. In addition to using paraformaldehyde and methanol fixation in most of immunofluorescence experiments we found PLP (paraformaldehyde-lysine-periodate) fixative and mild permeabilization with 0.01% saponin to be the most suitable for visualization of flotillin-1 in HeLa cells.

To check the involvement of clathrin-dependent endocytosis in the cellular uptake of CPP-protein complexes, in papers II and III we used fluorescently labelled transferrin known to enter cells via clathrin-coated pits. Although transferrin trafficks to the recycling pathway after cellular uptake, the excessive amount of the molecule can be directed to degradation and thus it can mark also lysosomes and give incorrect data about the cellular localization of CPP-protein complexes. Still, transferrin is a less ambiguous marker compared to the cholera toxin B subunit (CtxB), which we used in paper III. CtxB can enter cells via clathrin- (Torgersen *et al.*, 2001) or flotillin-mediated endocytosis (Glebov *et al.*, 2006), or via a clathrin- and caveolin-independent pathway in different cell lines (Orlandi and Fishman, 1998; Kirkham *et al.*, 2005). Since CtxB is one of a very few markers demonstrated to utilize flotillin-containing endocytic vesicles for cell entry, we used it as a positive control in studying the role of flotillin-mediated pathway in transportan/TP10-mediated protein delivery.

We used BODIPY-TR-C_5 ceramide as a marker of Golgi complex in live-cell imaging in paper II. In addition to the Golgi apparatus this probe stains also late endosomes and ER. To be more specific in studying the intracellular rout-

ing of CPP-protein complexes, we used antibody TGN46 against *trans*-Golgi network in paper IV.

The localization of CPP-complexes in relation to late endosomes and lysosomes was studied both in live and fixed cells. In paper IV acidic organelles were visualized by using live-cell marker LysoSensor DND, which fluorescence intensity increases along with the gradual decrease of pH, reflecting the acidity of the vesicle of interest. Compared to the labelling of degradative organelles with lysosome-specific protein LAMP 2 (paper II) LysoSensor provided more detailed information about the acidification process of CPP-containing vesicles. However, live cell markers might lack specificity that we observed when using LysoTracker (paper II). Therefore we applied the immunolabelling of lysosomes with LAMP2 antibody in parallel to enable more specific visualization of the CPP-protein complexes in relation to cellular acidic organelles.

3.6. Downregulation of protein levels in cells by siRNA treatment

Although the siRNA method became available recently, it has been widely used to down-regulate the protein of interest at the mRNA level. The mRNA is degraded by specific endogenous enzyme complexes RISC (Hammond *et al.*, 2000) and Dicer (Bernstein *et al.*, 2001) and the enzymatic degradation is initiated by the binding of one or several short complementary RNA sequences on the target mRNA. We used Western blot method in paper III to evaluate the rate of down-regulation of flotillin-1 and caveolin-1 proteins after siRNA treatment. Compared to the immunostaining of siRNA-treated cells with respective antibodies and subsequent analysis by FACS, Western blot showed that FACS analysis underestimated the efficiency of siRNA treatment. On the other hand, as the whole cell lysate was applied to Western blot analysis, differentiation between the efficiency of the down-regulation of protein at the membrane versus intracellular areas was impossible. To overcome this obstacle we analyzed flotillin-1 staining by confocal microscopy separately in the intracellular and membrane areas of siRNA-treated cells in paper III.

3.7. Electron microscopy

In spite of the advantages of fluorescence microscopy, this method also has several drawbacks. First, the resolution limit of fluorescence microscopy is determined by the wavelength of visible light. Therefore, it cannot provide detailed information about the actual size or shape of certain cellular organelles, like the endosomal vesicles or the plasma membrane. Analogously, fluorescence microscopy fails to distinguish between very closely situated objects that are clearly resolved by electron microscopy, best revealed as false-positive

colocalization by fluorescence techniques. In papers II and III we used transmission electron microscopy and pre-embedding method to characterize the intracellular localization of complexes of colloidal-gold labelled protein with CPP (Fig 2). As we also aimed to visualize proteins specific to endosomal structures in relation to CPP-protein complexes, immunocytochemical techniques were used for defining the type of endosomes. In paper II, cells were incubated simultaneously with CPP-protein complexes (labelled with 10 nm colloidal gold), and transferrin (20 nm colloidal gold), after which the cells were fixed and processed for flat embedding into epoxy resin. The flat embedding allows monitoring the largest area of the cell as the sections from cells are cut parallel to the growth substrate. In paper III we first incubated live cells with CPP-protein complexes labelled with colloidal gold (10 nm). After the incubation cells were fixed and mildly permeabilized, followed by the treatment with anti-flotillin-1 or anti-caveolin primary antibodies and respective secondary antibodies tagged with ultra small gold particles (1.4 nm). Application of two differently sized gold labels is not very often used as the silver deposition for the magnification of labels can unify the size of labels with initially different diameter. In addition, it might complicate the distinguishing of two labels situating very close to each other. As we aimed to visualize cellular caveolin and flotillin-1 localizing at the cytoplasmic side of the endosomes whereas CPP-protein complexes reside mostly intravesicularly, we considered the artefacts due to the close proximity of two markers to be of minor importance.

4. RESULTS AND DISCUSSION

4.1. Comparison of protein cargo delivery properties of cell-penetrating peptides (paper I)

As the ability of transportan (Pooga *et al.*, 1998a), penetratin (Derossi *et al.*, 1994), pVEC (Elmquist *et al.*, 2001) and pTat (Vivès *et al.*, 1997) to internalize into eukaryotic cells was demonstrated earlier, we selected these cell-penetrating peptides as representatives of the respective CPP families to evaluate their protein delivery properties. Using the non-covalent complexes of biotinylated peptide with fluorescently labelled avidin cargo instead of synthesizing covalently linked CPP-protein conjugates we compared the protein delivery efficiency of these peptides by fluorescence microscopy, flow cytometry and spectrofluorometry.

The redistribution of CPPs upon fixation of cells and the artefactual localization of peptides had been recently demonstrated (Lundberg and Johansson, 2002; Drin *et al.*, 2003; Richard *et al.*, 2003). Compared to the 2–3 kDa size of CPP, the molecular weight of CPP-avidin complex is considerably higher (~70 kDa) and the latter might be less easily redistributed in cells by aldehyde-fixation. In order to confirm the results with CPP-protein complexes obtained in fixed cells we assessed the influence of paraformaldehyde fixation to the cellular localization of transportan-protein complexes. We found that fixation of cells with 4% paraformaldehyde after a 30 min incubation with transportan-protein complexes does not lead to changes in the cellular localization of peptide-protein complexes.

4.1.2. CPPs vary in protein transduction ability

Incubation of HeLa cells with complexes of FITC-labelled avidin and biotinylated transportan, pTat, pVEC or penetratin for 30 minutes at physiological temperature yielded a homogenous population of labelled cells, which revealed 10–100-fold stronger cellular fluorescence as compared to the control cells incubated only with avidin, as demonstrated by FACS analysis. It indicated that all CPPs were able to mediate membrane interaction and cellular uptake of avidin. Since FACS measures the fluorescence of the whole cell, we prolonged the trypsin treatment of cells before FACS measurement in order to differentiate between the protein that had reached the cell interior from that remained attached to the outer leaflet of the plasma membrane only. Extensive trypsin treatment led to a marked decrease in the cellular fluorescence of all CPP-protein complexes, indicating that a considerable fraction of protein was accessible to trypsin digestion on the plasma membrane. pTat and pVEC were slightly more influenced by the treatment, suggesting that a higher amount of the protein was accessible to the enzyme on the plasma membrane when complexed with these CPPs. Incubation of cells with CPP-protein complexes for 4 hours resulted in the gradual increase of cellular fluorescence for all CPPs, being markedly higher in cells treated with pTat-protein complexes. However, after

24 hours the highest cellular fluorescence in relation to other peptide-protein complexes was detected for transportan-avidin complexes. The high efficiency of transportan during longer incubations was not hindered by the presence of serum, which inhibited the uptake of transportan-avidin complexes at shorter incubation times. Therefore, we propose that transportan is the most efficient of the studied CPPs for protein cellular delivery and the ranking of all studied CPPs in protein uptake efficiency is: transportan > pTat > pVEC > penetratin.

4.1.3. FACS and spectrofluorometry show different uptake kinetics of CPP-mediated protein delivery

During the first hour of incubation of cells with CPP-protein complexes, FACS measurements revealed a linear increase in cellular fluorescence and stable interaction of the complexes with the plasma membrane already during the first 15 minutes. Avidin complexed with transportan or pTat revealed a more rapid increase in fluorescence signal, which was, however, followed by a lag period and a subsequent rise after two hours. Uptake of protein coupled to penetratin and pVEC during 4 hours was less efficient as detected by FACS. As the FITC fluorescence decreases in the acidic environment (Ohkuma and Poole, 1978), we lysed the complexes-treated cells in alkaline buffer before spectrofluorometric measurements in order to avoid the quenching of FITC in cellular acidic organelles and the putative underestimation of the avidin concentration in cells. Spectrofluorometry revealed a continuous increase in fluorescence of complexes-treated cells, suggesting that the lag phase of the protein uptake with pTat- and transportan-protein detected by FACS reflected the quenching of fluorescence in the acidic environment. In addition, the protein uptake by pTat detected by spectrofluorometry after 4-hour incubation was only 3-fold higher than that of pVEC/penetratin-mediated avidin transport, whereas FACS detected a 10-fold difference between the protein delivery by transportan/pTat and penetratin/pVEC. This suggested that avidin transported into cells by pVEC and penetratin remains entrapped inside the endosomal vesicles where the fluorescence of FITC decreased. pTat and transportan, on the other hand, either inhibit the endosomal acidification and retain the fluorescence, or escape from acidic structures, or elude these vesicles (paper IV) and therefore avoid quenching of the signal.

4.1.4. Low temperature and energy depletion inhibit the cellular uptake of CPP-protein complexes

The cellular internalization of CPPs was in some cases shown to take place in the absence of cellular energy (Christiaens *et al.*, 2004; Thorén *et al.*, 2003), hence we used the low temperature (4 ˚C) and pretreatment of cells with non-metabolizable deoxyglucose and sodium azide to estimate the influence of energy depletion on CPP-mediated protein delivery. Incubation at 4 ˚C decreased

the cellular fluorescence for all CPP-protein complexes. In addition, when the low temperature was combined with the prolonged trypsin treatment, the drop in fluorescence was markedly stronger and revealed that at low temperature the majority of the complexes remained on the plasma membrane and were not taken up by cells. Cells incubated with penetratin-avidin showed the decrease of fluorescence almost to the basal level, suggesting that at lower temperature penetratin-avidin complexes are more loosely attached to the plasma membrane and not taken up by cells. It has been reported that tryptophan residues in the penetratin sequence are important in the interaction and cellular internalization of the peptide (Christiaens *et al.*, 2004). The decreased fluidity of the membrane caused by low temperature (Chapman, 1975) can interfere with the interaction of penetratin with the plasma membrane. On the other hand, the cargo molecule can also change or inhibit the uptake characteristics of CPPs. The cellular internalization of pVEC has been shown to decrease only marginally at 8 ˚C in Bowes cells (Elmquist *et al.*, 2006). However, when we assessed the uptake in the presence of deoxyglucose and sodium azide, a 3–6-fold decrease in cellular fluorescence for all CPP-protein complexes took place, revealing that the energy-driven processes are of high relevance in the CPP-mediated uptake. Still, even in the absence of cellular energy, about 2- to 5-fold more FITC-labelled avidin associated with cells with a biotinyl-CPP than without a carrier peptide, suggesting that the energy depletion does not completely block the insertion of complexes into the plasma membrane or the cellular uptake.

4.1.5. The clathrin mediated endocytosis and lipid raft driven endosomal processes are involved in the cellular uptake of CPP-protein complexes.

The clathrin-mediated endocytosis is the best characterized endosomal pathway. While low temperature should inhibit all endosomal processes by decreasing the fluidity of the membrane and abolishing energy-derived processes (Steinman *et al.*, 1974), the hyperosmolar environment interferes only with clathrin-mediated endocytosis (Heuser and Anderson, 1989). Therefore, we applied the hyperosmolar medium to assess the involvement of clathrin-mediated endocytosis in the protein delivery with CPPs. The use of hyperosmolar medium led to a decrease in the cellular fluorescence for all CPP-protein complexes, although the influence was not uniform for all peptides. After application of hyperosmolar medium we observed about 3-fold decrease in the fluorescence of cells incubated with penetratin-avidin complexes, while the drop in protein uptake with other peptides was about 1.5-fold. This result suggested that the clathrin-coated pits only partially mediate the cellular uptake of CPP-protein complexes.

Cholesterol is one of the main components of the plasma membrane where it is proposed to be responsible for the assembly of cellular microdomains by decreasing the membrane fluidity (Ikonen, 2008). Cholesterol is enriched also in caveolae and its extraction with methyl-β-cyclodextrin disrupts the formation of caveolae and

inhibits the formation of clathrin coated pits (Rodal *et al.*, 1999). In addition, cholesterol plays an essential role also in clathrin- and caveolin-independent endocytosis (Lamaze *et al.*, 2001; Nichols *et al.*, 2001; Lajoie and Nabi, 2007). The cholesterol depletion with MCD inhibited the avidin delivery with penetratin and pVEC about 2-fold, whereas the influence on transportan- and pTat-mediated uptake remained around 20%, showing that cholesterol-dependent processes contribute to the CPP-mediated protein cellular uptake. The relevance of cholesterol was further confirmed by our more recent experiments, where we observed a very strong inhibition of TP10-mediated protein delivery in cholesterol-depleted cells by confocal microscopy (Fig. 3). However, cholesterol depletion does not enable to differentiate between endocytosis via caveolae and lipid rafts. Caveolar pathway (Ferrari *et al.*, 2003; Fittipaldi *et al.*, 2003) and lipid raft-dependent macropinocytosis (Wadia *et al.*, 2004) are both suggested to participate in Tat-mediated protein delivery. The involvement of caveolae in CPP-mediated uptake was analyzed in more detail in paper III. Our results demonstrate that the CPP-complexes use different endosomal pathways in parallel.

