DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
197

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197

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Multiple faces of cell-penetrating peptides – their intracellular trafficking, stability and endosomal escape during protein transduction

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TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS

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

I. **Räägel, H.**, Säälik, P., Hansen, M., Langel, Ü., Pooga, M. (2009) CPP–protein constructs induce a population of non-acidic vesicles during trafficking through endo-lysosomal pathway. *J Control Release* 139(2):108–17

II. **Räägel, H.**, Kriiska, A., Säälik, P., Hein, M., Florén, A., Langel, Ü., Pooga, M. (2011) Peptide to cargo ratio dictates the internalization efficiency, resistance to degradation and photo-activatable endosomal escape of TP10b-StreptAvidin complexes. *Manuscript*

III. Holm, T.[1], **Räägel, H.** [1], El Andaloussi, S., Hein, M., Mäe, M., Pooga, M., Langel, Ü. (2011) Retro-inversion of certain cell-penetrating peptides causes severe cellular toxicity. *Biochim Biophys Acta* 1808(6):1544–51

IV. **Räägel, H.**, Lust, M., Uri, A., Pooga, M. (2008) Adenosine-oligoarginine conjugate, a novel bisubstrate inhibitor, effectively dissociates the actin cytoskeleton. *FEBS J* 275(14):3608–24

([1] these authors contributed equally to this work)

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

Ref. I designed and performed the experiments, analyzed the data and wrote the manuscript.

Ref. II designed and carried out the degradation experiments, analyzed the data and wrote the manuscript.

Ref. III designed and performed the experiments with fluorescence microscopy, participated in data analysis and in the writing of the paper.

Ref. IV performed the experiments, participated in data analysis and wrote the manuscript.

ADDITIONAL PUBLICATIONS

Räägel, H., Säälik, P., Pooga, M. (2010) Peptide-mediated protein delivery – which pathways are penetrable? *Biochim Biophys Acta* 1798(12):2240–8. Review.

Räägel, H., Säälik, P., Langel, Ü., Pooga, M. (2011) Mapping of protein transduction pathways with fluorescent microscopy. *Methods Mol Biol* 683:165–79

Räägel, H., Pooga, M. (2011) Peptide and protein delivery with cell-penetrating peptides. *Peptide and Protein Delivery*. Ed. Chris Van der Walle, Academic Press (Elsevier), London, UK; pp 221–46

ABBREVIATIONS

AP-2	adaptor protein 2
ARC	adenosine-oligoarginine conjugate
CCP	clathrin-coated pit
CCV	clathrin-coated vesicle
Cdc42	cell division cycle protein 42, a small GTPase
CLIC	clathrin-independent carriers
CME	clathrin-mediated endocytosis
CIE	clathrin-independent endocytosis
CLSM	confocal laser scanning microscopy
CPP	cell-penetrating peptide
EE	early endosome
EGF	epidermal growth factor
GEEC	GPI-enriched endosomal compartment
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GPI	glycosylphosphatidylinositol
GPI-AP	glycosylphosphatidylinositol-anchored protein
HA	hemagglutinin
HIV	human immunodeficiency virus
HSPG	heparin sulphate proteoglycan
LAMP-2	lysosome-associated membrane protein 2
L_d	liquid-disordered
LE	late endosomes
L_o	liquid-ordered
MAP	model amphipathic peptide
MLC	myosin light chain
NLS	nuclear localization sequence
NA	neutravidin
PEI	polyethyleneimine
PI	propidium iodide
PI3P	phosphatidylinositol-3-phosphate
$PI(4,5)P_2$	phosphatidylinositol-4,5-bisphosphate
PtdIns	phosphatidylinositol
Rac1	Ras-related C3 botulinum toxin substrate 1

3

RE	recycling endosome
RhoA	Ras homologue gene family member A, a small GTPase
RI	retro-inverso, retro-inversion
ROCK	Rho-associated kinase
SA	streptavidin
SDS	sodium dodecyl sulphate
SV40	simian virus 40
Tf	transferrin
TGN	trans-Golgi network

INTRODUCTION

Since the discovery that certain peptides are capable of enhancing the internalization of various bioactive molecules, the research to define the special characteristics or sequence(s) required for translocation and the different uptake routes exploited by the vectors has skyrocketed. Despite their rather different features, these peptidic carriers are collectively called cell-penetrating peptides or simply CPPs due to the initial reports on their propensity to penetrate directly through the plasma membrane and concentrate into the cytosol and the nucleus. To date, however, a consensus has been reached that CPPs, despite being capable of effective internalization, preferably still enter the cells mostly via an endocytic mechanism, especially when in complex with a cargo molecule.

In spite of the extensive research done on trying to delineate the distinct endocytic pathways involved in the uptake of either CPPs or CPP-cargo complexes, no agreement has yet been reached. This is perhaps due to the parallel utilization of and possible switching between different routes. Additionally, the cell entry depends on a number of factors starting from the characteristics of the peptide and ending with the size of the cargo, thus placing simply too many variables into the equation.

As the uptake occurs mainly via the capture of the CPPs or their complexes into the endocytic vesicles, the second relevant issue arising is their intracellular targeting and subsequent stability. Unlike the numerous reports claiming the revelation of the endocytic pathway(s) used in the uptake process, very few publications on the intracellular trafficking and the final destiny of the CPPs and/or the cargo attached to them are available.

Thus, the main purpose of this study was to dissect the events happening after cell entry by portraying the intracellular trafficking of different CPPs in complex with a protein cargo. The CPPs used in this study are mainly the representatives of the arginine-rich CPPs (Tat and nonaarginine) and the more amphipathic class of peptides (TP and its shortened analogue TP10) that have been defined as highly efficient transport vectors. Additionally, as at least a fraction of endosomes are intracellularly targeted to lysosomes where degradation events take over, the stability of the CPP-protein complexes was another issue addressed in this study. Furthermore, the cellular effects of degradation-resistant isoforms of common CPPs (M918, penetratin and Tat) were evaluated. In addition, the ability of a novel kinase inhibitor, an oligo-arginine conjugate (ARC), bearing the CPP-like oligoarginine sequence, to internalize, translocate into the cytosol and exert its effect in a cellular environment was described.

The knowledge gained from the present study could enhance the understanding of how the CPP-protein complexes act inside the cells. It could thus provide assistance in the development of the CPPs for biotechnological applications in order to enhance the overall levels of the CPP and the cargo reaching the cytosol, elevating subsequently the proportion of “effective” delivery.

1. LITERATURE OVERVIEW

1.1. Overcoming the plasma membrane barrier

The cellular plasma membrane imposes a selective barrier for compounds in the extracellular environment, controlling tightly their movement in (or out of) the cytoplasm. Because of this, the membrane blocks the internalization of a number of extracellularly applied (bio)molecules that due to either their large parameters and/or unfavorable chemico-physical characteristics cannot, on their own, enter the cell. Therefore, it is imperative, especially for the advancement of the field of biomedicine, to design effective transport vectors that would enhance the uptake of different therapeutics.

The widely used *in vitro* transfection systems (e.g. lipofectamine), physical membrane destabilization by electroporation or direct in-cell microinjection may all be powerful and effective tools in cell culture studies, yet, their *in vivo* use is often hampered by their high toxicity, the difficult if not impossible application, or the extremely low yield. Due to the above-mentioned limitations, numerous novel and rather dexterous methods have been proposed to elevate the level of (bio)molecules reaching the cellular cytoplasm in order to evoke the desired effects without the undesired responses. For example, the use of helper molecules like polyethylenimine (PEI) (Didenko *et al.*, 2005; Kitazoe *et al.*, 2005), encapsulating carrier systems like liposomes (Chonn and Cullis, 1995; Zelphati *et al.*, 2001) or viral vectors (Boeckle and Wagner, 2006) have greatly enhanced the delivery efficiency of different cargo molecules. However, just like the two opposing sides of a coin, the above-mentioned transporters also possess several drawbacks. These may be associated with, for instance, cytotoxicity, heterogeneous dispersion or immunogenicity. As a consequence, the field of effective transport calls for the emergence of alternative and even better strategies.

1.2. Cell-penetrating peptides (CPPs)

The ability of positively charged proteins, like histones, and homopolymers of L-arginine and L-lysine to stimulate the uptake of albumin was already discovered before the 1970s (Ryser, 1968). However, the field of enhanced membrane translocation really got off the ground about 20 years later, in 1988, when two independent research groups studying the human immunodeficiency virus (HIV) type 1 observed that its transcription trans-activating protein Tat displayed the ability to rapidly translocate into cells from the surrounding environment (Frankel and Pabo, 1988; Green and Loewenstein, 1988). This seemed to trigger the launch of the research area of cell-penetrating peptides (CPPs). Since then numerous studies have been conducted to find new, highly potent peptides for membrane translocation (Derossi *et al.*, 1994; Derossi *et al.*, 1998; Pooga *et al.*, 1998; Mitchell *et al.*, 2000; Morris *et al.*, 2001; Lundberg *et al.*,

2007) and/or to define the requisite characteristics evoking the translocation process (Vivés *et al.*, 1997; Wender *et al.*, 2000; Thorén *et al.*, 2003).

Considering the wide number of peptides assigned to the category of "cell-penetrating peptides", the term itself has not yet been defined in detail and thus still evokes a lot of debate. Despite this, most of these peptides do possess some common features. For instance, they are typically short, usually less than 40 amino acids in length, and capable of transporting various cargo efficiently into different cells (Langel, 2006). Additionally, the CPPs often contain basic amino acids in their sequence, which provides the peptide with a net positive charge under physiological pH. The positive charge of the peptide aids its association with the negatively charged plasma membrane components and induces its subsequent internalization. Due to the electrostatic forces that come to play in the attachment of the CPP to the membrane components, it has been verified that arginine carrying a positively charged bidentate guanidinium head-group is the most potent out of all the basic amino acids in eliciting the afore-mentioned effects (Mitchell *et al.*, 2000; Rothbard *et al.*, 2005; Åmand *et al.*, 2008).

At first, different CPPs were classified as:
(i) derived from naturally occurring proteins (e.g. Tat peptide from HIV (Vivés *et al.*, 1997) and penetratin from Drosophila Antennapedia homeodomain (Derossi *et al.*, 1994)),
(ii) chimeric peptides (e.g. transportan (Pooga *et al.*, 1998)), or
(iii) synthetic/artificial peptides (e.g. oligoarginine (Mitchell *et al.*, 2000)).

However, despite the fact that this classification reflects on the origin of the peptide under focus, it does not provide any valuable information about the actual chemico-physical attributes the peptide may possess. Thus, in 2008, Ziegler proposed a new classification for CPPs based on their membrane association features (Ziegler, 2008), dividing the large family of CPPs into:
(i) primary amphipathic,
(ii) secondary amphipathic, and
(iii) non-amphipathic CPPs.

1.2.1. Primary amphipathic CPPs

The term "amphipathicity" refers to molecules with both hydrophilic and hydrophobic parts. The primary amphipathic CPPs, in that case, are comprised of a primary structure with segmental amphiphilicity due to, for example, a highly hydrophilic N-terminus and a mainly hydrophobic C-terminus (Fernandez-Carneado *et al.*, 2004). The CPPs possessing this particular primary structure are usually longer than 20 amino acid residues, thus having long enough sequence to, at least theoretically, span the hydrophobic core of the membrane bilayer (Ziegler, 2008). Additionally, Ziegler et al. proposed that due to the ability of the primary amphipathic peptides to bind both neutral and

anionic lipids with a high affinity, the interaction between the peptides and the lipids is dominated by the hydrophobic, rather than the electrostatic interactions (Ziegler, 2008). However, on the plasma membrane the initial interactions could still be facilitated by the mere attraction of the reciprocal charges.

Transportan (TP) is one of the peptides belonging to the class of primary amphipathic peptides bearing several hydrophobic and hydrophilic blocks in its sequence. Transportan is a chimeric peptide consisting of 27 amino acids. It is generated by linking an N-terminal fragment of the neuropeptide galanin to a wasp venom peptide mastoparan via a Lys-residue (Pooga *et al.*, 1998). The lysine linker allows the attachment of either different reporter groups (fluorescent or nanogold markers) or other linkers (e.g. biotin moiety) to the peptide. Thus, these linkers make the peptide visible for microscopy studies or enable a convenient coupling of the cargo molecules to the side-chain amino group, respectively. Additionally, the lysine linker provides the peptide with a joint-like flexibility that may come in handy during its insertion into the membrane.

TP has been proven to be a highly efficient transport vector (Muratovska and Eccles, 2004; El-Andaloussi *et al.*, 2006). However, TP was demonstrated to be recognized by galanin receptors (because of the galanin fraction), and it shows an inhibitory effect on the basal GTPase activity in Bowes melanoma cell membranes (possibly due to the inclusion of mastoparan in its sequence). Although the inhibitory effect of TP is detectable at higher concentrations than commonly used in delivery experiments, this feature could be a drawback for the carrier peptide. Therefore, a series of deletion analogues of TP were synthesized and the search for an ultimate transportan analogue yielded an N-terminally truncated form of TP called TP10. TP10 has been demonstrated to result in equal levels of uptake into cells with lower toxicity than the parent peptide (Soomets *et al.*, 2000). In model membranes, transportan acquires an α-helical conformation, which assists in the submersion of the peptide into the lipid membrane (Magzoub *et al.*, 2001). Since the C- (and not the N-) terminus of transportan is shown to interact with the phospholipids using the hydrophobic face of the α-helix (Barany-Wallje *et al.*, 2004), it is possible that the membrane interaction of TP and its analogue TP10 is similar.

Pep-1 is another primary amphipathic peptide used in efficient delivery of the cargo into cells (Morris *et al.*, 2004; Munoz-Morris *et al.*, 2007). Pep-1 is a 21-residue peptide carrier, consisting of three domains:

(1) a hydrophobic tryptophan-rich motif containing five tryptophan residues required for efficient targeting to the cell membrane and for forming hydrophobic interactions with proteins,
(2) a hydrophilic lysine-rich domain derived from the nuclear localization sequence (NLS) of the simian virus 40 (SV40) large T antigen required for improving the intracellular delivery and the solubility of the peptide vector, and

(3) a spacer domain separating the two domains mentioned above, containing a proline residue that improves the flexibility and the integrity of both the hydrophobic and the hydrophilic domains (Morris *et al.*, 2001).

In the presence of phospholipids, Pep-1 (similarly to TP) adopts a helical conformation (Deshayes *et al.*, 2004), that assists in the peptide's interaction with the hydrophobic lipid acyl-chains.

1.2.2. Secondary amphipathic CPPs

Peptides categorized under the secondary amphipathic peptides display their amphipathic properties only through a change in their secondary structure upon interaction with lipids or other (surface) molecules. Their amphipathic properties, with all the polar residues pointing to one face and the nonpolar residues to the opposite side, are therefore apparent in either a helical wheel or a β-strand projection. As a secondary structure motif, the right-hand α-helix contains 3.6 residues per turn (Fernandez-Carneado *et al.*, 2004). The affinity of these secondary amphipathic peptides to the electrically neutral membranes has been shown to be rather poor. However, by increasing the anionic lipid content of the membrane, their affinity increases by several orders of magnitude (Ziegler, 2008).

Due to the fact that all of the above-mentioned primary amphipathic peptides, TP, TP10 and Pep-1, can upon interactions with lipids adopt a helical conformation, they are sometimes also classified under the secondary amphipathic peptides. However, the most thoroughly studied peptide belonging to this class is penetratin. Penetratin is a 16 amino acid long polypeptide corresponding to the third helix of the Drosophila Antennapedia homeodomain and has been shown to efficiently cross membranes and accumulate in different cells (Derossi *et al.*, 1994; Console *et al.*, 2003).

The phospholipid binding of penetratin depends on its helical amphipathicity, especially when the negative surface charge density of phospholipid vesicles is low (Drin *et al.*, 2001). However, recently, it was described that the ability of penetratin to acquire either the α-helical or the β-sheet structure or none at all depends highly on the distinct membrane lipid composition (Maniti *et al.*, 2010). The structural requirements for efficient cell entry have also been assigned to the positively charged amino acids in penetratin's sequence. The replacement of these basic residues by the uncharged alanine resulted in a marked decrease in the uptake of the penetratin analogues (Fischer *et al.*, 2000). Likewise, the tryptophan residues in penetratin are mandatory for internalization and their substitution hinders the translocation of the peptide (Dom *et al.*, 2003). Hence, the planar structure of the tryptophan could also participate in promoting the lipid-peptide interactions.

Another "classical" secondary amphipathic CPP is the α-helical amphipathic model peptide called MAP (short for model amphipathic peptide), which was

introduced by the group of Bienert (Oehlke *et al.*, 1998). MAP is designed on the basis of the helical wheel projection, thus adopting an "ideal" amphipathic helix as a secondary structure. Shortly after defining MAP as an efficient translocator, it was demonstrated that its amphipathicity and a chain length of at least 16 amino acids is necessary for the cellular internalization of the peptide (Scheller *et al.*, 1999). Despite its high efficiency in membrane translocation, a severe drawback is its high degree of toxicity (Oehlke *et al.*, 1998; Hällbrink *et al.*, 2001), arising possibly from its strong ability to interact with lipids.

