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PROTEIN TRANSDUCTION MECHANISMS OF TRANSPORTANS

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

II. Säälik, P., **Padari, K.**, Lorents, A., Hansen, M., Niinep, A., Jokitalo, E., Langel, Ü., Pooga, M. (2008) The endosomal pathways involved in transportan- and TP10-induced protein cellular delivery. *Submitted. FASEB J.*

III. 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

IV. Uri, A., Raidaru, G., Subbi, J., **Padari, K.**, Pooga, M. (2002) Identification of the ability of highly charged nanomolar inhibitors of protein kinases to cross plasma membranes and carry a protein into cells. *Bioorg Med Chem Lett.*19; 12(16):2117–20.

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My personal contribution to the articles referred to in this thesis is as follows:

Ref. I — designed and performed the electron microscopy experiments, participated in data analyses and in the writing of the paper.

Ref. II — performed the electron microscopy experiments, analysed the experimental data and participated in the writing of the paper

Ref. III — participated in the cellular uptake experiments, FACS analysis and in the writing of the paper.

Ref. IV — participated in the cellular uptake experiments and the fluorescence microscopy data analysis.

ABBREVIATIONS

Akt/PKB	Akt or protein kinase B
ARC	adenosine-oligoarginine conjugate
CLIC	clathrin independent carriers
CLSM	confocal laser scanning microscope
CME	clathrin mediated endocytosis
CPP	cell-penetrating peptide
CTB	cholera toxin B subunit
CtBP1/BARS	C-terminal binding protein 1/ Brefeldin A-dependent ADP-ribosylation substrate
dsDNA	double stranded DNA
EB1	endosomolytic penetratin analogue
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EGFR	EGF receptor
erbB-2	erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)
FACS	fluorescence activated cell sorter
FITC	fluoresceine isothiocyanate
GalR1	galanin receptor type 1
GEEC	GPI-enriched endosomal compartment
GFP	green fluorescent protein
GM1	monosialotetrahexosylganglioside 1
GPI	glycosylphosphatidylinositol
HIV	human immunodeficiency virus
HSPG	heparan sulfate proteoglycans
IL-2	interleukin 2
LAMP2	lysosome-associated membrane protein 2
LDL	low-density lipoprotein
MAP	model amphipathic peptide
NF-κB	nuclear factor kappa B
NLS	nuclear localization sequence
Pak1	p21-activated protein kinase 1
pI	isoelectric point
PI3K	phosphoinositide kinase-3
PI3P	phosphatidylinositol-3-phosphate
PKA	protein kinase A
PNA	peptide nucleic acid
pTat	HIV Tat protein derived cell-penetrating peptide
PTD	protein transduction domain
pVec	vascular endothelial cadherin derived CPP
ROCK-II	Rho-associated kinase II

siRNA	small interfering RNA
SV40	simian virus 40
TAT	HIV transactivator of transcription
TEM	transmission electron microscopy
TGFβ	transforming growth factor beta
TGN	trans-Golgi network
TP	transportan
TP10	transportan 10
VP22	CPP derived from herpes simplex virus-1 tegument protein VP22

INTRODUCTION

Introduction of plasmids, oligonucleotides, peptides, proteins, and other bioactive molecules or therapeutic agents into cells is a popular technique in cell biology and biomedical research to unravel the mechanisms of cell functions. The main obstacle of using large and water-soluble substances/therapeutics is their insufficient passage across the plasma membrane. The macromolecules of vital importance are taken up by cells using highly regulated processes, like endocytosis. However, for a vast majority of molecules no cell-entry route exists. To overcome this limitation, several techniques have been developed to gain access into cells and the carrier-mediated import is one of the most often used methods.

Cell-penetrating peptides (CPPs) have been introduced as vectors with a remarkable ability to translocate through cellular membranes and to deliver a wide variety of molecules and substances to the cell interior. Hence, CPPs may represent universal transport vectors for a range of biological and non-biological cargoes offering several advantages over conventional techniques because of their high internalization efficiency and low cytotoxicity.

The translocation mechanism of CPPs is still under debate despite more than a decade of intense studies. Some consensus has been reached relatively recently, suggesting that CPPs deliver cargo molecules mostly by inducing endocytosis. The effect of the cargo molecules in the cells depends on their endosomal escape, whereas in some cases direct translocation across the membrane may also take place. However, it is not clear yet to what extent CPPs employ the particular endocytotic pathways and how the properties of the cargo influence the efficiency and mechanism of the cellular uptake.

The main purpose of this study was to elucidate the protein-delivery mechanisms of transportan and its shorter analogue TP10. Transportan is a chimeric CPP that is constructed by connecting the N-terminal part of the neuropeptide galanin (1–12 amino acid residues) via a lysine residue to the wasp venom toxin mastoparan. Both TP10 and transportan translocate efficiently from the culture medium into living cells and have successfully been used as delivery vectors for peptide nucleic acid, peptides, and proteins. The characterization of interaction of transportans with the plasma membrane, the internalization pathways and the intracellular destination of proteins delivered by these CPPs was the main goal of this study. In addition, we compared the protein delivery efficiency and the internalization mechanisms of transportans with other commonly used CPPs. Furthermore, we characterized the internalization and the protein-delivery mechanism of the protein kinase inhibitors, which were designed by combining oligoarginine with an adenosine moiety.

The knowledge obtained from the current study might improve the development of cell-penetrating peptides as tools for efficient transduction of proteins and other macromolecules into cell interior in a non-invasive way.

1. LITERATURE OVERVIEW

1.1. Cellular transport processes

The plasma membrane of eukaryotic cells is composed of two layers of phospholipid molecules into which cholesterol and various proteins are inserted. In spite of the rapid lateral diffusion of these lipids and proteins in the dynamic membrane plane, the hydrophobic interior of the bilayer serves as a barrier and controls the movement of metabolites and nutrients between the cytoplasm and the extracellular environment. The cellular pathways for delivery of biomolecules over the plasma membrane to the cell interior can be divided into **passive** and **active** processes.

The **passive** transport does not require the metabolic energy or the aid of transport proteins, but depends on the permeability of the plasma membrane. Carbon dioxide, oxygen and small uncharged polar molecules, such as urea and ethanol, are among the few simple molecules that can cross the cell membrane by passive diffusion down to their chemical concentration gradient. For other small hydrophilic molecules and ions, there are channels and transporters that facilitate the diffusion across the plasma membrane.

The **active** transport processes, conversely, require cellular energy to move bioactive molecules and solutes across the membrane against their concentration gradient. Pumps, carriers and channels are only able to regulate the movement of relatively small molecules across the plasma membrane. Most of the large polar macromolecules, such as peptides and proteins, can enter cells via the energy-dependent and receptor-dependent or -independent process known as endocytosis.

1.2. Endocytosis

Endocytosis is a general term for processes whereby cells take up the extracellular substances by engulfing them with the plasma membrane and then budding the vesicles into the cytoplasm. Cells utilize different mechanisms for endocytosis which vary in the uptake mode, in the selectivity of internalized cargoes, and their intracellular fate. These mechanisms are commonly divided into two processes: **phagocytosis** and **pinocytosis** (Fig. 1).

Phagocytosis is an active and highly regulated actin-mediated process, that occurs only in a few cell types, like macrophages, neutrophils and dendritic cells, which specialize in ingestion of solid particles, such as pathogens, or infected and apoptotic cells (Conner and Schmid, 2003).

Pinocytosis, on the other hand, takes place in all cells and mediates the uptake of fluids, solutes and membrane components. At least four different

pinocytotic pathways can be distinguished. (1) The best characterized mechanism uses clathrin-coated pits for receptor-mediated endocytosis. (2) In recent years the caveolae-dependent pathway has also been characterized in more detailed manner (Nichols, 2003; Parton and Richards, 2003). The mechanisms of other types of pinocytotic pathways – (3) macropinocytosis and (4) nonclathrin/noncaveolae-dependent endocytosis – are still under intense studies (Conner and Schmid, 2003; Damm *et al.*, 2005; Kirkham and Parton, 2005; Payne *et al.*, 2007). These four mechanistically diverse and highly regulated endocytotic pathways carry out an essential cellular function of the uptake of nutrients and down-regulation of signalling receptors.

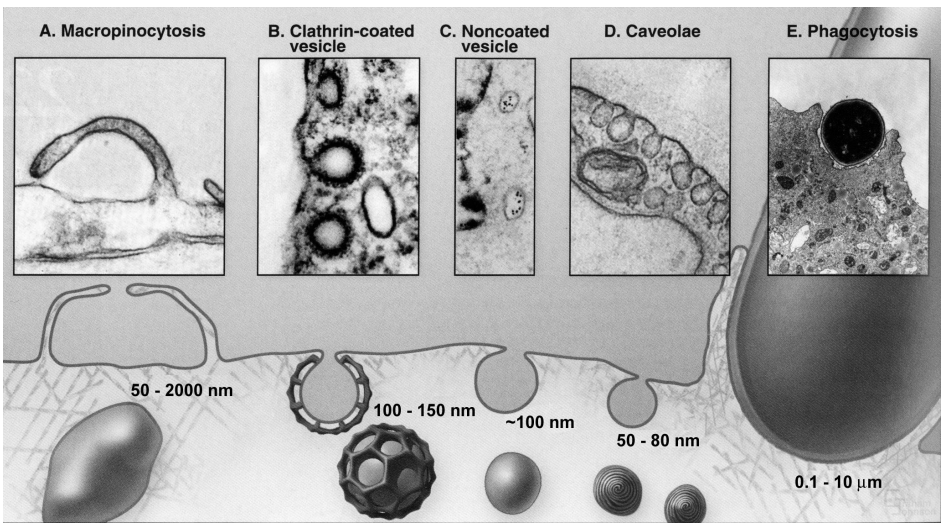

Figure 1. Electron micrographs and schematic illustrations that represent the different endocytotic processes used by mammalian cells (modified from Pollard and Earnshaw, 2008).

The intracellular endocytotic system sorts internalized ligands and receptors to different destinations. There are two principal post-internalization trafficking routes in the cell – recycling and lysosome-targeted pathway. The segregation of cargoes for recycling or degradation begins with their different sorting into distinct populations of early endosomes (Lakadamyali *et al.*, 2006; Pfeffer, 2003). Different endosomal compartments utilize specific sorting mechanisms to separate receptors, lipids or other molecules for either recycling, transporting to the trans-Golgi network (TGN), endoplasmic reticulum or targeting to lysosomes for degradation. However, intraluminal vesicles of multivesicular endosomes do not always end up in lysosomes but also contain proteins and lipids

that are not destined for degradation. They can fuse back with the limiting membrane of late endosomes, which sort specific proteins and lipids to the TGN and back to the plasma membrane before fusing directly with lysosomes (reviewed by van der Goot and Gruenberg, 2006). Within the acidic lumen of lysosomes, the hydrolytic enzymes degrade proteins, nucleic acids, and other large molecules into their monomeric form, which are then transported to the cytoplasm and reused to synthesise new macromolecules.

1.2.1. Clathrin-mediated endocytosis

By far the best researched mechanism for internalization of receptors and proteins from the cell surface is the clathrin-mediated endocytosis (CME). Receptor-mediated endocytosis via clathrin-coated pits can be constitutive or triggered by signals. Some membrane proteins, such as low-density lipoprotein (LDL) and transferrin receptors, are constitutively concentrated in coated pits, whereas others (e.g. EGFR) become concentrated upon ligand binding. Several surface molecules (various phospholipids and glycosphingolipids) can occasionally enter coated pits by lateral diffusion in the lipid bilayer and then be captured specifically by the coated-pit components (Mousavi *et al.*, 2004). The concentration of receptor-ligand complexes to the specific sites on the plasma membrane initiates the assembly of clathrin and adaptor proteins to form coated pits. The invagination and fission reaction during the later stages of CME is dependent on the large GTPase dynamin, which is a major regulator of several membrane trafficking events at the cell surface in most of the endocytotic processes (Conner and Schmid, 2003; Mousavi *et al.*, 2004; Santolini *et al.*, 2000).

1.2.2. Caveolin-dependent endocytosis

Caveolae are uniform flask-shaped invaginations of the plasma membrane that are considerably smaller (60–80 nm diameter) and morphologically distinct from the clathrin-coated pits. These invaginations are formed by the polymerization of caveolins and contain a subset of lipid-raft components, including cholesterol and sphingolipids (reviewed from Parton and Simons, 2007). In contrary to clathrin-coated pits, caveolae are present in some cell types (endothelial cells, smooth-muscle cells, adipocytes and fibroblasts), but have not yet been found in others (lymphocytes, many neuronal cells). The major protein component of caveolae, caveolin-1, is required for the formation of characteristic caveolar structures (Rothberg *et al.*, 1992).

Various ligands and receptors have been reported to localize in caveolae, including insulin, erbB-2, cholera and tetanus toxins, and many GPI anchored

proteins (Keller *et al.*, 1992; Pelkmans *et al.*, 2001). In contrast to the clathrin-mediated pathway, caveolin is immobilized in the caveolae and does not diffuse laterally in the plasma membrane. In addition, the internalization via caveoale is not a constitutive process (Thomsen *et al.*, 2002), as it occurs more slowly and only upon cell stimulation. Simian virus 40 (SV40), for example, stimulates the caveolar uptake by activating a signalling cascade that cross-links surface receptors in the caveolae. The intracellular routing of the SV40 is unusual, since it is delivered from the caveolae to the endoplasmic reticulum, bypassing the traditional endosome/lysosome system (Pelkmans *et al.*, 2001). The uptake of several raft molecules, such as glycosphingolipid GM1 binding the subunit of cholera toxin B and some GPI-anchored proteins, have shown to be mediated by the caveolae (Nichols, 2002). On the other hand, some raft proteins and lipids – for example TGFβ and GM1 – enter cells by both clathrin-coated pits and caveosomes (Nichols, 2002; Torgersen *et al.*, 2001). The glycosphingolipid GM1, upon binding of the cholera toxin, has been shown to be present in two different populations within the plasma membrane – one clearly internalizing by clathrin-coated pits, whereas another fraction is found in caveolin-positive cholesterol-sensitive clusters (Nichols, 2003). However, recent studies reveal that the role of caveolae and caveolin in endocytosis is more complex than was originally thought. For instance, caveolin-1 has been shown to act more as a regulator of receptor turnover and not always as an essential component of the caveolae/raft endocytotic pathway (Lajoie and Nabi, 2007; Nabi and Le, 2003; Nichols, 2003; Parton and Simons, 2007).

1.2.3. Macropinocytosis

Macropinocytosis is an actin-dependent process that is initiated by extensive plasma membrane reorganization and ruffling, which leads to the formation of large (0.5–2 μm) morphologically heterogeneous vesicular structures called macropinosomes. Macropinocytosis is a constitutive process in some tumour cell lines and specific cell types, such as neutrophils and macrophages, but in others it is only induced as a response to certain growth factors or other signals. Macropinocytosis is also utilized by microorganisms, including bacteria *Legionella* and *Listeria* or viruses such as HIV, to gain access to the cells (Francis *et al.*, 1993; Meier *et al.*, 2002).

Macropinocytosis is dependent on the activity of the phosphatidylinositol 3-kinase (PI3K) and Rho family small GTPases, which regulate actin rearrangements and can trigger membrane ruffling. Membrane ruffles, from which macropinosomes are derived, are enriched in polyphosphoinositides associated with specific lipid rafts. Lipid rafts have been suggested to take part in macropinosome formation since cholesterol depletion inhibits both membrane ruffling and macropinocytosis (Grimmer *et al.*, 2002).

Unlike clathrin- and caveolae-dependent endocytosis, the macropinocytosis is a dynamin-independent process. Instead of dynamin, the fission of the macropinosome requires a plasma-membrane recruitment and the protein CtBP1/BARS, as demonstrated by recent studies (Bonazzi *et al.*, 2005; Liberali *et al.*, 2007). The CtBP1/BARS is recruited to membrane ruffles and to the macropinocytotic cup fissioning sites during the EGF-receptor-induced macropinocytosis. The CtBP1/BARS controls the fissioning step of the formation of the macropinosomes downstream of the p21-activated kinase-1 (Pak1) and it needs to be phosphorylated by Pak1 before inducing the fission (Liberali *et al.*, 2007).

1.2.4. Clathrin- and caveolin-independent endocytosis

In addition to clathrin- and caveolin-dependent uptake and macropinocytosis, the activity of other less understood endocytotic routes is present in mammalian cells. For example, it has been demonstrated that an SV40 that internalizes via caveolae also can enter cells through a clathrin- and dynamin-independent mechanism in the absence of caveolin (Damm *et al.*, 2005). In addition, the subunit of cholera toxin B (CTB) can be internalized by both clathrin-coated pits as well as by caveolae (Torgersen *et al.*, 2001), but a significant fraction is also internalized via a clathrin- and dynamin-independent pathway (Kirkham and Parton, 2005).

The clathrin- and caveolin-independent pathways may act in a constitutive manner, like for the interleukine-2 (IL-2) receptor and for certain GPI-anchored proteins. The endocytosis of GPI-anchored proteins has been shown to take place by a clathrin-, caveolin-, and dynamin-independent mechanism using labile tubular endocytotic structures termed GEECs (GPI-enriched endosomal compartments) (Sabharanjak *et al.*, 2002). In later stages of internalization the GPI-anchored protein pathway converges with the classical clathrin-dependent pathway in endosomes. This process has shown to be dependent on the small GTPase Cdc42. The same pathway seems to be responsible for the dynamin-independent internalization of the CTB subunit into tubular and ring-shaped endocytotic structures, which have been termed CLICs (clathrin-independent carriers) (Kirkham and Parton, 2005).

The dynamin-dependent CLIC pathway is suggested to mediate the uptake of the beta subunit of the IL-2 receptors and delivery to late endosomes and lysosomes in cells with dominant-negative mutants of clathrin-associated protein Eps15 (Lamaze *et al.*, 2001). This pathway differs from the uptake of the GPI-anchored protein, it is dynamin-dependent and specifically inhibited by RhoA but not by Cdc42. On the other hand, since caveolar endocytosis does not necessarily require caveolin-1 protein (Le *et al.*, 2002; Nichols, 2002) (Nabi and Le, 2003), the IL-2 receptors might still use this pathway and accumulate in caveosomes. However, like caveolae, these CLIC pathways are dependent on the detergent-resistant microdomains of the plasma membrane.

The common unanimously acknowledged marker proteins for clathrin- and caveolin-independent pathways are missing so far. However, flotillin-1 was suggested as a putative marker for CLIC associated internalization routes (Glebov *et al.*, 2006) (Frick *et al.*, 2007). Flotillin-1 is present in the plasma membrane and in a specific population of endocytotic structures, which accumulate both GPI-linked proteins and CTB in a dynamin-independent manner (Glebov *et al.*, 2006). It was demonstrated very recently that coassembly of two similar proteins, flotillin-1 and flotillin-2, is sufficient for generation of new flotillin-positive plasma membrane microdomains and the formation of plasma membrane invaginations, which are morphologically similar to caveolae in the embryonic fibroblasts of caveolin-1 knockout mice (Frick *et al.*, 2007). Although the intracellular vesicles derived from those invaginations looked like caveolar rosettes or caveosomes, they were distinct from caveolae defined by caveolin-1. Moreover, several proteoglycan-binding ligands, including poly-arginine, have been shown to efficiently internalize into late endosomes by a mechanism that is clathrin- and caveolin-independent, but dynamin- and flotillin-dependent (Payne *et al.*, 2007). Heparane sulfate and other extracellular glycans are suggested to be favourable binding sites for many substances, such as lipoproteins, growth factors and probably also for cell-penetrating peptides, because of their anionic nature (Belting, 2003). The internalization pathway of the cationic proteoglycan-binding ligands is different from the clathrin-dependent endocytosis, since their trafficking to the late endosomes is microtubule-independent and does not require the phosphatidylinositol 3-phosphate kinase for sorting them from early endosomes (Payne *et al.*, 2007).