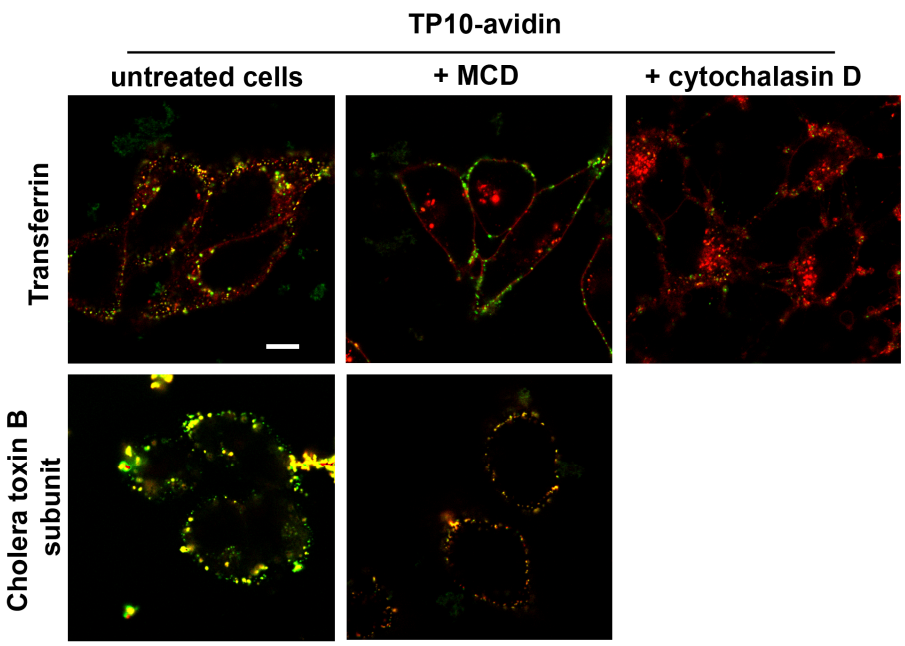

Figure 3. Internalization of TP10-avidin complexes by HeLa cells is inhibited by cholesterol depletion and actin depolymerization. Cells were incubated with complexes of 0.5 µM biotinylated TP10 and 0.15 µM avidin-Alexa Fluor (AF) 488 (green) after treatment with 10 mM methyl-β-cyclodextrin (MCD, middle panel) or 10 µM cytochalasin D (right panel). Along with CPP-protein complexes 25 µg/ml transferrin-AF 594 (upper panel, red) or 3 µg/ml cholera toxin B subunit-AF 594 (lower panel) were added into the cell medium. Scale bar 10 µM

4.2. Transportan-protein complexes enter cells via different endosomal pathways (paper II)

As the results in paper I focussed on the quantification of the uptake of CPP-protein complexes, we further aimed to characterize the cellular localization of complexes at ultrastructural level. Therefore, the cellular uptake and trafficking of biotinylated transportan and TP10 complexed to colloidal gold-labelled streptavidin was analyzed by transmission electron microscopy. We also included the confocal fluorescence microscopy analysis in the study in order to compare the electron microscopy data obtained from fixed cells with the protein uptake and localization in live cells. Therefore we used an analogous experimental setup, i. e. incubation of cells with fluorescently labelled avidin complexed to biotinylated CPP.

4.2.1. Complexes of CPP and protein interact with the cell membrane by various modes

The association of transportan- and TP10-streptavidin complexes with the plasma membrane was visualized in Bowes and HeLa cells by electron microscopy. The complexes of CPP-streptavidin interacted with both cell types already upon 5–10 minutes of incubation. Probably due to the tendency of transportan to form multimers in solution (Pooga *et al.*, 1998a), at higher concentration of the peptide (5 μM) large agglomerates of transportan-streptavidin complexes were detected on the plasma membrane. These aggregates had electron dense background, which was absent when complexes were formed in cell-free environment. This electron dense material could be the plasma membrane proteoglycans, as the HSPGs are reported to be essential for the CPP-mediated cargo delivery (Console *et al.*, 2003; Sandgren *et al.*, 2004; Payne *et al.*, 2007). HSPGs are shown to be very specific in binding of polyamines, compounds that are essential for cellular proliferation (Welch *et al.*, 2008). On the other hand, the ability of Tat peptide to interact with the HSPGs is proposed to depend only on its positive net charge (Mani *et al.*, 2007). On the physiological level the core protein of proteoglycan dictates the biological outcome of the interaction, leading either to lysosomal degradation (syndecan and perlecan) or recycling via endosomal and Golgi compartments (glypican) (Belting, 2003). Considering the high pI of avidin protein, the interaction between negatively charged HSPGs and peptide-protein complexes could be rather derived from electrostatic forces, but without more detailed study the role of HSPGs in protein delivery with transportans must be discussed with circumspection.

4.2.2. Transportan- and TP10-protein complexes enter cells using various vesicular carriers

Electron microscopy images revealed that transportan- and TP10-protein complexes interacted with the plasma membrane either as large agglomerates or small assemblies containing only a few gold particles. The interaction subsequently led to the formation of mainly vesicular carriers of different size and shape, of which the large aggregates induced membrane outgrowths and changes in the morphology of the adjacent plasma membrane. From that we presumed that the actin-dependent processes are involved in the uptake of CPP-protein complexes. Corroborating our hypothesis, we detected the protein complexed to transportans in close proximity to the actin cytoskeleton both by electron and fluorescence microscopy. Moreover, avidin transport with CPPs into cells treated with actin depolymerizing drug cytochalasin D was seriously hindered (Fig. 3). In addition, considering that several vesicular structures had a 0.5–2 μm diameter and could be classified by morphology as macropinosomes, and since macropinocytosis is an actin-driven endosomal process (Swanson and Watts, 1995), the participation of actin-mediated endosomal events in CPP-mediated protein uptake are probably of high importance. Actin polymerization has been shown to play an important role in the cellular uptake of oligoarginines (Nakase *et al.*, 2004; Nakase *et al.*, 2007) and of Tat-Cre fusion protein as well (Wadia *et al.*, 2004).

In addition to the localization in macropinosome-like vesicles, streptavidin-gold was also found in vesicles with regular shape, which we considered to be caveosomes and clathrin-coated vesicles. Therefore we proposed that the peptide-protein complexes were taken up by cells via different endosomal pathways in parallel. Our data from electron and fluorescence microscopy studies did not support the prevalence of the clathrin mediated pathway in the CPP-mediated protein delivery that was suggested for Tat-PNA conjugates earlier (Richard *et al.*, 2003). Little overlap between CPP-protein complexes with transferrin in cells also corroborated the results of our earlier uptake experiments where inhibition of clathrin-mediated endocytosis only partially inhibited the CPP-mediated protein delivery into cells.

However, electron microscopy revealed one more type of vesicles that contained transportan- or TP10-protein complexes, which had a discontinuous membrane and an electron density undistinguishable from that of the cytosol. We suggest that the membrane of such vesicles was destabilized by the peptide, enabling the escape of the complexes to the cytoplasm. This hypothesis was further supported by the detection of some apparently free gold-labelled streptavidin in the cytoplasm. The endosomal escape has been shown for anthrax and diphtheria toxin (Sandvig and Olsnes, 1980; Wesche *et al.*, 1998), several viruses (Gruenberg and van der Goot, 2006) and also for fibroblast growth factor (FGF), which is subsequently directed to the nucleus (Olsnes *et al.*, 2003). Although the endosomal translocation of FGF requires the activity of the cytosolic chaperone HSP90 (Wesche *et al.*, 2006) and its nuclear localization is driven by

NLS that is absent in transportan and TP10, the studies demonstrating the activity of TP10-delivered cargo in the nucleus (El-Andaloussi *et al.*, 2006; El-Andaloussi *et al.*, 2007a) support our results showing that the transportan/TP10-protein complexes can translocate to the cytoplasm. The oligonucleotide delivery with CPPs from Pep-family is also suggested either to use endosomal escape or direct translocation across the plasma membrane due to the subsequent cytosolic localization of the delivered cargo (Morris *et al.*, 2004; Morris *et al.*, 2007). The mechanism of the endosomal escape is still poorly understood, although the inhibition of the endosomal acidification by chloroquine and ammonium chloride (Cain and Murphy, 1986) is widely used and successfully applied to enhance the CPP-mediated cargo delivery (El-Andaloussi *et al.*, 2006; Abes *et al.*, 2007). It is proposed that the inhibition of the endosomal acidification destabilizes the endosomal membranes and initiates the release of vesicular contents. It has been shown on artificial membranes that the membrane perturbing effect of TP10 was significantly stronger in neutral than in negatively charged large unilamellar vesicles (Barany-Wallje *et al.*, 2007), suggesting that the endosomal escape of the peptide could be facilitated at a more neutral pH.

4.2.3. Majority of transportan and TP10-protein complexes are directed to lysosome-like organelles upon extended incubation

To analyze to which extent the peptide protein complexes are directed to the degradative organelles, we extended the incubation time of cells with complexes to 20–72 hours. Electron microscopy revealed that the majority of CPP-delivered protein was concentrating to the perinuclear vesicles with lysosomal morphology. Fluorescence microscopy confirmed the targeting of CPP-protein complexes to the degradative organelles after extended incubation since extensive colocalization with live cell marker LysoTracker or lysosomal marker protein LAMP2 was detected in cells after 12 hour chase experiment. However, in parallel with the increase in the accumulation of CPP-protein complexes in cells, even after 24 or 48 hours of incubation the fluorescence as well as electron microscopy revealed that at least some peptide-containing vesicles were present over the cytoplasm that did not stain for LAMP2 and LAMP1. This suggested that a fraction of vesicles do not recruit the lysosomal marker proteins even after extended incubations. The acidification of vesicles that contain the CPP-protein complexes was analyzed in more detail in paper IV.

4.2.4. Transportan and TP10-mediated protein delivery is abolished at 4 ˚C

In paper I we reported the ability of avidin complexed to some CPPs to interact and remain attached to the cell membrane even after the incubation at low temperature. Considering the cellular trafficking processes to be inhibited but not

totally stopped at 16 ˚C (Saraste *et al.*, 1986), we assessed the induction of endocytosis by transportan- and TP10-protein complexes at 20, 10 and 4 ˚C at the ultrastructural level. At 10 ˚C, the complexes interacted with the plasma membrane of cells and the gold-labelled protein was clearly detectable also in some vesicles of cortical cytoplasm. However, the complexes did not form big agglomerates as it was seen at physiological temperature, and the interaction with the membrane did not induce changes in the cellular morphology, indicating that induction of large pits and vesicles by CPP-protein complexes requires cellular energy. Incubation of the cells with CPP-protein complexes at 20 ˚C revealed almost comparable uptake of peptide protein complexes to that observed at 37 ˚C. On the contrary, the incubation at 4 ˚C diminished the membrane interaction and abolished cellular uptake of both transportan- and TP10-streptavidin complexes, suggesting the prevalence of energy-dependent processes in their cellular uptake. However, we cannot exclude that the profoundly different properties of the plasma membrane at 4 ˚C, like the decreased fluidity and interfered mixing of lipids etc. also plays a significant role in the absence of the cellular uptake of TP/TP10-protein complexes at 4 ˚C. In line with our studies, Console and collaborators observed an analogous temperature-dependent cellular uptake of streptavidin complexed with pTat or penetratin, suggesting the prevalence of energy-dependent mechanisms and the importance of plasma membrane fluidity in CPP-mediated protein uptake (Console *et al.*, 2003).

4.3. Protein delivery with transportans is mediated by caveolae rather than flotillin-dependent pathways (paper III)

We had suggested that several endosomal pathways including macropinocytosis, clathrin-mediated endocytosis and endocytosis requiring the membranous cholesterol are simultaneously active in the cellular uptake of CPP-protein complexes (paper I and II). However, the putative involvement of caveolar endocytosis was based only on the visual estimation of the morphology of complexes-containing vesicles (paper II) and on the markedly decreased CPP-protein uptake by the cholesterol-depleted cells (paper I). In addition to being one of the characteristic components of caveolae, membranous cholesterol is also crucial in the formation and functioning of different lipid rafts, shown to mediate the endosomal processes, which do not start from clathrin-coated pits and caveolae (Lajoie and Nabi, 2007). Recently, flotillin was reported as a novel marker for a particular type of non-caveolar endocytosis (Glebov *et al.*, 2006). Therefore we analyzed the role of flotillin-1 and caveolin-mediated endocytosis in CPP-mediated protein delivery.

4.3.1. A considerable amount of transportan- and TP10-mediated protein complexes use caveolin-dependent endocytosis for cellular uptake

Electron microscopy revealed a relatively high abundance of vesicles in HeLa cells that contained peptide-protein complexes and resembled caveolae by their morphology (paper II). To elucidate the involvement of caveolin-dependent endocytosis in the CPP-mediated protein transduction, we used immunocytochemical staining for visualizing caveolae in HeLa cells. Both the confocal and electron microscopy demonstrated a partial overlap of caveolin and avidin/neutravidin complexed either to transportan, TP10 or pTat. Quantification by electron microscopy revealed that 15–20% of all complexes-containing vesicles in cortical as well as perinuclear cytoplasm contained also caveolin, corroborating the fluorescence microscopy studies showing similar overlap. The partial utilization of caveolin-dependent endocytosis by CPP-complexes was further confirmed by the down-regulation of cellular caveolin-1 by siRNA treatment. The 70–80% depletion of cellular caveolin-1 led to 30–50% decrease in the uptake of transportan and TP10 protein complexes. Moreover, analogous results were obtained with caveolin-$1^{-/-}$ primary fibroblasts, assuring that the lack of caveolin in the plasma membrane inhibits the internalization of the CPP-protein complexes. Considering that the depletion of cellular cholesterol by MCD treatment removes the cholesterol also from caveolae, the inhibition of cellular uptake of CPP-protein complexes in cells treated with MCD (paper I) correlated well with our results on caveolin-deficient cells. The caveolar pathway is shown to participate in the cellular uptake of EGFP fused either to Tat protein (Ferrari *et al.*, 2003) or Tat peptide (Fittipaldi *et al.*, 2003). However, in both cases more extensive overlap of both constructs with cholera toxin subunit than caveolin-1 was observed. CtxB can enter cells by several pathways probably due to the distribution of its receptor GM1 not only to caveolae but also to other membrane microdomains (Parton and Richards, 2003). The more extensive colocalization of Tat fusion protein with the cholera toxin B subunit compared to caveolin suggests that the uptake of Tat-EGFP fusion proteins was not restricted to the caveolar pathway only.

4.3.2. Flotillin-1 marked endosomal pathway does not participate in the uptake of protein mediated by transportans

Flotillin-1 and -2-containing plasma membrane areas form microdomains that resemble caveolae by morphology in electron microscopy images (Frick *et al.*, 2007), and which mediate the endosomal uptake of cholera toxin B subunit to a certain extent (Glebov *et al.*, 2006). As clathrin- and caveolae-mediated endocytosis and macropinocytosis could not comprehend all the cellular uptake of CPP-protein complexes, we used immunocytochemical methods to evaluate the role of flotillin-mediated endocytosis in the protein delivery by transportans.

Somewhat unexpectedly we did not detect the complexes of protein with transportan, TP10 or pTat in flotillin-1-containing structures by fluorescence microscopy. Electron microscopy analysis revealed a negligible number of flotillin-1-positive perinuclear vesicles, which contained CPP-protein complexes. Next, to further assess the role of flotillin-1 in protein delivery by transportans we down-regulated flotillin-1 by siRNA treatment. The Western blot analysis revealed only a moderate down-regulation of flotillin-1 by 40–50%. The fluorescence microscopy demonstrated that flotillin-1 was unevenly down-regulated in different compartments of HeLa cells. The quantification of flotillin-1 at the plasma membrane and in the cell centre revealed more efficient down-regulation at the plasma membrane, reaching 75% efficiency of siRNA treatment. In spite of that, the lack of flotillin-1 did not inhibit the cellular uptake of transportan- or TP10-mediated avidin delivery as quantified by FACS analysis. This suggests that the flotillin-positive membrane areas and the respective endosomal vesicles do not participate in the CPP-mediated protein uptake. Unfortunately, no good marker has been discovered for flotillin-mediated endocytosis so far. Therefore, we used the cholera toxin B subunit as a marker for flotillin-mediated endocytosis, which can be taken up by cells via multiple pathways (Parton and Richards, 2003), making the quantification of the interference with the flotillin-1-dependent pathway impossible.