1.2.3. Non-amphipathic CPPs

Non-amphipathic peptides are defined as peptides that do not possess primary amphipathic properties and that are also unable to form a secondary amphipathic structure. The most prominent members of the group of non-amphipathic CPPs is the Tat peptide derived from the human immunodeficiency virus (HIV) type 1 transcription factor Tat, and the synthetic peptide oligoarginine. The fragment of the HIV Tat protein that was defined as the most effective sequence from the parent protein, carries also a nuclear localization signal, that aids in targeting the peptide to the nucleus after cell entry (Vivés *et al.*, 1997). Since the charge of the peptide plays a crucial role in the binding of the peptide to the negatively charged membrane components, and because the oligoarginines have demonstrated to be far better internalizers than other positively charged amino acids (Wender *et al.*, 2000), the abundant use of oligoarginines, instead of Tat, has emerged. Further studies defined the optimal number of arginines in the oligoarginine sequence to be from 6 to 9, since the shorter oligomers were poor translocators and the longer ones tended to display toxicity (Mitchell *et al.*, 2000). Regardless, both Tat and oligoarginine have been shown to be very efficient transport vectors, especially when attached to a cargo (as in the case of the Tat peptide) (Rothbard *et al.*, 2000; Langel, 2006).

As the non-amphipathic CPPs are exclusively cationic in their nature, they do not bind to lipid membranes unless a high fraction of anionic lipids is present. Thus, the main membrane interaction and binding comes from the electrostatic forces between the positively charged peptide and the negatively charged membrane constituents (Ziegler, 2008). However, a molecular-dynamics simulation presents evidence that arginine-rich peptides can, nevertheless, at high concentrations, sequester phosphate groups from neighboring phospholipids and create regions on the membrane that are crowded with peptides and phosphate groups. This crowding results in the attraction between the peptide and the phosphate groups on one bilayer and the phosphate groups in the distal bilayer thinning the membrane and facilitating the penetration across the membrane (Herce and Garcia, 2007; Herce *et al.*, 2009). Yet, it is important to keep in mind that these processes are taking place only at very high peptide to lipid ratios.

A recent addition to the family of cationic CPPs is a peptide called M918 derived from the tumor suppressor protein p14Arf amino acids 1–22 with its positions 3–8 inverted. This peptide was developed as a control peptide in a study where the parent peptide was used to mimic the activity of the Arf protein. Yet, quite surprisingly, M918 did not show any apoptotic effects, instead, displayed excellent cell-penetrating properties (El-Andaloussi *et al.*, 2007). M918 has approximately the same length (22 amino acids) as TP10, yet it shares the cationic nature of penetratin, having seven positively charged amino acids in its sequence. Unlike penetratin, however, it encompasses a low amphipathic moment. Therefore, it embodies traces of properties of each of the afore-mentioned subgroups of CPPs and could thus potentially act by using different mechanisms, e.g. during interactions with the lipid membranes. For example, due to its relatively long sequence compared to the other cationic peptides, it is possible that M918 is still capable of submerging into membranes similarly to penetratin. Regardless of the mechanism of membrane interaction, M918 has been shown to be highly effective in delivering cargo into cells (Lundin *et al.*, 2008; Mäger *et al.*, 2010).

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

CPP	Origin	Sequence	Reference
Primary amphipathic CPPs			
*Transportan (TP)	Galanin-Lys-mastoparan	QWTLNSAGYLLGKINLK ALAALAKKIL	(Pooga *et al.*, 1998)
*TP10	Truncated analogue of TP	AGYLLGKINLKALAALA KKIL	(Soomets *et al.*, 2000)
Pep-1	Dimerization domain of HIV-1 reverse transcriptase with NLS from SV40 large T antigen	KETWWETWWTEWSQPK KKRKV	(Morris *et al.*, 2001)
Secondary amphipathic CPPs			
*Penetratin	Drosophila Antennapedia homeodomain	RQIKIWFQNRRMKWKK	(Derossi *et al.*, 1994)
MAP	Model amphipathic peptide	KLALKLALKALKAALKLA	(Oehlke *et al.*, 1998)
Non-amphipathic CPPs			
*Tat	HIV-1 transcription activator Tat	GRKKRRQRRRPPQ	(Vivés *et al.*, 1997)
*nonaarginine (Arg_9)	Designed	RRRRRRRRR	(Mitchell *et al.*, 2000)
*M918	Tumor suppressor protein p14ARF	MVTVLFRRLRIRRACGPPR VRV	(El-Andaloussi *et al.*, 2007)

* CPPs used in this study

1.3. Internalization of CPPs and CPP-protein cargo complexes

The initial step of internalization requires the interaction of the peptide or peptide-cargo complex with the cellular plasma membrane. As a large number of different cellular membrane components carry negatively charged moieties, the first association between the peptide and the membrane is driven by the electrostatic forces. The matter of cell-surface molecules acting as potential electrostatic binding partners for CPPs is still under debate. Nevertheless, some reports have claimed that negatively charged plasma membrane surface sugars (e.g. heparin sulfate proteoglycans (HSPG)) are the binding sites for at least arginine-rich CPPs (Tyagi *et al.*, 2001; Fuchs and Raines, 2004; Kosuge *et al.*, 2008; Imamura *et al.*, 2011) acting as contributors to the recruitment of the peptide to the membrane.

Additionally, the presence of negatively charged lipids on the plasma membrane can also assist in the anchoring of the peptide to the membrane surface, as the affinity of CPPs towards anionic lipids has been reported repeatedly (Herce and Garcia, 2007; Ziegler, 2008; Cahill, 2009). Still, as the anionic lipids (e.g. phosphatidylserine) are mainly localized to the cytosolic leaflet of the plasma membrane and the CPPs bind with a higher affinity to the proteoglycans than to the membranes of low anionic lipid content (Ziegler, 2008), the direct peptide to lipid interactions could be considered rather unlikely, however, not impossible. Nonetheless, due to the far-extending side-chain lengths of the surface sugars, it is highly probable that indeed the sugars provide the cardinal hook for membrane binding. Nevertheless, it is clear that since charges play a vital role in the early membrane binding events, several different polyanions can be responsible for this.

1.3.1. Direct penetration or endocytosis

Upon membrane association, the components are internalized from the cell exterior into the cytoplasm. Whether the mode of internalization is a direct penetration across the membrane bilayer or of an endocytic nature is another issue under heavy dispute. It is rather tempting to support the hypothesis that CPPs are indeed capable of penetrating the membrane and transporting the attached cargo directly into the cytosol of the cells. In order to characterize the mechanism behind the direct membrane penetration, detailed knowledge about the membrane is needed.

The plasma membrane is composed of various lipids (phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PtdIns), phosphatidylserine (PS), phosphatidic acid (PC), sphingomyelin, etc.) (Warnock *et al.*, 1993; Pankov *et al.*, 2006) that are organized asymmetrically between the two membrane layers and can converge into either a liquid ordered (L_o) or liquid disordered (L_d) phase (van Meer *et al.*, 2008). The lipids with saturated chains

prefer to subcompartmentalize into the L_o phase, a specific lipid assemblage also called the lipid rafts (Simons and Ikonen, 1997; Lingwood and Simons, 2010). There their tight packing by proteins and cholesterol results in their elongation to a maximum extent, leading to thicker L_o domains compared to the L_d ones, which can cause energetic instabilities (Hurley *et al.*, 2010) that could be exploited by the CPPs to gain access into the cells. Indeed, recently, the role of ceramide blocks (formed from the enzymatic hydrolysis of sphingomyelin by acid sphingomyelinase) has been implemented in the rapid uptake of oligoarginine (Verdurmen *et al.*, 2010), hinting towards the enhancement of penetration near the boundary sites of membrane microdomains or phases. There the height mismatch could provide the imperative free energy for creating a hexagonal phase transition (Hurley *et al.*, 2010) leading to a direct translocation of the peptide into the cytosol (Ziegler, 2008).

However, usually, a relatively high extracellular concentration of the peptide needs to be used in order to bring about this mode of entry (Duchardt *et al.*, 2007; Tünnemann *et al.*, 2008). Furthermore, this uptake mode is more commonly established when CPPs are used alone (Luedtke *et al.*, 2003) rather than in a complex with a cargo molecule. Thus, when attempting to transport a (bio)molecule into the cells with CPPs, the exploitation of an endocytic mechanism appears to be prevalent (Console *et al.*, 2003; Ferrari *et al.*, 2003; Ignatovich *et al.*, 2003; Richard *et al.*, 2003).

Endocytosis is a natural way for a cell to sense its surrounding environment and acquire essential nutrients that cannot, in other ways, cross the barrier imposed by the plasma membrane. During endocytosis, the extracellular material is engulfed by the invagination of the plasma membrane to form a small vesicle that after budding from the membrane migrates to the cell interior (Lodish *et al.*, 2000). However, before the eventual uptake into membrane-bordered vesicles, membrane budding needs to be triggered at the site of cell entry. As stated by Hurley et al. “a dance between proteins and lipids leads to membranous buds” and the formation of spherical vesicles from flat membrane areas takes place (Hurley *et al.*, 2010). Therefore, both lipids and proteins contribute to the overcoming of the energetic barrier in order to create the necessary membrane curvature. The distinct mechanisms involved in different endocytic events will be further discussed below.

The endocytic machinery is comprised of a sophisticated array of different pathways that vary in cargo selectivity, intracellular trafficking and destination (Doherty and McMahon, 2009). Besides the phagocytic uptake that only occurs in certain cell types (e.g. macrophages), the endocytic routes can be subdivided into the clathrin-mediated, the caveolin-mediated, the clathrin- and caveolin-independent endocytosis, and the macropinocytosis.

1.3.2. Clathrin-mediated pathway

Clathrin-mediated endocytosis (CME) is the most prominent form of traffic from the plasma membrane to endosomes, a pathway by which ligands, such as transferrin, hormones and low-density lipoproteins with their receptors, enter the cells (Kirchhausen, 2000; Ehrlich *et al.*, 2004). Clathrin-coated vesicles (CCV) (usually 100–150 nm in diameter) are formed in confined plasma membrane areas called clathrin-coated pits (CCP) by progressive clustering of clathrin molecules that forces the membrane region to deform as it grows (Hinrichsen *et al.*, 2006). The clathrin molecule itself has a three-limbed shape which is called a triskelion (Greek: triskelion, meaning three-limbed or three-legged) (Lodish *et al.*, 2000). Upon membrane invagination in the CCPs, these triskelions polymerize to assemble into a cage-like structure, resembling the seam of a soccer ball, surrounding the membrane of a forming CCV (Kirchhausen, 2000).

Clathrin itself, however, does not interact with a lipid bilayer, and specific proteins must therefore recruit it to the membrane. Several adaptor proteins, adaptor protein complex 2 (AP-2) being the most comprehensively studied, are involved in the coat assembly of CCVs (Kirchhausen, 2000). The proteins in the AP-2 complex possess several different ligand binding moieties, which help to determine which proteins are specifically included in (or excluded from) the budding transport vesicle (Lodish *et al.*, 2000; Pearse *et al.*, 2000; Traub, 2009). The AP-2 complex, in turn, is recruited into the forming CCPs via interactions with a plasma membrane specific lipid, phosphatidylinositol(4,5)-bisphosphate ($PI(4,5)P_2$), which assists in the nucleation of AP-2 and clathrin at the membrane (Puertollano, 2004). Thus, in the presence of a cargo, $PI(4,5)P_2$, the adaptor protein complex and clathrin, a platform for CCP is generated.

The formation of the honeycomb-like CCV is terminated by its scission from the plasma membrane, where the membrane pinching by dynamin, a large GTPase, separates the fully formed coated vesicle from its parent membrane (Kirchhausen, 2009; Pucadyil and Schmid, 2009). Right after the formation of the CCV, the clathrin coat is shed, leaving the now uncoated vesicle free to be transported inside the cell to the site of vesicle fission.

As transferrin exploits the CME pathway as a gate for cell entry upon binding to its cell surface receptor (TfR), it is widely used as a marker for highlighting the vesicles formed by the CME inside the cells (van Dam and Stoorvogel, 2002) to study the internalization characteristics of CPPs and CPP-cargo complexes. Additional hallmark of the CME is its distinct electron microscopical structure (electron-dense bristled appearance). The involvement of the CME in the uptake process can also be studied using specific antibodies or inhibitors of the clathrin-mediated route (e.g. hyperosmolar conditions) and/or mutated proteins involved in or required for the assembly of the coat.

Using these conditions, it has been demonstrated that several cationic CPPs either on their own or with protein cargo can facilitate receptor internalization without displaying specificity (Fotin-Mleczek *et al.*, 2005), referring to at least

a partial utilization of the CME pathway in their uptake. Since HSPGs, as mentioned above, may act as membrane receptors for positively charged CPPs, it is highly possible that the CPP, as a ligand, clusters the surface sugars together initiating their internalization via the CME (Richard *et al.*, 2005). This is also supported by the discovery that extracellularly applied Tat protein can induce the binding and clustering of a receptor on CD8 T-cells, forcing it to internalize into the cells (Faller *et al.*, 2010). Furthermore, additional studies have confirmed that clathrin and the CME, indeed, play a role in the internalization of CPP or CPP-cargo complexes (Vendeville *et al.*, 2004; Padari *et al.*, 2005; Rinne *et al.*, 2007).

1.3.3. Caveolin-dependent pathway

The role of the caveolin-mediated pathway has been implicated in a number of different cellular processes, for instance endocytosis, transcytosis, calcium signaling, adhesion, motility and numerous other signal transduction events (Lisanti *et al.*, 1995; Pelkmans *et al.*, 2004; Parton and Simons, 2007; Howes *et al.*, 2010; Sinha *et al.*, 2011). However, unlike the CME, the exact role of the caveolin-dependent endocytosis inside the cells still remains somewhat mysterious. Despite the restricted knowledge about the function of the caveolin-mediated pathway, the structural components and features have been clarified over the past years.

Caveolae ("little caves") have been defined as flask-shaped 60–80 nm invaginations of the plasma membrane. An important milestone in the caveolae field was achieved with the immunocytochemical demonstration of caveolin (now caveolin-1) as a specific component of the ridges that make the caveolar coat (Rothberg *et al.*, 1992). Since then, caveolae have been exclusively defined as plasma membrane regions containing caveolins. As caveolins reside only in the strictly ordered membrane microdomains called lipid rafts (Tagawa *et al.*, 2005), the caveolin-containing structures assemble in membrane regions rich in raft lipids, caveolins and caveolin-associated cavins (Stan, 2005; Hansen and Nichols, 2010).

Caveolin itself is a transmembrane protein adopting a hairpin conformation into the membrane, leaving both the N- and the C-termini of the protein facing the cytoplasm (Dupree *et al.*, 1993). Caveolae contain a consistent number of caveolin molecules, ~144, which suggests the formation of a highly organized coat (Pelkmans and Zerial, 2005). Palmityolation, a posttranslational modification of caveolins at multiple residues promotes their constitutive association with cholesterol and other raft lipids (Pelkmans and Helenius, 2002). It has been hypothesized that the insertion of caveolin into the membrane shifts the intrinsic curvature of the membrane such that the positively curved bud is the low-energy state and the flat caveolin microdomain is the high-energy state (Hurley *et al.*, 2010), thus, the basis for a membrane bud is generated.

Little, however, is known about how the cargo is selected for the internalization via the caveolin-mediated pathway. In contrast to the CME, where specific adaptor proteins have been identified to recruit cargo to coated pits, no such well-defined adaptors have been established for the caveolin-dependent endocytosis so far. The potential mechanisms for cargo selection to caveolae have been divided into lipid- and protein-based mechanisms, although the various options within each type of mechanism are only beginning to be determined (Mayor and Pagano, 2007).

Ligands or membrane constituents that are reported to be internalized via caveolae include the commonly used caveolin-dependent endocytosis marker cholera toxin (Montesano *et al.*, 1982). Recently, however, it was discovered that cholera toxin is not a specific marker for caveolin-mediated endocytosis, as it can also exploit other clathrin-independent endocytosis (CIE) routes to gain entry to the cells (Howes *et al.*, 2010).

Additionally, several pathogens (e.g. the simian virus 40 (SV40)) are shown to take advantage of the caveolins in order to infect the target cells (Pelkmans *et al.*, 2001). Nevertheless, since SV40 binds the ganglioside GM1 on the plasma membrane (Ewers *et al.*, 2010) and the GM1 is demonstrated to only concentrate to the caveolae rather than exclusively reside there (Parton, 1994), the SV40 cannot be considered as a specific marker for caveolae either. Furthermore, SV40 has been demonstrated to still enter cells devoid of caveolin (Damm *et al.*, 2005), strengthening the notion that the virus can exploit different entry mechanisms.

The lack of specificity of different "markers" for caveolae makes it difficult to assess the exploitation of this particular pathway in the uptake of CPPs and CPP-cargo complexes. Alternatively, the use of raft disrupting agents (e.g. methyl-β-cyclodextrin), specific antibodies against caveolins, fluorescently tagged caveolin constructs, or caveolin-null cells has provided valuable information about the cell entry of different CPPs. Using these tools it has been demonstrated that the internalization of Tat-protein constructs relies heavily on the caveolin-dependent route (Ferrari *et al.*, 2003; Fittipaldi *et al.*, 2003). Furthermore, it was recently determined that this particular pathway may contribute upto 60–70% of uptake in the case of TP- or TP10-protein complexes (Säälik *et al.*, 2009).