1.3. Cell-penetrating peptides

The property of several short fragments of different proteins to efficiently traverse biological membranes was discovered in early nineties. These short sequences are now collectively named cell-penetrating peptides (CPPs), protein transduction domains (PTDs) or membrane translocation sequences (MTSs). The first protein discovered to translocate across cell membranes was the 86-amino acid protein TAT (transactivator of transcription) from human immunodeficiency virus type 1, which entered the mammalian cells in culture and activated transcription (Frankel and Pabo, 1988; Green and Loewenstein, 1988). Some years later the 60 amino acid homeodomain of the Antennapedia protein of Drosophila was also shown to be able to translocate across cell membranes (Joliot *et al.*, 1991). Subsequent studies led to the identification of short sequences within these proteins that are responsible for the translocating ability and resulted in the introduction of penetratin in 1994 (Derossi *et al.*, 1994) and the Tat peptide in 1997 (Vivés *et al.*, 1997). After these first reports, a range of other CPPs have been introduced, including protein-derived (pVec and VP22);

chimerical, which are combined from sequences of different sources (transportan and MPG); and peptide carriers with non-natural sequences (MAPs, polylysine, polyarginine) (Table 1).

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

CPP	Origin	Sequence	Reference
Protein derived			
*Penetratin	*Drosophila* Antennapedia homeodomain	RQIKIWFQNRRMK WKK	(Derossi *et al.*, 1994)
*Tat(48–60)	HIV-1 Tat	GRKKRRQRRRPPQ	(Vivés *et al.*, 1997)
VP22	HSV envelope protein 22	DAATATRGRSAARP TERPRAPARSASRPR RPVE	(Elliott and O'Hare, 1997)
hCT(9–32)	human calcitonin	LGTYTQDFNKFHTF PQTAIGVGAP	(Schmidt *et al.*, 1998)
*pVEC	murine vascular endothelial cadherin	LLIILRRRIRKQAHA HSK	(Elmquist *et al.*, 2001)
SAP, Proline-rich CPPs	N-terminal repetitive domain of maize gamma-zein	$(VRLPPP)_n$	(Fernandez-Carneado *et al.*, 2004)
Designed			
MAP	model amphipathic peptide	KLALKLALKALKAAL KLA	(Oehlke *et al.*, 1998)
MPG	fusion peptide of HIV-1 gp41 + NLS	GALFLGFLGAAGSTM GAWSQPKSKRKVC	(Morris *et al.*, 1997)
Pep-1	dimerization domain of HIV-1 reverse transcriptase + NLS	KETWWETWWTEWS QPKKKRKV	(Morris *et al.*, 2001)
*Transportan	Galanin-Lys(13)-masto-paran	GWTLNSAGYLLGKIN LKA LAALAKKIL	(Pooga *et al.*, 1998a)
*TP10	truncated analogue of transportan	AGYLLGKINLKALAA LAKKIL	(Soomets *et al.*, 2000)
oligoarginine	Arginine-rich CPPs	$(R)_n$	(Futaki *et al.*, 2001; Mitchell *et al.*, 2000)
Antimicrobial-derived peptides			
SynB	protegrin 1	RGGRLSYSRRRFST STGR	(Rousselle *et al.*, 2000)
$S4_{13}$-PV	dermaseptin S4 + NLS	ALWKTLLKKVLKAP KKKRKV	(Hariton-Gazal *et al.*, 2002)
Proline-rich CPPs	bactenecin 7	RRIRPRPPRLPRPRP RPLPFPRPG	(Sadler *et al.*, 2002)
LL-37	human cathelin-associated peptide	LLGDFFRKSKEKIGKE FKRIVQRIKDFLRNLV PRTES	(Sandgren *et al.*, 2004)

* CPPs used in this study

The distinguishing characteristic of the cell-penetrating peptides is their ability to deliver cargo molecules into cells. CPPs have been used to deliver different types of molecules with a wide range of size, such as small interfering RNA and peptide nucleic acid oligomers as well as full-length proteins and plasmids or even nanoparticles and liposomes (reviewed from Dietz and Bähr, 2004; El-Andaloussi *et al.*, 2005a; Snyder *et al.*, 2004).

Despite the broad acceptance of cell-penetrating peptides as molecular carriers, their internalization mechanism(s) is not completely understood and is still the subject of debate. It appears to involve two competing mechanisms: the fast penetration directly through the plasma membrane, and the slower mechanism by regulated endocytotic pathways.

Although CPPs form a very heterogeneous class of peptides regarding their origin and primary structures, they have some common characteristics. These include positive net charge and cargo-delivery property. Mainly, due to the heterogeneity and limited understanding of the exact internalization mechanisms, it has been difficult to formulate an unambiguous definition for CPPs and to find a general basis for their classification. However a general definition was recently proposed by Langel: "a cell-penetrating peptide is a relatively short peptide, 5–40 amino acids, with the ability to gain access to the cell interior by means of different mechanisms, including endocytosis, and with the capacity to promote the intracellular delivery of covalently or noncovalently conjugated bioactive cargoes" (Langel, 2006).

1.4. Classes of CPPs

1.4.1. Penetratins

The name penetratin was given to the 16 amino acid peptide derived from the third helix of Antennapedia homeodomain by the group of Prochiantz. This peptide, also denoted as pAntp(43–58), was the first reported CPP (Derossi *et al.*, 1994). In 1991, Joliot *et al*. discovered that the 60-amino acid homeodomain of Antennapedia, a *Drosophila* transcription factor, enters the neurons in culture, reaches the nuclei and induces neurite elongation (Joliot *et al.*, 1991). A comprehensive analysis by different substitutions and truncations led to the conclusion that the third helix (residues 43 to 58) is necessary and sufficient for penetration into cells (Le Roux *et al.*, 1993; Derossi *et al.*, 1994). Similarly to the homeodomain of Antennapedia, penetratin was shown to internalize by an energy-independent mechanism at both 4 and 37 °C into the cytoplasm and nucleus of primary cells from rat brain. Later, by deleting amino acids from the N-terminus of penetratin (43–58), it was found that the C-terminal segment (residues 52–58) was necessary and sufficient for efficient cell membrane translocation (Fischer *et al.*, 2000).

The secondary structure of penetratin appears to differ depending on the environment. The first studies described the α-helical structure of penetratin in a hydrophobic environment (Derossi *et al.*, 1994; Drin *et al.*, 2001), whereas others demonstrated transition of peptide to a β-structure when inserted into membranes (Bellet-Amalric *et al.*, 2000). Magzoub *et al*. have shown that the conformation of penetratin depends on the lipid/peptide ratio and on the surface charge of the vesicle. At a high lipid/ peptide ratio and low vesicle surface charge, the α-helical structure is dominating, and vice versa, α-helix transits to β-sheet when the lipid/peptide ratio is reduced or the membrane charge is increased (Magzoub *et al.*, 2002). However, the helical structure of penetratin is probably not required for translocation, because the substitution of one or three amino acids by prolines, which breaks the peptide helicity, did not alter internalization (Derossi *et al.*, 1996). Still, the conformational freedom of linear structure has some importance, since a cyclic analogue of penetratin does not enter cells (Fischer *et al.*, 2000).

The penetratin sequence contains several basic, positively charged amino acids throughout the molecule. The replacement of any of the charged residues in the penetratin sequence with alanine decreases the cellular uptake of peptide, indicating that the basic amino acids, Arg and Lys, within the sequence are essential for the cellular internalization (Drin *et al.*, 2001; Fischer *et al.*, 2000; Mazel *et al.*, 2001). In addition to basic residues, substitution of Trp 48 or Trp 56 with alanine reduces the cellular uptake of the peptides, suggesting that specific hydrophobic residues have an important role in the cellular translocation process of penetratin (Dom *et al.*, 2003). On the other hand, a penetratin mutant with two phenylalanines in place of Trp 48 and Trp 56 was not internalized, indicating that Trp, in particularly Trp 48, is crucial in the penetration process (Derossi *et al.*, 1994).

In conclusion, it has been proposed that charged residues have an essential role in the initial electrostatic interaction of the peptide with the lipid bilayer, and Trp 48 then destabilizes the membrane and initiates the translocation of penetratin (Christiaens *et al.*, 2004; Derossi *et al.*, 1996; Fischer *et al.*, 2000).

By now, it is clear that penetratin, like most of the other cationic CPPs, does not internalize only by non-endocytotic mechanism but also uses endocytotic pathways to enter cells. Non-endocytotic and endocytotic pathways might function in parallel, depending on the peptide concentration and the availability of alternative internalization routes (Duchardt *et al.*, 2007). Recently, penetratin analogues with endosomolytic properties (R_6-penetratin, EB1) were developed to ensure better accessibility and functionality of cargo molecules that are internalized through endocytotic pathways and therefore trapped to endosomal vesicles (Abes *et al.*, 2007; Lundberg *et al.*, 2007).

However, penetratin and its derivatives have been used as efficient delivery vectors for transporting different molecules, such as fluorophores (Fischer *et al.*, 2000), peptides (Snyder *et al.*, 2004), proteins (Console *et al.*, 2003), oligo-

nucleotides (Pooga *et al.*, 1998b; Villa *et al.*, 2000), and siRNA (Davidson *et al.*, 2004; Lundberg *et al.*, 2007; Moschos *et al.*, 2007) into cells.

1.4.2. Tat protein derived peptides

The highly cationic Tat(48–60) peptide from the human immunodeficiency virus 1 (HIV-1) TAT protein is one of the most well studied CPPs. The whole HIV TAT protein was reported to translocate into the cytosol and the nucleus of cells already in 1988 (Frankel and Pabo, 1988; Green and Loewenstein, 1988). Later, a fragment of the TAT protein, Tat(37–72), was shown to promote the cellular uptake of conjugated proteins, such as β-galactosidase (Fawell *et al.*, 1994). However, the most efficiently internalizing sequence, Tat(48–60), was reported shortly after the discovery of penetratin (Vivés *et al.*, 1997). This sequence of Tat contains motifs that are involved in many key functions of the protein, including the TAT RNA-binding and the nuclear localization signal.

The Tat peptide has been shown to deliver different cargoes ranging from small peptides and oligonucleotides to proteins and liposomes, plasmid DNA, and even nanoparticles to cells. pTat has often been used in recombinant fusion proteins. For example, the ability of the TAT-streptavidin fusion protein as a transporter for biotin or biotinylated molecules has been studied (Albarran *et al.*, 2005; Rinne *et al.*, 2007). Moreover, pTat has proven to be a highly efficient carrier for *in vivo* delivery of biologically active fusion proteins, such as β-galactosidase, which after the intraperitoneal injection was present in different tissues of a mouse, crossing even the blood-brain barrier (Schwarze *et al.*, 1999). The ability of the Tat peptide to cross the blood-brain barrier has led to its *in vivo* application for treatment of cerebral ischemia (Kim *et al.*, 2005). In addition, pTat has been efficiently used to introduce apoptotic proteins into cancer cells (Snyder *et al.*, 2004) and a biologically active antioxidant enzyme into pancreatic β cells to reduce oxidative stress (Eum *et al.*, 2004).

The cellular uptake mechanism of the Tat peptide has been extensively studied and different internalization routes have been suggested. The first step in the internalization process is an ionic interaction of the cationic CPP with negatively charged plasma membrane constituents (Vivés, 2003). The heparan sulfate proteoglycans of the cell surface might act as receptors for TAT protein and peptides. For instance, a full-length TAT protein that was fused with GFP failed to transduce cells that were genetically defective in biosynthesis of fully sulfated heparan sulfates (Tyagi *et al.*, 2001) and translocation of the Tat(48–60) was decreased when the cells were treated with an anti heparan sulfate antibody (Suzuki *et al.*, 2002a). Interaction of the arginine-rich peptides, including pTat, with the membrane associated proteoglycans might activate the intracellular signals that induce actin reorganization and endocytotic uptake (Nakase *et al.*, 2007).

Events that are characterized to follow the interaction of the Tat peptides with cells differ between reports and are sometimes contradictory. Some studies demonstrate that the Tat peptide internalizes rapidly in a receptor- and energy-independent manner (Futaki *et al.*, 2001; Ziegler *et al.*, 2005), whereas others suggest an active mechanism based on vesicular uptake (Nakase *et al.*, 2007; Wadia *et al.*, 2004; Vivés, 2003). Group of Giacca reported that Tat fusion protein with EGFP internalizes via a caveolar lipid raft-dependent endocytosis and is further targeted to the perinuclear area by actin cytoskeleton-mediated mechanism (Ferrari *et al.*, 2003; Fittipaldi *et al.*, 2003). On the contrary, Wadia *et al*. showed the uptake of the Tat-Cre fusion protein into cells by lipid raft-dependent macropinocytosis (Wadia *et al.*, 2004). In addition, Richard and co-workers demonstrated that fluorescently labelled Tat peptide and Tat-PNA constructs accumulate in endocytotic vesicles originating from clathrin-dependent endocytosis (Richard *et al.*, 2003). A few years later the same group emphasized the clathrin-dependent uptake rather for the unconjugated Tat peptide than for the Tat-protein conjugates (Richard *et al.*, 2005).

Such diversity of observed translocation mechanisms might be explained by the cargo-dependent targeting of the Tat peptide and other CPPs to different internalization routes (El-Andaloussi *et al.*, 2007a; Tünnemann *et al.*, 2006). A Tat peptide with a small cargo (<50 aa) or without a cargo might have the ability to pass through the plasma membrane but when a large cargo (>50 aa) is attached to the peptide, the uptake mechanism changes to endocytosis (Tünnemann *et al.*, 2006).

The cytotoxicity of the CPPs has also been suggested to depend on the size and type of the cargo. In general, it has been suggested that the cargoes, such as dsDNA and proteins, decrease the toxicity of CPP. However, in case of pTat, the fluorescein moiety appears to rather increase the toxicity of the peptide (El-Andaloussi *et al.*, 2007a), which usually is considered of low cytotoxicity even up to 100 μM peptide concentration (Suzuki *et al.*, 2002b).

1.4.3. Oligoarginines

Given that cationic residues in the pTat and the pAntp are important for cellular uptake, the homo-oligomers of arginine, lysine, ornitine, and histidine were tested for their ability to enter cells (Futaki *et al.*, 2001; Mitchell *et al.*, 2000; Wender *et al.*, 2000). It appeared that the positive charge is not the only determinant, since the uptake of peptides with an equivalent length/charge differed markedly (Mitchell *et al.*, 2000; Wender *et al.*, 2000). Homopolymers of arginine entered cells far more efficiently than other cationic polymers or the Tat(49–57) peptide itself (Wender *et al.*, 2000). Moreover, the replacement of any of the arginine residues in the Tat peptide with alanine reduced its uptake, confirming the importance of arginines in the cellular uptake of the peptide. The

length of arginine oligomers is also important, since oligomers with six to nine arginines have shown to be optimal for efficient translocation into cells (Futaki *et al.*, 2001; Mitchell *et al.*, 2000; Suzuki *et al.*, 2002b). In addition, longer poly-arginine chains also have higher cellular toxicity (Mitchell *et al.*, 2000).

The chirality of the backbone is not critical for the cellular uptake and D-arginine oligomers showed even a higher uptake than L-arginine oligomers (Mitchell *et al.*, 2000; Wender *et al.*, 2000). However, the equal uptake of the D- and L-oligoarginines in serum-free medium suggests that the higher cellular uptake of D-form is rather due to their better proteolytic stability than to a higher translocation ability (Wender *et al.*, 2000).

The main structural feature of polyarginines that ensures their more efficient cellular uptake compared to other polycations is the guanidine group of the arginine side-chain, which has the ability to transiently form bidentate hydrogen bonds with cell surface anions, such as phosphate or sulfate, enabling the adaptive diffusion of guanidine-rich transporters (Rothbard *et al.*, 2005). In addition, polyarginines have been shown to enter cells by endocytosis, most frequently by macropinocytosis. Nakase and co-workers demonstrated that the interaction of octaarginine and Tat peptide with the membrane-associated proteoglycans promotes actin rearrangements, which induce the formation of lamellipodia and macropinocytosis. Comparative uptake studies with branched-chain peptides indicate that the structure and charge density of the arginine-rich peptides might determine the interaction ability of the peptides with the cell-surface proteoglycans (Nakase *et al.*, 2007). On the other hand, a number of reports dispute the macropinocytotic uptake of polyarginines. For instance, Zaro *et al*. found no correlation between the cytosolic delivery of oligoarginine and the formation of macropinosomes or filopodia in HeLa cells (Zaro *et al.*, 2006) and Al-Taei *et al*. showed the uptake of octaarginine in haematopoietic K562 cells to be relatively insensitive to the treatment with amiloride, a known inhibitor of macropinocytosis (Al-Taei *et al.*, 2006).

In addition, the transition from one uptake mechanism to another depending on concentration or temperature can take place (Duchardt *et al.*, 2007; Fretz *et al.*, 2007). For instance, the uniform localization of L-octaarginine and its D-enantiomer in the cytosol and nucleus, which is characteristic for temperatures below 10 °C, changes to more vesicular above the threshold temperature of 12 °C (Fretz *et al.*, 2007). Moreover, different internalization mechanisms have also been demonstrated for oligoarginine-cargo complexes. Liposomes modified with low-density octaarginines were taken up by clathrin-mediated endocytosis, whereas a higher density of octaarginines on the liposomes stimulated their macropinocytosis-mediated uptake and resulted in gene expression from plasmid DNA, which was encapsulated in liposomes (Khalil *et al.*, 2006). In another study, the octaarginine-modified liposomes were shown to enter living cells via a specific pathway at 4 °C, which is distinct from energy-dependent vesicular transport (Iwasa *et al.*, 2006).

1.4.4. Transportans

Transportan as a CPP was discovered serendipitously in the search for novel galanin receptor ligands. The coupling of the galanin (1–13) fragment to the N-terminus of mastoparan yielded a 27 amino acid long galparan (Langel *et al.*, 1996), which in contrast to other ligands was able to internalize in a receptor-independent manner and activate G-proteins (Zorko *et al.*, 1998). In order to further characterize the mode of cellular uptake of galparan, the last amino acid from the galanin part (^{13}Pro) was replaced by Lys (Pooga *et al.*, 1998a). An amino group in Lys side chain provided a suitable attachment point for reporter groups, like biotin and fluorophores, enabling to form complexes with cargo molecules and/or visualize them in cells. The biotinylated galparan analogue, given the name transportan (TP), entered cells via an apparently non-endocytotic pathway localizing in the cytoplasm and nucleus of Bowes melanoma cells (Pooga *et al.*, 1998a).

Similarly to the predecessor molecule galparan, biotinylated transportan has a relatively high affinity for galanin receptors in Bowes cell membranes. In an attempt to minimize the affinity for galanin receptors and the interaction with G-proteins, and possibly to enhance cell penetration efficiency of the peptide, series of deletion analogues of transportan were designed (Soomets *et al.*, 2000).

The most promising candidate for further studies was transportan 10 (TP10), an analogue of TP, in which the first six N-terminal amino acids were removed. The TP10 retained its CPP activity showing comparable uptake with transportan, but did not bind to galanin receptors nor modulated the activity of the G-proteins. The C-terminal part of transportan and its analogues is necessary for the membrane translocation (Lindgren *et al.*, 2000; Magzoub *et al.*, 2001; Soomets *et al.*, 2000). The amphipathic character of the mastoparan sequence in the C-terminus of transportan, rather than its charge, is shown to be responsible for the association with membranes. Specifically, the C-terminus of transportan interacts with phospholipids using the hydrophobic face of the α-helix (Barany-Wallje *et al.*, 2004). Recently, Yandek *et al*. have proposed a model in which the binding of the TP10 to the membrane surface creates a mass imbalance across the lipid bilayer thereby perturbing the membrane and enabling peptides to transiently move into the hydrophobic core and cross the bilayer (Yandek *et al.*, 2007).