However, in addition to revealing the insignificant role of flotillin-marked endocytosis in CPP-mediated protein cellular delivery, our results demonstrate the functional difference of flotillin- and caveolin-containing membrane microdomains and the endocytic pathways deriving from these.

4.3.3. Transportan- and TP10-protein complexes are not trafficked through early endosomal pathways marked by Rab5 and PI3P

Early endosomes are the main organelles for processing the cargo internalized via clathrin-coated pits (Pollard, 2008). Rab5 and PI3P are both the mediators of the fusion of early endosomal vesicles by recruiting the early endosomal autoantigen (EEA1) to the membrane, which acts as a vesicle tethering factor (Clague, 1999). To elucidate the early steps in the internalization process of CPP-protein complexes, we used two markers of early endosomal pathways Rab5 and PI3P in our studies and characterized the cellular localization of avidin complexed with transportans in relation to these markers. However, we could not find transportan- and TP10-protein complexes in the endosomal carriers labelled by Rab5 or PI3P, indicating that the early endosomal pathway is not exploited by transportans in protein transduction. Still, our earlier studies had suggested the clathrin-mediated pathway as one route for CPP-mediated protein uptake. A possible explanation for this contradiction is that the CPP-protein complexes enter cells via a non-specific endocytosis, recruiting also extracellular transferrin during the vesicle formation. Another possibility is that the transportan/TP10-protein complexes enter cells in clathrin-independent vesicles,

which fuse with transferrin-containing vesicles after they have passed the Rab5 and PI3P positive structures. It is known that the uptake of GPI-anchored folate receptor is internalized by Cdc42-dependent endocytosis without entering Rab5-positive early endosomes (Sabharanjak *et al.*, 2002). In addition, the uptake of GPI-anchored folate receptor was inhibited by the depletion of cholesterol (Chadda *et al.*, 2007), in analogy with our results in CPP-mediated protein delivery presented in paper I. Therefore, the uptake by a clathrin- and caveolin-independent but cholesterol dependent pathway by some CPP-protein complexes cannot be excluded.

The studies of the Tat-EGFP fusion protein uptake by cells indicated a three-fold slower motion of the vesicles containing fusion construct compared to the transferrin-containing endosomes (Ferrari *et al.*, 2003) and the fluorescence of cells incubated with Tat11-EGFP increased at slower rate than that of cells incubated with fluorescent transferrin (Fittipaldi *et al.*, 2003). The cellular uptake of GPI-anchored proteins, on the contrary, takes place markedly quicker (Chadda *et al.*, 2007). We can conclude that the uptake route for a considerable amount of CPP-protein complexes is more similar to that of Tat-EGFP constructs.

4.4. CPP-protein complexes induce a population of endocytic vesicles with moderate acidity (paper IV)

In eukaryotic cells the endocytosed material in sorting endosomes is directed either to the degradative pathway, leading to the lysosomal digestion of internalized molecules, or delivered back to the plasma membrane via the recycling endosomal compartment. The data from our earlier experiments with CPP-protein complexes revealed the exploitation of several endosomal pathways for cellular internalization in parallel (paper II and III). To shed light to the further trafficking of peptide-protein complexes after cellular entry, we analyzed whether the vesicles containing CPP-protein complexes were directed to degradative or recycling pathway in cells.

4.4.1. CPP-protein complexes are not directed to the recycling endosomal pathway

We chose transportan, pTat and nonaarginine as the representatives of most studied CPPs to compare the intracellular trafficking of the attached avidin cargo. First, we addressed the localization of CPP-protein complexes in cells in relation to the endosomes of the recycling pathway. Endosomal recycling compartment is described to be a long-lived organelle of mainly tubular structures, from which most molecules return to the plasma membrane and its shape and localization in different cells can vary (Maxfield and McGraw, 2004). In Cos cells the recycling endosomes are spatially segregated from the degradative

ones, passing through the inside of the ring-shaped Golgi complex (Misaki *et al.*, 2007). We took advantage of this characteristic of Cos cells and assessed the localization of complexes of biotinylated CPP and avidin-Texas Red in relation to the Golgi complex using an antibody against *trans*-Golgi marker protein TGN46. Only about 10–15% of CPP-avidin complexes containing vesicles localized to the Golgi ring and even less inside, showing that if the recycling pathway is involved in the trafficking of the complexes, the role of this route is negligible. The small overlap between the complexes and TGN46 also corroborates the results obtained with HeLa cells and discussed in paper II, where we detected very little overlap between transportan-avidin and Golgi marker TR-ceramide.

4.4.2. CPP-avidin complexes are internalized into three populations of vesicles of different pH and concentration

The vesicles with cargo destined for degradation are gradually acidified by the Na+/H+ ATPases. The pH decrease accompanies the maturation of endosomes to lysosomes and enables the activity of catabolic enzymes and degradation of the lysosomal content. In Cos 7 cells, we mapped the acidity of endosomes containing CPP-protein complexes by using a live cell marker LysoSensor DND, which exhibits a pH-dependent increase in fluorescence intensity upon acidification. Visualization of the complexes of biotinylated CPP with avidin-Texas Red in cells after incubation of 1, 4 or 12 hours allowed us to define three distinct subpopulations of endosomes based on the pH of the carrier vesicle and the concentration of CPP-cargo inside these vesicles. First, the small vesicles with medium or slightly acidic pH and with low signal of avidin-Texas Red were mainly detected after one hour of incubation for all used CPPs. The second population consisted of larger perinuclear endosomes with low pH and high concentration of complexes emerging typically after 4 and 12 hour time period. The gradual growth in the size and intensity of complexes-containing acidic vesicles was probably due to the fusion of smaller endosomes. The third population of vesicles was characterized by a high concentration of complexes, but had a rather medium pH value. This population did not undergo the pH drop even during 12 hours and was present with all CPP-protein complexes, but a considerably higher number of such vesicles were typical for cells incubated with transportan-avidin complexes. The presence of long-living and acidification-resistant vesicles might suggest that nonaarginine, pTat and especially transportan are able to inhibit the acidification, as the population of vesicles with medium-pH vesicles was not present after 12 hours in cells incubated with avidin only. Still, upon longer incubation the complexes concentrated to the perinuclear acidic organelles, indicating that although the internalization took place via different routes involving the peculiar type of endosomes in which pH was reduced only slightly, the majority of internalized complexes reach lysosomal organelles. Our earlier results demonstrated that caveolin- as well as

clathrin-mediated endocytosis and macropinocytosis mediate the internalization of CPP-protein complexes. As transferrin could be taken up by cells in the same vesicles together with CPP-protein complexes, and as we observed only a minor amount of CPP-protein complexes in the recycling pathway, the colocalization of CPP-protein complexes with transferrin (paper III) was probably due to the targeting of both to the vesicles of degradative pathway.

Fluorescent dextran is used as a marker for both fluid phase uptake and macropinocytosis. However, the differentiation between fluid phase endosomes and bigger macropinosomes is based mostly on the size of the vesicles. We detected dextran mainly in the acidic vesicles that were markedly bigger than the suggested 0.1 μm diameter for fluid phase endosomes and therefore we defined these structures mostly as macropinosomes. Although we can not conclude from this result that macropinocytically ingested CPP-protein complexes are also directed to acidic endosomes and do not contribute to the population with medium pH, the more likely source for non-acidic endosomes is caveolin-dependent endocytosis. Caveosomes are demonstrated to have pH values close to neutral even in three hours after internalization of SV40 (Pelkmans *et al.*, 2001). We showed that caveolin-dependent endocytosis participates in CPP-mediated protein uptake (paper III) and therefore we propose that the non-acidic complexes-containing vesicles might result from caveolar endocytosis. On the other hand, application of 10 μM of peptide and 3 μM avidin increased the amount of acidic vesicles containing CPP-protein complexes, while the amount of non-acidic vesicles did not change markedly. This result is in concordance with the study by Di Guglielmo and coworkers, demonstrating that caveolin-1-positive lipid rafts mediate the turnover of TGF β receptor via Smad7-Smurf pathway. At the same time Smad2-SARA signalling is induced by the TGF β taken up by clathrin-coated pits (Di Guglielmo *et al.*, 2003). In addition, the EGF receptor internalization via caveolae is induced by high EGF concentration and subsequent ubiquitination of the receptor (Sigismund *et al.*, 2005).

SUMMARY

Studies on the cell-entry mechanisms of cell-penetrating peptides have revealed the very high complexity of the internalization process. Several pathways of cellular uptake are used in parallel and the internalization mode is changed when CPP-cargo complexes are applied instead of CPP alone. In this study the protein cargo delivery properties and endosomal routing of six cell-penetrating peptides, with the emphasis on transportans, was studied by using confocal fluorescence and electron microscopy, flow cytometry and spectrofluorometry. The internalization mechanisms of CPP-protein complexes were studied in different eukaryotic cell lines by inhibition of different cellular processes via energy depletion, application of various pharmacological agents or siRNA treatment. For the visualization of CPP-protein complexes in relation to the organelles of endocytic pathways, tracer molecules of different endosomal entry routes, and organelle-specific antibodies were used in live and fixed cells, respectively.

All the cell-penetrating peptides studied here were found to mediate the cellular uptake of protein cargo using mainly the endosomal pathways, of which we defined the use of three and non-involvement of one endosomal pathway. In addition, the degradative pathway was found to be the major destination of delivered CPP-protein complexes.

The main results of this study can be concluded as follows.

1) Protein cellular delivery with transportan, pTat, pVEC and penetratin is dependent on the cellular energy, temperature and the organization of lipids in the plasma membrane.
2) Transportan and Tat peptide are more efficient in the protein delivery than penetratin and pVEC.
3) Clathrin- and caveolin-dependent endocytosis and macropinocytosis are mediating the cellular uptake of CPP-protein complexes in parallel.
4) Flotillin-dependent endocytosis and early endosomal carriers defined by Rab5 and PI3P are not involved in the protein cellular delivery by CPPs.
5) The majority of CPP-protein complexes taken up by cells avoid the recycling endosomal pathway and are targeted to degradative organelles in a concentration-dependent manner.
6) A substantial fraction of endosomes that contain CPP-protein complexes resists the pH decrease and the complexes are not subjected to degradation even after 12 hours.
7) After longer incubation and at higher concentration transportan and TP10 are able to induce the endosomal escape of the internalized protein.

SUMMARY IN ESTONIAN

ENDOSOMAALSED RAJAD RAKKU SISENEVATE PEPTIIDIDEGA VAHENDATUD VALGUTRANSPORDIL

Eukarüootne rakk on eraldatud väliskeskkonnast plasmamembraaniga, mille moodustavad fosfolipiidne kaksikkiht ja sellesse sukeldatud või sellega ühenduses olevad valgud. Plasmamembraani peamiseks ülesandeks on olla selektiivne barjäär, mis vahendab rakku signaale ja erinevate biomolekulide transporti. Raku eluks vajalike ainete toimetamine läbi rakumembraani on täpselt reguleeritud protsess, kus ioonide ja väikese molekulmassiga ühendite sisenemist vahendavad põhiliselt kanalid ja pumbad. Suurte hüdrofiilsete molekulide peamiseks rakku pääsemise viisiks on nende transport plasmamembraanist punguvate vesikulaarsete kandjate abil, mille moodustumine algatatakse enamasti pärast ühendi seondumist plasmamembraanil asuvale retseptorile. Plasmamembraani selektiivsus tingib ka selle, et mitmete muidu efektiivsete ravimite kasutuselevõtt on takistatud – nende rakuline kättesaadavus on lihtsalt väga madal. Selliste ühendite rakku toimetamiseks on biotehnoloogias välja töötatud erinevaid mooduseid, millele hiljuti lisandus alternatiiv rakku sisenevate peptiidide (RSP) näol. Tegu on enamasti 5–40 aminohappest koosnevate peptiididega, mis omavad võimet läbida rakumembraani ja vahendada endaga liidetud suurte hüdrofiilsete molekulide rakku sisenemist. Ligi 15 aasta jooksul on identifitseeritud üle saja iseseisvalt rakku liikuva peptiidse järjestuse ning paljudega neist on veenvalt demonstreeritud ka rakku viidud ühendite bioloogilist aktiivsust. Samas eeldab RSPde kui transportmolekulide kasutuselevõtt põhjalikke teadmisi nii nende ühendite rakku sisenemisest kui ka rakusisesest suunamisest.

On näidatud, et paljude ühendite rakku sisestamine RSPdega toimub endotsütoosi teel, kuid teisalt on kirjeldatud ka mitte-endotsütootilist sisenemismoodust, mille täpsem mehhanism on veel siiani teadmata. Käesoleva väitekirja peamiseks eesmärgiks oli iseloomustada erinevate rakku sisenevate peptiidide efektiivsust ja sisenemisradu valgu rakkudesse toimetamisel avidiini, neutravidiini ja streptavidiini valkude näitel. Töös on käsitletud kuut erinevat peptiidi, millest põhirõhk on transportaani ja TP10 poolt vahendatud valgu rakku suunamise uurimisel. Transportaan on 27 aminohappe pikkune kimäärne peptiid, koosnedes N-otsas asuvast neuropeptiid galaniini fragmendist ja sellele liidetud herilasemürgi komponendist mastoparaanist. TP10 on transportaani N-otsast lühendatud analoog, mis vastupidiselt transportaanile ei inhibeeri rakkudes konstitutiivselt aktiivseid G-valke ja omab seega vähem võimalikke bioloogilisi kõrvalmõjusid. Transportaanidele lisaks on kaasatud töösse HIV-1 transaktivaatorvalgu transduktsioonidomeen, mis on tuntud Tat peptiidina; üheksast arginiinijäägist koosnev peptiid (R_9), Antennapedia homeovalgu homeodomeenist pärinev penetratiin ja vaskulaar-endoteliaalse kadheriini transmembraanse osa fragment pVEC. Tat peptiid, penetratiin ja nonaarginiin on ühed enim uuritud rakku sisenevad peptiidsed järjestused. pVEC, mida on

käsitletud esimeses publikatsioonis, kirjeldati rakku siseneva peptiidina hiljuti meie koostööpartnerite poolt ja selle peptiidi transpordiomadused olid senini vähe uuritud. Peptiidi ja valgu liitmiseks lisasime peptiidile biotiinimärgise, mis seostub nii avidiini, strept- kui ka neutravidiiniga väga kõrge afiinsusega, moodustades stabiilse kompleksi. RSPdega rakku viidud valgu koguse ja asukoha määramiseks kasutasime valguga liidetud fluorestsentsmärgise detekteerimist konfokaal-fluorestsentsmikroskoopia, spektrofluoromeetria ja läbivoolutsütomeetria abil. Peptiid-valk-komplekside tuvastamiseks rakus ultrastruktuuri tasemel kasutasime kolloidse kullaga märgitud valgu visualiseerimist elektronmikroskoopia meetodi abil.