1.3.4. Macropinocytosis

Macropinocytosis, an actin-driven endocytic process, represents a distinct pathway of endocytosis since it is not regulated through the direct actions of cargo/receptor-molecules coordinating the activity and recruiting the specific effector-molecules to particular sites at the plasma membrane, as in case of the CME or the caveolin-dependent endocytosis. The macropinosome was originally described as a large (diameter greater than 0.2 μm) heterogeneous phase-bright organelle observed to emanate from the base of the waving sheet-like extensions of the plasma membrane called membrane ruffles (Kerr and Teasdale, 2009). The ruffles are derived by a directed actin polymerization near the plasma membrane that generates a roughly planar extension of the cell surface. These dynamic protrusions provide the membrane and the energy required for the formation of the macropinosome. While most ruffles simply melt back into the plasma membrane, a few fold back forming fluid-filled cavities and undergo a membrane fission encapsulating large volumes of extracellular fluid (Doherty and McMahon, 2009). The directed actin polymerization is stimulated by small GTPases, e.g. Rac1, that regulate the advancement of the protrusion and the formation of the pinocytic cup (Ridley *et al.*, 1992; Hoppe and Swanson, 2004). Rac1 is shown to be activated early in the macropinocytic process and persists there until just after the cup closure (Swanson, 2008). The involvement of cholesterol in anchoring of the activated Rac1 to the sites of forming macropinosomes has been demonstrated (Grimmer *et al.*, 2002), referring to its role in the process.

Additionally, several lipids have been associated with the macropinocytic event, for example, the local levels of PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 are dramatically elevated in the forming macropinocytic cups (Okada *et al.*, 1996; Araki *et al.*, 2007). The substantial local accumulation of specific lipids at the heavily activated regions of the plasma membrane likely leads to the dramatic intracellular changes at these sites. The subsequent profound interplay between the plasma membrane organization and the cytoskeleton possibly accounts for the morphological features of this process (Doherty and McMahon, 2009).

The fission of the macropinocytic vesicle occurs, unlike in clathrin- and caveolin-mediated endocytosis, dynamin-independently and requires instead a CtBP1/BARS protein complex. The CtBP1/BARS is upon activation of macropinocytosis translocated to the macropinocytic cup and its surrounding membrane, where it is activated by phosphorylation and severs the bud neck (Liberali *et al.*, 2008).

Macropinocytosis is a constitutive process only in specialized cell types (dendritic cells and macrophages), where it plays a role during, for example, the immune response via either the presentation of antigens or the engulfment of pathogens or apoptotic bodies (Nobes and Marsh, 2000; Kerr and Teasdale, 2009). In other cell types, macropinocytosis is induced in response to certain growth factors, e.g. EGF, that trigger the activation of receptor tyrosine kinases leading to their uptake by macropinocytosis (Brunk *et al.*, 1976). Additionally,

several viruses, e.g. adenovirus, exploit the macropinocytic pathway for internalization and subsequent infection (Mercer and Helenius, 2009).

The involvement of macropinocytic uptake has also been described by demonstrating the participation of massive actin rearrangement in the uptake process of different CPP-cargo constructs (Nakase *et al.*, 2004). Moreover, the tendency of TP-protein complexes to favorably interact with the plasma membrane protrusions has been corroborated by electron microscopy studies (Padari *et al.*, 2005). It is thus possible that CPP-protein complexes associating with the extracellular face of the plasma membrane are capable of inducing the formation of these protrusions to evoke their internalization into the cells. Providing further evidence in support of this assumption, the ability of different CPPs to activate Rac1 and the consequent actin remodeling has been demonstrated repeatedly (Gerbal-Chaloin *et al.*, 2007; Imamura *et al.*, 2011). It has been suggested that the HSPGs, aiding the initial binding of the arginine-rich peptides onto the plasma membrane, can stimulate the activation of Rac1, the downstream actin polymerization and the membrane fluctuations (Futaki *et al.*, 2007). However, before any conclusive data is presented, this hypothesis remains strictly theorethical.

Because the disruption of the lipid rafts by the methyl-β-cyclodextrin treatment, abolished the cellular entry of the Tat peptide (Kaplan *et al.*, 2005), the role of cholesterol and the specific lipid microdomains in the internalization of CPPs was verified. Nevertheless, since caveolin also inhabits these lipid rafts, the mere destabilization of the microdomains does not provide convincing enough evidence about the involvement of the distinct pathways in the cell entry of CPPs. In any case, the role of macropinocytosis in the uptake process of CPP-protein complexes was further confirmed with the use of amiloride or EIPA, i.e. the Na^+/H^+ pump inhibitors widely used as selective inhibitors of macropinocytosis, which led to a marked decrease in the uptake of Tat-Cre constructs (Wadia *et al.*, 2004). However, contradictions arose when Zaro et al. demonstrated that in their experimental setup at least the membrane transduction of oligoarginine occurs separately from macropinocytosis in HeLa cells (Zaro *et al.*, 2006).

1.3.5. Clathrin- and caveolin-independent pathways

In addition to the CME, the caveolin-mediated endocytosis and the macropinocytosis, other, so-called clathrin- and caveolin-independent pathways facilitating the uptake of several receptors, molecules or pathogens, are active inside mammalian cells. The classification of this pathway was proposed on the basis of the small GTPases acting as master-switches in and associating specifically with the separate routes, namely RhoA, Cdc42, and Arf6 (Mayor and Pagano, 2007). However, since the detailed characterization of the particular routes is ongoing, the division is far from being complete. Furthermore, since the endocytic mechanisms falling into this category are, so far, the least understood, the

boundaries between routes classified under the clathrin- and caveolin-independent pathways are not always distinct and the required regulators of different pathways may have coinciding activities (Howes *et al.*, 2010).

Another characteristic associated with the clathrin- and caveolin-independent pathway is the inclusion of the lipid rafts that appears to be important for many surface components to be internalized via this particular pathway. For example, the interleukin receptor 2, that has been shown to enter cells via this pathway, partitions to detergent-resistant membrane regions (Lamaze *et al.*, 2001). Therefore, lipid raft components in combination with specific small GTPases facilitate the uptake via the clathrin- and caveolin-independent pathway.

In spite of the progress made in identifying the necessary components for this pathway, little is known about the driving force for the membrane deformation and fission during the generation of, for example, the clathrin-independent carriers (CLICs). GRAF1, identified as an ingredient of the CLIC pathway (Lundmark *et al.*, 2008), has the ability to generate membrane curvature via its BAR domain, yet compared to other BAR-containing proteins it is relatively inefficient in this respect. It might be that GRAF1 functions more as a sensor of curvature and thereby localizes to membrane tubules (produced by other proteins) and functions to stabilize their high curvature (Doherty and Lundmark, 2009).

The essentiality of dynamin in the process of vesicle neck scission was first invalidated when it was demonstrated that the inhibition of dynamin did not block the fluid phase uptake (Damke *et al.*, 1995), hinting that at least some pathways in the cell do not require the activity of dynamin for vesicle formation. However, at least the RhoA-dependent pathway utilized by some cytokine receptors still demands the presence of dynamin for vesicle severing (Lamaze *et al.*, 2001; Sauvonnet *et al.*, 2005). How the membrane scission takes place in the absence of dynamin is not clear, but the involvement of actin filaments and their spatial reorganization has been proposed (Liu *et al.*, 2006). This emphasizes the feature of the actin filaments to exert protrusive surface stresses on the forming bud and tubule. Additionally, the ability of actin to reorganize membranes and facilitate the subsequent bud neck scission was corroborated recently (Römer *et al.*, 2010).

Unlike the CME and the caveolin-mediated endocytic vesicles, where identification of the carriers is easy enough by only morphological characterization due to the distinct appearance of their coat, the vesicles formed by the non-clathrin and non-caveolar endocytosis do not seem to possess a coat and have been shown to display a rather diverse array of carrier morphology, i.e. small uncoated vesicles, ring-shaped structures or large tubular carriers (Lamaze *et al.*, 2001; Kirkham *et al.*, 2005; Römer *et al.*, 2007).

The most thoroughly studied clathrin- and caveolin-independent carriers arise from the internalization of the glycosylphosphatidylinositol (GPI)-anchored proteins. The proteins attached to the outer leaflet of the plasma membrane by a GPI lipid anchor are taken up by cells via the Cdc42-dependent

clathrin- and caveolin-independent pathway. Furthermore, their internalization appears to not require dynamin (Sabharanjak *et al.*, 2002; Mayor and Riezman, 2004). Upon entry, the GPI- anchored proteins (GPI-APs) are found inside primary carriers called CLICs, which are morphologically distinct from both CME intermediates and caveolae in that they have a tubular and ring-like appearance (Kirkham *et al.*, 2005). Inside the cells, the CLICs mature into tubulovesicular endosomes known as the GEECs (GPI-anchored-protein-enriched early endosomal compartments), which is why the pathway is often called the CLIC/GEEC pathway.

The sorting mechanism to the GEECs is to date relatively unclear, however, an original mechanism for the sorting of lipid-anchored proteins by a steric exclusion (and not cargo signals or lipid-structure factors) from the clathrin-coated pits and the eventual uptake by GEECs was recently established (Bhagatji *et al.*, 2009). Nevertheless, as mentioned above, several pathogens, e.g. SV40 (Damm *et al.*, 2005), and bacterial toxins, e.g. cholera toxin, can exploit this non-conventional uptake route for cell entry (Howes *et al.*, 2010). However, since their internalization occurs also via the caveolin-mediated pathway (as referred to already before in chapter 1.3.3.), their use as specific markers is limited. Since the specificity of the known markers has been jeopardized, the role of the CLICs and GEECs in CPP-mediated cargo delivery has not yet been analyzed to draw any conclusive resolutions. Despite of this, one cannot rule out the possibility of the use of this particular pathway in the transportan-mediated protein transport. The basis for this hypothesis comes from the notion that the TP- and TP10-avidin complexes were demonstrated not to be targeted to the conventional Rab5-positive early endosomes (Säälik *et al.*, 2009). As at least a certain type of GPI-APs are internalized via a clathrin-independent pathway leading to the trafficking of the endocytosed material to an unorthodox subgroup of Rab5-independent tubular vesicles (Sabharanjak *et al.*, 2002), it is possible that these two subgroups of Rab5-negative endosomes are formed via the same pathway. Recently, however, a new plasma membrane antigen CD44 was identified to use the CLIC/GEEC pathway during internalization (Howes *et al.*, 2010), thus, the role of the CLIC/GEEC pathway in the CPP-mediated uptake should be addressed once again.

The search for specific internalization pathways used by CPPs or CPP-cargo complexes has proven to be a difficult task due to the repeated recognition that several different endocytic routes can and probably are utilized in parallel (Säälik *et al.*, 2004; Melikov and Chernomordik, 2005; Räägel *et al.*, 2010). Furthermore, down-regulation or inhibition of specific routes may lead to the up-regulation of other pathway(s) and promote thus simply a switch from one endocytic type to another. Additionally, the varying membrane composition and levels of activity of different endocytic routes in different cell types makes the extrapolation of the data a rather complex task.

1.4. Intracellular trafficking of endocytosed material

After budding from the plasma membrane the cell-penetrating peptide-cargo complexes are confined in (some sort of) endocytic vesicles. As concluded above, the internalization route(s) utilized by the CPP-cargo complexes are fairly well documented, but the data available are ambiguous and often contradictory. The fate of the complexes after cell entry, however, is far less investigated, and thus, very little information exists on their intracellular trafficking. Because endocytosis is confirmed to play a major part in the internalization process of the CPPs, either alone or in complex with a cargo, it is essential to dissect in detail the events happening after endocytic capture at the plasma membrane.

In general, there are two major intracellular pathways initiating from the plasma membrane – the recycling and the endo-lysosomal pathway – and the specific intracellular pathway chosen determines the eventual fate of the endocytosed material. But how can the different endocytic organelles be distinguished from each other inside the cells when, for example, the clathrin coat is shed from the carrier right after its fission from the plasma membrane losing thus its characteristic appearance?

One of the early findings that paved the way for our current understanding of defining the specific intracellular vesicles was the observation that different Rab GTPases are localized to distinct organelles (Chavrier *et al.*, 1990) via submersion into the membrane upon their activation by their specific guanine nucleotide exchange factors (GEFs). Since then, the organelle identity of different intracellular vesicles has been assigned to the existence of specific Rab GTPases on their membrane (Chavrier *et al.*, 1990). For example, the Rab4 and Rab11 are associated with the recycling endosomes, Rab5 with the early endosomes, and Rab7 with the late endosomes (Pfeffer, 2001). In addition to marking the specific intracellular organelles, the different Rab GTPases function as molecular switches in controlling the vesicle maturation, transport, and fusion with the target membranes (Stenmark, 2009). Additionally, the existence of specific lipids, especially specific phosphatidylinositols (PtdIns-s), allows the deciphering of different organelles. The PtdIns that localizes, in abundance, to the endosomal compartments is the phosphatidylinositol-3-phosphate (PI3P) (Clague *et al.*, 2009).

Coming back to the two major intracellular pathways, both of the trafficking routes, the recycling and the endo-lysosomal one, are initiated at the early endosome (EE), where the initial intracellular sorting takes place. The EE is thus a hub for multiple pathways. The sorting events launched at this compartment determine the subsequent fate of the internalized proteins and lipids, destining them for either recycling to the plasma membrane, degradation in lysosomes, or delivery to the *trans*-Golgi network (TGN). The sorting of the endocytic cargo to the latter compartments is accomplished through the formation of distinct microdomains within the early endosomes through the coordinated recruitment

and assembly of the sorting machinery (Jovic *et al.*, 2010). The different Rab proteins that facilitate the formation of distinct carriers from the EE occupy distinct membrane microdomains (Sonnichsen *et al.*, 2000), creating a platform for fission of the specific vesicles.

At the recycling compartment membrane, proteins can be distributed further to the TGN or, in polarized cells, to the opposing plasma membrane domain. Most membrane proteins in the recycling compartment are, however, efficiently transported back to the plasma membrane (Mellman, 1996). The transferrin (Tf) receptor (TfR) and its ligand Tf have been used extensively as markers of the recycling pathway. Both Rab4 (van der Sluijs *et al.*, 1992) and Rab11 (Ullrich *et al.*, 1996) have been identified to function in the TfR recycling pathway. However, the two different endosomes (the Rab4- and the Rab11-positive ones) were characterized as two distinct populations of endosomes with different sorting function (Sheff *et al.*, 1999). It was demonstrated that the Rab4-containing endosomes facilitate a rather quick plasma membrane recycling, whereas the Rab11-positive structures are trafficked longer through the recycling endosomal compartment that resides in the perinuclear region of the cell.

The engulfed material that follows the classical endo-lysosomal pathway is, on the other hand, sorted from the EE to the Rab7-positive late endosomes (LE) and from thereon to the lysosomes, where it is degraded by the pH-sensitive enzymes. These endosomes of the degradative pathway do not exist as distinct stable organelles, but instead, undergo a maturation by changing their repertoire of membrane proteins, for example, by losing the Rab5 and gaining the Rab7, a hallmark for the transition of EE to LE (Rink *et al.*, 2005).

The significant remodeling of the endosomal membrane during endosomal maturation is guided by the arrival of SAND-1 which interacts with the Rab5 activator (i.e. a Rab5-specific GEF) called RabX-5 and displaces it from the endosomal membrane. This, in turn, initiates the replacement of Rab5 with Rab7 (Poteryaev *et al.*, 2010), promoting thus the conversion of EE to LE. The mechanism behind SAND-1 binding and initiation of the switch is not known. However, the relatively constant size with which the EEs undergo conversion could suggest that SAND-1 could either recognize a particular size or age of the endosome or the accumulation of specific factors, e.g. certain lipids, on the EE membrane (Poteryaev *et al.*, 2010).

Inside the EE, the cargo internalized e.g. via the receptor-mediated clathrin-dependent pathway, is dissociated from its receptor by the pH shift (to approximately 6.5), after which the cargo remains in the lumen of the endosome and the receptor accumulates in the membrane-rich tubular portions. From thereon the receptor finds its way back to the plasma membrane via the recycling endosomes (RE), whereas the cargo is directed to the lysosomes for degradation (Mellman, 1996; Pollard and Earnshaw, 2008).

Yet, not all material from the endo-lysosomal pathway is targeted for degradation. For example, the molecules internalized through the unconventional CLIC/GEEC pathway may be targeted inside the cell to either other compart-

ments, such as the Golgi apparatus, or fuse eventually with the early endosomes in a Rab5-dependent manner (Doherty and Lundmark, 2009). Additionally, budded caveolae have shown to accumulate to a distinct rosette-like intracellular structure called the caveosome (Pelkmans *et al.*, 2001; Nichols, 2002), which is distinguishable from the EE by its neutral pH. Whether the caveosome is a fusion station for budding caveolar vesicles or rather a sorting site is not yet clear. However, it is shown that material inside these structures sort their content also to the Golgi and the ER by the retro-grade transport (Nichols, 2002).

The characterization of the intracellular trafficking of CPPs or CPP-cargo complexes has turned out to be problematic due to the intermixing of different intracellular pathways, making it difficult to analyze a single pathway at once. A study characterizing the CPP-protein uptake by, for example, the caveolin-mediated pathway revealed that irrespective of the fact that caveosomes are able to target their content to the Golgi or ER, a specific localization of the complexes to these structures was not detected (Padari *et al.*, 2005). Yet, a portion of CPP-protein complexes was found right after internalization to reside in endosomes devoid of Rab5 and PI3P (Säälik *et al.*, 2009), hinting towards the use of some unconventional pathway for intracellular trafficking.