The TP10 and transportan have good translocation capabilities and have been successfully used as delivery vectors for peptide nucleic acid (PNA), peptides and proteins. Transportan and penetratin were used as delivery vectors for a 21-mer antisense PNA molecule to suppress the galanin receptor gene expression *in vivo* in the rat spinal cord (Pooga *et al.*, 1998b). Later, TP and TP10 were used for cellular transduction of PNA oligomers to define the regulatory regions of the GalR1 mRNA (Kilk *et al.*, 2005). Transportan, as well

as TP10, have repeatedly been used to deliver antiviral PNA molecules into cultured cells to efficiently block the TAT-protein mediated transactivation and HIV-1 replication (Chaubey *et al.*, 2005; Tripathi *et al.*, 2005; Turner *et al.*, 2007). The capability of transportans to deliver proteins, such as GFP, antibodies, and complexes of streptavidin-gold conjugate has also been demonstrated (Pooga *et al.*, 2001). The mechanisms by which transportans target proteins into cells are the main focus of the papers included in this thesis and will be discussed later.

1.4.5. Other CPPs

In addition to the cell-penetrating peptides discussed above, a variety of other CPPs have been described in literature. In the following subsection some more widely known representatives of the CPP family are introduced.

The investigations of **herpes simplex virus type 1 structural protein VP22** showed the unusual ability of this protein for intercellular translocation during infection and transient transfection. The 34 C-terminal amino acids of the VP22 were found to be responsible for its energy-independent translocation activity and VP22 was used to deliver oligonucleotides and peptides (Elliott and O'Hare, 1997), as well as functional proteins into cells (Morris *et al.*, 2002; Zheng *et al.*, 2006). However, later reports demonstrated that some earlier data with VP22 might be misleading due to the fixation artefacts (Lundberg and Johansson, 2001; Lundberg and Johansson, 2002). For instance, Aints *et al*. detected the expression of VP22-GFP fusion protein only in transfected cells and fixed recipient cells but not in live recipient cells (Aints *et al.*, 1999). By now, the ability of VP22-mediated intercellular trafficking has been reconfirmed not only by using more relevant live-cell confocal fluorescence microscopy (Lemken *et al.*, 2007) but also by *in vivo* experiments showing biological effect (Zheng *et al.*, 2006). In addition, GFP fused to the VP22, has been demonstrated to internalize via a lipid-raft mediated caveolae-independent endosomal pathway (Nishi and Saigo, 2007).

pVEC is an 18-amino acid CPP derived from the cell adhesion molecule of murine vascular endothelial cadherin (VE-cadherin). This peptide corresponds to the residues 615–632, comprising the hydrophobic fragment of 5 amino acids from the transmembrane region, and a hydrophilic part of 13 amino acids from the cytosolic tail of cadherin. The amphipathic nature of pVEC has been suggested to be essential for the cellular uptake (Elmquist *et al.*, 2001). Thus, the pVEC efficiently internalizes into a variety of cell lines by a receptor-independent mechanism (Elmquist *et al.*, 2001) and is able to deliver different cargoes, including PNA and 67 kDa protein streptavidin, at both physiological and low (4°C) temperatures (Elmquist and Langel, 2003). In addition, the D-form analogue of pVEC is able to enter cells with the same efficiency,

suggesting that binding to a chiral receptor is not necessary (Elmquist and Langel, 2003). Although these studies support the non-endosomal energy- and receptor-independent translocation process, we provide evidence for an endocytotic uptake mechanism of the pVEC, in paper III (Säälik *et al.*, 2004).

Recently, the atomic force microscopy analysis revealed that both the pVEC and its analogue W2-pVEC, increase the fluidity of the phase-separated supported phospholipid bilayers (SPBs) and induce the transformation from gel phase domains into a less ordered state (Herbig *et al.*, 2006). Since the pVEC has no influence on the plasma membrane permeability it probably enters cells without the formation of pores (Elmquist *et al.*, 2001). Despite the lack of toxicity of the pVEC in mammalian cells, it has shown to be one of the few CPPs with potent antimicrobial properties. The pVEC permeabilized bacterial cells of *Mycobacterium smegmatis* at concentrations as low as 2 μM, below the level that harmed human cells (Nekhotiaeva *et al.*, 2004).

The **model amphipathic peptide (MAP)**, composed only of the amino acids lysine, alanine, and leucine, is a typical amphiphilic peptide where the basic side chains and hydrophobic side chains are separated along the α-helix of the peptide. The MAP was originally designed by Steiner and colleagues (Steiner *et al.*, 1991) and was used for biophysical studies on interactions of bioactive helical amphipathic peptides with lipid membranes (Oehlke *et al.*, 1998). The MAP is able to enter cells by both energy-dependent and -independent mechanisms (Oehlke *et al.*, 1998; Oehlke *et al.*, 2004). The structure-activity relationship (SAR) studies and comparative cellular uptake of amphipathic and non-amphipathic analogues of MAPs suggested that amphipathicity and a chain length of at least 16 amino acids are essential for the cellular uptake of the MAP peptide (Oehlke *et al.*, 1998; Scheller *et al.*, 1999). Further studies showed that both amphipathic and non-amphipathic analogues were taken up by cells to about the same extent, indicating that amphipathicity prevented peptide washout, but was not essential for the internalization (Scheller *et al.*, 2000). The MAPs have been used to transport short peptides, oligonucleotides (Oehlke *et al.*, 2002; Oehlke *et al.*, 1998), and peptide nucleic acid (Oehlke *et al.*, 2004). Hällbrink and colleagues have compared the translocation efficiency of the MAP with other well known CPPs in Bowes human melanoma cells (Hällbrink *et al.*, 2001). Although MAP and transportan showed the fastest uptake and better cargo delivery efficiency than Tat(48–60) or pAntp, they also exhibited higher degree of cellular toxicity. In addition, the MAP was the most potent inducer of membrane leakage causing efflux from cells already at 1 μM concentration.

Membrane translocation sequences (MTS) have been designed in order to create a biologically active carrier peptide by combining various functional peptide segments with the signal sequences that contribute to their translocation activity. The hydrophobic region of different MTS peptides originates from different signal peptides, such as the signal sequences of Kaposi's sarcoma

fibroblast growth factor 1 (K-FGF) (Lin *et al.*, 1995), human integrin β_3 precursor signal sequence (Liu *et al.*, 1996), the immunoglobulin light chain Ig(v)40 of *Caiman crocodylus* (Chaloin *et al.*, 1997), etc. The MTSs, coupled to a nuclear localization signal (NLS), have been used to interfere with the intracellular trafficking of NF-κB (Lin *et al.*, 1995), to characterize the mechanisms of nuclear localization (Torgerson *et al.*, 1998), as well as for the delivery of oligonucleotides (Chaloin *et al.*, 1998).

The chimeric vectors **MPG** and **Pep-1** were designed based on the same principle as the MTS. These primary amphipathic peptides consist of three domains: (1) a hydrophobic domain, which interacts with the cargo molecules; (2) a hydrophilic lysine-rich domain derived from the nuclear localization signal (NLS) of the simian virus 40 (SV40) large T antigen, to improve the solubility of the peptide; (3) and a spacer region, which provides a flexible link connecting two domains. As a unique advantage over the covalent peptide-based cargo-delivery system, the MPG and Pep1 have been designed to form stable complexes/nanoparticles with cargoes without requiring any chemical covalent coupling.

The 28 amino acid amphipathic carrier peptide **MPG**, which contains sequences derived from the hydrophobic fusion peptide of HIV-1 gp41, and the hydrophilic NLS of SV40 large T antigen, was designed for the delivery of oligonucleotides into mammalian cells (Chaloin *et al.*, 1997; Morris *et al.*, 1997). The MPG was shown to efficiently promote the intracellular delivery of short oligonucleotides such as siRNAs via a non-endosomal pathway (Simeoni *et al.*, 2003).

The **Pep-1** comprises the hydrophobic sequence of the dimerization domain of HIV-1 reverse transcriptase (Morris *et al.*, 2001). The Pep family carriers have been applied successfully to deliver proteins, peptides, antibodies, and PNA oligomers in their biologically active form into mammalian cells (Morris *et al.*, 2004; Morris *et al.*, 2001). Recently, an *in vivo* application of the Pep carrier peptides has been demonstrated to block tumour growth by using CPP complexes with either PNA or a tumour suppressor protein (Morris *et al.*, 2004; Munoz-Morris *et al.*, 2007).

Both the MPG and Pep-1 are suggested to induce transient pore-like structures in the lipid bilayer that enables the internalization of several bioactive cargoes by a non-endosomal pathway (Deshayes *et al.*, 2004; Deshayes *et al.*, 2006). Interestingly, the biophysical study with Pep-1 showed no evidence of the formation of membrane pores in the lipidic bilayers (Henriques *et al.*, 2007). However, the non-endosomal pathway is active only for the specific particle size and the molar ratio of the CPP/cargo molecules (Munoz-Morris *et al.*, 2007).

A specific class of amphipathic CPPs has been designed based on **antimicrobial-derived peptides**, e.g. **SynB** peptides, derived from the antimicrobial peptide protegrin 1 (Rousselle *et al.*, 2000); **$S4_{13}$-PV**, containing

sequence from the dermaseptin S4 peptide (Hariton-Gazal *et al.*, 2002); **LL-37**, human cathelin-associated antimicrobial peptide (Sandgren *et al.*, 2004); and **proline-rich translocating peptides** from the antimicrobial peptide bactenecin (Sadler *et al.*, 2002). All these peptides interact with the surface of mammalian cells and cross the plasma membrane without cytotoxic or growth-inhibitory effects. In addition, sweet arrow peptide (SAP) $(VRLPPP)_3$ and other proline-rich sequences of gamma-zein $(VRLPPP)_n$, a storage protein of maize, have been introduced by the group of Giralt as amphipathic proline-rich CPPs with good non-viral and non-cytotoxic cellular uptake properties (Crespo *et al.*, 2002; Fernandez-Carneado *et al.*, 2004; Foerg *et al.*, 2005).

The N-terminally truncated derivatives of the peptide hormone **human calcitonin (hCT)** represent a class of weakly cationic CPPs, investigated by Tréhin *et al*. The calcitonin-derived peptides have deserved much attention because of their human origin and their ability to non-cytotoxically internalize into a fully differentiated epithelial cell lines (Trehin *et al.*, 2004). The hCT has been efficiently used to deliver several bioactive molecules across the cell membrane (reviewed in Neundorf and Beck-Sickinger, 2005).

1.5. Adenosine-oligoarginine conjugates (ARC)

Conjugates of oligoarginine peptides with adenosine are efficient inhibitors of several basophilic protein kinases (Enkvist *et al.*, 2006; Loog *et al.*, 1999; Viht *et al.*, 2003). While most of the inhibitors of protein kinases have been designed either to target the ATP-binding site or the peptide/protein-binding domain of the kinase, ARC was designed to interact with both the ATP and the substrate binding pocket of the enzyme.

This bisubstrate-analogue inhibitor is composed of two moieties, an adenosine-5'-carboxylic acid and an oligoarginine sequence which are connected via a linker chain that enables optimal association of both fragments with their binding sites (Fig. 2) (Loog *et al.*, 1999). Several derivatives of ARC, containing different nucleoside moieties as well as diverse structures of the linker and peptide fragment, have been synthesized (Enkvist *et al.*, 2006). The arginine-rich conjugates are able to inhibit several protein kinases *in vitro*, such as ROCK-II, PKA, Akt/PKB, etc, while showing moderate selectivity towards other basophilic protein kinases (Enkvist *et al.*, 2006).

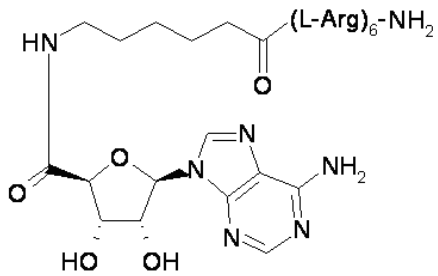

Figure 2. Structure of the adenosine-oligoarginine conjugate (ARC) containing adenosine-5'-carboxylic acid connected to the N-terminus of oligo-(L-arginine) via a linker chain.

The oligoarginine sequence of ARC ensures the membrane translocation ability and the high-affinity interaction of the conjugate with several protein kinases. The substitution of L-arginines with D-arginine residues resulted in ARC compounds with an increased stability to enzymatic degradation that showed even better cellular uptake than its L-arginine analogue (Enkvist *et al.*, 2006). The ARC-type compounds can enter cells of different origin and localize in the cytoplasm and nucleus (Uri *et al.*, 2002; Viht *et al.*, 2003). In addition, the biotin-tagged ARC is able to deliver fluorescently labelled avidin into cells.

Recently, the potency of the ARC conjugate with six arginine residues to modulate the activity of targeted kinases in living cells has been demonstrated (Räägel *et al.*, 2008). The ARC efficiently entered the cells and inhibited the formation of the actin cytoskeleton probably by interfering with the activity of Rho-associated serine/threonine kinase ROCK. Potentiation of the inhibitory effect of ARC by chloroquine, an inhibitor of the endosomal acidification, suggests the involvement of an endosomal pathway in the uptake of ARC, as indicated also before (Uri *et al.*, 2002; Viht *et al.*, 2003).

2. AIMS OF THE STUDY

In order to design and develop cell-penetrating peptides as efficient transport vectors for research and medicine, it is highly important to understand the mechanisms responsible for the internalization of CPPs. Hence, the primary interest of our research has been to elucidate and characterize the cargo-delivery mechanisms of transportan and its shorter analogue TP10. Although we have mainly examined the properties of transportans in delivering proteins to the cultured cells *in vitro*, several other cationic carrier peptides were also included in this study.

In addition to the main goal – to unravel the cargo delivery mechanisms of the transportans – the more specific aims of this thesis were:

- To characterize the membrane interaction, translocation mechanisms and cellular whereabouts of proteins coupled to cell-penetrating peptides transportan and TP10 (Paper I)
- To elucidate the transportan- and TP10-induced internalization pathways of proteins by immunocytochemical methods (Paper II)
- To compare the protein transduction ability of four most commonly used cell-penetrating peptides: penetratin, pTat, transportan, pVEC, and examine the influence of metabolic states of cells on the protein delivery efficiency (Paper III)
- To assess the cellular uptake of protein kinase inhibitors of adenosine-oligoarginine-type conjugates (ARC), and to characterize their potential as transporters for cellular delivery of proteins (Paper IV)

3. METHODOLOGICAL CONSIDERATIONS

The methods used in this study are described comprehensively in the respective papers. Here I only briefly comment on the choice of the particular methods used in the study underlying this thesis.

3.1. The cell-penetrating peptides used in the research

The membrane translocating peptide transportan has been in focus of studies of our research group since the discovery of its cell-penetrating and cargo-delivering abilities in 1998 (Pooga *et al.*, 1998a). For this reason, on the one hand, and because of its good vectorial properties, on the other, **transportan** is the main CPP that has been studied in this thesis. **TP10**, the shorter analogue of transportan with fewer side effects, is also included as an effective carrier peptide. In order to compare the cargo-delivery efficacy and mechanisms of transportans other peptides – **penetratin**, **pTat**, and **pVEC** – were selected as representatives of the most extensively studied CPP families. Peptides of the transportan family are amphipathic and less cationic than most of the other CPPs, like penetratin and pTat. The pVEC has, in addition to a highly positively charged region, a stretch of hydrophobic amino acids in N-terminus.

In paper IV we studied the cellular uptake of adenosine-oligoarginine conjugates (ARC) and the application as transporters for protein delivery. The ARCs are attractive for two reasons: their high positive charge and ability to deliver proteins into cells as well as their property to inhibit cellular protein kinases. However, a common characteristic for all the cell-penetrating sequences used in this study (presented in Table 1) is their ability to promote the cellular uptake of cargoes.

3.2. Cell cultures

Cell lines of different origins vary in their rate of metabolism, proliferation, protein expression, etc.

Four different cell lines were used for the experiments described in this thesis. **Human melanoma cells Bowes** originating from skin tissue were used in the earliest studies of transportan due to the presence of galanin receptor in these cells. Therefore, it was appropriate to conduct later experiments (in Paper I and IV) with transportan in the cell line already tested. Although it has been demonstrated that the cellular uptake of transportan does not depend on galanin receptors expressed by Bowes cells, we switched to using HeLa cells in further investigations (Paper I, II, III and IV).

The **HeLa** cells are derived from a human cervical carcinoma and have been used in a significant number of earlier studies of CPPs. Using the same cell line provides the possibility to complement and compare experimental data with other research groups in the field.

The **Jurkat cells**, an immortalized cell line of human T lymphocytes, and human hepatoma cell line **HepG2**, were used in parallel in experiments described in paper IV. The main reason to use cells of different origin was to ensure that the internalization of adenosine-polyarginine conjugates is a process that is a cell type independent.

Even though the cell lines have been considered an easily applicable model for many biological experiments, it is still not clear if the cells in a culture retain the properties typical in tissue conditions. Therefore, results based on experiments with cell cultures should be interpreted only with the greatest care. This is particularly true in the case of experiments with CPPs as transporters or drug carriers for a medical purpose.

3.3. Constructs of CPPs with cargo proteins

Different strategies for coupling cargo proteins to the carrier peptide have been applied, including direct synthesis of cargo and peptide sequence to the same polypeptide chain, recombinant expression of CPP-fusion protein, linkage by the labile disulphide bond, and covalent conjugation by bifunctional cross linkers. In this thesis, the selected CPPs were coupled to the proteins using a noncovalent interaction. We used biotinylated peptides and avidin, streptavidin, or neutravidin as the cargo tagged with fluorochrome or colloidal gold particles for facilitation of detection (Fig. 3). The complexes between biotinylated CPPs and these proteins are easy to form by simple mixing and co-incubation. Although noncovalent, the stability of these complexes is very high since biotin binds strongly to avidin, streptavidin, and neutravidin.

Avidin, an egg white glycoprotein, is heavily glycosylated which results in a high isoelectric point (pI > 10) and relatively high non-specific binding properties. A stable solution of gold-labelled avidin is not commercially available and we failed to produce avidin tagged with colloidal gold, probably due to its high pI value.

Streptavidin, a tetrameric protein from *Streptomyces avidinii*, binds biotin with the same affinity as avidin, but is less basic (pI 6.8–7.5) thus enabling tagging of this protein with colloidal gold. Unfortunately, streptavidin binds biotins coupled to CPP less efficiently than avidin, leading to the lower cellular uptake of complexes and necessitating the application of a higher concentration of the peptide.

In more recent studies (Paper II) we used the third biotin-binding protein – neutravidin. Neutravidin is an avidin derivative that contains no carbohydrate

and has a near-neutral isoelectric point (6.3), providing a very low nonspecific binding and a good applicability for gold-labelling.

All cargo proteins used in this study have four biotin-binding sites. Therefore we considered three aspects at choosing the optimal ratio between the concentration of biotinylated CPPs and cargo-proteins: (1) to achieve the detectable level of complexes in cells while avoiding cytotoxicity; (2) to occupy as many binding sites of protein with peptide as possible; and (3) to have no free peptide in the culture medium, which could compete with CPP-protein complexes in cellular uptake process. In addition, it is important to note that that transportan might multimerise in the solution. Therefore, the CPP-protein complexes may form bigger assemblies in or on the plasma membrane, and cells have to internalize particles, whose molecular weight exceeds that of one cargo protein.

