DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
220

DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
220

ANNELY LORENTS

Overcoming the plasma membrane barrier: uptake of amphipathic cell-penetrating peptides induces influx of calcium ions and downstream responses

TARTU UNIVERSITY PRESS

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

Dissertation is accepted for the commencement of the degree of Doctor of Philosophy in gene technology on June 21st, 2012 by the council of the Institute of Molecular and Cell Biology, University of Tartu

Supervisor: Prof. Margus Pooga
University of Tartu,
23 Riia Street, Tartu, Estonia

Opponent: Prof. Roland Brock
Nijmegen Centre for Molecular Life Sciences,
Radbound University Nijmegen,
Nijmegen, The Netherlands

Commencement: Room 217, 23 Riia Street, Tartu, Estonia,
at 12:00 on September 7th, 2012

Publication of this dissertation is granted by the Institute of Molecular and Cell Biology, University of Tartu and by the Graduate School in Biomedicine and Biotechnology created under the auspices of European Social Fund

ISSN 1024–6479
ISBN 978–9949–32–080–6 (trükis)
ISBN 978–9949–32–081–3 (PDF)

Tartu Ülikooli Kirjastus
www.tyk.ee
Tellimus nr 395

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. Palm-Apergi, C., **Lorents, A.**, Padari, K., Pooga, M., Hällbrink, M. (2009) The membrane repair response masks membrane disturbances caused by cell-penetrating peptide uptake. *FASEB J* 23(1): 214–23

II. Padari, K., Koppel, K., **Lorents, A.**, Hällbrink, M., Mano, M., Pedroso de Lima, M.C. Pooga, M. (2010) $S4_{13}$-PV cell-penetrating peptide forms nanoparticle-like structures to gain entry into cell. *Bioconjug Chem* 21(4): 774–83

III. **Lorents, A.**, Kodavali, P.K., Oskolkov, N., Langel, Ü., Hällbrink, M., Pooga, M. (2012) Cell-penetrating peptides split into two groups based on modulation of intracellular calcium concentration. *J Biol Chem* 287(20): 16880–9

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

Ref. I designed and performed the fluorescence microscopy and flow cytometry experiments, participated in the electron microscopy experiments, data analysis, and writing of the paper

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

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

ABBREVIATIONS

AM	acetoxymethyl
ARF6	adenosine diphosphate-ribosylation factor 6
Cdc42	cell division cycle protein 42
CLSM	confocal laser scanning microscopy
CPP	cell-penetrating peptide
FACS	fluorescence activated cell sorter
GAG	glycosaminoglycan
GPI	glycosylphosphatidylinositol
GTP	guanosine triphosphate
HSPG	heparan sulfate proteoglycan
LAMP-2	lysosome-associated membrane protein 2
MAP	model amphipathic peptide
MRR	membrane repair response
MVB	multivesicular body
NLS	nuclear localization signal
pGrB	peptide derived from granzyme B
pVEC	cell-penetrating peptide derived from vascular endothelial cadherin
Rac1	Ras-related C3 botulinum toxin substrate 1
RhoA	Ras homologue gene family member A
$S4_{13}$-PV	cell-penetrating peptide designed of dermaseptin antimicrobial peptide S4 and nuclear localization signal
$S4_{13}$-PVrev	$S4_{13}$-PV peptide with reversed nuclear localization signal
$S4_{13}$-PVscr	$S4_{13}$-PV peptide with scrambled sequence
$S4_{13}$-PVwt	wild-type $S4_{13}$-PV peptide
TGN	trans-Golgi network
TP	transportan
TP10	transpostan 10

INTRODUCTION

Many potential therapeutic molecules or other biologically active compounds that are efficient in solutions are not able to traverse eukaryotic cell membranes and, therefore, can not be utilized in living cells. To confer the barrier function of the plasma membrane, different delivery methods have been developed. However, many of these have at least some limitations, for example, they are generally invasive and/or can not be applied in living organisms.