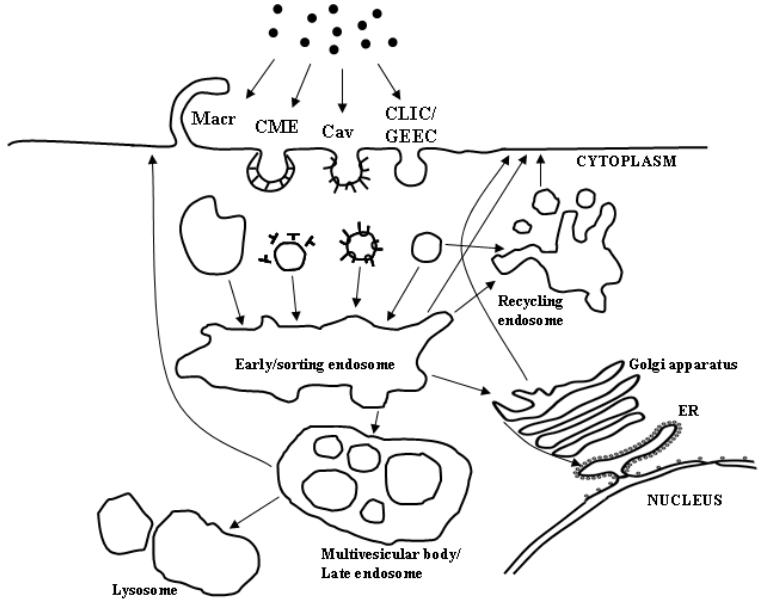

Figure 1. Endosomal entry and intracellular trafficking pathways available for exploitation by the CPP-protein complexes. Macr – macropinocytosis, CME – clathrin-mediated endocytosis, Cav – caveolin-mediated endocytosis, CLIC/GEEC – clathrin- and caveolin independent endocytosis.

Nevertheless, since several endocytic pathways ultimately target their content to acidic compartments, the accumulation of different CPP-cargo complexes to large acidic lysosomes has been corroborated repeatedly (Padari *et al.*, 2005; Al-Taei *et al.*, 2006; Laufer and Restle, 2008). Inside this acidic environment the CPPs and the cargo attached to them are likely to be destined for degradation. Because of this, it has become increasingly apparent that not the uptake but rather the intracellular trafficking is the bottleneck in achieving the effective cytosolic delivery in the CPP-mediated transport.

1.5. Induction of endosomal escape

The endosomal entry to the cells represents the two contradicting sides of one coin. On one hand, the endocytosis is a natural way for a cell to obtain molecules from the surrounding environment, being thus harmless to the cell. On the other hand, though, after endocytic uptake the CPP-cargo complexes are still isolated from the cell interior and are, as mentioned above, to a large extent probably targeted to degradation. Therefore, in order to complete the effective delivery, the transport peptide needs to somehow break loose from this "death row"-like confinement to facilitate the entry of the cargo into the cytosol of cells. Thus the key question still remains – how can CPPs and CPP-cargo complexes cross the membrane?

As mentioned above, the direct penetration of CPPs and especially the CPP-cargo complexes across the plasma membrane is relatively ineffective if not absent altogether. The situation inside the endocytic vesicles is actually quite similar with differences only in a couple of aspects. Yet, these discrepancies probably make all the difference and provide the required environment for effective escape.

Firstly, the capture of the internalized CPPs and CPP-cargo complexes into the endosomal lumen extensively elevates the local concentration of the peptide per unit area of the membrane. At high concentrations, the peptide accumulates and clusters at the membrane interfering with the regularity of its packing and inducing its subsequent leakage, which has been demonstrated, for example, with TP (Hällbrink *et al.*, 2001). Additionally, arginine-rich peptides can at higher peptide concentration induce membrane destabilization and pore formation (Herce *et al.*, 2009). Thus, it is plausible to conclude that the liberation of the CPPs and the CPP-cargo complexes occurs via the membrane destabilization and subsequently their slipping out of the leaky endosomes, as seen in the electron microscopy images (Padari *et al.*, 2005; Padari *et al.*, 2010). Whether the endosomes are ruptured entirely or they are just leaky is not known. Moreover, the exact amount of the peptide and cargo actually reaching the cytoplasm has not been defined, although it does seem to be the limiting step in CPP-mediated delivery today.

The second factor that has been implicated to play a role in the endosomal escape of the CPPs is the pH inside the vesicles, yet contradictory reports exist on the matter. One set of publications claim that the gradual drop of pH is needed to trigger the conformational changes of the peptide to induce its insertion into the membrane (Fischer *et al.*, 2004; Vendeville *et al.*, 2004; Abes *et al.*, 2008). This is similar to what is observed for several bacterial toxins (e.g. the diphtheria toxin (Falnes and Sandvig, 2000)) or viral proteins (e.g. the influenza hemagglutinin (Harrison, 2008)). Other publications, on the other hand, provide evidence that the inhibition of the endosomal acidification (by lysosomotrophic agents) elevates drastically the level of bioactivity of the cargo molecule. In that case the increased bioactivity arises from the increased delivery of the molecule into the cytosol of the cells, where it is able to interact with its intracellular target(s) (El-Andaloussi *et al.*, 2006; Abes *et al.*, 2007). Nevertheless, since low pH activates the lysosomal enzymes, it would be preferable if the complexes escaped before the low pH-activated enzymes render the cargo dysfunctional.

The third determinant possibly (also positively) affecting the translocation of the CPPs through the endosomal membranes (and not through the plasma membrane) is the lack of the reinforcing cytoskeleton or the framework of other structural proteins (e.g. clathrin) around the endosomes. On the contrary, at the plasma membrane, the meshwork of cortical actin cytoskeleton composed of a dense lattice-like network of filaments that underlie and attach to the plasma membrane (Henderson *et al.*, 1992) could elicit an additional hurdle for the CPPs. Thus the direct plasma membrane penetration could be impeded even if the peptide has been successful in submersion into the lipid environment. Nonetheless, also other properties, e.g. the specific lipid composition in the membrane, may additionally contribute to the heightened membrane affinity of different CPPs in the endosome. This hypothesis is, however, more thoroughly discussed in the results and discussion section.

Due to the limited endosomal escape facilitated by CPPs themselves, different chemical or physical methods for rupturing the endosomal membrane have been described to possibly enhance their delivery into the cytosolic compartment. As mentioned above, the hemagglutinin (HA) protein of the influenza virus coat is capable of acting as a fusogenic agent that is upon a pH drop converted to a hydrophobic helical conformation. This leads to the fusion of the viral membrane with the endosomal membrane resulting in the release of the viral content into the cytosol (Wiley and Skehel, 1987). A truncated peptide from HA has been successfully applied in the CPP-mediated transport by coupling the fragment to, for example, the Tat peptide (Wadia *et al.*, 2004).

The use of lysosomotrophic agents with a high buffering capability, such as chloroquine, mediate an effect called “the proton sponge effect”, where the chemical agent causes an extensive inflow of ions and water into the endosomal compartment upon its protonation. The massive inflow leads to the swelling of the endosomes and eventually to the rupture of the endosomal membrane

releasing its contents (Varkouhi *et al.*, 2010). The use of chloroquine in cell culture is easy and has proven to drastically increase the cytosolic delivery of CPPs and their attached cargo (El-Andaloussi *et al.*, 2006; Abes *et al.*, 2007). However, the use of this chemical enhancer is limited in the *in vivo* context. Nevertheless, the inclusion of chloroquine moieties into the sequence of the transport peptide has recently been efficiently applied in the delivery of siRNA (El Andaloussi *et al.*, 2011).

Additionally, the possibility of photochemically releasing biologicals from the endosomal pathway into the cytosol has been described as a technique called photochemical internalization. Photosensitizers can internalize into cells and localize primarily in the membrane of endosomes and lysosomes. After exposure to light, these photosensitizers induce the formation of reactive oxygen species that destroy the membrane they reside in (Berg *et al.*, 1999). The photostimulation (or photo-induction) technique has proven to be successful also in the redistribution of CPPs from endosomes to the cytoplasm and nucleus (Maiolo *et al.*, 2004). The different endosomal escape mechanisms effectively (or ineffectively) put to use in the CPP-mediated transport was recently reviewed by El-Sayed et al. (El-Sayed *et al.*, 2009).

1.6. Stability and toxicity of CPP and CPP-cargo complexes

Due to the massive accumulation of CPPs and their complexes in the acidic lysosomes after internalization (Padari *et al.*, 2005), their stability is another issue demanding attention in order to improve the transport efficiency of these peptidic vectors. A recent review by Aubry et al. collects the scarce information available on the intracellular stability of CPPs (Aubry *et al.*, 2010) and reports that, inside the cells, an arginine-rich peptide remains intact for upto 4 h, after which it starts to decrease slowly reaching to about 30% stability after 18 h incubation (during a continuous pulse experiment). However, as mentioned above, the data existing on the matter is relatively insufficient.

Additionally, the limitation in the stability of CPPs, coming to play during their *in vivo* use, is their fast degradation in the extracellular environment by e.g. the plasma enzymes. The composition of a large fraction of CPPs containing a high number of positively charged amino acids (as arginine and lysine) makes them highly susceptible to proteolysis by tryptic enzymes. As Tat, for example, has six potential trypsin cleavage sites (Chauhan *et al.*, 2007) and its cell penetration property relies heavily on the integrity of the highly basic sequence (Wender *et al.*, 2000), it is clear that the potential of Tat or similar peptides as mediators of intracellular delivery of therapeutic molecules could be significantly limited by their cleavage by the proteolytic enzymes. A recent publication reporting on the stability of the Tat peptide states that only 25% of Tat is present in its intact form after 10 minutes of incubation in the human

plasma (Grunwald *et al.*, 2009), corroborating its weakness against serum components.

In order to increase the stability of CPPs against degradative enzymes, several methods have been used, one of them being the replacement of the naturally occurring L-amino acids in the peptides' sequence with their D-counterparts. This replacement technique has been demonstrated to drastically enhance their preservation (Elmquist and Langel, 2003), resulting in their accumulation in the target cells (Wender *et al.*, 2000; Won *et al.*, 2010). An extension of the replacement strategy is the synthesis of the so-called retro-inverso (RI) peptides, where the D-amino acid sequence has been inverted creating a mirror-image of the peptide to better mimic the original chirality and the side chain topology of the L-isomer. Thus, the accompanied chain reversal yields a proteolytically stable RI peptide isomer, whose side chain topology, in the extended conformation, corresponds closely to that of a native sequence, and whose biological activity emulates that of a parent polypeptide (Howl *et al.*, 1999). The impact of the retro-inversion on the CPPs is not fully understood and conflicting data exists on this issue with some reports claiming the enhanced internalization properties of the RI-peptides (Brugidou *et al.*, 1995; Snyder *et al.*, 2004; Tünnemann *et al.*, 2008), others, instead, show their inability to internalize into cells altogether (Zhang *et al.*, 2004).

Increasing the stability of the vector possibly enhances the effects of the cargo both in total and over longer time periods, however, the gradual accumulation of the enzymatically stable peptide to the cell interior could eventually induce cytotoxic side-effects. The cytotoxicity of CPPs is usually studied as their ability to generate pores into membranes (detected as increased leakage of soluble cytosolic material (lactate dehydrogenase (LDH) or deoxyglycose (DOG)) into the extracellular medium or as the entrance of extracellularly applied chemicals (e.g. propidium iodide (PI) into cells) or their propensity to affect the mitochondrial activity of the treated cells. As the membrane activity of different peptides was already discussed above, the focus is now shifted towards the processes facilitating the cell death via the decrease in mitochondrial activity.

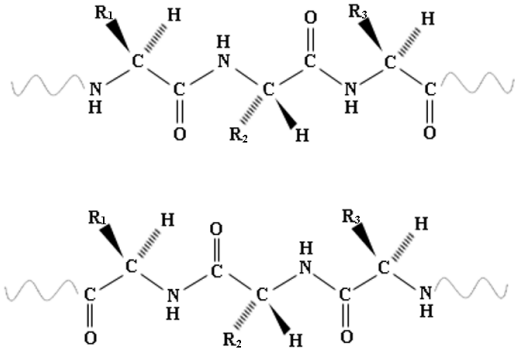

Figure 2. Retro-inversion of peptides. The L-amino acids in the parent peptide (upper) are replaced by their D-counterparts and the subsequent sequence inverted, yielding a retro-inverso peptide (lower) with a similar side-chain topology as the parent peptide, but possessing an elevated stability.

1.7. Cell death

Cell death is a key factor in a number of different biological processes, such as embryonic development, establishment of self-immune tolerance, regulation of cell viability by hormones or growth factors, to name a few. Thus, a controlled cell death provides both an organizational and functional basis for an organism. Cell death can be induced by several different mechanisms and it is most commonly visualized by morphology as extensive plasma membrane blebbing, granulated cytoplasm, chromatin condensation, and so on. Due to the large number of different pathways leading to cell death, here only the involvement of the mitochondria, with the emphasis on caspases and the Ca-ions, is brought into the spotlight.

In addition to their role as powerhouses of the cell, the mitochondria display an essential role as sensors of the health of the cell. If cells sense insults from which they cannot recover, the mitochondria trigger the intrinsic cell death pathway by releasing apoptogenic factors, which are otherwise stored safely inside the organelle. Upon a receipt of a death signal, the pro-apoptotic Bcl-2 family proteins Bax and Bak insert deeply into the mitochondrial outer membrane, where they form oligomers and, possibly via generation of membrane pores, cause its permeabilization. Permeabilized mitochondria lose their membrane potential and release the proteins of the intermembrane space (including cytochrome c) into the cytosol. Cytosolic release of cytochrome c results in the activation of the caspase cascade by removing the auto-inhibition and permitting thus the binding and autoactivation of the procaspase 9 to the apoptosome. The triggered caspase cascade results in the cleavage and subsequent activation of a number of procaspases, including the caspase-3, which amplifies the cas-

cade and terminates with the nuclear fragmentation and the demise of the cell (Pollard and Earnshaw, 2008).

Ca^{2+} is a global positive effector of mitochondrial function, and thus any perturbation in mitochondrial or cytosolic Ca^{2+} homeostasis will have profound implications for cell function, for example, at the level of ATP synthesis. At the same time, Ca^{2+} overload, frequently observed in a variety of pathological conditions, adversely affects mitochondrial function, and Ca^{2+} signals have been implicated in triggering the various forms of cell death. In this light, the mitochondrial Ca^{2+} homeostasis appears to be an important check point. A prolonged increase in cytosolic Ca^{2+} concentration can trigger the mitochondrial Ca^{2+} overload. This, in turn, leads to the opening of the mitochondrial permeability transition pores (PTP) ensuring the release of the caspase cofactors residing in the organelle and causing thus the translation of the Ca^{2+} signal into a death promoting process (Giorgi *et al.*, 2008). Therefore, an elevated cytoplasmic Ca^{2+} signal caused by either its release from the cell's reservoirs (e.g. the endoplasmatic reticulum) or by influx from the extracellular environment due to the compromised plasma membrane, can trigger the orchestrated collapse of the cell.

1.8. Rho-kinase (ROCK) and its inhibitors

Protein kinases act as molecular switches in most cellular processes by binding ATP and transferring its phosphate group to the specific substrate(s), thus their concentration and activity are tightly controlled. Additionally, as a number of different cellular events require the complex and precise regulation of the actin cytoskeleton, the research focusing on its governing is important. Rho-kinases or simply ROCKs have emerged as protein kinases involved in this process (Riento and Ridley, 2003). ROCKs are protein serine/threonine kinases that are activated by the binding of the small GTPase RhoA to the kinase. RhoA is upon stimulus from the outside environment converted from an inactive GDP-bound to an active GTP-bound form, transducing the signal from the plasma membrane to the ROCKs and downstream to the actin filaments (Ridley, 2001). The main substrates for ROCKs in the cellular environment are the myosin light chain (MLC) and the myosin phosphatase, which phosphorylation leads to the formation and strengthening of the actin fibers (Amano *et al.*, 2000).

As the frequent abnormal functioning of different protein kinases manifests during a number of diseases, inhibitors of protein kinases have attracted much attention as potential drugs. A widely used ROCK inhibitor, Y-27632, competes with ATP for the ATP-binding pocket of the enzyme molecule, inhibiting thereby the activity of the kinase to phosphorylate its substrates. Y-27632 has been shown to abolish the formation of the actin cytoskeleton and consequently the processes requiring the re-organization and existence of the cytoskeleton (Ishizaki *et al.*, 2000). Nevertheless, the ability of Y-27632 to inhibit also other

protein kinases (Davies *et al.*, 2000) attenuates the need for inhibitors with elevated affinity and specificity.

Conjugates of oligoarginine attached to an adenosine moiety have emerged as efficient inhibitors of basophilic protein kinases *in vitro* (Enkvist *et al.*, 2006). The most potent inhibitor of ROCK was named an adenosine-oligoarginine conjugate or simply ARC. ARC is able to block simultaneously the kinase's ATP- and substrate binding pockets via its adenosine moiety and oligoarginine sequence, respectively. Besides being the target for the kinase's substrate binding pocket, the ARC's oligoarginine sequence, much like a CPP, ensures perhaps also the effective membrane translocation ability of the conjugate.