Peptiididega vahendatud valgutranspordi efektiivsuse hindamisel ilmnes, et Tat peptiid ja transportaan on võrreldes penetratiini ja pVEC-ga võimelised rakkudesse suurema koguse avidiini toimetama. Energiast sõltuvaid rakulisi protsesse pärssides (madal temperatuur, metaboolse aktiivsuse inhibeerimine) leidsime, et peptiididega vahendatud valgutransport on sõltuv nii rakuenergiast kui ka temperatuurist. Mitmetest varem avaldatud tulemustest nähtus, et RSPde rakku sisenemine on takistatud, kui plasmamembraani lipiidide organiseeritus on häiritud. Selleks et hinnata plasmamembraani lipiidse organiseerituse mõju RSP rakku liikumisele juhul kui peptiid on liidetud valgumolekuliga, uurisime komplekside sisenemist rakkudesse, mille plasmamembraanist oli osaliselt eemaldatud kolesterool. Kolesterooli peetakse vastutavaks plasmamembraanis tihedamalt pakitud mikropiirkondade ehk lipiidsete parvede tekkimise eest, mis lisaks osalemisele endotsütootilistes protsessides arvatakse funktsioneerivat kui rakumembraanis asuvate retseptorite koondumiskohad ja paljude signalisatsiooniradade alguspunktid. Meie eksperimentidest selgus, et kolesterooli eemaldamise tagajärjel on RSPde vahendatud valgu rakkudesse suunamine tugevalt pärsitud, viidates sellele, et plasmamembraani lipiidne organiseeritus ja/või sellest tulenevad protsessid on RSPdega valgu rakkudesse suunamisel määrava tähtsusega.

Peptiididega rakku viidud valgu asukoha kirjeldamisest elektronmikroskoopia abil nähtus, et kompleksid asuvad rakus enamasti erineva suurusega vesikulaarsetes struktuurides. Kuna erinevaid endotsütootilisi vesiikuleid saab elektronmikroskoopilisel vaatlemisel morfoloogia alusel teatud määral alaliigiti eristada, järeldasime, et RSP-vahendatud valgu rakku suunamisel kasutatakse makropinotsütoosi ja nii klatriin- kui kaveoliin-sõltuvaid endosomaalseid radu. Samas ei võimalda vesiikulite elektronmikroskoopiline vaatlus vahet teha kaveoolidest lähtuvate ning klatriin- ja kaveoliin-sõltumatute vesikulaarsete kandjate vahel. Kuna kaveoole peetakse üheks lipiidsete parvede alaliigiks, otsustasime me selgitada lipiidsetest parvedest algavate endosomaalsete radade osalust RSP-vahendatud valgutranspordis, kasutades kahte tüüpi lipiidsete parvede markervalku, kaveoliini ja flotilliini. Flotilliini kui teatud tüüpi lipiidsete parvede ja sealt lähtuva endosomaalse raja vesiikulite pinnal asuvat valku on kirjeldatud suhteliselt hiljuti. Lisaks analüüsisime me varaste endosoomide osalust transportaani, TP10 ja Tat peptiidi vahendatud valgutranspordis, kasutades varaste endosoomide membraanis asuva valgu Rab5 ja lipiidi PI3P visuali-

seerimist RSP-valk-komplekside suhtes. Tulemustest nähtus, et arvestatav osa RSPga komplekseeritud valgust liigub rakkudesse kaveolaarset endotsütoosi kasutades, samas kui flotilliin-1, Rab5 ja PI3P sisaldavate vesiikulite kaudu komplekside rakku sisenemist praktiliselt ei toimu. Samuti leidsime, et komplekside suunamine ringlusraja endosoomidesse, organellidesse, mis oma sisaldise tagasi membraanile ja rakust välja suunavad, toimub väga vähesel määral ning valdav osa RSPdega rakku sisestatud valgust suunatakse kontsentratsioonsõltuvalt lagundava raja happelistesse organellidesse. Samas nägime, et märgatav osa peptiid-valk-komplekse sisaldavaid endosoome säilitas nõrgalt happelise või neutraalse keskkonna ka 12 tundi pärast kompleksidega inkubeerimise algust ja selliste endosoomide populatsioon oli suurem transportaan-avidiini komplekside korral. Transportaanide omadus aeglustada endosomaalsete vesiikulite pH langust võib olla seotud nende peptiidide võimega põhjustada endosomaalsete vesiikulite membraani suuremat läbilaskvust ja komplekside vabanemist tsütoplasmasse, mis oli eelkõige nähtav kõrgema kontsentratsiooniga ja pikemaajaliselt transportaan-streptavidiini kompleksidega töödeldud rakkude elektronmikroskoopilisel vaatlusel.

REFERENCES

Abes, S., Turner, J. J., Ivanova, G. D., Owen, D., Williams, D., Arzumanov, A., Clair, P., Gait, M. J., and Lebleu, B. (2007). Efficient splicing correction by PNA conjugation to an R6-Penetratin delivery peptide. Nucleic Acids Res *35*, 4495–4502.

Amsellem, S., Pflumio, F., Bardinet, D., Izac, B., Charneau, P., Romeo, P. H., Dubart-Kupperschmitt, A., and Fichelson, S. (2003). Ex vivo expansion of human hematopoietic stem cells by direct delivery of the HOXB4 homeoprotein. Nat Med *9*, 1423–1427.

Barany-Wallje, E., Andersson, A., Gräslund, A., and Mäler, L. (2004). NMR solution structure and position of transportan in neutral phospholipid bicelles. FEBS Lett *567*, 265–269.

Barany-Wallje, E., Gaur, J., Lundberg, P., Langel, Ü., and Gräslund, A. (2007). Differential membrane perturbation caused by the cell penetrating peptide Tp10 depending on attached cargo. FEBS Lett *581*, 2389–2393.

Belting, M. (2003). Heparan sulfate proteoglycan as a plasma membrane carrier. Trends Biochem Sci *28*, 145–151.

Bernstein, E., Caudy, A. A., Hammond, S. M., and Hannon, G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature *409*, 363–366.

Bishop, J. R., Schuksz, M., and Esko, J. D. (2007). Heparan sulphate proteoglycans fine-tune mammalian physiology. Nature *446*, 1030–1037.

Brunet, I., Weinl, C., Piper, M., Trembleau, A., Volovitch, M., Harris, W., Prochiantz, A., and Holt, C. (2005). The transcription factor Engrailed-2 guides retinal axons. Nature *438*, 94–98.

Cain, C. C., and Murphy, R. F. (1986). Growth inhibition of 3T3 fibroblasts by lysosomotropic amines: correlation with effects on intravesicular pH but not vacuolation. J Cell Physiol *129*, 65–70.

Chadda, R., Howes, M. T., Plowman, S. J., Hancock, J. F., Parton, R. G., and Mayor, S. (2007). Cholesterol-sensitive Cdc42 activation regulates actin polymerization for endocytosis via the GEEC pathway. Traffic *8*, 702–717.

Chaloin, L., Vidal, P., Lory, P., Mery, J., Lautredou, N., Divita, G., and Heitz, F. (1998). Design of carrier peptide-oligonucleotide conjugates with rapid membrane translocation and nuclear localization properties. Biochem Biophys Res Commun *243*, 601–608.

Chapman, D. (1975). Phase transitions and fluidity characteristics of lipids and cell membranes. Q Rev Biophys *8*, 185–235.

Chaubey, B., Tripathi, S., Ganguly, S., Harris, D., Casale, R. A., and Pandey, V. N. (2005). A PNA-transportan conjugate targeted to the TAR region of the HIV-1 genome exhibits both antiviral and virucidal properties. Virology *331*, 418–428.

Cheng, Z. J., Singh, R. D., Sharma, D. K., Holicky, E. L., Hanada, K., Marks, D. L., and Pagano, R. E. (2006). Distinct mechanisms of clathrin-independent endocytosis have unique sphingolipid requirements. Mol Biol Cell *17*, 3197–3210.

Christiaens, B., Grooten, J., Reusens, M., Joliot, A., Goethals, M., Vandekerckhove, J., Prochiantz, A., and Rosseneu, M. (2004). Membrane interaction and cellular internalization of penetratin peptides. Eur J Biochem *271*, 1187–1197.

Christoforidis, S., McBride, H. M., Burgoyne, R. D., and Zerial, M. (1999). The Rab5 effector EEA1 is a core component of endosome docking. Nature *397*, 621–625.

Clague, M. J. (1999). Membrane transport: Take your fusion partners. Curr Biol *9*, R258–260.

Console, S., Marty, C., Garcia-Echeverria, C., Schwendener, R., and Ballmer-Hofer, K. (2003). Antennapedia and HIV transactivator of transcription (TAT) "protein transduction domains" promote endocytosis of high molecular weight cargo upon binding to cell surface glycosaminoglycans. J Biol Chem *278*, 35109–35114.

Damm, E. M., Pelkmans, L., Kartenbeck, J., Mezzacasa, A., Kurzchalia, T., and Helenius, A. (2005). Clathrin- and caveolin-1-independent endocytosis: entry of simian virus 40 into cells devoid of caveolae. J Cell Biol *168*, 477–488.

Derossi, D., Calvet, S., Trembleau, A., Brunissen, A., Chassaing, G., and Prochiantz, A. (1996). Cell internalization of the third helix of the Antennapedia homeodomain is receptor-independent. J Biol Chem *271*, 18188–18193.

Derossi, D., Joliot, A. H., Chassaing, G., and Prochiantz, A. (1994). The third helix of the Antennapedia homeodomain translocates through biological membranes. J Biol Chem *269*, 10444–10450.

Deshayes, S., Gerbal-Chaloin, S., Morris, M. C., Aldrian-Herrada, G., Charnet, P., Divita, G., and Heitz, F. (2004a). On the mechanism of non-endosomial peptide-mediated cellular delivery of nucleic acids. Biochim Biophys Acta *1667*, 141–147.

Deshayes, S., Heitz, A., Morris, M. C., Charnet, P., Divita, G., and Heitz, F. (2004b). Insight into the mechanism of internalization of the cell-penetrating carrier peptide Pep-1 through conformational analysis. Biochemistry *43*, 1449–1457.

Di Guglielmo, G. M., Le Roy, C., Goodfellow, A. F., and Wrana, J. L. (2003). Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover. Nat Cell Biol *5*, 410–421.

Dietz, G. P., Kilic, E., and Bähr, M. (2002). Inhibition of neuronal apoptosis in vitro and in vivo using TAT-mediated protein transduction. Mol Cell Neurosci *21*, 29–37.

Drin, G., Cottin, S., Blanc, E., Rees, A. R., and Temsamani, J. (2003). Studies on the internalization mechanism of cationic cell-penetrating peptides. J Biol Chem *278*, 31192–31201.

Duchardt, F., Fotin-Mleczek, M., Schwarz, H., Fischer, R., and Brock, R. (2007). A comprehensive model for the cellular uptake of cationic cell-penetrating peptides. Traffic *8*, 848–866.

Dupree, P., Parton, R. G., Raposo, G., Kurzchalia, T. V., and Simons, K. (1993). Caveolae and sorting in the trans-Golgi network of epithelial cells. Embo J *12*, 1597–1605.

Edeling, M. A., Smith, C., and Owen, D. (2006). Life of a clathrin coat: insights from clathrin and AP structures. Nat Rev Mol Cell Biol *7*, 32–44.

El-Andaloussi, S., Johansson, H. J., Holm, T., and Langel, Ü. (2007a). A Novel Cell-penetrating Peptide, M918, for Efficient Delivery of Proteins and Peptide Nucleic Acids. Mol Ther *10*, 1820–1826.

El-Andaloussi, S., Johansson, H. J., Lundberg, P., and Langel, Ü. (2006). Induction of splice correction by cell-penetrating peptide nucleic acids. J Gene Med *8*, 1262–1273.

El-Andaloussi, S., Järver, P., Johansson, H. J., and Langel, Ü. (2007b). Cargo-dependent cytotoxicity and delivery efficacy of cell-penetrating peptides: a comparative study. Biochem J *407*, 285–292.

Elmquist, A., Hansen, M., and Langel, Ü. (2006). Structure-activity relationship study of the cell-penetrating peptide pVEC. Biochim Biophys Acta *1758*, 721–729.

Elmquist, A., Lindgren, M., Bartfai, T., and Langel, Ü. (2001). VE-cadherin-derived cell-penetrating peptide, pVEC, with carrier functions. Exp Cell Res *269*, 237–244.

Fawell, S., Seery, J., Daikh, Y., Moore, C., Chen, L. L., Pepinsky, B., and Barsoum, J. (1994). Tat-mediated delivery of heterologous proteins into cells. Proc Natl Acad Sci U S A *91*, 664–668.

Ferrari, A., Pellegrini, V., Arcangeli, C., Fittipaldi, A., Giacca, M., and Beltram, F. (2003). Caveolae-mediated internalization of extracellular HIV-1 tat fusion proteins visualized in real time. Mol Ther *8*, 284–294.

Fischer, R., Kohler, K., Fotin-Mleczek, M., and Brock, R. (2004). A stepwise dissection of the intracellular fate of cationic cell-penetrating peptides. J Biol Chem *279*, 12625–12635.

Fittipaldi, A., Ferrari, A., Zoppe, M., Arcangeli, C., Pellegrini, V., Beltram, F., and Giacca, M. (2003). Cell membrane lipid rafts mediate caveolar endocytosis of HIV-1 Tat fusion proteins. J Biol Chem *278*, 34141–34149.

Frankel, A. D., and Pabo, C. O. (1988). Cellular uptake of the tat protein from human immunodeficiency virus. Cell *55*, 1189–1193.

Fretz, M. M., Penning, N. A., Al-Taei, S., Futaki, S., Takeuchi, T., Nakase, I., Storm, G., and Jones, A. T. (2007). Temperature-, concentration- and cholesterol-dependent translocation of L- and D-octa-arginine across the plasma and nuclear membrane of CD34+ leukaemia cells. Biochem J *403*, 335–342.

Frick, M., Schmidt, K., and Nichols, B. J. (2007). Modulation of lateral diffusion in the plasma membrane by protein density. Curr Biol *17*, 462–467.

Futaki, S., Suzuki, T., Ohashi, W., Yagami, T., Tanaka, S., Ueda, K., and Sugiura, Y. (2001). Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. J Biol Chem *276*, 5836–5840.

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

Glebov, O. O., Bright, N. A., and Nichols, B. J. (2006). Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells. Nat Cell Biol *8*, 46–54.