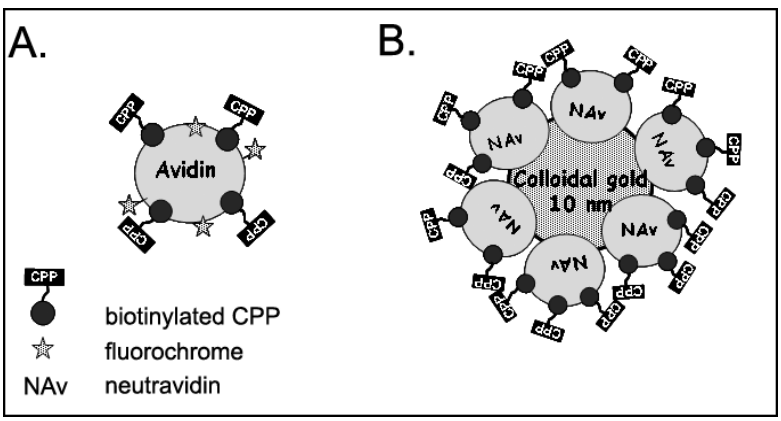

Figure 3. Schematic representation of differently labelled CPP-protein constructs used in this study. (A) In fluorescence microscopy experiments fluorochrome-labelled avidin is complexed with biotinylated CPPs. (B) For electron microscopy experiments the proteins (neutravidin or streptavidin) adsorbed to colloidal gold particle are complexed with biotinylated CPPs.

3.4. Delivery of CPP-protein complexes into cells

In order to estimate the ability of CPPs to deliver proteins into cells we used quantification by flow cytometry and spectrofluorimetry as well as qualitative monitoring by fluorescence and electron microscopy.

3.4.1. Quantification of the uptake of fluorescently labelled complexes

We used flow cytometry to quantify the amount of complexes of fluorescein-labelled avidin and biotinylated CPPs taken up by live cells. Although this method provides a fast and objective quantitative recording of the fluorescent signals from individual cells, the method can not distinguish between the signal from the inside of the cells and the signal of avidin-CPP complexes bound to the plasma membrane or to the extracellular structures. Therefore the extensive trypsinization of cells in order to degrade the non-internalized complexes is necessary in the flow cytometric measurements. Hence, in paper III we examined the effect of different duration of trypsin treatment on the liberation of the complexes from the cell surface.

Another aspect that has to be considered in the interpretation of results obtained by quantitative FACS analyses in living cells is the quenching of the fluorescence signal in a cellular environment. The quantum yield of the fluorescein emission is dramatically decreased at pH < 7, which is typical for endolysosomal organelles. Moreover, the fluorescence signal of the fluorescein-labelled CPPs is reduced several-fold upon binding to the DNA or negatively charged glycosaminoglycans (Ziegler and Seelig, 2007a). Therefore, the quenching of the fluorescence emission is very difficult to reliably quantify when studying the uptake efficiency and accumulation of CPP-cargo complexes to the subcellular compartments that have different pH.

The advantage of using a spectrofluorimeter is the ability to measure the cellular uptake of CPP-protein complexes in cell lysates in highly alkaline conditions, which is optimal for fluorescence emission and guarantees a reliable quantification. In flow cytometric measurements we use live cells that enable a comparison of the signals cell-by-cell. Thus, to determine the differences in uptake efficiency that might be due to the decreased fluorescence signal in the acidic vesicles recorded by FACS, we supplemented the fluorescence measurements on a flow cytometer with the quantification of the labelled protein on a spectrofluorimeter (Paper III).

3.4.2. Fluorescence microscopy

Although the methods to quantify delivery of CPP and the attached cargo gives us valuable information about the kinetics and efficiency of the internalization mechanism, it is necessary to use a microscopic examination in order to characterize the cellular localization of CPP-protein complexes. On the other hand, the reliability of the results obtained with fixed biological material, which is prerequisite for classical immunomicroscopy, has often been questioned. One of our tasks was to confirm that the distribution of complexes of fluorescein-

labelled CPPs and cargo-proteins in cells was not affected upon fixation with regularly used 4 % paraformaldehyde (Paper III). In paper I and II, we compared the localization of labelled CPP-protein complexes in relation to the different endosomal structures by using immunocytochemical staining of the marker proteins of the respective endocytotic routes.

In parallel with immunocytochemical methods, other approaches were used to examine the mechanisms of CPPs or CPP-cargo constructs, including inhibiting agents. In our studies (Paper III) we used low temperature (4°C) to decrease the fluidity of the plasma membrane and a hyperosmolar solution of sucrose to abolish the clathrin-dependent endocytosis. Since initially the translocation of the CPPs was reported to be an energy-independent process, we also examined the ability of CPP-protein constructs to internalize into energy-depleted cells, inhibiting the oxidative phosphorylation and glycolysis with sodium azide and deoxyglucose.

The first step in the cell translocation process of the CPPs or CPP-cargo complexes is the interaction with the plasma membrane. We used the extraction of cholesterol from the plasma membrane with methyl β-cyclodextrin to study the impact of integrity of membrane microdomains on the internalization ability of CPP-protein complexes.

To further specify the role of the membrane rafts we used the RNA interference mechanism. Small interfering RNAs, introduced into the cells, can suppress the expression of a gene of interest, making the siRNA a valuable research tool to identify the components necessary for particular cellular processes. In paper II we combined the colocalization experiments by fluorescence microscopy with RNA interference to assess the role of some endocytotic pathways initiated by caveolae and membrane rafts on the internalization efficiency of CPP-protein complexes.

Still, the most preferable method for studying the cellular distribution of CPPs and their constructs with cargo is the confocal microscopy in live cells, even when taking into account the better recordability of the location of complexes and the internal structures in the fixed cells. We used CLSM to map the localization pattern of CPP-protein complexes in relation to markers of cellular organelles. In the experiments with live cells, we used fluorescently labelled transferrin for studying clathrin mediated endocytosis, and we used LysoTracker for detecting lysosomes, and fluorescent ceramide complex for visualizing plasma membrane microdomains and the Golgi apparatus.

3.4.3. Electron microscopy

Electron microscopy has a number of advantages over the fluorescence microscopy. Even though the floursescence microscopy gives an overall view of the location of the molecules of interest in relation to the cellular structures or

other macromolecules, and can successfully be used for quantitative analysis, it has its limitations.

First, the resolution of light microscopy is not sufficient to distinguish smaller objects in a detailed manner, like the shape and size of the particles, membranes of internal structures of cells, as well as direct relation between these structures. Therefore, the separate structures or molecules in close proximity to each other that are labelled with different fluorescence dyes might erroneously be interpreted as colocalizing.

Secondly, the ratio of the signals from the fluorescently labelled markers and CPP-cargo complexes must be optimized with great care, in order to avoid signal cross-talk and background.

Therefore, we used electron microscopy techniques that provide a higher magnification and resolving power than a light microscopy. In order to characterize the interaction sites and localization of proteins delivered into cultured cells by CPPs, we applied a conventional transmission electron microscopy (TEM) for the best preservation of the morphology of cellular ultrastructure (Paper I and II). Briefly, for TEM the living cells were incubated with complexes of colloidal-gold labelled protein and CPP. The cells with respective complexes were then fixed and processed for flat embedding into epoxy resin. The flat embedding allows monitoring the largest view of the cell, since the sections from cells are cut in parallel to the substrate where cells are growing.

In addition to the characterization by morphology, we needed an immunocytochemical technique of EM for defining the endocytotic vesicular structures and for mapping of the internalization routes. The suitable immunocytochemical methods include traditional post-embedding electron microscopy of resin-embedded specimens and the immunolabelling of ultrathin frozen sections. The main drawback to these techniques is the incompatibility of immunochemical staining with good preservation of both antigenicity and ultrastructural morphology. Due to the lack of equipment for cryo-electron microscopy we applied a pre-embedding double labelling method using protein tagged with colloidal gold and nanogold particles in parallel, combined with a silver enhancement.

Application of two different sizes of gold tags has not often been used, since their distinguishing after the magnification of labels by a silver deposition is complicated. In our study (Paper II), we first incubated live cells with CPP-protein complexes labelled with colloidal gold (10 nm). The cells were fixed, mildly permeabilised, then incubated with primary antibodies and thereafter treated with the respective secondary antibody tagged with ultra small gold particles (1.4 nm).

The “classical” glutaraldehyde fixative and extensive detergent treatment necessary for the penetration of gold conjugates in pre-embedding techniques might lead to the major loss of ultrastructure and antigenicity (Hayat, 2000). Therefore we used PLP (Paraformaldehyde-Lysine-Periodate) fixative and a

mild permeabilization with 0.01 % saponin, which is sufficient to guarantee the accessibility of the antibody labelled with Nanogold™ to the intracellular antigens. The following silver enhancement step yields two types of particles: smaller and larger, which are quite well distinguishable. In principle, if both labels are in very close proximity, the silver-enhancement might mask different markers due to the formation of one particle instead of magnifying two particles. However, the antibodies used in our study labelled different regions of the endocytotic compartments than the CPP-protein complexes did, allowing a reliable detection of both tags in different endocytotic compartments.

4. RESULTS AND DISCUSSION

4.1. Transportan-protein complexes translocate into cells by different endocytotic routes (Paper I and unpublished data)

In paper I, our first objective was to perform the morphological/descriptive analysis of the transportan-mediated protein uptake in order to better understand the mechanism(s) responsible for it. In parallel with fluorescence microscopy we used the advantage of electron microscopy to gain insight into ultrastructural details of the interaction of transportan-protein complexes with the plasma membrane, the cell translocation, the intracellular distribution and the final localization/destination of proteins in the cells.

4.1.1. Transportan-protein complexes associate with membrane protrusions and actin cytoskeleton

Electron microscopy revealed that the complexes of gold-labelled streptavidin with biotin-transportan are detectable as large assemblies on the electron dense background between the cells and the filopodia. Since the transportan-protein complexes, which were formed in a cell-free culture medium, did not have such background and did not form big aggregates, we suggest that the background results rather from the association of complexes with extracellular components. Although we do not know yet the exact origin of this electron dense material surrounding the peptide-protein aggregates, it might represent the proteins of an extracellular matrix and/or cell surface proteoglycans that have interacted with CPPs. Considering the fact that heparan sulfates have been suggested as receptors for several positively charged CPPs, the involvement of heparan sulfates in cellular translocation of transportan-protein complexes is highly probable. Moreover, it has been suggested that the binding of ligands to HSPG induces aggregation and ligand clustering, so that CPPs are concentrated at the cell surface as dense aggregates for subsequent internalization (Ziegler *et al.*, 2005; Ziegler and Seelig, 2007b).

However, based on the observations of EM we distinguished two morphologically different modes of interaction between transportan-streptavidin complexes and the cell surface. As a prevailing mode, the aggregates of the peptide-protein complexes associated preferentially with filopodia and/or other extensions (Fig. 4A), but also with flat areas (Fig. 4B) of the plasma membrane in HeLa as well as in Bowes cells and induced the formation of membrane invaginations. The second interaction mode was typical for a smaller fraction of complexes containing only a few or even a single gold particle, which was in close contact with the plasma membrane at the flat areas of the cell surface and

did not induce detectable changes in the morphology of the plasma membrane. The different modes seem to depend on the concentration of complexes and the size/rate of the aggregation.

It is not clear yet, whether the transportan-protein complexes prefer to associate with the region of a high membrane activity, displaying numerous protrusions and extensions of the cell surface or if the membrane ruffling and the morphological changes are induced upon interaction of TP-protein complexes. However, the membrane extensions and protrusions associating with the transportan-protein complexes are supported by actin cytoskeleton as visualized with Texas Red-labelled phalloidin by fluorescence microscopy.

4.1.2. Internalization and localization of proteins delivered into cells by transportans

Different membrane interaction modes of transportan-protein complexes described above lead to different uptake mechanisms. First, the peptide-protein aggregates that induce the formation of membrane invaginations are further shifted deeper to the cytoplasm and engulfed finally into endocytotic vacuoles of irregular shape and varying diameter of 0.5 – 2.0 μm. In addition, the cell transduced transportan-protein aggregates resided also in other types of smaller vesicular structures, including small caveosome-like structures, multivesicular bodies, and rarely in clathrin-coated vesicles. The results obtained by confocal laser scanning microscopy revealed the localization of transportan-avidin complexes as smaller or bigger fluorescent punctuate structures suggesting vesicular uptake of complexes and confirming the EM data.

The involvement of various actin-dependent cell surface extensions in the translocation process of transportan-protein complexes, the following membrane invagination and localization in large heterogeneous vesicular structures are the features resembling at most the macropinocytosis of some pathogenic bacteria (Ammendolia *et al.*, 2004; Garcia-Perez *et al.*, 2003). In addition, macropinocytosis has been proposed to be a translocation route for several CPPs (El-Andaloussi *et al.*, 2007b; Magzoub *et al.*, 2006; Nakase *et al.*, 2007; Wadia *et al.*, 2004). However, the fact that transportan-streptavidin complexes are trapped also in vesicular structures that obviously belong to other endocytotic pathways, suggest that transportan-mediated protein delivery takes place by several different mechanisms concurrently. The contribution of different translocation pathways acting in parallel is probably not typical only for transportan-protein complexes. Recently, involvement of three different endocytotic pathways, which act in parallel, was suggested for antennapedia peptide, nona-arginine, and Tat peptide without cargo as well as for these CPPs coupled to cargo (Duchardt *et al.*, 2007).

The second mode of internalization, characteristic for smaller complexes that did not induce detectable changes in the plasma membrane upon interaction, was also detected in addition to the vesicular uptake. In this case single gold particles located freely in the cortical cytoplasm of HeLa and Bowes cells. However, the second type of internalization was detected only very rarely and we could never catch the moment of single particle penetrating through the intact plasma membrane despite the fine ultrastructural analysis. Therefore we can not exclude the possibility of artificial redistribution of transportan-protein complexes during the fixation and embedding for EM.

On the other hand, the translocation of small transportan-protein complexes via short-living pore-like structures as proposed for carrier peptides MPG, Pep-1 (Deshayes *et al.*, 2004; Deshayes *et al.*, 2006), and Tat peptide (Herce and Garcia, 2007) can not be ruled out. In line with the latter hypothesis is the simple model in which TP10 binds to the membrane surface without forming specific aggregates, and allows peptide monomers to insert transiently into its hydrophobic core due to the membrane perturbation by the mass imbalance, but not because of the formation of "hole" in the bilayer (Yandek *et al.*, 2007). Even though we do not know whether the uptake mechanisms and conformation/ structure of molecules characterized for CPPs alone could be valid also for CPP-cargo complexes, the model proposed for TP10 correlates with our findings that revealed the second type of internalization being more typical for TP10-protein than for transportan-protein complexes.

4.1.3. Protein transduction by transportans is temperature-dependent

Lowering of the incubation temperature below 18 °C, reduces the flexibility and fluidity of the plasma membrane, slows the membrane trafficking and blocks or drastically decreases the endocytosis. We used electron microscopy to obtain detailed information about the internalization of transportan-protein complexes at low temperature.

Consistent with the earlier studies of transportan-protein complexes (Pooga *et al.*, 2001; Säälik *et al.*, 2004), only a negligible amount of gold particles were associated with the plasma membrane after the incubation of cells with the transportan-streptavidin complexes on ice for 1 h, and no complexes were detected inside the cells. This suggests the involvement of energy-dependent processes as well as the effect of membrane fluidity on the internalization of CPP-protein complexes. On the other hand, the fact that the cellular translocation and vesicular transport of the transportan-streptavidin complexes was not completely blocked at temperatures of 8–10 °C, but the amount of internalized complexes had only decreased significantly, suggests that some uptake pathways are still active at low temperatures (Ziegler *et al.*, 2005).

4.1.4. Destination of vesicles mediating the uptake of transportan-protein complexes

The fact that the uptake of transportan-protein complexes into different vesicular structures was temperature-dependent pointed to the involvement of endocytotic pathways. In order to specify the intracellular trajectories and structures of endocytotic pathways that the CPP-protein complexes utilize to translocate into cells, we used double-labelling for visualization of the organelles involved in endocytosis and subsequent cellular trafficking of CPP complexes simultaneously. To find out whether the internalization of proteins by transportan or TP10 is mediated by the classical clathrin-dependent pathway, we used fluorescently or gold labelled transferrin.

The confocal microscopy observations demonstrated only a partial overlap of transportan-avidin complexes with the marker protein of clathrin-dependent endocytosis. A more detailed analysis by EM confirmed the fluorescence microscopy data revealing no marked colocalization of the transportan-streptavidin complexes with transferrin at the plasma membrane of HeLa cells. Still, some overlap between transferrin and transportan-protein complexes in a particular type of vesicular structures suggests that a fraction of complexes might co-internalize with transferrin or it may suggest that structures containing peptide-protein complexes fuse in later steps with vesicles (multivesicular bodies, late endosomes) of a clathrin-mediated pathway.

Whether the cell-transduced proteins are finally targeted to the cellular degradative pathway or remain in the cytoplasm is the question of the highest importance for the application of CPPs as delivery vectors for functional cargo proteins. The ultrastructural analysis revealed that transportan-streptavidin complexes accumulated in structures resembling late endosomes and lysosomes upon 20–27 h of incubation in Bowes cells. Staining of lysosomes with the antibody against lysosome-associated membrane protein-2 (LAMP2) or LysoTracker in fluorescence microscopy showed that the transportan-protein complexes concentrated mostly to late endosomes and lysosomal structures after 20 h.

Our unpublished data of electron microscopy correlate well with fluorescence microscopy observations confirming the localization of transportan/ TP10-protein complexes in vesicles labelled by LAMP2 after 24 h (Fig. 4E, arrow). However, a fraction of complexes-containing vesicles retained their localization distinct from the degradative organelles marked by LAMP2 antibody and no specific accumulation of the transportan-protein complexes to ER or Golgi network was observed by fluorescence or electron microscopy.

4.1.5. Transportan and TP10 can promote the endosomal release of proteins

The endosomal capture and lysosomal degradation of therapeutic proteins or other molecules, delivered into cells by CPPs, has been recognized as the main factor limiting their bioactivity. Hence, the biological activity of delivered molecules in cells is not merely dependent on their translocation efficacy but depends largely on the ability of CPPs to induce destabilization of endolysosomal membranes and promote the endosomal escape.

Despite the fact that a significant amount of proteins delivered by transportans concentrate to late endosomes/lysosomes (Fig. 4D, arrows), we presume that the transportan and TP10 have the potential to induce the endosomal escape of the cargo proteins.

Our first finding arguing for the membrane disturbance property of transportans was the observation that transportan-streptavidin complexes localized often in vesicles with a discontinuous membrane or localized apparently freely in the cytosol at close proximity to the vesicles with a broken membrane. These electron microscopy data that were obtained in Bowes cells at a relatively high peptide concentration (5 μM) were later confirmed, when we documented the analogous effect induced by the complexes of 3 μM TP10 and neutravidin (indicated by arrowheads and arrows in Fig. 4F) after 24 h of incubation with HeLa cells (unpublished data). However, during 1 h-incubation we have not observed the escape of complexes from vesicles into the cytosol.

These results permit us to assume that the endosomolytic properties of transportans manifest mostly upon longer incubation and at a higher concentration of peptides. More importantly, it has been shown that the type of cargo molecule and the structure of the CPP-cargo construct, as well as the cargo coupling strategy, seem to have the main implications for destabilization of endolysosomal membranes (Lundberg *et al.*, 2007). The different ability of transportans to induce the endosomal escape depending on the properties of cargo molecule could be suggested also based on the recent publications, where siRNA targeted into cells with TP10 did not have the expected biological effect (Lundberg *et al.*, 2007), whereas double stranded DNA delivered by TP10 retained its activity (El-Andaloussi *et al.*, 2005b).