2. AIMS OF THE STUDY

The ability of CPPs to facilitate the uptake of various cargo molecules attached to them has been long recognized, yet, to date the bottleneck in CPP-mediated delivery has been their limited endosomal escape and release into the cytosol of treated cells. As the intracellular trafficking defines the eventual fate of the endocytosed material and thus also the destiny of the CPP-cargo complexes inside the cells, the elucidation of this aspect is imperative in order to enhance the level of "effective" delivery. Additionally, since the lack of stability of CPPs due to enzymatic digestion extracellularly (by serum components) and intracellularly (after targeting to low pH organelles) has emerged as a drawback in their use, a demand for more resistant isoforms has emanated. Nevertheless, the gained stability may also cause undesired side-effects, such as cytotoxicity, which needs to be addressed whenever using a new peptide. Briefly, thus, the main focus of this study was to elucidate the intracellular trafficking, stability, endosomal escape and efficiency of the used compounds.

More precisely, the specific aims of the study were:

- to map and characterize the time-dependent intracellular targeting of CPP-protein complexes in relation to the two main intracellular pathways – the recycling and the endo-lysosomal route (Paper I)
- to define the kinetics of CPP-protein degradation inside the cells and establish the attributes required for effective endosomal escape (Paper II)
- to determine the mechanism(s) behind the cellular toxicity arising from the treatment of cells with the degradation-resistant retro-inverso isoforms of CPPs (Paper III)
- to assess the efficiency of the application of the CPP-like sequence containing protein kinase inhibitor ARC in a cellular environment (Paper IV)

3. METHODOLOGICAL CONSIDERATIONS

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

3.1. Cell Cultures

Five cell lines have been used throughout the studies described in this thesis. In paper I, Cos-7 cells derived from the African green monkey kidney cells were used. It was recently revealed that the two major intracellular pathways – the recycling and the endo-lysosomal pathway – are, unlike in other cell lines, spatially segregated inside Cos-1 cells. There the endosomes of the recycling pathway localize predominantly inside and the organelles of the endo-lysosomal pathway reside outside of the trans-Golgi ring-like structure (Misaki *et al.*, 2007). A spherical Golgi structure was corroborated to exist also in Cos-7 cells (Paper I). Thus, due to the better cultivation properties of the latter, Cos-7 cells were exploited in the unveiling of the intracellular trafficking of the CPP-protein complexes.

Chinese hamster ovary (CHO) cells were used in the studies conducted in Paper II. The ability of different peptides to efficiently internalize into these cells has been demonstrated repeatedly (Richard *et al.*, 2003; El-Andaloussi *et al.*, 2007; Zaro *et al.*, 2009). Therefore, the intracellular fate and stability of the CPP-protein complexes were studied using this cell-line.

MDA MB 231 cells are derived from a human breast cancer and are widely used due to their human origin. In our hands, the MDA MB 231 cells have shown to be less robust and thus more affected by different treatments than other commonly used cell lines. In this light, these particular cells were used in Paper III with the emphasis on the different cytotoxic side-effects caused by the degradation-resistant retro-inverso-peptides.

In paper IV, human cervical carcinoma cells (HeLa) and mouse embryonic fibroblast cells (NIH 3T3) were used due to their ability to assemble a distinguishable meshwork of the actin cytoskeleton upon a transfection with a fluorescently-tagged actin-containing plasmid. As paper IV elucidates the cellular effects of a novel inhibitor of ROCK, a protein kinase responsible for the formation of the actin cytoskeleton, the visualization of the actin filaments inside these cells was an imperative attribute in these experiments.

3.2. Cell-penetrating peptides and the adenosine-oligoarginine conjugate

Transportan (TP) has been one of the main CPPs under focus in our research group since the discovery of its cell-penetrating and cargo-transporting ability (Pooga *et al.*, 1998), which is why this peptide was included in the study in

Paper I. Nona-arginine and Tat were used as comparative CPPs owing to their slightly different nature from the more hydrophobic TP. As TP10, a shortened analogue of TP, has shown similar cell-transporting efficiency as TP, but lacks the unwanted side-effects (Soomets *et al.*, 2000), this peptide was used in the further studies addressing the intracellular stability of CPP-cargo complexes in Paper II.

Additionally, in Paper III, where severe cytotoxic effects came into the picture in the case of the degradation-stable retro-inverso-peptides, the sequence of a relatively new, but highly effective M918 was used. In order to define the characteristics behind the evoked toxicity, the most common secondary amphipathic peptide penetratin was used as comparison, as well as the arginine-rich Tat due to its structural dissimilarity compared to the two previous peptides. An oligo-arginine sequence containing conjugate ARC was used in Paper IV to determine the ability of this novel inhibitor to hinder the activity of its substrate ROCK in a cellular environment.

3.3. Complexes of CPPs with a protein cargo

The cargo molecule can be attached to the CPP in many different ways. Furthermore, the coupling method could also depend on the nature of the cargo, whether it is an oligonucleotide, a peptide, a protein, or something else. In case of a protein cargo, as used in this study, the CPP can be connected to the cargo during a recombinant expression of the CPP-fusion protein, via a linkage by a reducible disulfide bond, etc. Possibly the easiest strategy, however, is the simple mixing of the peptide carrier with the cargo molecules. Yet, in order to yield a substantial level of complex formation, high affinity between the two compounds is required. The strongest known non-covalent interaction between a protein and a ligand has been determined to be the biotin-binding affinity of avidin ($K_d = 10^{-15}$M) (Green, 1975), which was exploited in this study, using avidin as the protein cargo and biotinylated CPPs as the carrier peptides. Avidin is a heavily glycosylated egg white protein forming a 66–69 kDa tetramer under normal conditions. Since every subunit of avidin can bind a single biotin molecule, then as a tetramer, the avidin protein is capable of interacting with 4 biotins simultaneously. Due to extensive glycosylation and the presence of a number of positively charged amino acids in its sequence, the protein possesses a high isoelectric point (pI) of ~10.5, giving avidin a high positive charge under physiological conditions and a tendency to adhere non-specifically via electrostatic interactions.

Thus, less positively charged analogues of avidin have emerged, such as streptavidin (SA). SA is a bacterial protein synthesized by *Streptomyces avidinii*, which forms a 53 kDa tetramer, but unlike avidin, contains a number of negatively charged amino acids and bears, overall, a much lower pI value of ~5.5. The pI value under 7 causes the protein to be slightly negatively charged

under physiological conditions. Nonetheless, its ability to bind biotin is equivalent to avidin.

Another widely used analogue of avidin is neutravidin (NA), a deglycosylated form of avidin that has a pI near 6.3, owning thus a slightly negative charge just like SA. In its 60 kDa tetrameric form NA binds 4 biotins, analogously and with a comparable affinity to avidin and SA. Thus, the three proteins have matching biotin-binding properties and also rather similar molecular weights, yet their charge and subsequently the interactions with either peptides or cell surface molecules is quite different.

In order to visualize the protein cargo, fluorescently labeled avidin, SA and NA were used throughout the study. The fluorescent dyes conjugated to the protein cargo were either green (FITC, Oregon Green), red (Texas Red) or far-red (Alexa Fluor 633).

As mentioned above, biotinylated CPPs are used in this study to create stable complexes with the protein cargo. As all the proteins described above contain 4 biotin binding pockets in their native tetrameric form, the peptide to cargo ratio of 3:1 (used in paper I and paper II) represents the situation where all the peptide is bound to the cargo leaving no free peptide in the solution. In paper II, however, the effect of the peptide concentration on the uptake and stability of the complexes was addressed, which is why higher peptide to cargo ratios (5:1 and 8:1) were also applied. Under these conditions, there is excess peptide in the solution capable of associating with the complexes. Nevertheless, due to the different pI values and subsequently also the different charges of the used protein cargos, the ability of the excess peptide to bind to the complexes is probably different.

3.4. Visualization by fluorescence microscopy

Fluorescence microscopy, and especially confocal laser scanning microscopy, enables the evaluation of cellular uptake of CPP-cargo complexes on the organelle level, providing valuable information about their intracellular localization and targeting. Besides visualizing fixed and permeabilized cells in case of classical immunolabelling and immunofluorescence microscopy, different probes for live-cell imaging were used in order to avoid possible fixation-derived artifacts, an issue that gained much attention in the early 2000s (Richard *et al.*, 2003). Thus, live-cell imaging was used as much as possible in this study.

3.4.1. Analysis of the CPP-protein complexes containing vesicles

Paper I analyzed the involvement of different intracellular trafficking pathways in cellular targeting of the CPP-protein complexes. For this, the trans-Golgi network (TGN) separating the vesicles of the recycling pathway from the endo-lysosomal pathway in Cos-7 cells was visualized either with a specific antibody

TGN46 or fluorescently labeled ceramide in both fixed and live cells, respectively. The organelles of the endo-lysosomal pathway were further highlighted with a pH sensitive probe LysoSensor DND, which intensity rises gradually as the pH of the environment drops, allowing analysis of the intravesicular milieu of the endosomes that contain the CPP-cargo complexes.

The detailed analysis of the different endosomes of the endo-lysosomal pathway containing the CPP-protein complexes was done with the Olympus special software FV10-ASW, which enables the marking of single structures (containing the CPP-protein complexes) and the visualization of the intensity plots of the two overlapping signals. The threshold of the signals was established in a way that would eliminate the bleed-through of one signal to the other channel, avoiding thus misinterpretation of the data. As three distinct populations emerged during earlier time-points – termed pop1, pop2 and pop3 – the results were depicted the same way throughout the experiment.

The proportion of CPP-cargo complexes (in paper I) or ARC (in paper IV) ending up in lysosomal organelles was analyzed using antibody against the lysosomal membrane protein LAMP-2.

3.4.2. Defining the cellular events elicited by the cytotoxic CPPs

In Paper III, where cytotoxic side-effects of degradation-resistant CPPs came into play, different mechanisms involved in the process of cell death were analyzed. For example, the mitochondrial activity and its membrane potential was assessed using a live-cell imaging probe MitoTracker, which passively diffuses across the plasma membrane and accumulates in active mitochondria. Additionally, the integrity of the plasma membrane was determined with Ca-sensitive dye Fluo-4, which is before the experiment loaded into cells. Upon Ca^{2+} influx from, for example, the extracellular environment, the Fluo-4 probe gains fluorescence intensity, which can be measured and further analyzed. As cell death evokes, in several cases, also the activation of the caspase cascades, the up-regulation of this cascade was measured using antibody against an activated form of Caspase-3.

3.4.3. Cellular uptake and effects of the adenosine-oligoarginine conjugate

The uptake of an adenosine-oligoarginine conjugate (ARC) into the cells and its intracellular targeting were detected using tetramethylrhodamine(TAMRA)-labeled ARC in combination with antibodies against either the early endosomal compartment with EEA1 or the lysosomal organelles with LAMP-2.

Additionally, paper IV observed the ability of ARC to inhibit the functions of ROCK in a cellular environment. The affinity of ARC towards endogenous ROCK was confirmed using first an affinity purification protocol with avidin

beads that were saturated with biotinylated ARC and then a Western Blot analysis using a specific antibody against ROCK. As ROCK activity is required for the formation of the actin cytoskeleton, the ability of ARC along with a widely used ROCK inhibitor Y-27632 to suppress this process was determined in cells that were transiently expressing actin that was tagged with a fluorescent green protein (actin-GFP). The fact that the actin-GFP is indeed capable of being recruited to the actin filaments was corroborated beforehand. Since the formation of the actin cytoskeleton is dependent on direct phosphorylation of myosin light chain (MLC), a reaction where ROCK plays a significant role, the ability of both ARC and Y-27632 to suppress this phosphorylation was assessed using Western Blot with an antibody against a phosphorylated form of MLC.

3.5. Visualization by fluorescence imager

Analysis of the decay patterns of the CPP-protein complexes, addressed in Paper II, required, first, the separation of the cellular material by SDS-PAGE, which segregates proteins by their size (and charge) resulting in their distinct velocities inside the polyacrylamide gel. Then, the complexes and their degradation fragments were analyzed with a fluorescence imager Typhoon (GE Healthcare), which reads the fluorescence intensities from the gel and allows quantification of the bands by special software (ImageQuant).

The main drawback of this method, as any other method collecting data only from the fluorescence intensities, is the fact that the protein(s) or its fragments that lack the fluorescent dye, due to perhaps an enzymatic digestion, are not included in the data, resulting in a possible underestimation of the proportions of the different bands.

4. RESULTS AND DISCUSSION

4.1. CPP-protein complexes are intracellularly targeted through different pathways (Paper I)

Upon internalization, the endocytosed material is encapsulated inside endocytic vesicles that follow different pathways inside the cell. The route taken depends on a number of factors, for example, the specific endocytic uptake pathway chosen by the material, its signal sequences that may direct its further targeting, to name a few. As CPPs (alone or with a cargo molecule) have been demonstrated to enter cells via different pathways, which are possibly also exploited in parallel, it is essential to analyze their intracellular targeting in order to define their final destinations and perhaps also the route of effective endosomal escape. The intracellular trafficking of the CPP-protein complexes in the present study was assessed in Cos-7 cells due to the spatial segregation of the two major pathways – the recycling and the endo-lysosomal pathway – inside these particular cells. To draw data on different CPPs, we used the three commonly used transport peptides – Tat, oligoarginine and TP – complexed to an avidin cargo molecule to characterize in detail the intracellular transport of the CPP-protein complexes.

4.1.1. CPP-protein complexes evade the recycling pathway during their intracellular trafficking

It was demonstrated recently that in Cos-1 cells, the recycling endosomes conventionally reside inside the ring-like Golgi structure, whereas the vesicles of the endo-lysosomal pathway are located outside of it (Misaki *et al.*, 2007). The same phenomenon was established in Cos-7 cells and due to better cultivation properties, these cells were used in the following experiments. Thus, advantage of the special characteristics of these cells was taken to analyze the colocalization between the CPP-protein complexes and the Golgi structure, using either an antibody against the *trans*-Golgi protein TGN46 in fixed cells or fluorescently labeled ceramide as the Golgi marker in live cells.

We discovered that even though a fraction of the complexes were transported to a close proximity of the outer border of the ring-like *trans*-Golgi network (TGN), only 10–12% of the complexes-containing vesicles were established to reside inside the structure. The negligible role of the recycling pathway was confirmed for both TP and nonaarginine as well as for Tat. Thus, other pathways are predominantly used by the complexes during their intracellular trafficking.

4.1.2. CPP-protein complexes are trafficked inside the vesicles of the endo-lysosomal pathway where they induce the formation of three distinct populations of vesicles with varying pH and intravesicular concentration of the complexes

Since colocalization with the recycling endosomes was found to be rather scant, the assessment of the role of the endo-lysosomal pathway in the trafficking of the CPP-protein complexes was imperative. During the targeting of its contents, the endosomes following the pathway to lysosomes gradually acquire an acidic pH due to the presence of active proton pumps on their membranes that concentrate protons from the cytosol into the lumen of the vesicles. Subsequently, the decrease in pH activates the pH-sensitive hydrolytic enzymes residing in these vesicles and leads to the degradation of the intravesicular material. The drop in pH in the intracellular vesicles can be visualized with the aid of fluorescent probes, for example LysoSensor dyes, which are internalized into cells via the endocytic pathway where they emit fluorescent light depending on the pH of the environment. Thus, more acidic endosomes appear brighter and more intense than the endosomes that carry a neutral pH.

Strong colocalization between the CPP-protein complexes and the LysoSensor signal was detected already after 1h of internalization, persisting inside the cells for longer than 12h. After internalization, some of the CPP-avidin complexes were still found near the plasma membrane, whereas others were already being trafficked towards the perinuclear region, to where most of the degradative compartments localize. At first, most of the complexes-containing structures were small and of lower avidin content, however, a population of vesicles encompassing a lower pH and a higher concentration of complexes was also evident. Over time the complexes-bearing vesicles became larger with an elevated concentration of the complexes inside, suggesting that during their intracellular trafficking the initial small vesicles had fused together, localizing increasingly to the low pH compartments. The eventual targeting and accumulation of the complexes to large acidic organelles, indicates that, indeed, as demonstrated before (Padari *et al.*, 2005; Al-Taei *et al.*, 2006), a portion of CPP-cargo complexes are destined for degradation.