Glenney, J. R., Jr. (1989). Tyrosine phosphorylation of a 22-kDa protein is correlated with transformation by Rous sarcoma virus. J Biol Chem *264*, 20163–20166.

Gonçalves, E., Kitas, E., and Seelig, J. (2005). Binding of oligoarginine to membrane lipids and heparan sulfate: structural and thermodynamic characterization of a cell-penetrating Peptide. Biochemistry *44*, 2692–2702.

Green, M., and Loewenstein, P. M. (1988). Autonomous functional domains of chemically synthesized human immunodeficiency virus tat trans-activator protein. Cell *55*, 1179–1188.

Gruenberg, J., and van der Goot, F. G. (2006). Mechanisms of pathogen entry through the endosomal compartments. Nat Rev Mol Cell Biol *7*, 495–504.

Hammond, S. M., Bernstein, E., Beach, D., and Hannon, G. J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature *404*, 293–296.

Harding, A. S., and Hancock, J. F. (2008). Using plasma membrane nanoclusters to build better signaling circuits. Trends Cell Biol *18*, 364–371.

Hariton-Gazal, E., Feder, R., Mor, A., Graessmann, A., Brack-Werner, R., Jans, D., Gilon, C., and Loyter, A. (2002). Targeting of nonkaryophilic cell-permeable peptides into the nuclei of intact cells by covalently attached nuclear localization signals. Biochemistry *41*, 9208–9214.

Heuser, J. E., and Anderson, R. G. (1989). Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. J Cell Biol *108*, 389–400.

Hill, M. M., Bastiani, M., Luetterforst, R., Kirkham, M., Kirkham, A., Nixon, S. J., Walser, P., Abankwa, D., Oorschot, V. M., Martin, S., *et al.* (2008). PTRF-Cavin, a conserved cytoplasmic protein required for caveola formation and function. Cell *132*, 113–124.

Howl, J., Jones, S., and Farquhar, M. (2003). Intracellular Delivery of Bioactive Peptides to RBL-2H3 Cells Induces b-Hexosaminidase Secretion and Phospholipase D Activation. ChemBioChem *4*, 1312–1316.

Hällbrink, M., Florén, A., Elmquist, A., Pooga, M., Bartfai, T., and Langel, Ü. (2001). Cargo delivery kinetics of cell-penetrating peptides. Biochim Biophys Acta *1515*, 101–109.

Ikonen, E. (2008). Cellular cholesterol trafficking and compartmentalization. Nat Rev Mol Cell Biol *9*, 125–138.

Jones, A. T. (2007). Macropinocytosis: searching for an endocytic identity and role in the uptake of cell penetrating peptides. J Cell Mol Med *11*, 670–684.

Kang, S. H., Cho, M. J., and Kole, R. (1998). Up-regulation of luciferase gene expression with antisense oligonucleotides: implications and applications in functional assay development. Biochemistry *37*, 6235–6239.

Kaplan, I. M., Wadia, J. S., and Dowdy, S. F. (2005). Cationic TAT peptide transduction domain enters cells by macropinocytosis. J Control Release *102*, 247–253.

Khalil, I. A., Futaki, S., Niwa, M., Baba, Y., Kaji, N., Kamiya, H., and Harashima, H. (2004). Mechanism of improved gene transfer by the N-terminal stearylation of octaarginine: enhanced cellular association by hydrophobic core formation. Gene Ther *11*, 636–644.

Kirkham, M., Fujita, A., Chadda, R., Nixon, S. J., Kurzchalia, T. V., Sharma, D. K., Pagano, R. E., Hancock, J. F., Mayor, S., and Parton, R. G. (2005). Ultrastructural identification of uncoated caveolin-independent early endocytic vehicles. J Cell Biol *168*, 465–476.

Kosuge, M., Takeuchi, T., Nakase, I., Jones, A. T., and Futaki, S. (2008). Cellular internalization and distribution of arginine-rich peptides as a function of extracellular peptide concentration, serum, and plasma membrane associated proteoglycans. Bioconjug Chem *19*, 656–664.

Kumari, S., and Mayor, S. (2008). ARF1 is directly involved in dynamin-independent endocytosis. Nat Cell Biol *10*, 30–41.

Lajoie, P., and Nabi, I. R. (2007). Regulation of raft-dependent endocytosis. J Cell Mol Med *11*, 644–653.

Lakadamyali, M., Rust, M. J., and Zhuang, X. (2006). Ligands for clathrin-mediated endocytosis are differentially sorted into distinct populations of early endosomes. Cell *124*, 997–1009.

Lamaze, C., Dujeancourt, A., Baba, T., Lo, C. G., Benmerah, A., and Dautry-Varsat, A. (2001). Interleukin 2 receptors and detergent-resistant membrane domains define a clathrin-independent endocytic pathway. Mol Cell *7*, 661–671.

Lamaze, C., and Schmid, S. L. (1995). The emergence of clathrin-independent pinocytic pathways. Curr Opin Cell Biol *7*, 573–580.

Langel, Ü. (2006). Handbook of Cell-Penetrating Peptides, 2nd edn (Boca Raton, London, New York, Washington, CRC Press).

Langel, Ü., Pooga, M., Kairane, C., Zilmer, M., and Bartfai, T. (1996). A galanin-mastoparan chimeric peptide activates the Na+,K(+)-ATPase and reverses its inhibition by ouabain. Regul Pept *62*, 47–52.

Lau, A. W., and Chou, M. M. (2008). The adaptor complex AP-2 regulates post-endocytic trafficking through the non-clathrin Arf6-dependent endocytic pathway. J Cell Sci *121*, 4008–4017.

Lesaffre, B., Joliot, A., Prochiantz, A., and Volovitch, M. (2007). Direct non-cell autonomous Pax6 activity regulates eye development in the zebrafish. Neural Dev *2*, 2.

Lundberg, M., and Johansson, M. (2002). Positively charged DNA-binding proteins cause apparent cell membrane translocation. Biochem Biophys Res Commun *291*, 367–371.

Lundberg, P., El-Andaloussi, S., Sütlü, T., Johansson, H., and Langel, Ü. (2007). Delivery of short interfering RNA using endosomolytic cell-penetrating peptides. Faseb J *21*, 2664–2671.

Lundin, P., Johansson, H., Guterstam, P., Holm, T., Hansen, M., Langel, Ü., and El-Andaloussi, S. (2008). Distinct uptake routes of cell-penetrating peptide conjugates. Bioconjug Chem *19*, 2535–2542.

Lundmark, R., Doherty, G. J., Howes, M. T., Cortese, K., Vallis, Y., Parton, R. G., and McMahon, H. T. (2008). The GTPase-activating protein GRAF1 regulates the CLIC/GEEC endocytic pathway. Curr Biol *18*, 1802–1808.

Macia, E., Ehrlich, M., Massol, R., Boucrot, E., Brunner, C., and Kirchhausen, T. (2006). Dynasore, a cell-permeable inhibitor of dynamin. Dev Cell *10*, 839–850.

Maiolo, J. R., Ferrer, M., and Ottinger, E. A. (2005). Effects of cargo molecules on the cellular uptake of arginine-rich cell-penetrating peptides. Biochim Biophys Acta *1712*, 161–172.

Mani, K., Sandgren, S., Lilja, J., Cheng, F., Svensson, K., Persson, L., and Belting, M. (2007). HIV-Tat protein transduction domain specifically attenuates growth of polyamine deprived tumor cells. Mol Cancer Ther *6*, 782–788.

Mano, M., Teodosio, C., Paiva, A., Simoes, S., and Pedroso de Lima, M. C. (2005). On the mechanisms of the internalization of S4(13)-PV cell-penetrating peptide. Biochem J *390*, 603–612.

Maxfield, F. R., and McGraw, T. E. (2004). Endocytic recycling. Nat Rev Mol Cell Biol *5*, 121–132.

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

Meier, O., Boucke, K., Hammer, S. V., Keller, S., Stidwill, R. P., Hemmi, S., and Greber, U. F. (2002). Adenovirus triggers macropinocytosis and endosomal leakage together with its clathrin-mediated uptake. J Cell Biol *158*, 1119–1131.

Mercer, J., and Helenius, A. (2008). Vaccinia virus uses macropinocytosis and apoptotic mimicry to enter host cells. Science *320*, 531–535.

Misaki, R., Nakagawa, T., Fukuda, M., Taniguchi, N., and Taguchi, T. (2007). Spatial segregation of degradation- and recycling-trafficking pathways in COS-1 cells. Biochem Biophys Res Commun *360*, 580–585.

Mitchell, D. J., Kim, D. T., Steinman, L., Fathman, C. G., and Rothbard, J. B. (2000). Polyarginine enters cells more efficiently than other polycationic homopolymers. J Pept Res *56*, 318–325.

Morris, M. C., Chaloin, L., Choob, M., Archdeacon, J., Heitz, F., and Divita, G. (2004). Combination of a new generation of PNAs with a peptide-based carrier enables efficient targeting of cell cycle progression. Gene Ther *11*, 757–764.

Morris, M. C., Depollier, J., Mery, J., Heitz, F., and Divita, G. (2001). A peptide carrier for the delivery of biologically active proteins into mammalian cells. Nat Biotechnol *19*, 1173–1176.

Morris, M. C., Gros, E., Aldrian-Herrada, G., Choob, M., Archdeacon, J., Heitz, F., and Divita, G. (2007). A non-covalent peptide-based carrier for in vivo delivery of DNA mimics. Nucleic Acids Res *35*, e49.

Morris, M. C., Vidal, P., Chaloin, L., Heitz, F., and Divita, G. (1997). A new peptide vector for efficient delivery of oligonucleotides into mammalian cells. Nucleic Acids Res *25*, 2730–2736.

Mueller, J., Kretzschmar, I., Volkmer, R., and Boisguerin, P. (2008). Comparison of cellular uptake using 22 CPPs in 4 different cell lines. Bioconjug Chem *19*, 2363–2374.

Muñoz-Morris, M. A., Heitz, F., Divita, G., and Morris, M. C. (2007). The peptide carrier Pep-1 forms biologically efficient nanoparticle complexes. Biochem Biophys Res Commun *355*, 877–882.

Möbius, W., van Donselaar, E., Ohno-Iwashita, Y., Shimada, Y., Heijnen, H. F., Slot, J. W., and Geuze, H. J. (2003). Recycling compartments and the internal vesicles of multivesicular bodies harbor most of the cholesterol found in the endocytic pathway. Traffic *4*, 222–231.

Myrberg, H., Zhang, L., Mäe, M., and Langel, Ü. (2008). Design of a tumor-homing cell-penetrating peptide. Bioconjug Chem *19*, 70–75.

Nakase, I., Niwa, M., Takeuchi, T., Sonomura, K., Kawabata, N., Koike, Y., Takehashi, M., Tanaka, S., Ueda, K., Simpson, J. C.*, et al.* (2004). Cellular uptake of arginine-rich peptides: roles for macropinocytosis and actin rearrangement. Mol Ther *10*, 1011–1022.

Nakase, I., Tadokoro, A., Kawabata, N., Takeuchi, T., Katoh, H., Hiramoto, K., Negishi, M., Nomizu, M., Sugiura, Y., and Futaki, S. (2007). Interaction of arginine-rich peptides with membrane-associated proteoglycans is crucial for induction of actin organization and macropinocytosis. Biochemistry *46*, 492–501.

Nichols, B. J. (2002). A distinct class of endosome mediates clathrin-independent endocytosis to the Golgi complex. Nat Cell Biol *4*, 374–378.

Nichols, B. J., Kenworthy, A. K., Polishchuk, R. S., Lodge, R., Roberts, T. H., Hirschberg, K., Phair, R. D., and Lippincott-Schwartz, J. (2001). Rapid cycling of lipid raft markers between the cell surface and Golgi complex. J Cell Biol *153*, 529–541.

Nielsen, P. E., Egholm, M., Berg, R. H., and Buchardt, O. (1993). Peptide nucleic acids (PNAs): potential antisense and anti-gene agents. Anticancer Drug Des *8*, 53–63.

Oehlke, J., Scheller, A., Wiesner, B., Krause, E., Beyermann, M., Klauschenz, E., Melzig, M., and Bienert, M. (1998). Cellular uptake of an alpha-helical amphipathic model peptide with the potential to deliver polar compounds into the cell interior non- endocytically. Biochim Biophys Acta *1414*, 127–139.

Ohkuma, S., and Poole, B. (1978). Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. Proc Natl Acad Sci U S A *75*, 3327–3331.

Olsnes, S., Klingenberg, O., and Wiedlocha, A. (2003). Transport of exogenous growth factors and cytokines to the cytosol and to the nucleus. Physiol Rev *83*, 163–182.

Orlandi, P. A., and Fishman, P. H. (1998). Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains. J Cell Biol *141*, 905–915.

Owen, D. J., Collins, B. M., and Evans, P. R. (2004). Adaptors for clathrin coats: structure and function. Annu Rev Cell Dev Biol *20*, 153–191.

Padari, K. (2008). Protein transduction mechanisms of transportans, University of Tartu, Tartu.

Parton, R. G. (2003). Caveolae - from ultrastructure to molecular mechanisms. Nat Rev Mol Cell Biol *4*, 162–167.

Parton, R. G., and Richards, A. A. (2003). Lipid rafts and caveolae as portals for endocytosis: new insights and common mechanisms. Traffic *4*, 724–738.

Payne, C. K., Jones, S. A., Chen, C., and Zhuang, X. (2007). Internalization and trafficking of cell surface proteoglycans and proteoglycan-binding ligands. Traffic *8*, 389–401.

Pelkmans, L., Burli, T., Zerial, M., and Helenius, A. (2004). Caveolin-stabilized membrane domains as multifunctional transport and sorting devices in endocytic membrane traffic. Cell *118*, 767–780.

Pelkmans, L., and Helenius, A. (2002). Endocytosis via caveolae. Traffic *3*, 311–320.

Pelkmans, L., Kartenbeck, J., and Helenius, A. (2001). Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. Nat Cell Biol *3*, 473–483.

Pelkmans, L., and Zerial, M. (2005). Kinase-regulated quantal assemblies and kiss-and-run recycling of caveolae. Nature *436*, 128–133.

Pokorny, A., Birkbeck, T. H., and Almeida, P. F. (2002). Mechanism and kinetics of delta-lysin interaction with phospholipid vesicles. Biochemistry *41*, 11044–11056.

Pollard, T. D., Earnshaw, W., C., Lippincott-Schwartz, J. (2008). Cell Biology, 2nd Edition (USA, Saunders Elsevier).

Pooga, M., Hällbrink, M., Zorko, M., and Langel, Ü. (1998a). Cell penetration by transportan. FASEB J *12*, 67–77.

Pooga, M., Soomets, U., Hällbrink, M., Valkna, A., Saar, K., Rezaei, K., Kahl, U., Hao, J. X., Xu, X. J., Wiesenfeld-Hallin, Z., *et al.* (1998b). Cell penetrating PNA constructs regulate galanin receptor levels and modify pain transmission in vivo. Nat Biotechnol *16*, 857–861.