4.2. Contribution of different endosomal pathways to the TP- and TP10-induced protein delivery (Paper II)

The results of paper I implicated that complexes of transportan or TP10 with protein can translocate into cells by different mechanisms in parallel. The continuously accumulating data about the complexity of the processes of endocytosis and involvement of its different types in CPPs' internalization mechanisms

necessitated a further specification of the endosomal pathways responsible for transportan-mediated protein delivery. In paper II we used antibodies against marker proteins of different endocytotic pathways and attempted to characterize the extent of different internalization routes in CPP-mediated protein delivery.

4.2.1. Caveolin-1-dependent pathway is the prevailing route for transportan-mediated protein delivery in HeLa cells

The involvement of a raft-dependent caveolar endocytosis in the Tat-mediated cargo delivery has been demonstrated earlier (Ferrari *et al.*, 2003; Fittipaldi *et al.*, 2003). Our results obtained by EM showed that TP/TP10-protein complexes enter cells often in small caveosome-like or non-coated vesicles (arrows in Fig. 4B and C). The immunofluorescence and immunoelectron microscopy analyses revealed that some TP/TP10-protein complexes were present in caveolin-1-positive vesicles. However, not all of the caveosomes contained TP/TP10-protein complexes and, surprisingly, the amount of caveolin-1-positive vesicles carrying CPP-protein complexes was slightly different when examined by EM or CLSM. By confocal microscopy, the colocalization of caveolin-1 and CPP-avidin complexes was detected mainly at the plasma membrane or in the cortical cytoplasm of the HeLa cells and the visual estimation gave about 30 % of the colocalization.

By electron microscopy, on the other hand, the caveosomes that contained CPP-neutravidin complexes were detected rather in the cytoplasm than underneath the plasma membrane and the degree of colocalization was lower (approx. 20 %). The ultrastructural analysis showed that the CPP-neutravidin complexes and caveolin-1 localized often in a very close proximity, which could be undistinguishable by CLSM and lead to the overestimation of colocalization.

Another explanation for the lower colocalization detected by EM could be the difference in pI value of neutravidin (pI 6.3) and avidin (pI 10). Avidin is more cationic and has a higher affinity to negatively charged components on the cell surface, which may facilitate the electrostatic interactions of complexes with the plasma membrane. As evident from FACS analysis, the down-regulation of caveolin-1 in the HeLa cells strongly inhibited the internalization of CPP-protein complexes. This suggests the caveolin-1-dependent pathway is one of the main routes for transportan-mediated protein delivery. Since the down-regulation of caveolin-1 expression affects also the turnover of cholesterol to the plasma membrane (Uittenbogaard and Smart, 2000), we can not exclude the impact of lower cholesterol concentration in the assembly of other types of membrane rafts putatively involved in the internalization of the CPP-protein complexes. However, it is known that in the absence of caveolin, the caveolar endocytosis is not blocked and the cholesterol-rich detergent-resistant membrane microdomains retain their capacity to invaginate and form endocytotic structures that are morphologically equivalent to the caveolae (Le *et al.*, 2002; Nichols, 2002).

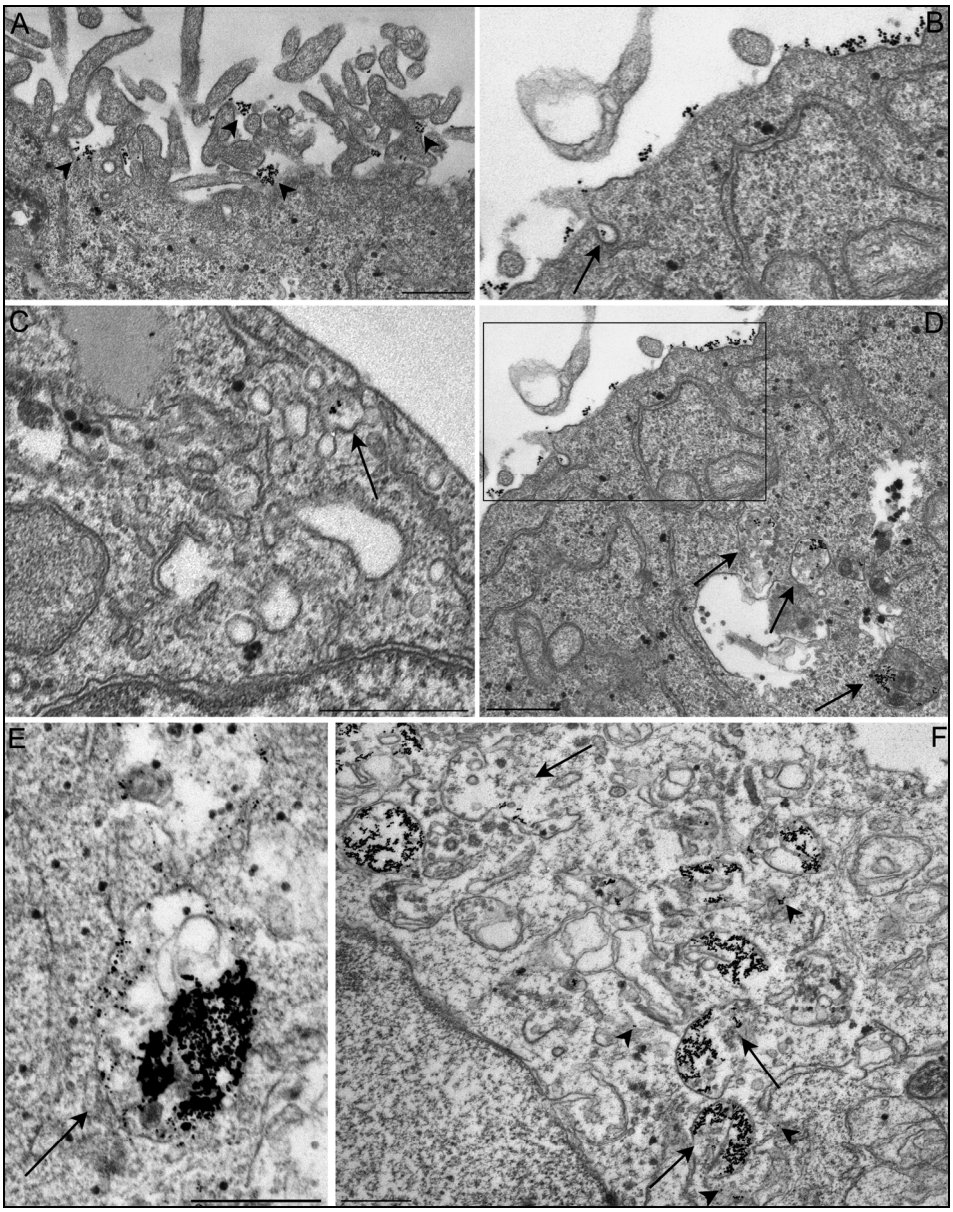

Figure 4. Interaction of transportan- and TP10-protein complexes with cell surface. Internalization and intracellular trafficking of complexes. (A-D) HeLa cells were incubated with complexes of neutravidin-gold (10 nm) and 3 μM biotinylated transportan for 1 h. B – enlarged section of D. **(E)** HeLa cells incubated with complexes of neutravidin-gold (10 nm, big gold particles) and 3 μM biotinylated transportan for 4 h, followed by a chase period of 20 h. Lysosomes were visualized by staining them with LAMP2 antibody and nano-gold-labelled (1.4 nm, small gold particles) anti mouse antibody. **(F)** Escape of neutravidin-gold (10 nm) complexed with 3 μM biotinylated TP10 from vesicles into the cytosol after 24 h of incubation in HeLa cells. Bar – 0.5 μm.

4.2.2. Flotillin-containing rafts do not participate in transportan-mediated protein delivery

Apparently, the caveolin-1-dependent pathway is not the only route for cell entry of transportan-protein complexes. As noted above, we have detected complexes of transportan and TP10 with protein also in other types of vesicular structures including macropinosomes and clathrin-coated vesicles (Paper I). In addition, a substantial fraction of small caveosome-resembling vesicles, which mediate the internalization of TP/TP10-protein complexes were not recognized by caveolin-1 antibodies. A novel marker protein for a particular type of lipid rafts, flotillin-1, was suggested recently (Glebov *et al.*, 2006).

Coassembly of flotillin-1 and flotillin-2 into microdomains induces membrane curvature, the formation of plasma-membrane invaginations morphologically similar to caveolae, and the accumulation of intracellular vesicles, as shown by ultrastructural analysis (Frick *et al.*, 2007). These results are in accordance with our electron microscopy observations and initiated us to address the question whether the microdomains organized by flotillins could mediate the cellular translocation of transportan-protein complexes. Unexpectedly, we could not find the colocalization of CPP-protein complexes neither with flotillin-1 nor with flotillin-2 on the plasma membrane or inside the cells by immunocytochemical analysis by both confocal and electron microscopy. Moreover, down-regulation of flotillin-1 using siRNA silencing rather increased than decreased the CPP-protein uptake, corroborating the microscopy results. Obviously, the role of flotillin-dependent endocytotic pathway in CPP-mediated protein delivery is not considerable.

The fact that complexes show equal or even better uptake in partially flotillin-1-depleted cells suggests that the non-raft areas of the plasma membrane might also play a role in CPP internalization mechanisms. Alternatively, the absence of flotillin-1 in plasma membrane may influence the cellular transport machinery and induce the uptake of TP/TP10-protein complexes by other pathways.

4.2.3. Transportan and TP10 bypass the early endosomes in delivering cargo protein

Our earlier results showed that after a long-time incubation the majority of the transportan-protein complexes accumulate in structures containing LAMP2 in perinuclear region and are probably targeted to degradation (Paper I). In order to find the possibilities for releasing cargo molecules before lysosomal degradation, we focused on the characterization of the early internalization steps of TP/TP10 avidin complexes.

We used the small GTPase Rab5 and PI3P as markers for early endosomes. The confocal microscopy analysis showed that only a negligible fraction of

complexes colocalized with Rab5 or PI3P. Moreover, the treatment of cells with wortmannin, a specific inhibitor of the phosphoinositide-3-kinases, did not inhibit the CPP-induced protein delivery into HeLa cells at a detectable level. This confirmes the independence of transportan-mediated protein delivery on the PI3P-regulated endosomal pathway.

However, the question how transportan-protein complexes are targeted into lysosomes without involvement of common endosomal pathways still remains to be answered. Considering the significant role of caveolae in the internalization of CPP-protein complexes, one might find similarity with the internalization mode of the SV40, which is delivered from caveolae to the endoplasmic reticulum, bypassing the endosomes and the Golgi apparatus (Pelkmans *et al.*, 2001). Finding that the SV40 colocalizes with β-COP, the common marker of both Golgi and caveosomes, but not with the more discriminating Golgi marker BODIPY-ceramide (Norkin and Kuksin, 2005), is consistent with our results, which showed no specific colocalization of CPP-protein complexes with BODIPY-TR-C_5 ceramide (Paper I).

Based on the results of paper I and paper II, we can conclude that the caveolae-mediated endocytosis gives a higher contribution than other known endocytotic pathways into TP/TP10-mediated protein delivery in HeLa cells. The clathrin-dependent endocytosis has a clearly minor role and macropinocytosis seems to prevail in Bowes rather than in HeLa cells (Fig. 5).

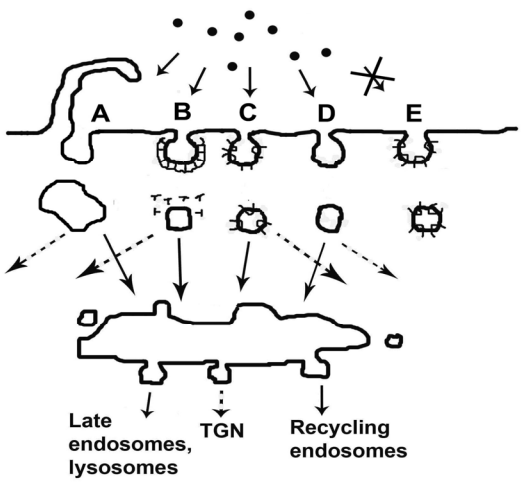

Figure 5. Endocytotic pathways used for cellular entry of proteins delivered by transportan and TP10. The complexes of peptide and protein can enter cells via (A) macropinocytosis, (B) clathrin-, or (C) caveolae-mediated endocytosis, and (D) clathrin- and caveolin-independent endocytosis. The flotillin-mediated pathway (E) is not used. Intracellular trafficking routes are indicated as solid arrows, putative directing is represented as dashed arrows. TGN – trans-Golgi network.

4.3. Comparison of protein delivery efficiency of different CPPs (Paper III)

In order to gain more information about the internalization mechanisms of cell-penetrating peptides and to quantitatively compare the carrier abilities of transportan with other CPPs, we studied the cellular uptake and cargo delivery kinetics of four commonly used cell-penetrating peptides: penetratin, pTat, transportan, and pVEC. The influence of metabolic and endocytosis inhibitors on the uptake efficiency of CPP-cargo complexes was examined to assess the involvement of endocytosis in the import mechanisms of cationic CPPs with cargoes.

Additionally, at that time the question concerning fixation artefacts was not less important for us. A year before this study, Richard *et al*. reported that even mild fixation could lead to the artefactual results in characterization of the uptake of CPPs (Richard *et al.*, 2003), which initiated the re-evaluation of cellular uptake mechanism of highly cationic peptides. In order to find out whether fixation of cells induces artefactual redistribution of CPP complexes with cargo molecule, we conducted a CLSM study. In contrary to the Tat peptide, the localization of transportan-avidin complexes was not influenced by fixation of cells with aldehyde.

4.3.1. CPPs vary in protein transduction ability

As evident from the FACS analysis, all CPPs studied in this paper were able to deliver FITC-labelled avidin into the HeLa cells. Protein delivery efficiency of the studied CPPs was the following: penetratin < pVec < Tat < transportan. Thus, transportan and pTat induced cellular uptake of protein more efficiently than penetratin or pVec.

Quantitative analysis of the cellular uptake of fluorescently labelled CPPs by flow cytometry requires trypsination to ensure that peptides associated with the plasma membrane are removed. Without this step, the detected protein levels were markedly higher than the actual protein uptake for all tested CPPs. The effect of an extensive trypsin treatment (15 min) on the liberation of the complexes from cell surface was most apparent for penetratin-avidin and pVec-avidin complexes, revealing their slower passage across the plasma membrane into the cytoplasm or better accessibility to the enzyme in the plasma membrane. The prolongation of the incubation time with CPP-avidin complexes up to 4 h increased the cellular fluorescence gradually, indicating a continuous internalization of CPP-avidin complexes, which were initially loosely bound to the plasma membrane. The uptake of Tat-protein complexes was slightly more favoured during the long-lasting incubation compared to complexes with transportan or other CPPs. Nevertheless, transportan showed a unique ability to

increase the protein uptake at high speed up to 24 h, while the other studied CPPs slowed the internalization considerably after 4 h.

4.3.2. FACS and spectrofluorimetry show different uptake kinetics of complexes

Using fluorescence-based methods, one of the concerns is the misinterpretation of results due to the decrease of fluorescence signal in acidic environment (Ziegler and Seelig, 2007a), which is typical for endolysosomal organelles. The importance of relevant analysis and critical assessment of CPP-mediated protein uptake in that respect becomes very evident in this study. The FACS analysis revealed deceleration in the uptake of fluorescein-labelled CPP-avidin complexes after 1 h, followed by an ongoing raise after 2 h. Spectrofluorimetry, instead, revealed linear increase in the uptake of fluorescent CPP-avidin complexes during the first 4 h. Moreover, spectrofluorimetry showed an about 2–3-fold difference in the transduction efficiency between pTat and penetratin, whereas FACS analysis suggested an almost 10-fold difference.

The discrepancy in the results is probably caused by the different experimental conditions applied to the cells. While by flow cytometry we measure the fluorescence in live cells, spectrofluorimetric measurements are carried out in cell lysates in alkaline conditions. The temporary deceleration in the cellular uptake detected by FACS could reflect the translocation of complexes into acidic compartment in cells and the ability of transportan and pTat to disrupt the acidic structures and escape into the cytoplasm more efficiently than penetratin or pVec upon longer incubation.

4.3.3. Protein delivery by CPPs is inhibited by low temperature and depletion of cellular energy

All endocytotic processes are blocked at low temperature and in energy-deficient cells (Nichols and Lippincott-Schwartz, 2001). As shown by flow cytometry, lowering the incubation temperature from 37 to 4°C decreased the fluorescence signal from cells for all CPP-protein complexes about 10-fold, except for pTat-avidin, which was less dependent on temperature. Moreover, we can assume that at low temperature the majority of complexes are not internalized, but remain associated with the plasma membrane, since the extensive trypsination removed about 80 – 90 % of FITC-labelled avidin from the membrane surface of HeLa cells. The effect of a low temperature on the uptake of CPP-protein complexes obtained by quantitative analysis is in good accordance with observations of qualitative monitoring by fluorescence and electron

microscopy (Paper I), confirming the mainly energy-dependent uptake process of proteins delivered by transportan.

The involvement of other energy-dependent pathways in the uptake of transportan-avidin complexes was corroborated by the depletion of cellular energy by inhibiting oxidative phosphorylation and glycolysis with the inhibitors of cellular metabolism, sodium azide, and deoxyclucose. Blockage of oxidative phosphorylation with sodium azide reduced the internalization of transportan-avidin complexes about 2 – 5-fold, while deoxyclucose affected the uptake less. The co-application of inhibitors had an additive inhibitory effect on the cellular translocation of CPP-avidin complexes leading to a decrease of about 3 – 6-fold. Still, a clearly detectable fraction of CPP-protein complexes remained associated with the cells in the presence of both inhibitors and at low temperature even after prolonged trypsination, suggesting that at least the step of interaction of peptide-protein complexes with cells is not completely dependent on the cellular energy.

4.3.4. Role of clathrin-mediated endocytosis and cholesterol of plasma membrane in cellular translocation of CPP-protein complexes

The inhibition of the most efficient pathway, clathrin-dependent endocytosis, by using the hyperosmolar sucrose containing medium decreased the cellular uptake of FITC-labelled avidin complexed with CPPs only slightly, about 1.5-fold, as revealed by the FACS analysis. The internalization of penetratin-avidin complexes was somewhat exceptional showing an approximate 3-fold decrease under hyperosmolar conditions. However, the minor inhibition of the internalization of CPP-avidin complexes in the hyperosmolar medium indicates that the clathrin-dependent endocytosis is neither the only nor the major uptake mechanism used by CPPs and other clathrin-independent pathways are also involved. The co-localization experiments with transferrin, performed in paper I, corroborated the minor role of clathrin-dependent endocytosis in the protein delivery by transportan.

Cholesterol in cells has multiple roles. It has been reported that caveolae- and raft-mediated pathways can be selectively blocked by depletion of cholesterol (Simons and Toomre, 2000). In addition, the cholesterol removal disorganizes not only the membrane microdomains but also deeply invaginated clathrin-coated pits (Rodal *et al.*, 1999) as well as inhibits membrane ruffling and macropinocytosis (Grimmer *et al.*, 2002). Treatment of HeLa cells with methyl-β-cyclodextrin decreased the delivery efficiency of different CPPs to a different extent as demonstrated by the FACS analysis. Although the staining of cells with filipin indicated that not all of the cholesterol was removed from the plasma membrane, the uptake of avidin complexes with penetratin and pVec decreased about 2-fold, whereas that of transportan and pTat complexes by only

about 20 %. Even though the cholesterol extraction affects multiple cellular processes, we suggest that the organization of the membrane lipids is essential for protein delivery mediated by transportan and other CPPs.