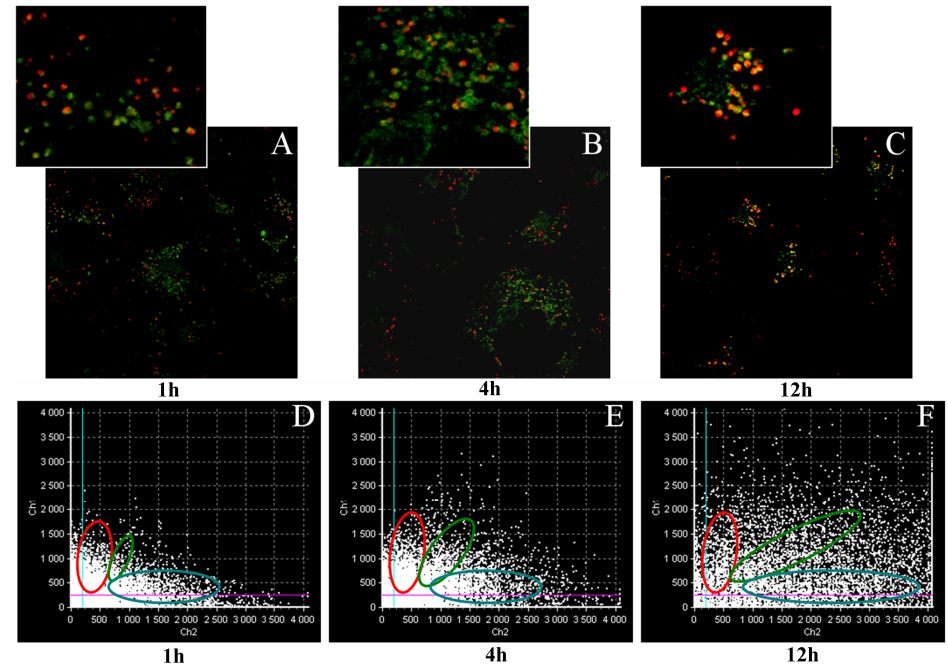

Figure 3. Colocalization of TP-avidin complexes (red in A-C, Ch2 in D-F) in relation to the endo-lysosomal pathway highlighted by the pH sensitive LysoSensor (green in A-C, Ch1 in D-F). Time-dependent intracellular trafficking of TP-avidin complexes revealed the gradual accumulation of the complexes to strongly acidic compartments (yellow in upper panels (A–C), green area (pop2) in lower panels (D–F)), yet, a relatively large proportion of complexes was evident in near-neutral endosomes even after 12h (bright red in upper panels (A–C), blue area (pop3) in lower panels (D–F)). Red area in D–F represents smaller vesicles with low concentration of complexes and a varying pH termed pop1 in this paper.

Plotting of the pixel intensities of the two overlapping signals (Figure 3, lower panels (D–F)) revealed, however, the formation of three distinct populations of intracellular vesicles (pop1, pop2 and pop3) with varying pH and concentration of the complexes. In our study, pop1 represents the small vesicles containing a relatively low concentration of the complexes that possess a varying pH from near-neutral to acidic. Pop2 represents the lysosomes with a strongly acidic environment and a high concentration of the complexes. Pop3-type vesicles, alternatively, encapsulate an elevated quantity of complexes, yet, for some reason do not undergo the pH shift. The proportions of these distinct three populations depend on the time, the CPP and the concentration of complexes used.

Since fusion of vesicles was observed to take place during the intracellular trafficking of the CPP-protein complexes, the amount of pop1-type vesicles decreased, giving rise to either the pop2 or pop3 vesicles. Additionally, TP (out

of all the CPPs used) displayed the highest potency of inducing the population of near-neutral pop3-type vesicles. Nevertheless, an elevation of the concentration of the complex applied to the cells resulted in an increased targeting of the cargo to the pop2 lysosomes, for each and every one of the CPP-protein complexes used.

One of the most prominent and probably also the most significant difference between the pop2- and the pop3-type vesicles is that inside the pop3-endosomes, the peptide as well as the cargo is not being digested, suggesting a favorable environment for extended functionality. However, unlike the vesicles of pop2, classified as lysosomes due to their low intravesicular pH, the origin and also the fate of the near-neutral pop3 vesicles remain unclear.

As dextran, the fluid phase uptake marker that enters cells via the macropinocytic pathway was extensively targeted to the low pH pop2-type organelles, it can be concluded that macropinocytic uptake does not generate these near-neutral vesicles. Similarly, EGF, a ligand internalizing through the receptor-mediated clathrin-dependent pathway (Sorkin and Von Zastrow, 2002) was detected inside the pop2-organelles, suggesting that the participation of also this particular pathway in the formation of the neutral vesicles can be excluded. With the macropinocytosis and the clathrin-mediated endocytosis (CME) out of the picture, the role of the caveolin-dependent endocytosis was hypothesized. Caveolin-mediated endocytosis, in particular, has been demonstrated to play a significant role in the uptake of the CPP-cargo complexes (Ferrari *et al.*, 2003; Fittipaldi *et al.*, 2003), and especially for TP-protein complexes (Säälik *et al.*, 2009). Further evidence rises from the fact that endocytosis stemming from the caveolin-enriched invaginations is shown to give rise to a non-acidic non-digestive subpopulation (Pelkmans *et al.*, 2001; Nabi and Le, 2003; Parton and Richards, 2003). Since caveolar carriers budding from the plasma membrane can during cellular trafficking be fused to each other to form a so-called rosette-like caveosome (Parton and Simons, 2007), it could also explain the elevated concentration of the CPP-protein complexes inside these vesicles. Thus, it is highly probable that caveolin-dependent endocytosis could be the prevalent route in the formation of these near-neutral vesicles containing a high concentration of the CPP-cargo complexes.

4.2. Peptide to cargo ratio dictates the internalization efficiency, stability and endosomal escape of CPP-cargo complexes (Paper II)

As noted before by several groups, and confirmed in Paper I, a large amount of the CPP-protein complexes were in time targeted to the low pH compartments where they become susceptible to enzymatic degradation. Since degradation eliminates the activity of both the peptide carrier and the cargo molecule, it is essential to analyze their decay pattern(s) in order to pinpoint the exact time-

window of their ability to function inside the cells. Therefore, the cells were incubated with different peptide to protein-cargo ratios (3:1, 5:1 and 8:1) with a pulse-chase experiment, allowing the characterization of the time-dependent degradation. Since avidin and avidin-like molecules (in their tetrameric form) have four biotin binding sites, the 3:1 ratio depicts the situation, where all the CPP is bound to the complexes leaving no free peptide in the solution. Correspondingly, in 5:1 and 8:1 ratio samples, there is an increasing amount of excess peptide that could associate with the complexes. TP10, a truncated analogue of TP was used as the CPP in this study mainly due to its high cargo-transporting ability (El-Andaloussi *et al.*, 2005). Fluorescently labeled avidin, neutravidin (NA) and streptavidin (SA) were used as models of protein cargo due to their similar weight, yet a relatively different charge under physiological conditions. The charge of the protein probably ordains a number of different aspects involved in its intracellular delivery, starting from its influence on the binding affinities between the cationic peptide and the protein cargo during the formation of the complexes, and exerting influence throughout the process.

4.2.1. Elevation of the peptide ratio enhances the internalization efficiency of the protein cargo

Degradation patterns of a protein cargo were established in CHO cells, where the uptake of the CPP-cargo complexes has been determined to be of high efficiency. SDS-PAGE technique in combination with fluorescence imaging was used to analyze in detail the degradation fragments of the protein cargos.

The initial quantification of the data revealed that the utilization of a higher peptide concentration resulted in an increased uptake of the cargo protein, irrespective of the charge possessed by the protein. However, the influence of the peptide concentration on the uptake was the most prominent in the case of SA with its internalization elevated over 9-fold (under 8:1 ratio) compared to the level reached by the 3:1 ratio. The drastic differences arise probably from the low pI value of SA (pI ~5.5) giving the protein a slightly negative charge under physiological conditions. As TP10 carries a number of positively charged amino acids, it is, besides being able to occupy the biotin-binding pockets of SA (under 3:1 ratio conditions), also capable of associating with the complex via electrostatic interactions when a higher peptide concentration was used. The gradual masking of the negative charges on SA by the positive peptide elevates thus the affinity of the complex towards the negatively charged plasma membrane making it more prone to attach to the membrane components there and internalize into the cells.

Avidin, on the other hand, possesses a high pI value of ~10.5 and is thus probably less attracted to the excess cationic CPP. Indeed, the elevation of TP10 concentration in the solution did not markedly enhance the uptake of the complexes. However, a slight increase compared to the 3:1 ratio was detected with

the 5:1 ratio, indicating that at least some of the excess peptide could still associate with the avidin despite the charge-related repulsion of the peptide and the cargo. As the TP10 used in this study contains a biotin tag on its N-terminus, the helical mastoparan sequence remains relatively free for association/ multimerization with the excess peptide. The multimerization of the peptide could possibly occur via the hydrophobic interactions between the leucine residues similarly to the leucine-zipper mechanism used by many transcription factors for oligomerization and binding to DNA (Pollard and Earnshaw, 2008). Nevertheless, other mechanisms cannot be excluded either.

Additionally, the peptide that is not attached to the biotin-binding pockets of the cargo protein could be more flexible and unrestrained in its interactions with the membrane components (Dunkin *et al.*, 2011), resulting in a greater membrane binding and subsequent internalization of the entire complex. Still, the question remains unanswered whether the enhanced uptake driven by the stronger binding of the increasingly cationic complexes to the plasma membrane in case of higher peptide concentration brings about the up-regulation of some specific transport pathway(s) or the entire endocytic machinery in general.

4.2.2. Elevation of the peptide concentration secures a better preservation of the cargo protein

Additional data analysis revealed a longer stability of the protein cargo when a higher peptide concentration was used. As avidin and avidin-like proteins are natively found in a tetrameric form and could thus be perceived as models for bioactive proteins requiring dimerization for functioning (for example p53, which has to adopt a tetrameric form in order to be active (Jeffrey *et al.*, 1995)), the band corresponding to the size of the tetramer (see, e.g. Figure 1 in Paper II) gained our attention.

The amount of protein (avidin, SA, NA) in its native (tetrameric) form was determined to peak around 12 h, after which the cargo inside the cells was slowly but steadily subjected to degradation (observed as decrease in the intensity of first the larger bands and then the smaller ones). Nevertheless, the levels of the tetrameric cargo were drastically different, being the highest for SA and the lowest for avidin. Avidin, unlike SA, also displayed a strong aggregation inside the cells originating perhaps from the high pI value of avidin. The high pI of avidin makes it highly attractive for the negatively charged plasma membrane components (such as the HSPGs or other proteoglycans) due to the strong electrostatic interactions. The results of our experiments illustrate that the aggregation that probably already takes place on the plasma membrane, persists in cells for at least 12h, suggesting that the conglomerate is rather tightly packed hindering thus the access of degradative enzymes to the complexes. These massive aggregates were determined not to be intracellularly dissociated into the native tetrameric form, but were instead dissociated or degraded into

smaller fragments, which were, judged purely by their size, probably the monomeric forms of the protein.

The ability of all the used protein cargos (SA, NA and avidin) to persist inside the cells for extended periods of time (up to 12h) either in the presence or absence of intense aggregates indicates that something may protect them from the enzymatic digestion. It was, thus, hypothesized that the dense packing of the cargo with the peptide could shield against the degrading effect and postpone thus the decay of the cargo molecule. Therefore, the fate of the CPP in the complex was further analyzed using double-labeled complexes with TP10 carrying a fluorescent tag at its lysine7 side-chain. As the best preservation of cargo was acquired with the 8:1 ratio with SA as the protein cargo, the peptide associated with these particular complexes came into focus.

The data revealed that a portion of the peptide remained attached to the tetrameric form of SA, whereas a large amount of TP10 dissociated from the complex already after 1h, moving faster in the polyacrylamide-gel. Since excess peptide was used in the making of these complexes, the peptide associated with avidin indirectly (not through the biotin-binding pocket) was probably loosely associated and became detached from the cargo, an effect even more prominent in the denaturing environment created by the SDS-PAGE. Unfortunately, however, no conclusions can be drawn from our current data on whether the excess peptide was simply dissociated or enzymatically cut from the complex.

On the other hand, the peptide bound to SA directly via the biotin moiety is intracellularly perhaps not as easily dissociated, but instead possibly released from the complex after enzymatic digestion. Still, the influence of the denaturing SDS on the stability of the biotin-binding pocket on SA cannot be overlooked since denaturing of the protein may result in the loss of its biotin-binding ability, giving an overestimation of the amount of dissociated peptide. Nevertheless, it is highly probable that since the peptide in the complex is surrounding the cargo molecule, it provides probably an easier access for the degradative enzymes, becoming the primary targets for digestion before the protein cargo is exposed. The fact that the peptide in the complex is dissociated and also degraded faster than the cargo, has been noted before (Amantana *et al.*, 2007). Thus, the peptide, but at least to some extent also the proteoglycans, could perhaps be perceived as the armor around the complex guarding its stability.

4.2.3. Higher peptide to cargo ratio results in a time-dependent photo-induced endosomal escape of the complexes

An additional feature arising from the use of elevated peptide to cargo ratio was the ability of the complexes to escape the endosomal constriction upon exposure to (UV or laser) light. The complexes of, for example, biotinylated TP10 and fluorescently labeled SA (in 8:1 ratio) created a massive diffuse cytosolic signal already after 6h of treatment and a brief illumination in ~90% of the treated

cells. A lower ratio of 5:1 also generated the photo-induction phenomenon, although in that case, a slightly longer chase period was necessary to evoke such events. The NA in complex with TP10 resulted in the photo-induction phenomenon mostly at 8:1 peptide to cargo ratio, showing a much narrower time-window of endosomal escape only after a 6h chase period, where ~70% of the cells were diffusely stained with the complexes. Alternatively, avidin, as cargo, was not as efficiently released from the endosomes as SA and NA. Nevertheless, a detailed examination revealed that a very minor cytoplasmic relocation was still evident even for avidin.

Since the membrane instability resulting in the rupture of the endosome occurred exclusively with higher peptide to cargo ratios, it is likely caused by the excess peptide. The peptide could upon disengagement from the complex better submerge into the endosomal membrane, causing its destabilization and disruption upon an energy input created by the photo-excitation. As a slight time-dependent partitioning of TP10 into the membrane-bound fraction was detected, the hypothesis of the CPP deserting the complex and becoming progressively membranophilic gains further credit.

Because this photo-induction phenomenon was discovered to take place with SA and NA and only to a very low extent also with avidin as the protein cargo molecule, questions about the special requirements or, on the contrary, the impediments for endosomal escape emerged. The differences in the used proteins and their subsequent endosomal escape arise potentially from the propensity of the strongly positively charged avidin to aggregate extensively under cellular conditions (e.g. with the HSPGs and other proteoglycans on the plasma membrane). As was described above, the avidin complex remains tightly associated with these membrane components also inside the endosomes, making perhaps the escape of the peptide harder from the intense agglomerates. Furthermore, the entanglement with the negatively charged surface sugars makes the complexes rather bulky, restraining the escape of avidin even if the photo-induction and membrane destabilization were successful. Since SA in complex with TP10 is far less positive than the TP10-avidin, the SA-peptide complexes bind to the negatively charged surface sugars with a much lower affinity. Because of this the TP10-SA complexes are thus also more readily dissociable from the negatively charged sugars inside the endocytic environment. Hence, this could, indeed, be one of the reasons why the photo-induction phenomenon was the most extensive with SA as the cargo molecule. As SA and avidin are depicted as the extremes of this experiment, NA with an intermediate pI value of ~6.3 demonstrated also intermediate results, speaking further in favor of this hypothesis.

Additionally, since the level of photo-induction increased drastically over time, peaking at 6 to 12h post incubation, some sort of time-dependent mechanism(s) must be involved in this process. Firstly, the step-wise accumulation of CPP-cargo complexes to successively fewer and larger endosomes, as shown in Paper I, results in a local high concentration of the peptide, which has

shown to strongly distort the arrangement of lipids (Huang, 2006; Herce and Garcia, 2007; Herce *et al.*, 2009) and may thus be one of the prerequisite conditions preceding the endosomal membrane destabilization event.

Another factor that could explain the time-dependence of this process is the specific lipid composition that the endosomes acquire during their maturation. It has been demonstrated that Tat, for example, can destabilize the liposomes resembling the intraluminal vesicles of the multivesicular bodies, highly enriched in anionic phospholipids, whereas the liposomes composed of lipids simulating the plasma membrane are not affected (Yang *et al.*, 2010). Also penetratin possesses a higher affinity towards the negatively charged phospholipids (Salamon *et al.*, 2003), which is why it is fair to assume that other CPPs may too have a preference for a specific type of lipids for interaction. The gradual accumulation of these specific, preferably anionic, phospholipids into the endosomal membrane (Kobayashi *et al.*, 2002) may create a platform for increased clustering of the peptide to the membrane and facilitate thus the subsequent membrane disturbance. As the acquisition of anionic lipids is a feature of mainly maturing late endosomes and multivesicular bodies (Kobayashi *et al.*, 2002), the involvement of the large more acidic compartments described in Paper I could provide the environment for the observed endosomal escape. Furthermore, as the pH shift neutralizes slightly the negative charges of the proteoglycans, it subsequently decreases their affinity towards the CPP-cargo complexes, promoting thus the transition of the association of the complexes towards the anionic lipids. Because a pH shift and a gradual endosomal maturation to a multivesicular body seem to be required for effective endosomal membrane destabilization by the CPPs, the ability of the cargo released by the photo-induction to still exert its biological effects needs to be evaluated in the future.

Whether also the near-neutral organelles (described in detail in Paper I) could play a role in the endosomal escape of the CPP-cargo complexes is not known. Furthermore, the mechanism(s) behind the membrane destabilization there remains obscure.