Potocky, T. B., Menon, A. K., and Gellman, S. H. (2003). Cytoplasmic and nuclear delivery of a TAT-derived peptide and a beta-peptide after endocytic uptake into HeLa cells. J Biol Chem *278*, 50188–50194.

Prochiantz, A. (2000). Messenger proteins: homeoproteins, TAT and others. Curr Opin Cell Biol *12*, 400–406.

Richard, J. P., Melikov, K., Brooks, H., Prevot, P., Lebleu, B., and Chernomordik, L. V. (2005). Cellular uptake of unconjugated TAT peptide involves clathrin-dependent endocytosis and heparan sulfate receptors. J Biol Chem *280*, 15300–15306.

Richard, J. P., Melikov, K., Vivès, E., Ramos, C., Verbeure, B., Gait, M. J., Chernomordik, L. V., and Lebleu, B. (2003). Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. J Biol Chem *278*, 585–590.

Rinne, J., Albarran, B., Jylhävä, J., Ihalainen, T. O., Kankaanpää, P., Hytönen, V. P., Stayton, P. S., Kulomaa, M. S., and Vihinen-Ranta, M. (2007). Internalization of novel non-viral vector TAT-streptavidin into human cells. BMC Biotechnol *7*, 1.

Rodal, S. K., Skretting, G., Garred, O., Vilhardt, F., van Deurs, B., and Sandvig, K. (1999). Extraction of cholesterol with methyl-beta-cyclodextrin perturbs formation of clathrin-coated endocytic vesicles. Mol Biol Cell *10*, 961–974.

Roth, T. F., and Porter, K. R. (1964). Yolk Protein Uptake in the Oocyte of the Mosquito Aedes Aegypti. L. J Cell Biol *20*, 313–332.

Rothbard, J. B., Garlington, S., Lin, Q., Kirschberg, T., Kreider, E., McGrane, P. L., Wender, P. A., and Khavari, P. A. (2000). Conjugation of arginine oligomers to cyclosporin A facilitates topical delivery and inhibition of inflammation. Nat Med *6*, 1253–1257.

Rothberg, K. G., Heuser, J. E., Donzell, W. C., Ying, Y. S., Glenney, J. R., and Anderson, R. G. (1992). Caveolin, a protein component of caveolae membrane coats. Cell *68*, 673–682.

Ryser, H. J., and Hancock, R. (1965). Histones and basic polyamino acids stimulate the uptake of albumin by tumor cells in culture. Science *150*, 501–503.

Sabharanjak, S., Sharma, P., Parton, R. G., and Mayor, S. (2002). GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway. Dev Cell *2*, 411–423.

Sahlin, S., Hed, J., and Rundquist, I. (1983). Differentiation between attached and ingested immune complexes by a fluorescence quenching cytofluorometric assay. J Immunol Methods *60*, 115–124.

Sallusto, F., Cella, M., Danieli, C., and Lanzavecchia, A. (1995). Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. J Exp Med *182*, 389–400.

Sandgren, S., Wittrup, A., Cheng, F., Jonsson, M., Eklund, E., Busch, S., and Belting, M. (2004). The human antimicrobial peptide LL-37 transfers extracellular DNA plasmid to the nuclear compartment of mammalian cells via lipid rafts and proteoglycan-dependent endocytosis. J Biol Chem *279*, 17951–17956.

Sandvig, K., and Olsnes, S. (1980). Diphtheria toxin entry into cells is facilitated by low pH. J Cell Biol *87*, 828–832.

Saraste, J., Palade, G. E., and Farquhar, M. G. (1986). Temperature-sensitive steps in the transport of secretory proteins through the Golgi complex in exocrine pancreatic cells. Proc Natl Acad Sci U S A *83*, 6425–6429.

Schorey, J. S., and Bhatnagar, S. (2008). Exosome function: from tumor immunology to pathogen biology. Traffic *9*, 871–881.

Schulze, H., Kolter, T., and Sandhoff, K. (2009). Principles of lysosomal membrane degradation Cellular topology and biochemistry of lysosomal lipid degradation. Biochim Biophys Acta *1793*, 674–683.

Schwarze, S. R., Ho, A., Vocero-Akbani, A., and Dowdy, S. F. (1999). In vivo protein transduction: delivery of a biologically active protein into the mouse. Science *285*, 1569–1572.

Sigismund, S., Woelk, T., Puri, C., Maspero, E., Tacchetti, C., Transidico, P., Di Fiore, P. P., and Polo, S. (2005). Clathrin-independent endocytosis of ubiquitinated cargos. Proc Nat Acad Sci USA *102*, 2760–2765.

Silverstein, S. C., Steinman, R. M., and Cohn, Z. A. (1977). Endocytosis. Annu Rev Biochem *46*, 669–722.

Simons, K., and Ikonen, E. (1997). Functional rafts in cell membranes. Nature *387*, 569–572.

Simonsen, A., Lippe, R., Christoforidis, S., Gaullier, J. M., Brech, A., Callaghan, J., Toh, B. H., Murphy, C., Zerial, M., and Stenmark, H. (1998). EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. Nature *394*, 494–498.

Solis, G. P., Hoegg, M., Munderloh, C., Schrock, Y., Malaga-Trillo, E., Rivera-Milla, E., and Stuermer, C. A. (2007). Reggie/flotillin proteins are organized into stable tetramers in membrane microdomains. Biochem J *403*, 313–322.

Soomets, U., Lindgren, M., Gallet, X., Hällbrink, M., Elmquist, A., Balaspiri, L., Zorko, M., Pooga, M., Brasseur, R., and Langel, Ü. (2000). Deletion analogues of transportan. Biochim Biophys Acta *1467*, 165–176.

Steiner, V., Schar, M., Bornsen, K. O., and Mutter, M. (1991). Retention behaviour of a template-assembled synthetic protein and its amphiphilic building blocks on reversed-phase columns. J Chromatogr *586*, 43–50.

Steinman, R. M., Silver, J. M., and Cohn, Z. A. (1974). Pinocytosis in fibroblasts. Quantitative studies in vitro. J Cell Biol *63*, 949–969.

Stuermer, C. A., Lang, D. M., Kirsch, F., Wiechers, M., Deininger, S. O., and Plattner, H. (2001). Glycosylphosphatidyl inositol-anchored proteins and fyn kinase assemble in noncaveolar plasma membrane microdomains defined by reggie-1 and -2. Mol Biol Cell *12*, 3031–3045.

Sugiyama, S., Di Nardo, A. A., Aizawa, S., Matsuo, I., Volovitch, M., Prochiantz, A., and Hensch, T. K. (2008). Experience-dependent transfer of Otx2 homeoprotein into the visual cortex activates postnatal plasticity. Cell *134*, 508–520.

Swanson, J. A., and Watts, C. (1995). Macropinocytosis. Trends Cell Biol *5*, 424–428.

Zaro, J. L., and Shen, W. C. (2005). Evidence that membrane transduction of oligoarginine does not require vesicle formation. Experimental Cell Research *307*, 164–173.

Ziegler, A. (2008). Thermodynamic studies and binding mechanisms of cell-penetrating peptides with lipids and glycosaminoglycans. Adv Drug Deliv Rev *60*, 580–597.

Ziegler, A., Nervi, P., Dürrenberger, M., and Seelig, J. (2005). The cationic cell-penetrating peptide CPP(TAT) derived from the HIV-1 protein TAT is rapidly transported into living fibroblasts: optical, biophysical, and metabolic evidence. Biochemistry *44*, 138–148.

Zoncu, R., Perera, R. M., Balkin, D. M., Pirruccello, M., Toomre, D., and De Camilli, P. (2009). A phosphoinositide switch controls the maturation and signaling properties of APPL endosomes. Cell *136*, 1110–1121.

Zorko, M., Pooga, M., Saar, K., Rezaei, K., and Langel, Ü. (1998). Differential regulation of GTPase activity by mastoparan and galparan. Arch Biochem Biophys *349*, 321–328.

Théry, C., Zitvogel, L., and Amigorena, S. (2002). Exosomes: composition, biogenesis and function. Nat Rev Immunol *2*, 569–579.

Thomsen, P., Roepstorff, K., Stahlhut, M., and van Deurs, B. (2002). Caveolae are highly immobile plasma membrane microdomains, which are not involved in constitutive endocytic trafficking. Mol Biol Cell *13*, 238–250.

Thorén, P. E., Persson, D., Isakson, P., Goksör, M., Onfelt, A., and Nordén, B. (2003). Uptake of analogs of penetratin, Tat(48–60) and oligoarginine in live cells. Biochem Biophys Res Commun *307*, 100–107.

Torgersen, M. L., Skretting, G., van Deurs, B., and Sandvig, K. (2001). Internalization of cholera toxin by different endocytic mechanisms. J Cell Sci *114*, 3737–3747.

Toro, A., and Grunebaum, E. (2006). TAT-mediated intracellular delivery of purine nucleoside phosphorylase corrects its deficiency in mice. J Clin Invest *116*, 2717–2726.

Tünnemann, G., Martin, R. M., Haupt, S., Patsch, C., Edenhofer, F., and Cardoso, M. C. (2006). Cargo-dependent mode of uptake and bioavailability of TAT-containing proteins and peptides in living cells. Faseb J *20*, 1775–1784.

Wadia, J. S., Stan, R. V., and Dowdy, S. F. (2004). Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. Nat Med *10*, 310–315.

Vassilieva, E. V., Ivanov, A. I., and Nusrat, A. (2009). Flotillin-1 stabilizes caveolin-1 in intestinal epithelial cells. Biochem Biophys Res Commun *379*, 460–465.

Welch, J. E., Bengtson, P., Svensson, K., Wittrup, A., Jenniskens, G. J., Ten Dam, G. B., Van Kuppevelt, T. H., and Belting, M. (2008). Single chain fragment anti-heparan sulfate antibody targets the polyamine transport system and attenuates polyamine-dependent cell proliferation. Int J Oncol *32*, 749–756.

Wesche, J., Elliott, J. L., Falnes, P. O., Olsnes, S., and Collier, R. J. (1998). Characterization of membrane translocation by anthrax protective antigen. Biochemistry *37*, 15737–15746.

Wesche, J., Malecki, J., Wiedlocha, A., Skjerpen, C. S., Claus, P., and Olsnes, S. (2006). FGF-1 and FGF-2 require the cytosolic chaperone Hsp90 for translocation into the cytosol and the cell nucleus. J Biol Chem *281*, 11405–11412.

Wittrup, A., Sandgren, S., Lilja, J., Bratt, C., Gustavsson, N., Morgelin, M., and Belting, M. (2007). Identification of proteins released by mammalian cells that mediate DNA internalization through proteoglycan-dependent macropinocytosis. J Biol Chem *282*, 27897–27904.

Vivès, E., Brodin, P., and Lebleu, B. (1997). A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. J Biol Chem *272*, 16010–16017.

Yao, Y., Hong, S., Zhou, H., Yuan, T., Zeng, R., and Liao, K. (2009). The differential protein and lipid compositions of noncaveolar lipid microdomains and caveolae. Cell Res *19*, 497–506.

Yin, H., Moulton, H. M., Seow, Y., Boyd, C., Boutilier, J., Iverson, P., and Wood, M. J. (2008). Cell-penetrating peptide-conjugated antisense oligonucleotides restore systemic muscle and cardiac dystrophin expression and function. Hum Mol Genet *17*, 3909–3918.

ACKNOWLEDGEMENTS

This work has been carried out at the Institute of Molecular and Cell Biology, University of Tartu, and at the Estonian Biocentre. Some experiments were also performed at the Department of Neurochemistry and Neurotoxicology, at the Stockholm University.

Several people have guided me during my PhD studies to whom I owe a big „thank you“ because without them I hadn't reached writing these words.

Without the bold decision of my supervisor Prof. Margus Pooga to take me in his research group I wouldn't had the opportunity to become acquainted with the enigmatic world of cell-penetrating peptides. It has been an honour and privilege to be his student and I hope that I have been able to aqcuire at least a piece of his thoroughness, devotion to science and skills of scientific thinking during this time.

Great thanks to Prof. Toivo Maimets for allowing me to perform my studies at the department of cell biology, and to my former supervisor Dr. Rein Sikut for introducing me to cell biology during my first lab year. The secretary of the institute Tiiu Rootslane, the master of solving all the administrative problems, many thanks!

My lab-mates Kärt, Külliki, Helin, Annely, Kaida and Aira, thank you for all the talks in all thinkable topics in and outside lab – I have been very lucky to know you and to work with you. Especially I am grateful to ever-optimistic Kärt, for being such a great friend and for making the high-quality ultra-structural artwork for our publications. Helin, for your endless kindness and help in my broken english. Annely, for your refreshing sarcasm and help in counting numbers for statistics.

All the colleagues around me on two floors of the lab – Signe, Daima, Tõnis, Arnold, Kersti, Lili, Ade, Viljar, Janeli, Jaak, Riho, Tambet, Merly, Marko, Kuldar, Keiu, Martti, Sulev I., Sulev S. and Anneli Kukk, for your good company, help and advice.

The topic of CPPs has brought me together with great scientists whose second home is or has been the department of Neurochemistry at the Stockholm University. Many thanks to Prof. Ülo Langel for allowing me to spend two months during my master studies in his lab at Arrheniusväg, and for being very supportive co-author through all the years. Mats, thanks for all the peptides and for introducing me to the great people from Tõravere gadgetville. Külliki, my neuro-mummy in Stockholm and colleague at present, your perfectionism in the lab and in the kitchen have made my world remarkably wider (and repeatedly tastier). And this list would be very incomplete without the chemical personalities Kalle and Ursel.

I also want to thank Prof. Michael Bienert and Dr. Johannes Oehlke for enabling me to perform some experiments in the Leibnitz Institute of Molecular Pharmacology in Berlin.

My friends in and outside the lab. Signe, somehow (well, mostly due to your wild ideas) we have managed to do so many funny and odd things together

during our studies. Thank you for that! Triin K., Hanna-Liisa, Liina K., Karmen I. and Anri, thanks for all the discussions about science and life! My good companions in the choir are too numerous to list here, but their routine-free opinions about the molecular biology have helped me to keep in touch with the world outside lab bench.

Suur tänu mu armsale perekonnale, kes on mind tingimusteta toetanud kogu õpingute aja.

PUBLICATIONS

CURRICULUM VITAE

Pille Säälik

Date of birth: 07.05.1979
Nationality: Estonian
Contact: University of Tartu, Institute of Molecular and Cell Biology, 23 Riia Street, 51010, Tartu, Estonia.
E-mail: pillesk@ut.ee

Education and professional employment

1994–1997	Palamuse secondary school
1997–2002	University of Tartu, BSc (molecular biology and genetics),
2002–2004	University of Tartu, MSc (cell biology)
2004 – present	University of Tartu, PhD student in the Institute of Molecular and Cell Biology
2005 – present	University of Tartu, Institute of Molecular and Cell Biology, extraordinary scientist at Department of Developmental Biology

Special courses

2003	Performing experiments in Stockholm University, in the department of Neurochemistry and Neurotoxicology
2005	FACS Users Meeting, Stockholm
2005	FEBS lecture course "Cellular and molecular biology of biological membranes", Corsica, France
2006	Performing experiments in the Leipzig Institute of Molecular Pharmacology Institute, Berlin, Germany
2008	FEBS workshop "Lipids as regulators of cell function", Spetses, Greece

Professional organizations

Member of Estonian Biochemical Society

Scientific work

Since 2001 my main scientific interests have focused on the internalization mechanisms of transportan and other cell-penetrating peptides, and their application for protein cellular delivery.