4.4. Adenosine-oligoarginine conjugates mediate the cellular delivery of proteins (Paper IV)

Paper IV was published in collaboration with group of Dr. A. Uri, who had developed the adenosine-oligoarginine-type conjugates (ARC) – bisubstrate analogue inhibitors for basophilic protein kinases. The ARCs contain moieties targeting the ATP binding site, adenosine-5'-carboxylic acid, and oligo-(L-arginine) directed to the protein substrate domain (Loog *et al.*, 1999). Our contribution to this study was to assess the cellular uptake of a fluorescently labelled ARC derivative and its complex with avidin in order to estimate the applicability of these protein kinase inhibitors for further experiments with living cells. In analogy with many other CPPs, adenosine-polyarginine conjugates have a high positive charge and a hydrophilic nature.

Fluorescently labelled ARC (ARC-BODIPY) was able to penetrate into different cell types, distribute diffusely over the cytoplasm, and enter into nuclei. In addition, localization to the reticular and vesicular structures, preferentially in HepG2 cells, was also observed. Co-application of ARC-BODIPY with a membrane-specific dye FM 1–43 or MitoTracker Red revealed different localization of these compounds in relation to each other, suggesting that ARC-BODIPY does not concentrate specifically into endosomal-lysosomal structures and mitochondria.

Next we addressed the question of whether the adenosine-oligoarginine conjugates could also serve as transporters for cellular delivery of proteins. We used non-covalent complexes of biotinylated ARC derivative with avidin-FITC, which were detectable in the plasma membrane already in 5–10 min after their application to the culture medium of cells. The peripheral localization of internalized complexes in the beginning of cellular translocation changed to more perinuclear within 1–2 h. In contrary to ARC-BODIPY, the ARC-avidin complexes did not show a diffuse uniform distribution in cells, but retained the vesicular-granular localization pattern mainly suggesting the prevalence of endocytotic uptake. However, despite the similar localization pattern of ARC-avidin and membrane specific marker FM 1–43, different populations of vesicular and tubular structures seem to mediate their internalization.

The ability of the ARC-type protein kinase inhibitors to cross cell membranes in a cell type independent manner and to deliver protein cargo into cells resembles the properties of several cationic CPPs. This prompted us to examine how this process depends on temperature. Lowering the incubation temperature

to 4 °C did not abolish the internalization of ARC-BODIPY, but decreased significantly the translocation of ARC-avidin complexes. Similarly to transportan-protein complexes, ARC-avidin conjugates retained their ability to associate with the plasma membrane and, to some extent, localize to peripheral cytoplasm, but were not able to move towards the cell centre. This suggests that the uptake of the ARC compounds is mediated by endocytotic pathways, which in turn are dependent on the membrane fluidity and requires cellular energy.

We observed that the cellular uptake of adenosine-oligoarginine conjugates with four arginine residues was more efficient than that of fluorescein-labelled tetra-arginine (Futaki *et al.*, 2001; Mitchell *et al.*, 2000; Wender *et al.*, 2000). Considering the planar structural similarity between the AdoC moiety of ARC and the tryptophan residue in penetratin (Derossi *et al.*, 1994), it is possible that analogous to tryptophan, adenosine facilitates the transport of ARC-type conjugates into cells. To the best of our knowledge, this was the first study where the ability of highly charged protein kinase inhibitors, such as ARCs, to enter living cells was demonstrated and their applicability for regulation the processes in living cells has been demonstrated by now (Räägel *et al.*, 2008).

SUMMARY AND CONCLUSIONS

The mechanisms used by CPPs for cell entry are more complex than was originally thought. Instead of one universal internalization route, the CPPs use several mechanisms, which often act in parallel. This study focused on the characterization of the protein cellular delivery by transportan and its shorter analogue TP10. In addition, we described the cellular entry of the ARC-type protein kinase inhibitors, which were designed using the oligoarginine moiety.

We studied the cellular transduction mechanisms of proteins using complementary methods, such as transmission electron microscopy, live cell confocal microscopy and quantification of the uptake by FACS and fluorimetry. In parallel, we used the inhibitors of endocytosis, tracer molecules for endocytotic routes, and organelle specific antibodies.

All the studies we have performed demonstrate that transportan and TP10 deliver proteins into cells mainly by inducing endocytosis. We were able to map three known endocytotic pathways which, to different extents, contribute to transportan-mediated protein delivery and one route, which does not contribute. Also, the concentration of CPPs as well as the type of cells seems to be important factors in determining the uptake mechanism of transportan-protein complexes.

To summarize the results presented in this thesis, we conclude the following:

1. The internalization of protein complexes with cell-penetrating peptides (transportans, penetratin, pTat, pVec) is dependent on the cellular energy, the temperature, and the organization of the membrane lipids.
2. The cellular delivery of proteins by transportan and TP10, is mediated by different types of vesicular structures, suggesting the involvement of different endocytotic mechanisms:
   - The **caveolin-1-dependent endocytosis** contributes to a higher extent than other uptake pathways to transportan and TP10-mediated protein delivery in HeLa cells.
   - The **clathrin-dependent endocytosis** has a minor role in transportan- and TP10-induced protein delivery.
   - The cellular uptake of transportan- and TP10-protein complexes by **macropinocytosis** is more typical for Bowes than for HeLa cells.
3. **Flotillin-containing rafts** do not participate in the transportan- and TP10-induced protein delivery.
4. A small fraction of protein-CPP complexes enters cells by a **non-vesicular mechanism**, which is more typical for TP10 than for transportan.
5. The cargo proteins delivered into cells by transportan and TP10 bypass the early endosomes, but most of them concentrate to degradative organelles later.
6. Transportan and TP10 can induce the endosomal escape of the cargo proteins upon longer incubation and at a higher concentration of peptides.

7. A comparative study of different CPPs shows that transportan and pTat induce cellular uptake of protein more efficiently than penetratin or pVec.
8. The ARC-type protein kinase inhibitors have properties characteristic for CPPs: they enter living cells and can deliver proteins by an energy-dependent vesicular pathway.

SUMMARY IN ESTONIAN

Valkude suunamine rakkudesse transportaanide abil

Transportaan on 27 aminohappest koosnev kimäärne peptiid, mille N-terminaalne osa pärineb neuropeptiid galaniinist ja C-terminaalne osa vastab herilasemürgis leiduva peptiidi, mastoparaani järjestusele. Transportaani 13. positsioonis paikneva lüsiini kõrvalahelasse saab siduda teisi molekule, mis on transportaani uurimisel oluline nii erinevate märgete kui last-molekulide liitmiseks. Transportaani analoog TP10, millel puudub kuus N-terminaalset aminohapet, ei pärsi erinevalt transportaanist kõrgetel kontsentratsioonidel summaarset GTP-aasset aktiivsust rakumembraanides ning mõjutab raku elutegevust seega minimaalselt. Oma võime tõttu läbida plasmamembraani, tungida erinevatesse rakutüüpidesse ning transportida rakkudesse teisi molekule, kuuluvad transportaan ja TP10 rakku penetreeruvate peptiidide perekonda.

Rakku penetreeruvateks peptiidideks (RPP) nimetatakse rühma erineva päritolu ja primaarstruktuuriga suhteliselt lühikesi (5–40 ah) peptiidseid järjestusi, mille ühiseks omaduseks on võime siseneda rakkudesse ning indutseerida nendega kovalentselt või mittekovalentselt seotud molekulide liikumist rakkudesse. Vaatamata arvukatele uuringutele, pole seniajani üheselt kindlaks tehtud, milliseid mehhanisme RPP-d rakkudesse sisenemiseks ning oma last-molekulide transportimiseks kasutavad. Esialgsed hüpoteesid, mis kirjeldasid RPP-de internaliseerumist kui ATP energiast sõltumatut rakku tungimist otse läbi plasmamembraani, on nüüdseks asendunud arusaamaga, mille kohaselt sisenevad need peptiidid rakkudesse peamiselt erinevaid endotsütootilisi protsesse kasutades. Kuigi taoliste katioonsete peptiidide rakkudesse sisenemise mehhanismid pole veel lõplikult selged, peetakse nende kasutamist tulevikus efektiivseks ja mitteinvasiivseks võimaluseks transportimaks rakkudesse erinevaid funktsionaalseid molekule ja ravimeid nii biotehnoloogias, geeniteraapias kui meditsiinis.

Käesoleva väitekirja peamiseks eesmärgiks oli selgitada transportaani ja TP10 poolt vahendatud valgu transpordimehhanisme erinevates rakutüüpides. Selleks kasutasime biotinüleeritud transportaani, mis moodustab väga stabiilse kompleksi avidiini, streptavidiini ja neutravidiiniga. Esialgne analüüs ultratsruktuuri tasemel näitas, et peptiid-valk kompleksid seostuvad sageli, kuid mitte alati, filopoodide ning väiksemate rakujätketega. Sõltuvalt transportaani kontsentratsioonist ning peptiid-valk komplekside suurusest, indutseerib edasine interakteerumine rakupinnaga kaht erinevat tüüpi internaliseerumist: (1) enamikel juhtudel põhjustavad omavahel koondunud kompleksid plasmamembraanis sissesopistumist ning peptiid-valk kompleksid satuvad neist moodustuvatesse rakusisestesse vesiikulitesse; (2) väikesed transportaani/TP10 ja valgu kompleksid on aga tõenäoliselt võimelised läbima plasmamembraani, põhjusta-

mata selles olulisi morfoloogilisi muutusi ning paiknema vabalt kortikaalses tsütoplasmas.

Raku metaboolse energia defitsiidi korral ning temperatuuri alandamisel väheneb märgatavalt transportaanide poolt indutseeritud valgu transport rakku, mis viitab energiast sõltuvatele internalisatsiooniprotsessidele. Klatriin-sõltuva endotsütoosi inhibeerimine hüperosmolaarse söötmega ja kolesterooli eemaldamine plasmamembraanist vähendab valkude rakkudesse sisenemise efektiivsust, kuid ei peata seda täielikult. Eelpool kirjeldatud tulemuste põhjal võib väita, et valkude sisenemine rakkudesse toimub valdavalt endotsütoosi teel.

Põhjalikum immunotsütokeemiline analüüs erinevate endotsütoosi markervalkudega näitas, et transportaan-valk kompleksid sisenevad HeLa rakkudesse tõenäoliselt mitmete endotsütoosi radade kaudu samaaegselt. Kõige suurem osakaal RPP-dega vahendatud valgu transpordis on kaveoliin-sõltuval endotsütoosil. Klatriin-sõltuva endotsütoosi teel siseneb rakkudesse vaid minimaalne osa kompleksidest ning flotilliini sisaldavad membraani mikropiirkonnad ehk ”parved” (rafts) ei osale transportaanidega vahendatud valgu transpordis. Makropinotsütoosi teel valkude sisenemist on transportaan ilmselt võimeline indutseerima vaid kõrgetel kontsentratsioonidel ning pigem Bowes kui HeLa rakkudes. Seega indutseerivad transportaan ja TP10 valkude internaliseerumist vähemalt kolme erineva endotsütoosiprotsessi kaudu samaaegselt (joonis 4), kusjuures erinevate mehhanismide osakaal sõltub nii rakutüübist kui peptiidi kontsentratsioonist. Lisaks kolmele tuvastatud endotsütoosiprotsessile siseneb teatav osa kompleksidest vesiikulite kaudu, mida meil seni pole immunotsütokeemiliselt õnnestunud defineerida. Seega on tõenäoline, et transportaan-valk kompleksid kasutavad rakkudesse liikumiseks veel neljandatki mehhanismi – sisenemist nn. „katmata” vesiikulite kaudu.

Valkude edasine rakusisene suunamine ja lõplik saatus ei ole veel kaugeltki selge. Uurimuse tulemused näitavad, et kuigi transportaan-valk kompleksid ei läbi klassikalist endosomaalset rada, satub valdav osa neist pikema aja jooksul lüsosomaalsetesse organellidesse, kus nad suure tõenäosusega degradeeritakse. Siiski, seniste avaldamata andmete põhjal on alust oletada, et transportaanil on võime destabiliseerida vesiikulite membraane ning indutseerida lastmolekulide vabanemist tsütosooli.

Viimases väitekirja aluseks olevas artiklis on käsitletud adenosiin-oligoarginiini konjugaatide (ARC) rakku sisenemise ning avidiini transportimise mehhanismi. Tegemist on bisubstraatse proteiinkinaaside inhibiitoriga, mis sisaldab efektiivset rakku penetreeruvat järjestust nelja arginiini näol. Töö tulemused näitavad, et lisaks kinaase pärssivale aktiivsusele võimaldab ARC sarnaselt transportaanile ning paljudele teistele RPP-dele makromolekulide efektiivset rakku transportimist.

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Eriline tänu minu armsale pojale, kelle siiras lapsemeelsus on aidanud mul elutervema ja tasakaalukama kõrvalpilguga oma doktoranditegemistele vaadata.

ORIGINAL PUBLICATIONS I–IV

I

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The endosomal pathways involved in transportan- and TP10-induced protein delivery

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ABSTRACT

Cellular delivery of large bioactive cargoes with cell-penetrating peptides (CPPs) takes place mostly via endocytic processes. Here we map the cellular pathways used by transportan and transportan 10 (TP10) in protein transduction in HeLa cells. We used caveolin-1, flotillin-1, Rab5, and PI3P as marker compounds to determine the involvement of different endosomal pathways in protein uptake process. Confocal laser scanning and electron microscopy demonstrate that 20–30% of avidin/neutravidin complexed with biotinylated transportans colocalizes with caveolin-1, a caveolar marker, but only a negligible overlap with the raft marker flotillin-1 and early endosomal markers Rab5 and PI3P can be detected. Partial downregulation of caveolin-1 by siRNA treatment inhibits the CPP-mediated protein uptake by 50% but the lack of flotillin-1 rather increases than decreases the CPP-mediated protein transport. We demonstrate that caveolin- and clathrin-mediated endocytosis and macropinocytosis contribute to the cellular entry of transportan/TP10-protein complexes in various ratios, and that the caveolin-mediated pathway plays the main part among the observed endocytic pathways.

Key words: Cell-penetrating peptides, endocytosis, flotillin-1, caveolin-1, macropinocytosis

Abbreviations: TP – transportan, TP10 – transportan 10, GFP – green fluorescent protein, siRNA – small interfering RNA, FACS – fluorescence-activated cell sorter, PI3P – phosphatidyl inositol-3-phosphate, TEM – transmission electron microscopy, CLSM – confocal laser scanning microscopy

INTRODUCTION

The cellular transport of bioactive but low-permeable molecules using cell-penetrating peptides (CPPs) has been under intense investigation as an alternative mode for drug delivery for past fifteen years. During this period the permanently growing amount of data about peptides with putative membrane translocating abilities has led to the broadening of the meaning of the term CPP towards less uniform nature and characteristics (1). Currently a CPP is defined as a relatively short peptide being capable of gaining access to the cell interior by means of different mechanisms, including endocytosis, and having the capacity to promote the intracellular delivery of covalently or non-covalently conjugated bioactive cargoes. The main focus of CPP studies has now shifted to the specificity of targeting and functionality of the delivered cargo. However, understanding of the detailed cell entry mechanism(s) is still of great importance as the mode of internalization can become a critical obstacle in addressing the cargo attached to CPP.

Numerous reports indicate the prevalence of vesicular internalization mode as the pathway utilized by peptide-cargo constructs. In parallel, non-vesicular cellular entry is shown to be the major mechanism for CPPs themselves in some conditions (2–4). However, in all of the above mentioned studies the peptide was not carrying a cargo but a fluorescent tag and the observed diffuse cellular staining, which is thought to reflect non-vesicular internalization mode, was detected only at high peptide concentrations. Moreover, the rapidly accumulating knowledge about the mechanisms of endocytosis in general urges more exact definition of different types of vesicular transport pathways involved in CPP mediated cargo delivery.

Clathrin-mediated endocytosis (CME) is the most common mechanism for downregulation of a variety of plasma membrane receptors. Although different receptors may utilize highly specifically regulated mechanisms, the CME seems to be the general route of uptake (5–7). Without the specification of the exact mode of CME, this pathway has been shown to be active in CPP-mediated cargo delivery in several experimental systems, although with different efficacy (8, 9). The uptake via caveolae has also been demonstrated to participate in CPP-mediated cargo delivery. Caveolae are abundant in some cell types and their functions extend from endocytosis to cell signaling (10). The internalization of Tat-GFP fusion protein has been demonstrated to proceed using caveolar pathway (11, 12), but has also been reported to use macropinocytosis (13). Macropinocytosis is thought to occur in cells in response to certain stimuli (14) and it is reported that CPP entry activates the cellular signalling cascades and subsequent macropinocytosis in analogy to growth factors as stimuli (15, 16). The least characterized type of vesicular transport, clathrin- and caveolin-independent endocytic pathway, has been described for pathogen internalization as well as for physiological processes. Simian virus 40 (SV40) has been shown

to enter Cav-1 knockout cells, which also express dominant-negative Eps15, interfering with the assembly of the clathrin-coated pits and the formation of caveolae, where the internalization was demonstrated to be cholesterol-, microtubule- and Arf1-dependent (17). Moreover, the internalization of cholera toxin b subunit (CtxB) is not inhibited in cells depleted of functional dynamin and Arf6, where it takes place by inducing of the tubular network that is not detected in cells possessing the dynamin and Arf6 activity (18).

The network formation dependence on cholesterol (18) allows to assume that lipid rafts play a significant role in this type of cellular trafficking. However, the lack of adequate methods to investigate membrane dynamics in living cells at the level of lipids limits the studies to using mainly the raft-associated marker proteins (19). One such membrane-organizing protein suggested to be a marker of particular type of lipid rafts is flotillin. Endocytosis of CtxB subunit via flotillin-positive domains takes place independently of dynamin and the formed vesicles do not contain proteins characteristic to CME or caveosomes (20).

We have described the cellular routing of complexes of transportan and TP10 with protein earlier (9, 21). However, a fraction of observed vesicles containing complexes of protein and transportan/TP10 could not be identified by any of the used cellular markers and endocytic inhibitors. Since both the novel endocytic pathway and the CPP-mediated cellular delivery are cholesterol dependent and not overlapping with clathrin- or caveolin dependent route, we analyzed here the cellular trafficking mode of complexes of transportan/TP10 and avidin/neutravidin in relation to flotillin-1 marker. We also included caveolin-1 in the studies, as a marker of specific type of membranous rafts in cells lacking the typical caveolae. In addition, the cellular localization of complexes in relation to early endosomal markers Rab5 and phosphatidyl-inositol-3 phosphate (PI3P) was mapped in order to disclose the early steps of transportan-mediated delivery. The expression of flotillin-1 and caveolin-1 was downregulated using siRNA, to further elucidate the role of cholesterol rich membrane microdomains and the respective proteins in transportan/TP10 mediated protein transport. The Tat peptide (pTat) was included to the studies as one of the best described CPPs.

MATERIALS AND METHODS

Reagents

For immunomicroscopy experiments the following antibodies were used: mouse monoclonal anti-PI3P (Echelon Biosciences, UT), rabbit polyclonal anti-caveolin1 (BD Transduction Laboratories, Belgium), anti-Rab5 (AbCam, UK), and polyclonal anti-flotillin-1 (Santa Cruz Biotechnology, Heidelberg, Germany). Alexa Fluor 488-conjugated anti-mouse and anti-rabbit antibodies, Texas Red-avidin and Oligofectamine were bought from Invitrogen (UK). FITC-labeled avidin, saponin and sodium thiosulfate pentahydrate were from Sigma-Aldrich and Nanogold-Fab' fragments of goat anti-rabbit IgG and HQ Silver kit were purchased from Nanoprobes (Yaphank, NY). siRNA against flotillin-1, caveolin-1 and nonspecific siRNA were acquired from Santa Cruz Biotechnology.