4.3. Increasing the stability of CPPs with retro-inversion could be accompanied by cytotoxic side-effects (Paper III and unpublished data)

As the stability of the transport vector in serum and also inside the cells (analyzed in detail in Paper II) has been a significant concern in the field of CPP-mediated delivery, different approaches have been applied in order to make them less susceptible to degradation. The retro-inversion method, or simply the synthesis of peptides with D-amino acids in reversed order, yields a peptide with an analogous side chain topology as the parent peptide, yet with an elevated stability (Chorev *et al.*, 1979). The same modification was applied to a potent new CPP M918 (El-Andaloussi *et al.*, 2007) in order to expand the time-

window of its ability to function inside the cells. Surprisingly, however, the attachment of the RI-M918 peptide to the PNA cargo molecule did not increase splice correction as expected, but instead, abolished the process altogether. This observation prompted us to investigate the matter further in order to elucidate the reasons behind this aberration. To rule out the possibility of the toxic effects rising only with RI-M918, the most commonly used CPPs – Tat and penetratin – were also synthesized in their RI-isoforms and used as comparison.

It was discovered that RI-M918 along with RI-penetratin induced significant cytotoxic effects in cells visualized as granulated cytoplasm and nuclear condensation after 4h of incubation. During the attempts to identify the mechanism behind the cellular death process evoked by the RI-CPPs, it was discovered that the initial process could be the disruption of the mitochondrial network via the loss of its membrane potential inside the treated cells. Since the experiments also revealed an ability of the two RI-CPPs to destabilize the plasma membrane (visualized as the influx of propidium iodide into cells) and cause a subsequent influx of Ca^{2+}-ions into the cytoplasm, it is possible that the mitochondrial membrane potential was obliterated by the massive cytosolic accumulation of Ca^{2+}, which has been shown to cause the permeabilization of the mitochondrial membrane (Giorgi *et al.*, 2008).

Furthermore, since the plasma membrane was, to an extent, destabilized by both the RI-M918 and RI-penetratin, it is possible that these peptides can intracellularly also interact directly with the mitochondrial membranes, inducing further the effects that lead to the disruption of the mitochondrial potential. The ability of some polylysine complexes to perturb the mitochondrial membrane potential has, indeed, been shown (Symonds *et al.*, 2005). Additionally, some CPPs, for example mitoparan, have been implicated in the permeabilization of the mitochondrial membrane once reaching the cellular cytosol (Jones *et al.*, 2008). As the perturbation of the mitochondrial potential and the concomitant mitochondrial membrane permeation leads to the release of the proteins residing in the intermembrane space and the launch of the caspase cascade (Pollard and Earnshaw, 2008), the activation of caspase 3, involved in that chain reaction, was examined. Indeed, a slight activation of the procaspase 3 into its functional form inside the RI-CPP-treated cells was detected.

Because none of these effects were observed with RI-Tat, the hypothesis that the length of the peptide and also the inclusion of some hydrophobic residues in their sequence could be the main characteristics causing the cytotoxic effects came to the forefront. The aspect of the peptide length was corroborated with a truncated version of RI-penetratin with only the 9 first amino acids still present, which did not possess any of the cytotoxic effects on cells even at a very high concentration. The importance of the peptide length on its cytotoxicity has been demonstrated, for example, for oligoarginine (Mitchell *et al.*, 2000). Additionally, removal of four amino acids from a membrane active amphipathic CPP called model amphipathic peptide (MAP) abolished its cytotoxic effects (Scheller *et al.*, 1999). Thus, it is probable that the peptide length as well as its

hydrophobic amino acids, could determine the depth of membrane insertion and subsequently also the range of membrane destabilization. Further evidence for this arise from a study with TP analogues, where the peptide length determined the membrane submersion properties with longer peptides spanning the whole lipid bilayer whilst the shorter ones were capable of diving only into the outer membrane leaflet (Soomets *et al.*, 2000).

Additionally, the adoption of secondary structures could provide the peptide with the necessary conformation to better interact with the membrane fraction. Thus, as the peptide in RI-form are not degraded by either the intra- or extracellular enzymes, these peptides could irreversibly aggregate to membranes and cause the above described cytotoxic events. Penetratin, when residing at the surface of a membrane, has shown to be chameleon-like in terms of its induced structure. For example, at low membrane surface charge density and low peptide concentration penetratin favors mainly an α-helical conformation. Nevertheless, at high charge density and peptide concentration it dominantly prefers to adopt a β-sheet structure (Magzoub *et al.*, 2002). M918, likewise, was recently demonstrated to trigger a conformational change from a random coil to a β-structure upon the elevation of the concentration of the negatively charged phospholipids in the membrane (Eiriksdottir *et al.*, 2010). The high number of positively charged amino acids on both of these peptides could thus provide the initial membrane binding bringing about the adoption of membrane active secondary structures. This, in turn, could drive the membrane destabilization process either directly on the plasma membrane or intracellularly, e.g. at the mitochondrial membrane.

4.4. The CPP-strategy can be efficiently utilized in the design of bioactive molecules for modulating intracellular processes (Paper IV)

As cell-penetrating peptides usually carry a high positive charge and possess the ability to facilitate their entry to cells, these characteristics were further exploited in the design of a new class of inhibitors for different basophilic protein kinases. Since these basophilic kinases provide a binding pocket for substrates with a highly positively charged target site, they can be perceived as strong candidates for inhibitors bearing such sequences. Additionally though, to be a potent inhibitor, the compound also needs to have a moiety that is able to compete with ATP for the kinase's ATP-binding pocket. Keeping these guidelines in mind, a novel inhibitor with an oligoarginine as the peptide sequence attached to the 5' carbon atom of the adenosine sugar moiety was designed and due to its composition termed as an adenosine-oligoarginine conjugate (or simply ARC) (Loog *et al.*, 1999).

ARC has been demonstrated to be highly efficient in inhibiting the activity of several basophilic protein kinases *in vitro*, showing the highest affinity and

inhibitory effect towards a Rho-associated kinase called ROCK (Enkvist *et al.*, 2006). However, since the intracellular milieu is a rather different environment than the one in a test tube, the ability of ARC to function also in the cellular system was addressed.

4.4.1. ARC internalizes into cells and induces its leakage out of the entrapping endosomes, an event enhanced by the application of a lysosomotrophic agent

Since its target kinase(s) in cells reside in the cytosol, the compound ARC needs to find its way into the cytosol to exert its effects in a cellular environment. Therefore, the uptake of ARC was assessed, revealing the use of an endocytic mechanism during the cell entry process. The gradual targeting of the conjugate to successively fewer and larger vesicles was observed, as the organelles entrapping the conjugate appeared enlarged with an elevated intensity of the signal from the fluorescent dye tagged to the inhibitor. Yet, at a closer look, only a 20% colocalization between ARC and the lysosomal marker LAMP-2 was detected after 2h of intracellular targeting. This could mean that a portion of ARC, much like CPPs, is not following the classical endo-lysosomal route inside the cells, but rather concentrates to some other endosomal compartments, such as the near-neutral vesicles described in Paper I.

Additionally, a slight leakage of ARC from the endosomes after cell entry was detected. It was further apparent that the fraction of ARC reaching the cytosol was increasing in a time-dependent manner, hinting towards the "ripening" of the entrapping endosomes during their intracellular trafficking, out of which the release was induced. Since ARC strongly resembles the characteristics of the arginine-rich CPPs, it could suggest that ARC, just like these CPPs, can upon entry induce its leakage from the endosomes it accumulates to. As described and discussed in Paper I and Paper II, a time-dependent mechanism for endosomal maturation into a specific construction or form (e.g. a high concentration of the compound, a specific lipid composition, etc.), where the endosomal membrane could be readily destabilized by the CPPs, is necessary for their escape process.

Additionally, it was determined that chloroquine, a lysosomotrophic agent, enhances the cytosolic accumulation of ARC during the shorter incubation times. The ability of chloroquine to evoke an elevated release of ARC from the entrapping endosomes arises probably mostly from its capacity to promote the swelling of endosomes making their membrane more prone for destabilization. However, since chloroquine amplified the leakage of ARC only at shorter timepoints and similar endosomal escape was reached after 2h incubation with ARC only or in combination with chloroquine, the data implies that after a certain time of intracellular trafficking, the compound is, on its own, capable of inducing an analogous level of membrane destabilization. Altogether these results

prove that ARC, and probably also some CPPs, are indeed capable of intrusion to the cellular cytosol after entry through an endocytic process.

4.4.2. ARC efficiently binds to and inhibits the activity of ROCK inside the cellular environment, observed as the reduction of myosin light chain phosphorylation and actin depolymerization

Once reaching the cytosol, ARC is able to bind its target(s) and manifest its inhibitory effects. Thus, first of all, the ability of ARC to bind and concentrate ROCK was established, corroborating the *in vitro* experiments (Enkvist *et al.*, 2006) and giving further evidence that even in the presence of other cellular components, including other protein kinases, ARC still retains its affinity towards the target kinase. During its binding to ROCK, ARC presumably occupies the substrate- and the ATP-binding pocket of the enzyme, suppressing subsequently the binding and phosphorylation of the cellular targets of the kinase, as shown, for instance, with another ARC compound in its binding to cyclic AMP-dependent protein kinase (PKA) (Vaasa *et al.*, 2009).

As myosin light chain (MLC) has been defined as one of the main recipients of the phospho-group supplied by ROCK, its levels of phosphorylation in control, ARC- or Y27632-treated cells was evaluated and compared. Indeed, the quantification of phosphorylated MLC levels in these cells exposed that ARC was more potent than the commonly used ROCK inhibitor Y-27632, displaying a stronger inhibition of the phosphorylation process.

Since the activity of ROCK and the subsequent phosphorylation of MLC are necessary for the formation and arrangement of the actin cytoskeleton (Amano *et al.*, 2000; Ridley, 2001), the inhibitors were tested in light of their ability to hinder the assembly of the actin filaments. Both ARC and Y-27632 efficiently abolished the genesis of the actin network, yet, their cellular effects were rather different. For example, in Y-27632-treated cells, the actin cytoskeleton was missing entirely, whereas ARC-treatment inflicted the disappearance of only the centrally located actin fibers, leaving the cortical ones unaffected. As it has previously been demonstrated that inside the cells the ROCK-mediated actin remodeling is spatially restricted, being active more in the central regions (Totsukawa *et al.*, 2000), it can be assumed that ARC is, not only *in vitro* but also in the cellular environment, a more potent and specific inhibitor of ROCK than the commonly used Y-27632. Thus, the CPP strategy can be efficiently applied also to other fields of science, and exploited not only as mediators of uptake.

SUMMARY

The plasma membrane creates a selective barrier for the movement of molecules in (and out) of the cells. Because of this, a number of extracellularly applied (bio)molecules, including therapeutics, cannot enter the cells and therefore lose their potential to elicit their inherent activity. Thus, limitations in effective delivery call for the development of transporters that could enhance the internalization of these compounds across the cell membrane.

Cell-penetrating peptides as alternative non-invasive vehicles for the intracellular delivery of bioactive compounds have found both recognition and wide application in molecular biotechnology. Their ability to exploit different endocytic pathways in parallel and possibly switch between the uptake routes, however, makes the investigation of the involvement of distinct pathways a rather complex task. Furthermore, the gradual intermixing of the endocytic vesicles of different origin inside the cell raises another complication that the scientists in search of the intracellular trafficking and endosomal escape of CPPs have to face. Thus, in this study the endosomal routing, stability and endosomal escape of different CPPs attached to a protein cargo was put in the spotlight.

Fluorescently labeled proteins were used as the cargo in the complex to visualize their intracellular targeting by fluorescence microcopy, but to also determine their degradation patterns by fluorescence imaging after SDS-PAGE. As the low stability of the CPPs as well as of the cargo emerges as a possible drawback during extended intracellular targeting, the cellular effects of degradation-resistant CPPs were explored. Additionally, the cellular effects of a novel kinase inhibitor ARC containing a CPP-like moiety were addressed to confirm the ability of the CPP-like conjugate to effectively escape the endosomal restraint and carry out its activity.

The gradual targeting of the CPP-protein complexes to the endo-lysosomal pathway and eventually into low-pH organelles was observed, causing a progressive decay of the complexes, the cargo and the peptide. Nevertheless, a fraction of the complexes are still for extended periods detected inside endosomes with a relatively neutral pH, and the most significant degradation of the cargo commences only after 12h of internalization. Also, the concentration of the CPP in the complex was discovered to be one of the key determinants in inducing the endosomal escape.

Conclusively, the main results of this study are as follows:

1) The CPP-protein complexes taken up by cells avoid the recycling pathway and are instead targeted through the endo-lysosomal pathway (Paper I).
2) A large fraction of the CPP-protein complexes is targeted to degradative organelles (Paper I).
3) A fraction of the endosomes containing the CPP-protein complexes does not acquire a lysosome-associated low pH even after 12h, resisting a fast degradation (Paper I).

4) The stability of the complexes depends on the concentration of CPPs used during the formation of the complexes, being the highest with 8:1 ratio. The most prominent decay of the complexes, however, starts only after 12h of internalization (Paper II).
5) A higher peptide to cargo ratio, a prolonged (≥6h) intracellular trafficking, and a brief exposure to UV or laser light ensure an efficient endosomal escape of the complexes from the entrapping endosomes (Paper II).
6) A higher concentration of the degradation-resistant retro-inverso isoforms of CPPs causes destabilization of the plasma membrane, the loss of mitochondrial activity and the collapse of the cell depending on their length and level of amphipathicity (Paper III).
7) An adenosine-oligoarginine conjugate (ARC) possessing a CPP-like sequence element is effectively internalized into cells, where it, on its own or with the help of a lysosomotrophic agent chloroquine, is able to escape into the cytosol and inhibit the activity of its substrate ROCK, illustrating that an endosomal membrane destabilization is indeed taking place (Paper IV).

15

SUMMARY IN ESTONIAN

Rakku sisenevate peptiidide (rsp) mitu nägu ehk RSP-valk komplekside rakusisene suunamine, stabiilsus ning endosoomidest vabanemine

Eukarüootset rakku ümbritseb lipiidsest kaksikkihist koosnev plasmamembraan, mis on selektiivseks barjääriks raku sees ja väljas paiknevatele ühenditele. Kuna membraanne kaksikkiht on oma olemuselt vett hülgav ehk hüdrofoobne, siis ei ole see läbitav paljudele laetud hüdrofiilsetele raku väliskeskkonda lisatud (bio)molekulidele nagu mitmed ravimid, valgud või oligonukleotiidid, mistõttu nende toime rakkudes on vähene või puudub üldse. Et suurendada biomolekulide võimet ületada plasmamembraanset barjääri ja seeläbi võimendada nende toimet rakkudes, on kasutusele võetud mitmeid erinevaid abistavaid ühendeid. Üheks tõhusamaks mooduseks, mille abil parandada erinevate lastmolekulide transporti rakkudesse, on viimase paari aastakümne jooksul tõusnud rakku sisenevate peptiidide (RSP-de) kasutamine. Rakku sisenevate peptiidide perekonda kuulub tohutul hulgal erinevaid peptiidseid ühendeid, mis pärinevad kas looduslikult esinevatest valkudest (näiteks Tat peptiid, mis pärineb inimese immuundefitsiitsust põhjustava HI-viiruse valgust) või on täielikult sünteetilised (näiteks arginiinist koosnevad järjestused). Vaatamata nende transporterite erinevale päritolule võib kokkuvõtvalt öelda, et RSP-d on enamasti kuni 40 aminohappejäägi pikkused sageli mitmeid positiivse laenguga aminohappeid sisaldavad peptiidsed ühendid, mis suudavad efektiivselt rakkudesse viia suuruselt ja omadustelt erinevaid lastmolekule. Positiivse laenguga aminohapete sisaldumine järjestuses aitab peptiidil raku pinnale paremini seonduda, kuna seal leidub rohkelt erinevaid negatiivselt laetud membraanseid komponente, näiteks tugevalt glükosüleeritud membraanivalke, mis tagavad peptiidi ja peptiidi külge seondunud lastmolekuli ankurdumise plasmamembraanile. Kuigi järgnev rakku sisenemine võib toimuda ka otsese membraani läbimise teel, on korduvalt näidatud, et sisenemine toimub siiski pigem vesikulaarsete kandjate ehk endotsütoosi abil, eriti kui tegu on peptiidiga, mille küljes on lastmolekul. Seega toimub peptiidi ja lastmolekuli komplekside seostumisel raku pinnaga plasmamembraani piirkonna sissesopistumine, millele järgneb komplekse sisaldavate spetsiifiliste endosomaalsete vesiikulite moodustumine. Kuna endotsütoos on rakkudele iseloomulik moodus omandada ümbritsevast väliskeskkonnast nii informatsiooni kui toitaineid, siis sedalaadi sisenemistee on rakkudele üldjuhul kahjutu.