List of publications

Säälik, P., Elmquist, A., Hansen, M., Padari, K., Saar, K., Viht, K., Langel, Ü. and Pooga, M. (2004) Protein cargo delivery properties of cell-penetrating peptides, a comparative study. Bioconj Chem. Nov-Dec; 15(6):1246–53

Pooga, M., Padari, K., Säälik, P., Koppel, K., Hansen, M., Raid, R., Langel, Ü. (2005). Mechanisms of protein cellular delivery with transportans. FEBS J, 272, 525

Padari, K., Säälik, P., Hansen, M., Koppel, K., Raid, R., Langel, Ü. and Pooga, M. (2005) Cell transduction pathways of transportans. Bioconj Chem. 2005 Nov-Dec; 16(6):1399–410.

Säälik, P.; Hansen, M.; Langel, Ü.; Pooga, M. (2006). Actin-dependent CPP-mediated protein cellular delivery takes place via induced endocytosis. J Pept Sci, 12(Suppl), 236

Säälik, P., Padari, K., Hansen, M., Lorents, A., Niinep, A., Langel, Ü. and Pooga, M. (2009) "Protein delivery with transportans is mediated by caveolae rather than flotillin-mediated pathways." Bioconj Chem. Apr 20(5): 877–887

Social activities:

Council and board member of the students' science journal Akadeemiake
Member of Tartu University Female Choir

CURRICULUM VITAE

Pille Säälik

Sünniaeg:	07.05.1979
Kodakondsus:	Eesti
Kontaktandmed:	Molekulaar- ja Rakubioloogia Instituut, Tartu Ülikool, Riia 23, 51010, Tartu, Eesti
E-post:	pillesk@ut.ee

Haridus ja erialane teenistuskäik

1994–1997	Palamuse O. Lutsu nimeline Keskkool
1997–2002	Tartu Ülikool, Molekulaar- ja Rakubioloogia Instituut, BSc molekulaarbioloogia ja geneetika erialal
2002–2004	Tartu Ülikool, Molekulaar- ja Rakubioloogia Instituut, MSc rakubioloogia erialal
2004–	Tartu Ülikool, Molekulaar- ja Rakubioloogia Instituut, doktoriõpingud rakubioloogia erialal
2005–	Tartu Ülikool, Molekulaar- ja Rakubioloogia Instituut, Arengubioloogia õppetooli erakorraline teadur

Erialane enesetäiendus

2003	Eksperimentide teostamine Stockholmi ülikooli Neurokeemia ja Neurotoksikoloogia Instituudis
2005	Läbivoolutsütomeetri kasutajate konverents "BD FACS Users Meeting"
2005	FEBS'i loengukursus "Cellular and molecular biology of biological membranes", Korsika, Prantsusmaa
2006	Eksperimentide teostamine Saksamaal, Berliinis, Molekulaarse Farmakoloogia Instituudis
2008	FEBS'i *workshop* "Lipids as regulators of cell function", Spetses, Kreeka

Teadusorganisatsioonid

Eesti Biokeemia Seltsi liige

Teadustöö

Alates 2001. aastast on uurimistöö põhisuunaks olnud transportaani ja teiste rakku sisenevate peptiidide internalisatsioonimehhanismid ning nende poolt vahendatud valgu transport rakkudesse.

Teaduspublikatsioonid

Säälik, P., Elmquist, A., Hansen, M., Padari, K., Saar, K., Viht, K., Langel, Ü. and Pooga, M. (2004) Protein cargo delivery properties of cell-penetrating peptides, a comparative study. Bioconj Chem. Nov-Dec; 15(6):1246–53

Pooga, M., Padari, K., Säälik, P., Koppel, K., Hansen, M., Raid, R., Langel, Ü. (2005). Mechanisms of protein cellular delivery with transportans. FEBS J. 272, 525

Padari, K., Säälik, P., Hansen, M., Koppel, K., Raid, R., Langel, Ü. and Pooga, M. (2005) Cell transduction pathways of transportans. Bioconj Chem. 2005 Nov-Dec; 16(6):1399–410.

Säälik, P.; Hansen, M.; Langel, Ü.; Pooga, M. (2006). Actin-dependent CPP-mediated protein cellular delivery takes place via induced endocytosis. J Pept Sci. 12(Suppl), 236

Säälik, P., Padari, K., Hansen, M., Lorents, A., Niinep, A., Langel, Ü. and Pooga, M. (2009) "Protein delivery with transportans is mediated by caveolae rather than flotillin-mediated pathways." Bioconj Chem. Apr 20(5): 877–887

Ühiskondlik tegevus

Õpilaste teadusajakirja Akadeemiake nõukogu ja kolleegiumi liige
TÜ Akadeemilise Naiskoori liige

DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS

1. **Toivo Maimets**. Studies of human oncoprotein p53. Tartu, 1991, 96 p.
2. **Enn K. Seppet**. Thyroid state control over energy metabolism, ion transport and contractile functions in rat heart. Tartu, 1991, 135 p.
3. **Kristjan Zobel**. Epifüütsete makrosamblike väärtus õhu saastuse indikaatoritena Hamar-Dobani boreaalsetes mägimetsades. Tartu, 1992, 131 lk.
4. **Andres Mäe**. Conjugal mobilization of catabolic plasmids by transposable elements in helper plasmids. Tartu, 1992, 91 p.
5. **Maia Kivisaar**. Studies on phenol degradation genes of *Pseudomonas* sp. strain EST 1001. Tartu, 1992, 61 p.
6. **Allan Nurk**. Nucleotide sequences of phenol degradative genes from *Pseudomonas sp.* strain EST 1001 and their transcriptional activation in *Pseudomonas putida.* Tartu, 1992, 72 p.
7. **Ülo Tamm**. The genus *Populus* L. in Estonia: variation of the species biology and introduction. Tartu, 1993, 91 p.
8. **Jaanus Remme**. Studies on the peptidyltransferase centre of the *E.coli* ribosome. Tartu, 1993, 68 p.
9. **Ülo Langel**. Galanin and galanin antagonists. Tartu, 1993, 97 p.
10. **Arvo Käärd**. The development of an automatic online dynamic fluorescense-based pH-dependent fiber optic penicillin flowthrought biosensor for the control of the benzylpenicillin hydrolysis. Tartu, 1993, 117 p.
11. **Lilian Järvekülg**. Antigenic analysis and development of sensitive immunoassay for potato viruses. Tartu, 1993, 147 p.
12. **Jaak Palumets**. Analysis of phytomass partition in Norway spruce. Tartu, 1993, 47 p.
13. **Arne Sellin**. Variation in hydraulic architecture of *Picea abies* (L.) Karst. trees grown under different enviromental conditions. Tartu, 1994, 119 p.
13. **Mati Reeben**. Regulation of light neurofilament gene expression. Tartu, 1994, 108 p.
14. **Urmas Tartes**. Respiration rhytms in insects. Tartu, 1995, 109 p.
15. **Ülo Puurand**. The complete nucleotide sequence and infections *in vitro* transcripts from cloned cDNA of a potato A potyvirus. Tartu, 1995, 96 p.
16. **Peeter Hõrak**. Pathways of selection in avian reproduction: a functional framework and its application in the population study of the great tit (*Parus major*). Tartu, 1995, 118 p.
17. **Erkki Truve**. Studies on specific and broad spectrum virus resistance in transgenic plants. Tartu, 1996, 158 p.
18. **Illar Pata**. Cloning and characterization of human and mouse ribosomal protein S6-encoding genes. Tartu, 1996, 60 p.
19. **Ülo Niinemets**. Importance of structural features of leaves and canopy in determining species shade-tolerance in temperature deciduous woody taxa. Tartu, 1996, 150 p.

20. **Ants Kurg**. Bovine leukemia virus: molecular studies on the packaging region and DNA diagnostics in cattle. Tartu, 1996, 104 p.
21. **Ene Ustav**. E2 as the modulator of the BPV1 DNA replication. Tartu, 1996, 100 p.
22. **Aksel Soosaar**. Role of helix-loop-helix and nuclear hormone receptor transcription factors in neurogenesis. Tartu, 1996, 109 p.
23. **Maido Remm**. Human papillomavirus type 18: replication, transformation and gene expression. Tartu, 1997, 117 p.
24. **Tiiu Kull**. Population dynamics in *Cypripedium calceolus* L. Tartu, 1997, 124 p.
25. **Kalle Olli**. Evolutionary life-strategies of autotrophic planktonic microorganisms in the Baltic Sea. Tartu, 1997, 180 p.
26. **Meelis Pärtel**. Species diversity and community dynamics in calcareous grassland communities in Western Estonia. Tartu, 1997, 124 p.
27. **Malle Leht**. The Genus *Potentilla* L. in Estonia, Latvia and Lithuania: distribution, morphology and taxonomy. Tartu, 1997, 186 p.
28. **Tanel Tenson**. Ribosomes, peptides and antibiotic resistance. Tartu, 1997, 80 p.
29. **Arvo Tuvikene**. Assessment of inland water pollution using biomarker responses in fish *in vivo* and *in vitro*. Tartu, 1997, 160 p.
30. **Urmas Saarma**. Tuning ribosomal elongation cycle by mutagenesis of 23S rRNA. Tartu, 1997, 134 p.
31. **Henn Ojaveer**. Composition and dynamics of fish stocks in the gulf of Riga ecosystem. Tartu, 1997, 138 p.
32. **Lembi Lõugas**. Post-glacial development of vertebrate fauna in Estonian water bodies. Tartu, 1997, 138 p.
33. **Margus Pooga**. Cell penetrating peptide, transportan, and its predecessors, galanin-based chimeric peptides. Tartu, 1998, 110 p.
34. **Andres Saag**. Evolutionary relationships in some cetrarioid genera (Lichenized Ascomycota). Tartu, 1998, 196 p.
35. **Aivar Liiv**. Ribosomal large subunit assembly *in vivo*. Tartu, 1998, 158 p.
36. **Tatjana Oja**. Isoenzyme diversity and phylogenetic affinities among the eurasian annual bromes (*Bromus* L., Poaceae). Tartu, 1998, 92 p.
37. **Mari Moora**. The influence of arbuscular mycorrhizal (AM) symbiosis on the competition and coexistence of calcareous crassland plant species. Tartu, 1998, 78 p.
38. **Olavi Kurina**. Fungus gnats in Estonia (*Diptera: Bolitophilidae, Keroplatidae, Macroceridae, Ditomyiidae, Diadocidiidae, Mycetophilidae*). Tartu, 1998, 200 p.
39. **Andrus Tasa**. Biological leaching of shales: black shale and oil shale. Tartu, 1998, 98 p.
40. **Arnold Kristjuhan.** Studies on transcriptional activator properties of tumor suppressor protein p53. Tartu, 1998, 86 p.

41. **Sulev Ingerpuu.** Characterization of some human myeloid cell surface and nuclear differentiation antigens. Tartu, 1998, 163 p.
42. **Veljo Kisand.** Responses of planktonic bacteria to the abiotic and biotic factors in the shallow lake Võrtsjärv. Tartu, 1998, 118 p.
43. **Kadri Põldmaa.** Studies in the systematics of hypomyces and allied genera (Hypocreales, Ascomycota). Tartu, 1998, 178 p.
44. **Markus Vetemaa.** Reproduction parameters of fish as indicators in environmental monitoring. Tartu, 1998, 117 p.
45. **Heli Talvik.** Prepatent periods and species composition of different *Oesophagostomum* spp. populations in Estonia and Denmark. Tartu, 1998, 104 p.
46. **Katrin Heinsoo.** Cuticular and stomatal antechamber conductance to water vapour diffusion in *Picea abies* (L.) karst. Tartu, 1999, 133 p.
47. **Tarmo Annilo.** Studies on mammalian ribosomal protein S7. Tartu, 1998, 77 p.
48. **Indrek Ots.** Health state indicies of reproducing great tits (*Parus major*): sources of variation and connections with life-history traits. Tartu, 1999, 117 p.
49. **Juan Jose Cantero.** Plant community diversity and habitat relationships in central Argentina grasslands. Tartu, 1999, 161 p.
50. **Rein Kalamees.** Seed bank, seed rain and community regeneration in Estonian calcareous grasslands. Tartu, 1999, 107 p.
51. **Sulev Kõks.** Cholecystokinin (CCK) — induced anxiety in rats: influence of environmental stimuli and involvement of endopioid mechanisms and erotonin. Tartu, 1999, 123 p.
52. **Ebe Sild.** Impact of increasing concentrations of O_3 and CO_2 on wheat, clover and pasture. Tartu, 1999, 123 p.
53. **Ljudmilla Timofejeva.** Electron microscopical analysis of the synaptonemal complex formation in cereals. Tartu, 1999, 99 p.
54. **Andres Valkna.** Interactions of galanin receptor with ligands and G-proteins: studies with synthetic peptides. Tartu, 1999, 103 p.
55. **Taavi Virro.** Life cycles of planktonic rotifers in lake Peipsi. Tartu, 1999, 101 p.
56. **Ana Rebane.** Mammalian ribosomal protein S3a genes and intron-encoded small nucleolar RNAs U73 and U82. Tartu, 1999, 85 p.
57. **Tiina Tamm.** Cocksfoot mottle virus: the genome organisation and translational strategies. Tartu, 2000, 101 p.
58. **Reet Kurg.** Structure-function relationship of the bovine papilloma virus E2 protein. Tartu, 2000, 89 p.
59. **Toomas Kivisild.** The origins of Southern and Western Eurasian populations: an mtDNA study. Tartu, 2000, 121 p.
60. **Niilo Kaldalu.** Studies of the TOL plasmid transcription factor XylS. Tartu 2000. 88 p.