Peptide Synthesis

Transportan (GWTLNSAGYLLGKINLKALAALAKKIL-NH_2), TP10 (AGYLLGKINLKALAALAKKIL-NH_2), and pTat (GRKKRRQRRRPPQ-NH_2) were synthesized stepwise on a 0.1 mmol scale on an automated peptide synthesizer
(Applied Biosystems Model 431A) using the *t*-Boc solid phase peptide synthesis strategy. *tert*-Butyloxycarbonyl amino acids were coupled as hydroxybenzotriazole esters to a *p*-methylbenzylhydrylamine resin (Neosystem, Strasbourg, France) to obtain C-terminally amidated peptides. Biotin was coupled manually to the side-chain of Lys13 or Lys7 in transportan and TP10, respectively, or to N-terminus of pTat. The final cleavage and purification of peptides was performed, as described earlier (9). The molecular weights of biotinylated peptides were determined by MALDI-TOF mass spectroscopy (prO-TOF 2000, PE Biosciences) and calculated molecular weights were obtained each time.

Cell Culture

Human cervical carcinoma cell line HeLa (ATCC CCL-2) was cultured in a humidified atmosphere containing 5% CO_2 at 37°C in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, Invitrogen, UK) supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories, Austria), 100 IU/mL penicillin and 100 µg/mL streptomycin (Gibco, Invitrogen, UK).

Confocal laser scanning microscopy

5 x 10^4 HeLa cells were seeded one day before experiments onto round glass coverslips (Ø 12 mm, Menzel-Glazer, Germany) in 24-well plate. Cells were incubated with 0.15 µM Texas Red labeled avidin and 0.5 µM biotinyl-CPP in serum-free IMDM at 37 ºC for 30 min. After incubation the medium with CPP-

avidin complexes was removed, the cells were rinsed twice with PBS and fixed for two hours either with PLP fixative (2% formaldehyde, 0.01 M sodium periodate, and 0.075 M lysine-HCl in 0.075 M phosphate buffer, pH 7.4) or with 4% paraformaldehyde for 30 min for staining with flotillin-1/Rab5 or PI3P/caveolin-1 antibodies respectively. The cells were permeabilized either with 0.1% Triton X-100 (anti-caveolin), 0.5% saponin (anti-PI3P) for 5 min or with a solution of 0.01% saponin and 0.1% BSA in sodium phosphate buffer for 8 min, which also acted as blocking solution (anti-flotillin1, anti-Rab5). Nonspecific binding sites were blocked with 10% w/v non-fat dry milk solution in PBS for 1h (anti-caveolin) or with 10% heat-inactivated goat serum (anti-PI3P). After treatment with the respective fluorochrome labeled secondary antibodies the coverslips were mounted with 30% glycerol in PBS on preparate glasses. Images were obtained with Nikon EZ-Z1 confocal microscope using exitation at 488 nm (for FITC and Alexa Fluor 488) and 543 nm (for Texas Red). Images from different channels were recorded separately to avoid crosstalk. Obtained images were processed with Adobe Photoshop 7.0

siRNA experiments

Cells were grown in 6- or 12-well culture plates and transfected with duplex RNA oligonucleotides against flotillin-1 or caveolin-1, or non-specific siRNA using oligofectamine (Invitrogen), according to the manufacturer's instructions in two consecutive days. On day 4 cells were incubated with peptide-protein complexes at the same conditions as for confocal microscopy experiments. The complexes-treated cells were trypsinized, fixed with PLP fixative for 2 h and analyzed by FACS. To check the efficiency of siRNA cells were detached by scraping, fixed with PLP fixative and permeabilized with 0.01% saponin and 0.1% BSA in 0.1 M sodium phosphate buffer (pH 7.4) for 8 min. Permeabilized cells were stained with primary antibodies against flotillin-1 or caveolin-1 and subsequently with appropriate fluorochrome labeled secondary antibodies in the same buffer supplemented with 2 mM EDTA.

Electron Microscopy

HeLa cells were seeded onto glass coverslips in 24-well plates and grown to 90–100% confluency. Cells were incubated with complexes of biotinylated transportan, TP10 or pTat and colloidal gold-labeled neutravidin at 37°C for 1 h. CPP-protein complexes were preformed by incubating neutravidin-gold (d 10 nm, 1:100 or 1:200 dilution) (21) with biotinyl-CPP at room temperature in a minimal volume for 5 min. Solution with complexes was diluted with culture medium to the final peptide concentration of 1.5–3 μM according to experiment and applied to cells.

Pre-embedding immunolabeling

The cells treated with CPP-neutravidin complexes were fixed for immuno-electron microscopy with PLP fixative at room temperature for 2 h. Cells were permeabilized using 0.01% saponin and 0.1% BSA in sodium phosphate buffer for 8 min. After permeabilization, the cells were first incubated with caveolin-1 (1:50) or flotillin-1 antibodies (1:30) for 1 h and treated with 1.4 nm nanogold-conjugated Fab' fragment against rabbit IgG diluted to 1:60 for an additional 1 h. The nano-gold label was magnified by silver enhancement according to manufacturer's protocol for 2–5 min, followed by gold-toning with gold chloride. Briefly, for gold-toning cells were washed with 2% sodium acetate for 3×5 min, treated with 0.05% gold-chloride on ice for 10 min and washed with 0.3% sodium thiosulfate pentahydrate on ice for 2×10 min. After postfixation with 2% osmium tetroxide in the 0.1 M sodium cacodylate buffer cells were dehydrated and embedded in epoxy resin. Ultrathin sections were cut in parallel with the coverslip, poststained with uranyl acetate and lead citrate, and examined with a JEM-100S (JEOL, Japan) or FEI Tecnai 10 (Philips) transmission electron microscope at 80 kV. The scanned electron microphotos were analyzed and processed with Adobe Photoshop 7.0.

RESULTS

Transportan and TP10 do not direct the cargo protein to flotillin-1-dependent endosomal pathways.

To unravel the internalization mode of transportan-mediated cargo delivery, we mapped the localization of the peptide-protein complexes in relation to flotillin-1, previously described as a marker of specific type of lipid rafts (20). The flotillin-1 (Flot-1) positive areas showed almost no overlap with complexes of avidin and biotinylated transportan or TP10 (Fig. 1*A-B*). Very low colocalization could be detected inside cells whereas some overlap was observed at the plasma membrane after 30 min incubation with peptide-protein complexes. At the plasma membrane, colocalization was mostly detected as a smaller flotillin-marked focuses covered with bigger irregular peptide-protein aggregate (Fig. 1*B-C*). The loci of flotillin-1 were detected as a dense punctate staining on the plasma membrane, in the cytoplasm and on the cellular microspikes while the majority of complexes assembled into larger structures, which were associated with the outer membrane, as detected by confocal microscopy (Fig. 1*A–C*).

In parallel to confocal microscopy (CLSM) we used transmission electron microscopy (TEM), which allows identification of cellular structures by morphology at the higher resolution level. The complexes of biotinylated CPP and neutravidin were detected as bigger particles, which localized mostly to the extracellular side of the plasma membrane, cell protrusions and vesicular structures in the cytoplasm (arrows in Fig. 1*D–F*). Flot-1 protein (smaller

particles), on the contrary, was detected in specific loci at the cytoplasmic side of the plasma membrane (arrowheads in Fig. 1*E* and *F*), but also on the membranes of multivesicular bodies (arrowheads in Fig. 1*D*) and lysosomes in the cytoplasm. Although the complexes of CPP-neutravidin were found close to the location sites of flotillin-1 protein, we could not detect colocalization between Flot-1 and CPP-protein complexes at the plasma membrane by TEM (Fig. 1*E* and *F*). However, a small population of the intracellular vesicles containing TP/TP10-protein complexes included also Flot-1 protein. The same results were obtained with biotinylated Tat peptide complexed with avidin (CLSM) or neutravidin-gold (TEM) (Fig. 1*C, F*). For all CPP-protein complexes, overlapping structures with Flot-1 could be estimated to remain around 1–5% of the total number of complexes-containing vesicles and membrane areas. We also performed experiments using shorter incubation time as peptide-protein complexes might pass the flotillin-positive vesicles earlier. However, after 10 min of incubation very few complexes had internalized and the colocalization between Flot-1 and CPP-protein complexes was rarely detected on the plasma membrane (data not shown).

Transportan and TP10 avoid the early endosomal pathway marked by Rab5 and PI3P in the cellular delivery of cargo protein.

In order to see whether the early endosomal pathway is exploited in peptide-mediated protein transport, we characterized the cellular localization of CPP-avidin complexes in comparison to Rab5 and PI3P, markers of early endosomes. The analogous scarce intracellular colocalization of CPP-protein complexes as was seen with flotillin-1 was also detected with PI3P (Fig. 2*A, B*) and Rab5 (Fig. 2*C, D*) as observed by CLSM after 30 min incubation. Though both the complexes and markers were confined to punctate structures in cells, they represented a different population of vesicles. Somewhat surprisingly the shorter (10 min) incubation time also revealed very little colocalization between the internalized CPP-protein complexes and Rab5 or PI3P cellular markers (see the Supplementary data). Again, in case of rare overlap, the focuses stained by anti-Rab5 or anti-PI3P antibody were situated in the middle of large regions of aggregates of CPP-protein complexes. The negligible role of PI3P-dependent pathway in CPP-mediated protein delivery (Fig. 2*A, B*) correlates well with our unpublished results showing that internalization of TPb/TP10b-avidin complexes was not inhibited in wortmannin-treated HeLa cells where the activity of PI3P is restrained (data not shown).

A fraction of CPP-protein complexes is targeted to caveolae.

Earlier reports have demonstrated the involvement of caveolae-dependent endocytosis in both the CPP internalization and the CPP-mediated cargo delivery (11, 12). Our results obtained by electron microscopy have shown that TP/TP10-protein complexes enter cells often in small (50–100 nm in diameter)

morphologically caveosome-like or non-coated vesicles. Therefore we looked for the presence of Cav-1 in these structures by both immunofluorescence and immunoelectron microscopy. Indeed, a fraction of CPP-protein complexes colocalized with caveolin-1 on the plasma membrane and in cells. A higher number of colocalization could be detected on the plasma membrane than in the cytoplasm for all peptide-protein complexes. About 30% of CPP-avidin complexes on the membrane and 20% inside the HeLa cells exhibited similar localization to Cav-1 by visual estimation of CLSM images (Fig. 3*A, B*). The results of electron microscopy revealed that most of the small vesicles or bigger rosette-like structures had caveolin-1 on the cytosolic side (small particles in Fig. 3*D*). These structures were always in contact or in close proximity to actin fibres. Although the caveosomes were abundant in the cortical cytoplasm, most of them did not contain the TP/TP10-neutravidin complexes (big particles). However, several of the TP/TP10-protein, as well as pTat-protein complexes, were found to reside in the Cav-1-positive vesicles in the perinuclear region (arrows in Fig. 3*G* and 3*H*). The presence of both, TP/TP10-neutravidin complexes and Cav-1 was also detected in bigger vesicles (0.4 μm), which had small caveosomes at close proximity or were connected to them (indicated by arrowheads in enlarged section of Fig. 3*E*). The amount of pTat-protein complexes in vesicles covered with Cav-1 was comparable to that of TP/TP10-protein and Cav-1, reaching to about 20–30% of all Cav-1 containing vesicles. Slightly greater overlap between Cav-1 and pTat-protein complexes was found in the cortical vesicles as compared to TP/TP10. In addition, about one-third of all CPP-protein-complexes-containing vesicles were found in close proximity to caveosomes as shown in Fig. 3*F*, which might lead to some overestimation of the degree of colocalization when using confocal microscopy (Fig. 3*A–C*).

Partial downregulation of flotillin-1 and caveolin-1 confirms the participation of caveolin-1-dependent endocytosis in transportan/TP10-mediated protein delivery.

In addition to microscopy experiments we downregulated the Flot-1 with siRNA to see whether the lack of cellular Flot-1 interferes with the uptake of peptide-protein complexes. The used siRNA decreased the Flot-1 concentration by 20–25% as estimated by FACS (Fig. 4*A*). Although the downregulation was too small to draw clear conclusions about the impact on protein delivery by CPPs, partial downregulation of Flot-1 rather slightly enhanced than decreased the internalization of TP/TP10-avidin complexes (Fig. 4*B*). The results from microscopy prompted us to check whether depletion of Cav-1 from the plasma membrane influences the TP/TP10-mediated protein delivery. By using siRNA treatment we reached about 50% downregulation of Cav-1 protein expression as confirmed by FACS (Fig. 4*C*). The decrease in Cav-1 concentration inhibited the uptake of TP/TP10-protein complexes around 40–50% (Fig. 4*C*). When observing the protein uptake into caveolin-1-downregulated cells by CLSM,

less colocalization between the CPP-protein complexes and caveolin-1 was detected for both peptides, possibly because of the smaller amount of membranous Cav-1 (data not shown). However, the amount of membrane-associated complexes seemed unchanged compared to controls, indicating that the interaction with plasma membrane is not inhibited in the absence of Cav-1. Nevertheless, the extensive trypsin digestion removed CPP-protein-complexes from the cell surface yielding in reduced uptake quantified by flow cytometry.

DISCUSSION

The plasma membrane is a complex cellular organelle, being simultaneously a portal and a barrier for all metabolites. Cell-penetrating peptides are under intense study as possible mediators for crossing this barrier together with biologically active cargo, but their putative ability to penetrate through membranes represents also an interesting scientific phenomenon *per se*. On the other hand, endocytic processes have proven to be gates for CPP/CPP-cargo entry in most cases and the role of pathways that originate from the lipid rafts and/or caveolae is often stressed (11, 13, 22). Involvement of rafts is usually suggested based on the decreased cellular uptake of CPPs by cells depleted of cholesterol in the plasma membrane after treatment with methyl-ß-cyclo-dextrine. However, it does not enable to distinguish the caveolar and raft-mediated pathways, which have different cellular destinations. Moreover, removal of cholesterol influences also other trafficking processes and might lead to changes in cell morphology (23). Continuously accumulating data about the nature and complexity of endocytosis and involvement of its different types in CPP mechanisms prompted us to reassess the initial steps of CPP-mediated protein delivery. We chose flotillin-1 and caveolin-1 as markers for two types of plasma membrane microdomains, and Rab5 and PI3P for characterization of early endosomal vesicles, to elucidate their involvement in TP/TP10 mediated protein delivery into HeLa cells.

Cholesterol and sphingolipid-rich rafts represent highly heterogeneous populations of functionally distinct membrane domains. The availability of caveolae-independent but morphologically similar endocytic pathway involved in the SV40 cell entry was recently demonstrated by two groups (17, 18). In analogy with CPP mediated delivery, this type of endocytosis is dependent on cholesterol and dynamin. Flotillins are shown to be responsible for the assembly of a particular type of rafts, which participate in clathrin- and caveolin-independent endocytosis (20). Coassembly of flotillin-1 and flotillin-2 into microdomains induces formation of membrane invaginations that are morphologically reminiscent of caveolae and considered to be capable of forming intracellular vesicles, which localize in the cortical cytoplasm, look similar to caveosomes, but lack caveolin-1 (24). According to our electron microscopy results, the

vesicular pathway of TP/TP10-mediated protein uptake resembles by morphology this noncaveolar, flotillin-1-dependent internalization mode described by Frick and coworkers. Therefore, we assessed the putative involvement of flotillin-1-dependent internalization pathway in the cellular translocation of TP/TP10-protein complexes. However, somewhat unexpectedly we could not find the colocalization of CPP-protein complexes with flotillin-1 on the plasma membrane or inside cells by immunocytochemical analysis either by confocal or electron microscopy. Moreover, downregulation of flotillin-1 using siRNA silencing rather increased than decreased the CPP-protein uptake, corroborating the microscopy results. It seems that flotillin-1-dependent pathway has a negligible if any role in CPP-mediated protein delivery. For the sake of clarity, we also checked the localization of cell transduced protein in relation to flotillin-2 but in analogy with flotillin-1 no significant overlap was detected (data not shown). On the other hand, based on the experiments with flotillin-1 and 2, we can not exclude the participation of lipid rafts in delivery by CPPs. Moreover, the possibility that complexes indeed show equal or even better uptake in partially flotillin-1-depleted cells suggests that the phospholipids of non-raft areas of the plasma membrane might also play a role in CPP internalization mechanisms (25, 26) in addition to the cell surface proteoglycans. Considering the ability of transportan to modulate the dynamics of the membrane lipids (27), and that the location and function of cell surface proteoglycans is probably interfered in flotillin-1-deficient cells, one can imagine that the changed properties of the plasma membrane could lead to the vesicular uptake mode(s) that is not common to cells with a properly organized membrane.

The involvement of another raft-dependent pathway, caveolar endocytosis, has been demonstrated in both, the cellular uptake of Tat peptide and pTat-mediated cargo delivery (11, 12). Recent data about caveolae not just merely as a subtype of rafts but as an independent membrane structure (28–30), encouraged us to include it in the study. Our CLSM results demonstrated that approximately 30 % of CPP-avidin complexes exhibited similar localization to Cav-1 and a higher overlap was observed at the plasma membrane than inside cells. Unexpectedly electron microscopy revealed somewhat lower degree of colocalization (about 20%) of complexes with caveolin-1-positive vesicles, especially in the cortical cytoplasm where the main fraction of caveosomes was found. Electron microscopy results reveal that the CPP-neutravidin complexes and caveolin-1 localized often in very close proximity, which might have lead to the overestimation of the degree of colocalization by CLSM. However, we cannot exclude that the lower colocalization detected in electron microscopy could be caused by different isoelectric points of avidin and neutravidin, and by the higher affinity of avidin to negatively charged components of the plasma membrane. Still, a strong inhibition of CPP-mediated protein uptake after caveolin-1 downregulation was detected by FACS analysis, which stresses the

importance of caveolin-1-containing membrane areas in the internalization process. However, the 50% inhibition of the cell-entry of transportan/TP10-avidin complexes after reduction of caveolin-1 concentration in HeLa cells by 50% cannot be interpreted as a linear correlation and that only the caveolin-1 dependent pathway is responsible for the uptake of the complexes. The confocal micrographs reveal that siRNA treatment lead to a major loss of caveolin-1 from the plasma membrane but not from intracellular structures. Therefore the caveolar endocytic pathway was down-regulated by siRNA at markedly higher extent than 50% and other transport routes also contributed to the uptake of CPP-protein complexes. In addition, the shortage of caveolin in cells might also reduce the concentration of cholesterol in the plasma membrane thereby inhibiting the CPP-mediated delivery, since caveolins are known to mediate the transfer of cholesterol to the plasma membrane (31). Our earlier results demonstrated that around 10–15% TP/TP10-protein complexes localized in transferrin-positive vesicles and a fraction of cell-transduced protein was confined in macropinosome-like vesicles suggesting that more than one endocytic pathway is active in CPP-mediated protein transduction (21). Based on this we propose that in conditions where the functioning of one entry route is restrained, TP/TP10 target the cargo into different endocytic pathways, although with smaller efficiency.