Pärast pungumist rakumembraanilt suunatakse endosoomides sisalduv materjal mööda erinevaid radasid raku erinevatesse organellidesse, kusjuures rakusisene saatus sõltub suuresti kasutatud endotsütoosiraja valikust. Seetõttu on eriti oluline kindlaks teha, milliseid endotsütoosiradasid RSP-d lastmolekulide rakku transportimiseks kasutavad. On näidatud, et kompleksid suudavad kasutada mitmeid radu paralleelselt. Kuna aga raku tsütoplasmas toimub mitmete endotsütoosiradade segunemine ning selle üksikasjadest teatakse tegelikult suhteliselt vähe, siis muudab see RSP-lastmolekulide komplekside rakusisese

saatuse uurimise veelgi keerulisemaks. Antud töö keskendubki eelkõige sellele, millistesse endosoomidesse RSP-d koos valgulise lastmolekuliga pärast rakkudesse sisenemist suunatakse, kui stabiilsed nad seal on ning kas kompleksid on suutelised ka endosoomidest vabanema. See on aga vajalik selleks, et bioaktiivne lastmolekul saaks raku sisemuses oma sihtmärgiga seonduda ning oma toimet avaldada. Rakkudesse transporditavaks molekuliks oli antud töös avidiin või avidiiniga sarnane valk, mida kasutati eelkõige seetõttu, et moodustada võimalikult lihtsalt ja kiiresti stabiilseid komplekse biotinüleeritud transporteritega. Töös on kasutatud transporteritena erinevaid peptiide, näiteks arginiinirikaste peptiidide grupist (Tat ja arginiinijääkidest koosnev peptiid R_9) ning hüdrofoobsemate peptiidide rühmast (transportaan (TP) ning selle lühendatud versioon TP10). Kuna looduslikud peptiidid, mis sisaldavad oma järjestuses L-aminohappeid, on mitmete ensüümide poolt nii rakuvälises kui -siseses keskkonnas suhteliselt kiiresti lagundatavad, siis testiti antud töös ka erinevate lagundamisele vähem vastuvõtlike peptiidide toimet rakkudele. Täpsemalt kasutati nn retro-inverso peptiide nii uudsest efektiivsest RSP-st M918 kui ka rohkem tuntud peptiididest nagu Tat ja penetratiin. Samuti on töös kasutatud arginiinijääkidest koosnevat järjestust sisaldavat uudset kinaaside inhibiitorit nimega ARC, mille peptiidne osa võib tänu sarnasusele RSP-dega vahendada ühendi rakkudesse sisenemist. ARC-iga tehtud katsete eesmärgiks oli kinnitada ühendi võimet siseneda rakkudesse, vabaneda endosoomidest ning inhibeerida tema rakulise sihtmärgi Rho-kinaasi aktiivsust.

Peptiid-valk komplekside rakkudesse sisenemine toimus, nagu eelnevalt kirjeldatud, eelkõige endotsütootilise raja kaudu, kuna kompleksid koondusid vesikulaarsetesse struktuuridesse. Antud komplekside rakusisese suunamise uurimisel ilmnes, et ringlusraja endosoomid ei ole kompleksidega märkunud, mistõttu võib järeldada, et endosoomid, mis suunavad endas sisalduva materjali rakupinnale tagasi, ei osale RSP-valk-komplekside rakusiseses transpordis. Seega uuriti edasi teise olulise endotsütootilise raja – endo-lüsosomaalse raja – rolli antud protsessis. Selgus, et valdav enamus rakkudesse sisenenud komplekse järgib just seda teed. Kuna antud rada suunab endas sisalduva materjali järk-järgult madala pH-ga lüsosoomidesse, kus toimub vesiikulis oleva materjali lagundamine, siis uuriti komplekse sisaldavate organellide pH-d lähemalt. Sõltuvalt endosoomis sisalduvate komplekside hulgast ning vesiikulisisesest pH-st oli võimalik eristada kolme erinevat populatsiooni vesiikuleid – väikesed vähe komplekse sisaldavad varieeruva pH-ga vesiikulid (pop1), suured, rohkelt komplekse sisaldavad happelised struktuurid (pop2) ning suured, rohkelt komplekse sisaldavad nõrgalt happelised vesiikulid (pop3). Pop1-tüüpi vesiikulite hulk ajas väheneb, mis tähendab tõenäoliselt seda, et need on esmased endosomaalsed vesiikulid, mis hiljem ühinevad ja muutuvad pop2- või pop3-tüüpi vesiikuliteks. Pop2-tüüpi vesiikulid meenutasid enim lüsosomaalseid struktuure. Pop3-vesiikulite päritolu aga oli suhteliselt selgusetu. Kuna on teada, et kaveoliin-vahendatud endotsütoosirada suunab osa sisenenud materjali pikaajaliselt neutraalseks jäävatesse struktuuridesse ning kuna pop3-tüüpi endosoomid

püsisid rakus isegi 12 tundi, on võimalik, et sisenemine just selle raja kaudu tekitab pop3-tüüpi vesiikulid. Antud hüpoteesile lisab tuge fakt, et kaveoliin-vahendatud endotsütoosiraja osalust eriti TP-vahendatud transpordis on näidatud ka varem ning et suurim osakaal pop3 vesiikuleid moodustus just TP-valk komplekside sisenemisel.

Kuna eelnevast selgus, et suur osa komplekse suunatakse endo-lüsosomaalse raja kaudu siiski pop2-tüüpi hapudesse organellidesse, kontrolliti komplekside stabiilsust rakulises keskkonnas. Antud katsetest selgus, et sõltuvalt lastmolekuli keemilistest omadustest ja laengust on kompleksid rakkudes stabiilsed kuni 12 tundi, misjärel nad järk-järgult väiksemateks juppideks lagundatakse. Samuti leiti, et positiivse laenguga avidiin agregeerub rakuliste komponentidega oluliselt tugevamini kui kergelt negatiivse laenguga avidiiniga sarnane streptavidiin, ning et moodustunud agregaadid püsivad rakus samuti vähemalt 12 tundi. Kasutades komplekside moodustumisel ülehulgas transportpeptiidi, avastati, et kuue tunni pärast olid kompleksid võimelised valguse toimel vesiikulitest vabanema, märkides hajusalt kogu tsütoplasma. Sellest võib eeldada, et peptiid, mis oli kompleksidega vabamalt seotud, lahkus kompleksist ning sukeldus endosoomi membraani, põhjustades muudatusi selle korrapäras. Lisaenergia andmisel (valguskiirgusega) aga kutsus see omakorda esile endosoomi membraani katkemise. Antud fenomen oli kõige tugevam TP10-streptavidiini komplekside puhul, samas kui avidiiniga komplekside puhul oli antud efekt suhteliselt tagasihoidlik. Võimalik, et avidiiniga kompleksid olid vesiikulis tugevalt agregeerunud negatiivselt laetud rakumembraani komponentidega, takistades peptiidi vabanemist ja membraaniga seondumist. Samuti ei saa välistada, et endosoomide membraan oli siiski lekkiv, kuid kompleksid ei suutnud oma liialt suurte mõõtmete tõttu sealt lihtsalt välja pugeda. Kuna endosoomidest vabanemine oli tugevalt ajast sõltuv protsess, siis vajas see lisaks komplekside koondumisele järjest suurematesse vesiikulitesse tõenäoliselt ka mingitlaadi endosoomide küpsemist, milleks võis olla näiteks spetsiifiliste negatiivselt laetud lipiidide kogunemine endosoomide membraani. Oma tugeva positiivse laengu tõttu võivad peptiidid selliste lipiididega paremini seonduda ning põhjustada seega tugevamat membraani häiritust. Kuna aga selliste lipiidide lisandumine endosoomi membraani on üheks näitajaks, et vesiikul on küpsenud hiliseks hapustunud endosoomiks, siis võib see vihjata sellele, et valguse poolt indutseeritud vabanemine toimus eelkõige just pop2-tüüpi vesiikulitest, kus tõenäoliselt toimub järk-järguline materjali lagundamine. Seetõttu oleks vaja üle kontrollida, kas antud tingimustel vabanenud kompleksid säilitavad rakus oma bioaktiivsuse. See, kas ja kuidas võiks antud vabanemine toimuda ka neutraalsetest pop3-tüüpi vesiikulitest, jääb aga hetkel veel lahtiseks.

Kuna peptiidide stabiilsus on äärmiselt vajalik nende transpordivõime säilimiseks, vaatlesime lagundamisele vastupidavamate retro-inverso-peptiidide toimet rakkudele. Avastasime üllatusena, et mitmed kasutatud stabiilsed peptiidid omasid rakkudele toksilist mõju, põhjustades plasmamembraani häiritust, kaltsiumi-ioonide rakku sisenemist, mitokondrite aktiivsuse kadumist ning apop-

toosi algatava signalisatsiooniraja aktiveerumist. Kuna aga näiteks RI-Tat ei olnud rakkudele toksiline, siis järeldasime, et toksilisteks osutunud pikemad ja hüdrofoobsemad retro-inverso-peptiidid suudavad sukelduda sügavamale membraani sisemusse ning seetõttu sellega ka tugevamalt seonduda, põhjustades nii pöördumatuid membraanikahjustusi. Avastus, et osad lagundamisele vastupidavamad peptiidid on rakkudele toksilised, kinnitab aga veelkord erinevate peptiidide toksilisuse kontrollimise olulisust.

Viimaks vaatlesime ka RSP-tüüpi peptiidset järjestust sisaldava uudse inhibiitori ARC rakusisest toimet ning leidsime, et nagu RSP-dki, siseneb ARC rakkudesse endotsütoosiraja kaudu, lekkides suhteliselt efektiivselt endosoomidest välja ning seondudes oma sihtmärgi Rho-kinaasiga. Seondudes kinaasile ühelt poolt tänu oma ATP-ga konkureeriva adenosiini-motiiviga ning teisalt oma substraadiga konkureeriva arginiinirikka peptiidse järjestusega, takistab ARC antud kinaasi aktiivsusest sõltuvat müosiini kerge ahela fosforüleerimist ning sellest lähtuvat aktiini tsütoskeleti moodustumist. Antud tulemused tõestavad, et RSP-d on võimelised raku endotsütoosiradasid ekspluateerides efektiivselt rakkudesse sisenema, destabiliseerima neid kandvate vesiikulite membraane ning vabanema seejärel endosomaalsest vangistusest.

16

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Toomas, I am endlessly grateful for your love and friendship and for appreciating me with all my little quarks. You and Charlie have brought happiness and joy to my days and also been the stress relievers, helping me to stay chipper and motivated (at least for the most of the time).

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say that friendship is “the one thing that makes the good times great and the bad times more tolerable”. So thank you for keeping me on the road.

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And finally, warm greetings go to all my international friends, whom I’ve met during conferences or just travelling – I wish you all the best wherever you are…

I did it! ☺

PUBLICATIONS

CURRICULUM VITAE

Helin Räägel

Date of Birth:	07.04.1983
Nationality:	Estonian
Contact:	University of Tartu, Institute of Molecular and Cell Biology, 23 Riia Street, 51010, Tartu, Estonia
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Education and professional employment

1998 – 2001	Pärnu Co-Educational Gymnasium (graduated with high honors)
1999 – 2000	Annie Wright School, WA (USA), exchange student (on honor roll)
2001 – 2005	University of Tartu, BSc in transgenic technology (*cum laude*)
2005 – 2007	University of Tartu, MSc in molecular and cell biology, minor in economics
2007 – ...	University of Tartu, Ph.D student at the Institute of Molecular and Cell Biology
2010 – ...	Competence Centre of Reproductive Medicine and Biology (CCRMB), Tartu, Estonia; researcher

Special courses

2011	University of Tartu conference "Peptide Vectors and Delivery of Therapeutics", Tallinn, Estonia (oral presentation)
2011	EMBO/FEBS joint lecture course "Protein Transport Systems" in Santa Margherita di Pula, Italy (oral presentation)
2010	EMBO Global Exchange Lecture Course "Molecular Mechanism of Protein Transport" in Bangalore, India BIT Lifesciences conference "PepCon-2010: After a Solution for the Machines of Life" in Beijing, China (oral presentation)
2009	EMBO Practical Course "Quantitative FRET, FRAP and FCS" in Heidelberg, Germany Practical Course in Advanced Microscopy "Winterschool 2009: Life Cell Microscopy" in Zurich, Switzerland
2008	FEBS Workshop "Mechanics and Dynamics of the Cytoskeleton" in Potsdam, Germany
2007	FEBS Workshop "Invadopodia, Podosomes and Focal Adhesions in Tissue Invasion" in Ortona, Italy
2006	FEBS Course "FEBS Special Meeting: Cellular Signaling – Dubrovnik 2006" in Dubrovnik, Croatia

Professional organizations

Member of board of the Faculty of Science and Technology of University of Tartu Member of the Estonian Biochemical Society

Scientific work

The focus of my research is defining the internalization routes, intracellular trafficking, degradation, endosomal escape and mechanisms of action of cell-penetrating peptides (CPPs) with the emphasis of their improvement for bio-technological application.

List of publications

Räägel, H., Pooga, M. (2011) Peptide and protein delivery with cell-penetrating peptides. Peptide and Protein Delivery. Ed. Chris Van der Walle, Academic Press (Elsevier), London, UK; pp 221–46

Räägel, H., Säälik, P., Langel, Ü. and Pooga, M. (2011) "Chapter 12. Mapping of protein transduction pathways with fluorescent microscopy" in Cell-Penetrating Peptides. Methods and Protocols. Methods in Molecular Biology, Ed. by Ü. Langel, pp 165–79

Räägel, H. (2010) "The trojan – a beneficial intruder?" popular science article in collaboration with Atomium Culture (the permanent platform for European excellence)

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Holm, T., Räägel, H., El Andaloussi, S., Hein, M., Mäe, M., Pooga, M. and Langel, Ü. (2010) "Retro-inversion of certain cell-penetrating peptides causes severe cellular toxicity" Biochimica et Biophysica Acta – Biomembranes (in press)

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Räägel, H., Lust, M., Uri, A. and Pooga, M. (2008) "Adenosine-oligoarginine conjugate, a novel bisubstrate inhibitor, effectively dissociates the actin cytoskeleton" FEBS Journal 275(14):3608–24

Social activities

Member of the Tartu University Academic Sports Club (badminton)

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Haridus ja erialane teenistuskäik

1998–2001	Pärnu Ühisgümnaasium (kuld medal)
1999–2000	Annie Wright School, WA (USA), vahetusõpilane Avatud Eesti Fondi stipendiumiga
2001–2005	Tartu Ülikool, Molekulaar- ja rakubioloogia instituut, BSc transgeense tehnoloogia erialal (*cum laude*)
2005–2007	Tartu Ülikool, Molekulaar- ja rakubioloogia instituut, MSc molekulaar- ja rakubioloogia erialal, majandus kõrvalerialana
2007– ...	Tartu Ülikool, Molekulaar- ja rakubioloogia instituut, doktoriõpingud rakubioloogia erialal
2010– ...	Reproduktiivmeditsiini ja -bioloogia Teaduse Arenduskeskuse AS, Tartu, teadur

Erialane enesetäiendus

2011	Tartu Ülikooli korraldatav konverents "Peptide Vectors and Delivery of Therapeutics", Tallinn, Eesti (suuline ettekanne)
2011	EMBO/FEBS ühendkursus "Protein Transport Systems", Santa Margherita di Pula, Itaalia (suuline ettekanne)
2010	EMBO globaalne teadmistevahetuskursus "Molecular Mechanism of Protein Transport", Bangalore, India BIT Lifesciences konverents "PepCon-2010: After a Solution for the Machines of Life", Peking, Hiina (suuline ettekanne)
2009	EMBO praktiline kursus "Quantitative FRET, FRAP and FCS", Heidelberg, Saksamaa Praktiline mikroskoopia täienduskursus "Winterschool 2009: Life Cell Microscopy", Žürich, Šveits (suuline ettekanne)
2008	FEBS'i kursus "Mechanics and Dynamics of the Cytoskeleton", Potsdam, Saksamaa
2007	FEBS'i kursus "Invadopodia, Podosomes and Focal Adhesions in Tissue Invasion", Ortona, Itaalia
2006	FEBS'i kursus "FEBS Special Meeting: Cellular Signaling – Dubrovnik 2006", Dubrovnik, Horvaatia

Teadusorganisatsioonid

2009– ... Tartu Ülikooli Loodus- ja tehnoloogiateaduskonna nõukogu liige

2006– ... Eesti Biokeemia Seltsi liige

Teadustöö

Minu uurimistöö põhisuunaks on rakku sisenevate peptiidide ja nendega seotud valgulise lastmolekuli komplekside rakkudesse sisenemine, rakusisene suunamine, stabiilsus, võimalik toksilisus ning endosoomidest vabanemine ehk protsess, mis on vajalik bioaktiivse lastmolekuli rakusisese toime avaldumiseks.

Teaduspublikatsioonid

Räägel, H., Pooga, M. (2011) Peptide and protein delivery with cell-penetrating peptides. Peptide and Protein Delivery. Ed. Chris Van der Walle, Academic Press (Elsevier), London, UK

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Räägel, H. (2010) "The trojan – a beneficial intruder?" popular science article in collaboration with Atomium Culture (the permanent platform for European excellence)

Räägel, H., Säälik, P. and Pooga, M. (2010) "Peptide-mediated protein delivery – which pathways are penetrable?" Biochimica et Biophysica Acta - Biomembranes

Holm, T., Räägel, H., El Andaloussi, S., Hein, M., Mäe, M., Pooga, M. and Langel, Ü. (2010) "Retro-inversion of certain cell-penetrating peptides causes severe cellular toxicity" Biochimica et Biophysica Acta - Biomembranes (in press)

Räägel, H., Säälik, P., Hansen, M., Langel, Ü. and Pooga, M. (2009) "CPP-protein constructs induce a population of non-acidic vesicles during trafficking through endo-lysosomal pathway" Journal of Controlled Release

Räägel, H., Lust, M., Uri, A. and Pooga, M. (2008) "Adenosine-oligoarginine conjugate, a novel bisubstrate inhibitor, effectively dissociates the actin cytoskeleton" FEBS Journal

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