61. **Dina Lepik.** Modulation of viral DNA replication by tumor suppressor protein p53. Tartu 2000. 106 p.
62. **Kai Vellak.** Influence of different factors on the diversity of the bryophyte vegetation in forest and wooded meadow communities. Tartu 2000. 122 p.
63. **Jonne Kotta.** Impact of eutrophication and biological invasionas on the structure and functions of benthic macrofauna. Tartu 2000. 160 p.
64. **Georg Martin.** Phytobenthic communities of the Gulf of Riga and the inner sea the West-Estonian archipelago. Tartu, 2000. 139 p.
65. **Silvia Sepp.** Morphological and genetical variation of *Alchemilla L.* in Estonia. Tartu, 2000. 124 p.
66. **Jaan Liira.** On the determinants of structure and diversity in herbaceous plant communities. Tartu, 2000. 96 p.
67. **Priit Zingel.** The role of planktonic ciliates in lake ecosystems. Tartu 2001. 111 p.
68. **Tiit Teder.** Direct and indirect effects in Host-parasitoid interactions: ecological and evolutionary consequences. Tartu 2001. 122 p.
69. **Hannes Kollist.** Leaf apoplastic ascorbate as ozone scavenger and its transport across the plasma membrane. Tartu 2001. 80 p.
70. **Reet Marits.** Role of two-component regulator system PehR-PehS and extracellular protease PrtW in virulence of *Erwinia Carotovora* subsp. *Carotovora*. Tartu 2001. 112 p.
71. **Vallo Tilgar.** Effect of calcium supplementation on reproductive performance of the pied flycatcher *Ficedula hypoleuca* and the great tit *Parus major,* breeding in Nothern temperate forests. Tartu, 2002. 126 p.
72. **Rita Hõrak.** Regulation of transposition of transposon Tn*4652* in *Pseudomonas putida*. Tartu, 2002. 108 p.
73. **Liina Eek-Piirsoo.** The effect of fertilization, mowing and additional illumination on the structure of a species-rich grassland community. Tartu, 2002. 74 p.
74. **Krõõt Aasamaa.** Shoot hydraulic conductance and stomatal conductance of six temperate deciduous tree species. Tartu, 2002. 110 p.
75. **Nele Ingerpuu.** Bryophyte diversity and vascular plants. Tartu, 2002. 112 p.
76. **Neeme Tõnisson.** Mutation detection by primer extension on oligonucleotide microarrays. Tartu, 2002. 124 p.
77. **Margus Pensa.** Variation in needle retention of Scots pine in relation to leaf morphology, nitrogen conservation and tree age. Tartu, 2003. 110 p.
78. **Asko Lõhmus.** Habitat preferences and quality for birds of prey: from principles to applications. Tartu, 2003. 168 p.
79. **Viljar Jaks.** p53 — a switch in cellular circuit. Tartu, 2003. 160 p.
80. **Jaana Männik.** Characterization and genetic studies of four ATP-binding cassette (ABC) transporters. Tartu, 2003. 140 p.
81. **Marek Sammul.** Competition and coexistence of clonal plants in relation to productivity. Tartu, 2003. 159 p

82. **Ivar Ilves.** Virus-cell interactions in the replication cycle of bovine papillomavirus type 1. Tartu, 2003. 89 p.
83. **Andres Männik.** Design and characterization of a novel vector system based on the stable replicator of bovine papillomavirus type 1. Tartu, 2003. 109 p.
84. **Ivika Ostonen.** Fine root structure, dynamics and proportion in net primary production of Norway spruce forest ecosystem in relation to site conditions. Tartu, 2003. 158 p.
85. **Gudrun Veldre.** Somatic status of 12–15-year-old Tartu schoolchildren. Tartu, 2003. 199 p.
86. **Ülo Väli.** The greater spotted eagle *Aquila clanga* and the lesser spotted eagle *A. pomarina*: taxonomy, phylogeography and ecology. Tartu, 2004. 159 p.
87. **Aare Abroi.** The determinants for the native activities of the bovine papillomavirus type 1 E2 protein are separable. Tartu, 2004. 135 p.
88. **Tiina Kahre.** Cystic fibrosis in Estonia. Tartu, 2004. 116 p.
89. **Helen Orav-Kotta.** Habitat choice and feeding activity of benthic suspension feeders and mesograzers in the northern Baltic Sea. Tartu, 2004. 117 p.
90. **Maarja Öpik.** Diversity of arbuscular mycorrhizal fungi in the roots of perennial plants and their effect on plant performance. Tartu, 2004. 175 p.
91. **Kadri Tali.** Species structure of *Neotinea ustulata*. Tartu, 2004. 109 p.
92. **Kristiina Tambets.** Towards the understanding of post-glacial spread of human mitochondrial DNA haplogroups in Europe and beyond: a phylogeographic approach. Tartu, 2004. 163 p.
93. **Arvi Jõers.** Regulation of p53-dependent transcription. Tartu, 2004. 103 p.
94. **Lilian Kadaja.** Studies on modulation of the activity of tumor suppressor protein p53. Tartu, 2004. 103 p.
95. **Jaak Truu.** Oil shale industry wastewater: impact on river microbial community and possibilities for bioremediation. Tartu, 2004. 128 p.
96. **Maire Peters.** Natural horizontal transfer of the *pheBA* operon. Tartu, 2004. 105 p.
97. **Ülo Maiväli.** Studies on the structure-function relationship of the bacterial ribosome. Tartu, 2004. 130 p.
98. **Merit Otsus.** Plant community regeneration and species diversity in dry calcareous grasslands. Tartu, 2004. 103 p.
99. **Mikk Heidemaa.** Systematic studies on sawflies of the genera *Dolerus, Empria,* and *Caliroa* (Hymenoptera: Tenthredinidae). Tartu, 2004. 167 p.
100. **Ilmar Tõnno.** The impact of nitrogen and phosphorus concentration and N/P ratio on cyanobacterial dominance and N_2 fixation in some Estonian lakes. Tartu, 2004. 111 p.
101. **Lauri Saks.** Immune function, parasites, and carotenoid-based ornaments in greenfinches. Tartu, 2004. 144 p.

102. **Siiri Rootsi.** Human Y-chromosomal variation in European populations. Tartu, 2004. 142 p.
103. **Eve Vedler.** Structure of the 2,4-dichloro-phenoxyacetic acid-degradative plasmid pEST4011. Tartu, 2005. 106 p.
104. **Andres Tover.** Regulation of transcription of the phenol degradation *pheBA* operon in *Pseudomonas putida*. Tartu, 2005. 126 p.
105. **Helen Udras.** Hexose kinases and glucose transport in the yeast *Hansenula polymorpha*. Tartu, 2005. 100 p.
106. **Ave Suija.** Lichens and lichenicolous fungi in Estonia: diversity, distribution patterns, taxonomy. Tartu, 2005. 162 p.
107. **Piret Lõhmus.** Forest lichens and their substrata in Estonia. Tartu, 2005. 162 p.
108. **Inga Lips.** Abiotic factors controlling the cyanobacterial bloom occurrence in the Gulf of Finland. Tartu, 2005. 156 p.
109. **Kaasik, Krista.** Circadian clock genes in mammalian clockwork, metabolism and behaviour. Tartu, 2005. 121 p.
110. **Juhan Javoiš.** The effects of experience on host acceptance in ovipositing moths. Tartu, 2005. 112 p.
111. **Tiina Sedman.** Characterization of the yeast *Saccharomyces cerevisiae* mitochondrial DNA helicase Hmi1. Tartu, 2005. 103 p.
112. **Ruth Aguraiuja.** Hawaiian endemic fern lineage *Diellia* (Aspleniaceae): distribution, population structure and ecology. Tartu, 2005. 112 p.
113. **Riho Teras.** Regulation of transcription from the fusion promoters generated by transposition of Tn*4652* into the upstream region of *pheBA* operon in *Pseudomonas putida*. Tartu, 2005. 106 p.
114. **Mait Metspalu.** Through the course of prehistory in india: tracing the mtDNA trail. Tartu, 2005. 138 p.
115. **Elin Lõhmussaar.** The comparative patterns of linkage disequilibrium in European populations and its implication for genetic association studies. Tartu, 2006. 124 p.
116. **Priit Kupper.** Hydraulic and environmental limitations to leaf water relations in trees with respect to canopy position. Tartu, 2006. 126 p.
117. **Heili Ilves.** Stress-induced transposition of Tn*4652* in *Pseudomonas Putida.* Tartu, 2006. 120 p.
118. **Silja Kuusk.** Biochemical properties of Hmi1p, a DNA helicase from *Saccharomyces cerevisiae* mitochondria. Tartu, 2006. 126 p.
119. **Kersti Püssa.** Forest edges on medium resolution landsat thematic mapper satellite images. Tartu, 2006. 90 p.
120. **Lea Tummeleht.** Physiological condition and immune function in great tits (*Parus major* l.): Sources of variation and trade-offs in relation to growth. Tartu, 2006. 94 p.
121. **Toomas Esperk.** Larval instar as a key element of insect growth schedules. Tartu, 2006. 186 p.

122. **Harri Valdmann.** Lynx (*Lynx lynx*) and wolf (*Canis lupus*) in the Baltic region: Diets, helminth parasites and genetic variation. Tartu, 2006. 102 p.
123. **Priit Jõers.** Studies of the mitochondrial helicase Hmi1p in *Candida albicans* and *Saccharomyces cerevisia*. Tartu, 2006. 113 p.
124. **Kersti Lilleväli.** Gata3 and Gata2 in inner ear development. Tartu, 2007. 123 p.
125. **Kai Rünk.** Comparative ecology of three fern species: *Dryopteris carthusiana* (Vill.) H.P. Fuchs, *D. expansa* (C. Presl) Fraser-Jenkins & Jermy and *D. dilatata* (Hoffm.) A. Gray (Dryopteridaceae). Tartu, 2007. 143 p.
126. **Aveliina Helm.** Formation and persistence of dry grassland diversity: role of human history and landscape structure. Tartu, 2007. 89 p.
127. **Leho Tedersoo.** Ectomycorrhizal fungi: diversity and community structure in Estonia, Seychelles and Australia. Tartu, 2007. 233 p.
128. **Marko Mägi.** The habitat-related variation of reproductive performance of great tits in a deciduous-coniferous forest mosaic: looking for causes and consequences. Tartu, 2007. 135 p.
129. **Valeria Lulla.** Replication strategies and applications of Semliki Forest virus. Tartu, 2007. 109 p.
130. **Ülle Reier**. Estonian threatened vascular plant species: causes of rarity and conservation. Tartu, 2007. 79 p.
131. **Inga Jüriado**. Diversity of lichen species in Estonia: influence of regional and local factors. Tartu, 2007. 171 p.
132. **Tatjana Krama.** Mobbing behaviour in birds: costs and reciprocity based cooperation. Tartu, 2007.
133. **Signe Saumaa.** The role of DNA mismatch repair and oxidative DNA damage defense systems in avoidance of stationary phase mutations in *Pseudomonas putida.* Tartu, 2007. 172 p.
134. **Reedik Mägi**. The linkage disequilibrium and the selection of genetic markers for association studies in european populations. Tartu, 2007. 96 p.
135. **Priit Kilgas.** Blood parameters as indicators of physiological condition and skeletal development in great tits (*Parus major*): natural variation and application in the reproductive ecology of birds. Tartu, 2007. 129 p.
136. **Anu Albert**. The role of water salinity in structuring eastern Baltic coastal fish communities. Tartu, 2007. 95 p.
137. **Kärt Padari.** Protein transduction mechanisms of transportans. Tartu, 2008. 128 p.
138. **Siiri-Lii Sandre.** Selective forces on larval colouration in a moth. Tartu, 2008. 125 p.
139. **Ülle Jõgar.** Conservation and restoration of semi-natural floodplain meadows and their rare plant species. Tartu, 2008. 99 p.
140. **Lauri Laanisto.** Macroecological approach in vegetation science: generality of ecological relationships at the global scale. Tartu, 2008. 133 p.
141. **Reidar Andreson**. Methods and software for predicting PCR failure rate in large genomes. Tartu, 2008. 105 p.

142. **Birgot Paavel.** Bio-optical properties of turbid lakes. Tartu, 2008. 175 p.
143. **Kaire Torn.** Distribution and ecology of charophytes in the Baltic Sea. Tartu, 2008, 98 p.
144. **Vladimir Vimberg.** Peptide mediated macrolide resistance. Tartu, 2008, 190 p.
145. **Daima Örd.** Studies on the stress-inducible pseudokinase TRB3, a novel inhibitor of transcription factor ATF4. Tartu, 2008, 108 p.
146. **Lauri Saag.** Taxonomic and ecologic problems in the genus *Lepraria* (*Stereocaulaceae*, lichenised *Ascomycota*). Tartu, 2008, 175 p.
147. **Ulvi Karu.** Antioxidant protection, carotenoids and coccidians in greenfinches – assessment of the costs of immune activation and mechanisms of parasite resistance in a passerine with carotenoid-based ornaments. Tartu, 2008, 124 p.
148. **Jaanus Remm.** Tree-cavities in forests: density, characteristics and occupancy by animals. Tartu, 2008, 128 p.
149. **Epp Moks.** Tapeworm parasites *Echinococcus multilocularis* and *E. granulosus* in Estonia: phylogenetic relationships and occurrence in wild carnivores and ungulates. Tartu, 2008, 82 p.
150. **Eve Eensalu.** Acclimation of stomatal structure and function in tree canopy: effect of light and CO_2 concentration. Tartu, 2008, 108 p.
151. **Janne Pullat**. Design, functionlization and application of an *in situ* synthesized oligonucleotide microarray. Tartu, 2008, 108 p.
152. **Marta Putrinš.** Responses of *Pseudomonas putida* to phenol-induced metabolic and stress signals. Tartu, 2008, 142 p.
153. **Marina Semtšenko.** Plant root behaviour: responses to neighbours and physical obstructions. Tartu, 2008, 106 p.
154. **Marge Starast.** Influence of cultivation techniques on productivity and fruit quality of some *Vaccinium* and *Rubus* taxa. Tartu, 2008, 154 p.
155. **Age Tats.** Sequence motifs influencing the efficiency of translation. Tartu, 2009, 104 p.
156. **Radi Tegova.** The role of specialized DNA polymerases in mutagenesis in *Pseudomonas putida.* Tartu, 2009, 124 p.
157. **Tsipe Aavik.** Plant species richness, composition and functional trait pattern in agricultural landscapes – the role of land use intensity and landscape structure. Tartu, 2008, 112 p.
158. **Kaja Kiiver.** Semliki forest virus based vectors and cell lines for studying the replication and interactions of alphaviruses and hepaciviruses. Tartu, 2009, 104 p.
159. **Meelis Kadaja.** Papillomavirus Replication Machinery Induces Genomic Instability in its Host Cell. Tartu, 2009, 126 p.
160. **Pille Hallast.** Human and chimpanzee Luteinizing hormone/Chorionic Gonadotropin beta (*LHB/CGB*) gene clusters: diversity and divergence of young duplicated genes. Tartu, 2009, 168 p.

161. **Ain Vellak.** Spatial and temporal aspects of plant species conservation. Tartu, 2009, 86 p.
162. **Triinu Remmel.** Body size evolution in insects with different colouration strategies: the role of predation risk. Tartu, 2009, 168 p.
163. **Jaana Salujõe.** Zooplankton as the indicator of ecological quality and fish predation in lake ecosystems. Tartu, 2009, 129 p.
164. **Ele Vahtmäe.** Mapping benthic habitat with remote sensing in optically complex coastal environments. Tartu, 2009, 109 p.
165. **Liisa Metsamaa.** Model-based assessment to improve the use of remote sensing in recognition and quantitative mapping of cyanobacteria. Tartu, 2009, 114 p.