The further intracellular targeting of CPP-transduced cargos is even more important than the starting point. Upon long-time incubations the cargo protein delivered into cells by CPPs accumulates in LAMP2 containing structures in perinuclear region (21) and is targeted to digestion (3). Therefore, mapping of the early steps of CPP-mediated delivery might provide valuable information for finding a way to induce liberation of cargo molecules from the endosomal structures before direction to degradation. The small GTPase Rab5 and PI3P are both typical constituents of early endosomes and both of them are also reported to take part in macropinocytosis (32). In addition, the lateral transport between early endosomal vesicles and caveosomes has been shown to be Rab5-dependent (29). However, most of CPP-protein complexes were observed to evade early endosomes since only a negligible fraction of complexes showed colocalization with Rab5 or PI3P after 10 or 30 min. Furthermore, the treatment of cells with wortmannin, a specific inhibitor of phosphoinositide 3-kinases, did not inhibit the CPP-induced protein transport into HeLa cells. Although early endosomes are reported to be capable of receiving contents from caveosomes (29), the fraction of TP/TP10 protein complexes that internalized in caveolin-1 positive vesicles might avoid early endosomes by using other transport route(s), probably analogously to SV40 (33). Still, the caveolar pathway is considered to lead to the Golgi complex but only a minor part of TP/TP10-protein complexes is targeted there as we showed earlier. Although the CPP-protein complexes seem to avoid being trapped into early endosomal structures after translocation

into HeLa cells, upon longer incubation they accumulate in vesicles containing endolysosomal marker protein LAMP2 (21).

Recently, it has been reported that at least three endocytic pathways are used for cellular entry by CPPs: macropinocytosis, clathrin-mediated and caveolar/ lipid raft mediated endocytosis (4). Our results about protein cellular delivery are well in line with this study suggesting analogous concurrently acting endocytic uptake mechanisms and in our system the caveolin-1-dependent pathway but not flotillin-1-mediated route is preferentially used. However, the intracellular targeting of complexes seems to avoid canonical trafficking pathways (schematic representation in Fig. 5). Additionally, there is a fraction of small vesicles induced by CPPs and mediating the internalization of cargo molecules, which we have not been able to classify with markers of known endocytic pathways and flotillin-1 did not help with filling this gap.

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FIGURE LEGENDS

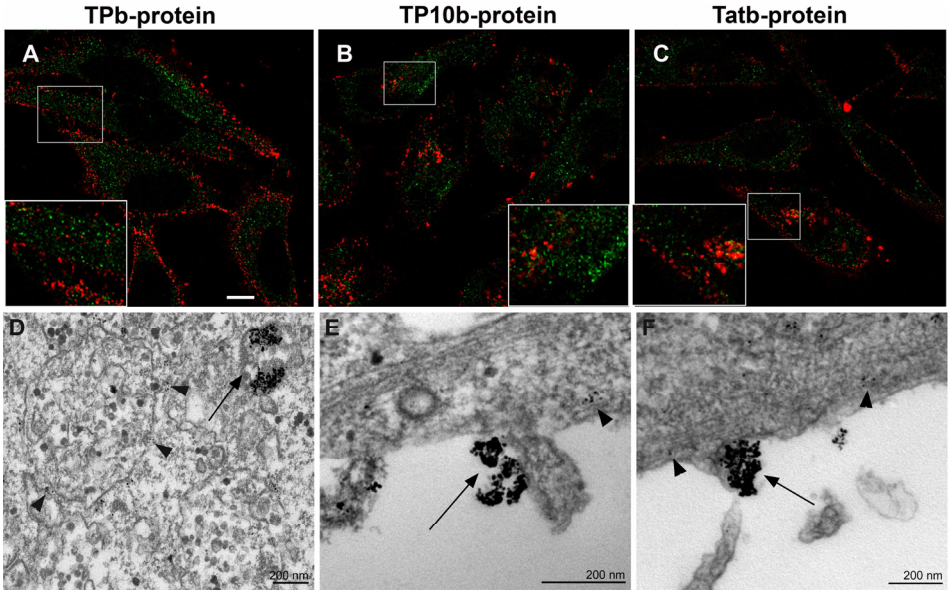


Fig. 1. Localization of CPP-protein complexes in HeLa cells in relation to flotillin-1. For CLSM experiments cells were incubated for 30 min with 0.5 µM biotinylated transportan (*A*), TP10 (*B*) or pTat (*C*) complexed with 0.15 µM avidin-Texas Red (red). Flotillin-1 was stained with anti-flotillin-1 pAb and with appropriate Alexa Fluor 488 conjugated secondary Ab (green). (*D-F*) TEM images of cells incubated with neutravidin-gold (10 nm) complexed with 1.5 µM biotinylated transportan (*D*), 3 µM TP10 (*E*), or pTat (*F*) for 1h. Flotillin-1 is visualized with antibodies tagged with small nanogold (1.4 nm) particles (arrowheads in *D-F*). The bar in CLSM images represents 10 µM.

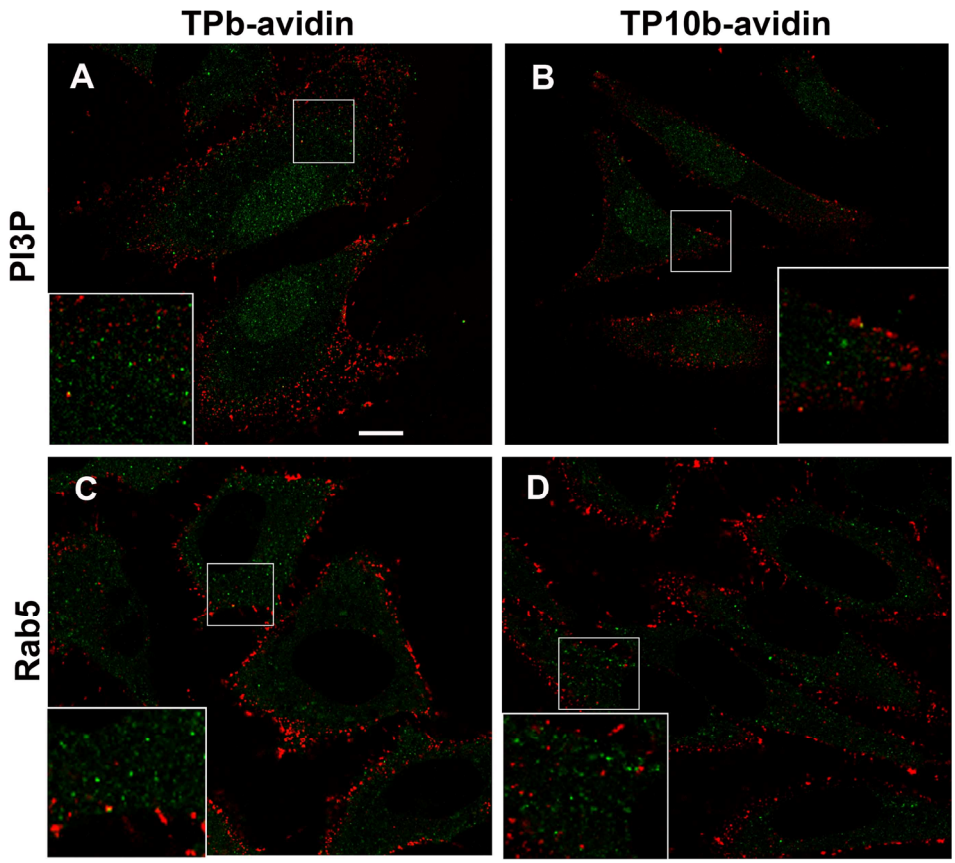


Fig. 2. Localization of transportan- and TP10-protein complexes in HeLa cells in relation to Rab5 and PI3P. Cells were incubated for 30 min with 0.5 μM biotinylated transportan (*A, C*) or TP10 (*B, D*) complexed with 0.15 μM avidin-Texas Red (red). To visualize PI3P and Rab5, cells were stained with anti-PI3P mAb (*A, B*) or anti-Rab5 (*C, D*) and with appropriate Alexa Fluor 488 conjugated secondary Ab (green). The bar represents 10 μM.

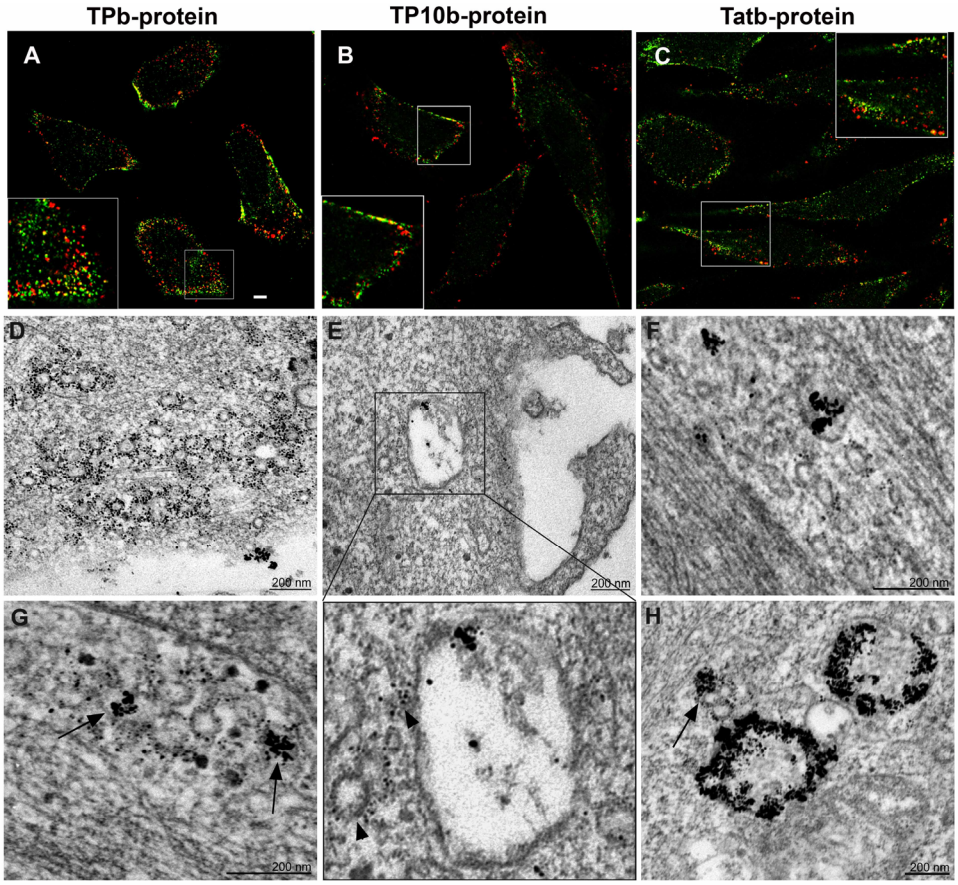


Fig. 3. Localization of CPP-protein complexes in HeLa cells in relation to caveolin-1. Cells were incubated for 30 min with 0.5 μM biotinylated transportan (*A*), TP10 (*B*) or pTat (*C*) complexed with 0.15 μM avidin-Texas Red (red). To visualize the cellular caveolin-1, cells were stained with anti-caveolin-1 pAb and with appropriate Alexa Fluor 488 conjugated secondary Ab (green). (*D-H*) TEM images of cells incubated with complexes of neutravidin-gold (10 nm) and 1.5 μM biotinylated transportan (*D, G*), TP10 (*E*) or pTat (*F, H*) for 1 h. Caveolin-1 is visualized with small nano-gold (1.4 nm) particles. The bar in CLSM images represents 10 μM.

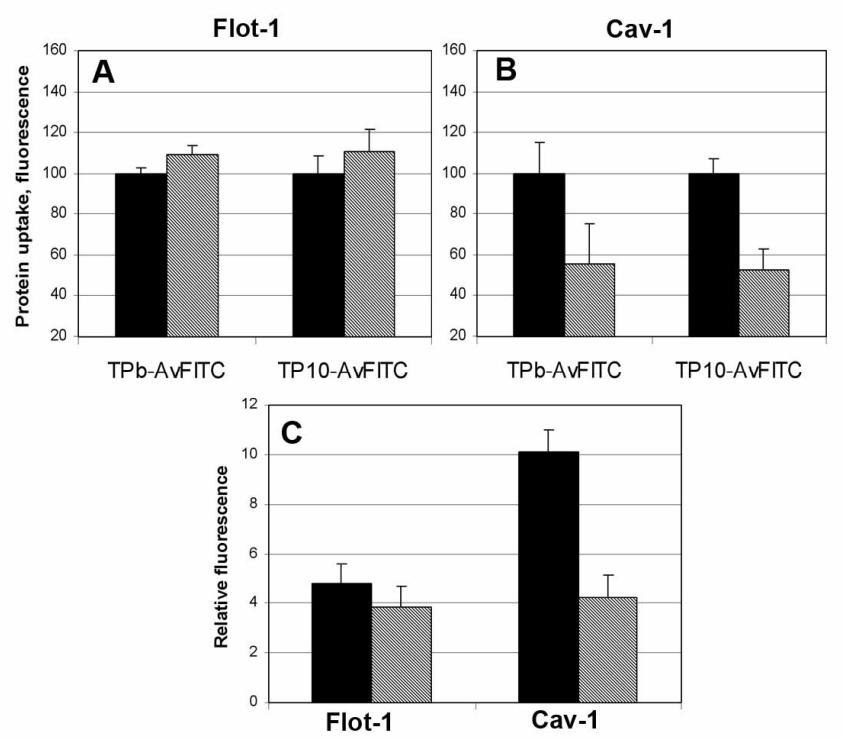


Fig. 4. Influence of siRNA downregulation of flotillin-1 and caveolin-1 on cellular protein delivery by transportan and TP10. HeLa cells were treated with siRNA specific to flotillin-1 (*A*) or caveolin-1 (*B*) (grey bars) or with non-specific siRNA (black bars) as control, incubated with complexes of biotinylated TP/TP10 and avidin-FITC for 30 min and analyzed by FACS. Protein delivery efficiency into cells treated with non-specific siRNA is represented as 100 %. (*C*) Estimation of the siRNA efficiency. Cells treated with siRNA specific to flotillin-1 or caveolin-1 (grey bars) or non-specific siRNA (black bars) were stained for Flot-1 or Cav-1 and applied for FACS analysis. Results are shown as fluorescence ratio to cells treated with Alexa Fluor 488-labeled secondary Ab.

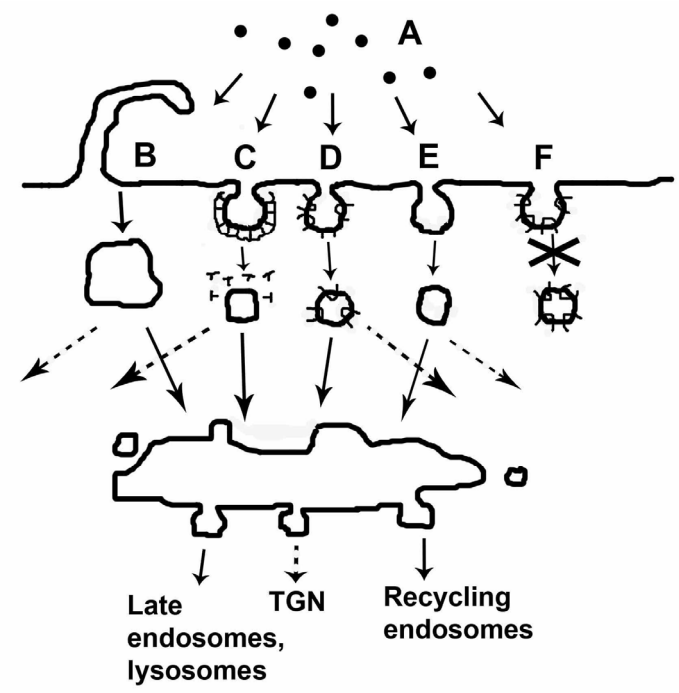
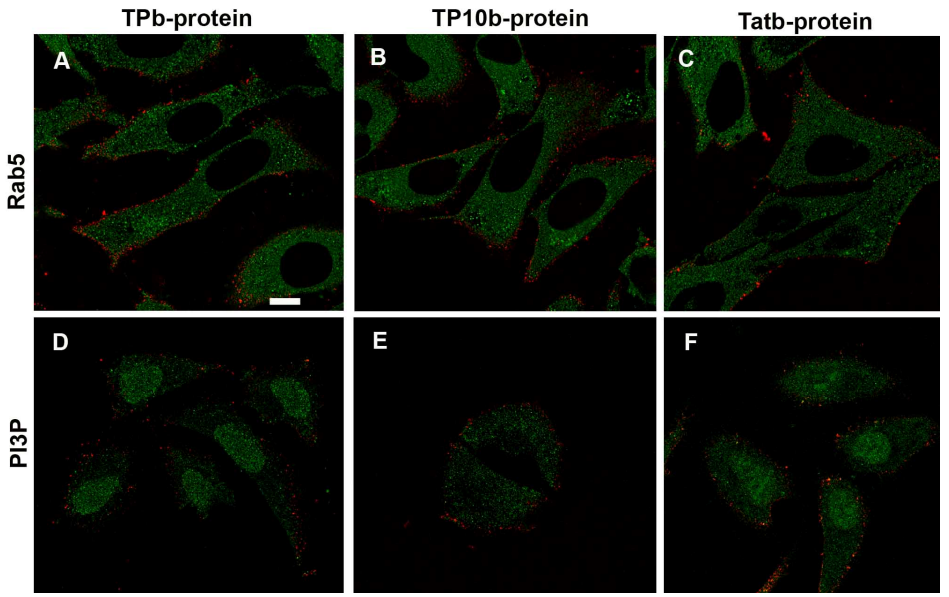


Fig. 5. Endocytic pathways used for cellular entry by transportan/TP10-protein complexes. The complexes of peptide and protein (*A*) can enter cells via macropinocytosis (*B*), clathrin- (*C*), or caveolae (*D*)-mediated endocytosis, and clathrin- and caveolin-independent endocytosis (*E*). The flotillin-mediated pathway (*F*) is not utilized. Intracellular trafficking routes are indicated as solid arrows, putative directing is represented as dashed arrows.



Supplementary Fig. S1. Localization of transportan- and TP10-protein complexes in HeLa cells in relation to Rab5 and PI3P. Cells were incubated for 10 min with 0.5 µM biotinylated transportan (*A, D*), TP10 (*B, E*) or pTat (*C, F*) complexed with 0.15 µM avidin-Texas Red (red). To visualize PI3P and Rab5, the cells were stained with anti-Rab5 (*A, B, C*) or anti-PI3P mAb (*C, D*) and with appropriate Alexa Fluor 488 conjugated secondary Ab (green). The bar represents 10 µM.

III

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

IV

Uri, A., Raidaru, G., Subbi, J., **Padari, K.**, Pooga, M. (2002)
Identification of the ability of highly charged nanomolar inhibitors
of protein kinases to cross plasma membranes and carry a protein into cells.
*Bioorg Med Chem Lett.*19; 12(16):2117–20.

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Scientific publications

Padari, K.; Säälik, P.; Hansen, M.; Koppel, K.; Raid, R.; Langel, Ü.; Pooga, M. (2005). Cell transduction pathways of transportans. Bioconjugate Chemistry, 16(6), 1399–1410.

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. Bioconjugate Chemistry, 15(6), 1246–1253.

Viht, K.; Padari, K.; Raidaru, G.; Subbi, J.; Tammiste, I.; Pooga, M.; Uri, A. (2003). Liquid-phase synthesis of a pegylated adenosine-oligoarginine conjugate, cell-permeable inhibitor of cAMP-dependent protein kinase. Bioorganic & Medicinal Chemistry Letters, 13(18), 3035–3039.

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SCANDEM Nordic Microscopy Society ning Eesti Biokeemia Seltsi liige

Teadustöö

Alates 1999 aastast on uurimustöö põhisuunaks olnud transportaani ja teiste rakku penetreeruvate peptiidide internalisatsioonimehhanismid ning nende poolt vahendatud valgu transport rakkudesse.

Teaduspublikatsioonid

Padari, K.; Säälik, P.; Hansen, M.; Koppel, K.; Raid, R.; Langel, Ü.; Pooga, M. (2005). Cell transduction pathways of transportans. Bioconjugate Chemistry, 16(6), 1399–1410.

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