Discovery of sequences derived from full proteins that display cell-penetrating properties, however, has helped to pave the way for the development of efficient peptidic transport vectors. These so called cell-penetrating peptides (CPPs, also known as protein transduction domains or membrane translocating sequences) are able to efficiently gain access into cells and, furthermore, transport various payloads attached to them into intracellular compartments in a non-invasive manner. To date, CPPs have been used to deliver different types of molecules with a wide range of size, such as small interfering RNA and peptide nucleic acid oligomers as well as full length proteins and plasmids or even nanoparticles and liposomes. Moreover, their potential as efficient delivery vehicles has repeatedly been demonstrated both *in vitro* and *in vivo*.

Before these promising transport vectors can be used in therapeutic biomedicine, their mechanism of entry needs to be carefully elucidated. At present, there is no unambiguous agreement on the uptake pathways exploited by CPPs, because characteristics of the peptide, its concentration, and cargo molecule may all play a role in the selection of uptake pathway. Nevertheless, it appears that several well-known CPPs are able to interact with membrane lipids and induce disturbances in the plasma membrane before they are taken up by cells. This, in turn, may be harmful for cells, and it is not known how cells can cope with the potential stress caused by the uptake of CPPs.

Therefore, the main focus of this study was to elucidate the membrane destabilizing capacity of several CPPs belonging to different families, including protein-derived, chimeric, and artificial ones. Since disturbances in the lipid bilayer might hinder the barrier function of the plasma membrane, we assessed whether CPPs are able induce the influx of calcium ions into cells. Furthermore, activation of different downstream responses that would help cells to reseal the damaged cell surface regions and deal with the detrimental influx of calcium were studied. In addition, we characterized the interaction of relatively novel $S4_{13}$-PV peptides with the cell surface, their uptake pathways, and intracellular localization in order to reveal the determinants responsible for their different membrane disturbing effects and uptake efficiency.

The knowledge obtained from the present study might help to improve the understanding of processes involved in the uptake of CPPs and, therefore, gives valuable information for the ongoing development of CPPs as transport vectors. Furthermore, it helps to recognize that not all seemingly harmful side-effects hamper the utilization of CPPs, but there are means of overcoming these drawbacks.

1. LITERATURE OVERVIEW

1.1. Plasma membrane barrier

Plasma membrane is a dynamic and complex structure that defines the boundaries of eukaryotic cells. It is mainly composed of various lipids (*e.g.* phospholipids, glycolipids, cholesterol) that self-assemble into a bilayer because of their amphipathic nature and hydrophobic interactions. The polar head-groups of lipids interact with water, while the hydrophobic tails favor contact with one another, forming a double-layer sheet-like structure with a hydrophobic core that acts as a permeability barrier. This barrier prevents essential cytoplasmic components from leaking out and, at the same time, protects cells from harmful molecules from diffusing in. In addition to lipids, specific proteins are embedded into the bilayer that mediate distinctive functions of the plasma membrane. Selective permeability of the membrane, for example, is facilitated by proteins that act as pumps or channels for small uncharged polar molecules or ions. Yet, these transport systems are not able to assist the internalization of larger hydrophilic compounds.

In biomedicine and scientific research, however, insertion or delivery of potential bioactive molecules into cells, in order to modulate cellular processes and functions, has become of high importance. Since these compounds are generally not taken up by cellular transport systems, numerous methods have been developed to bypass this obstacle. Direct microinjection into cells, electroporation, utilization of virus-based delivery vectors, or cationic lipids (lipoplexes), to name a few, are all relevant approaches to transport various compounds into mammalian cells. At the same time, application of these methods has lead to the discovery of their various drawbacks and side-effects. For example, many of these methods can be employed only in *in vitro* systems, and their usage in cellular tissues or organisms is rather complicated or not feasible at all. In addition, other aspects (such as the stability of the carrier systems in blood stream, low or heterogeneous yield, and acute toxicity) have to be carefully considered before their application *in vivo*. However, the discovery of relatively short amino acid sequences with cell-penetrating properties in mid-1990s has become a considerable alternative to the methods mentioned above.

1.2. Cell-penetrating peptides

CPPs represent a large family of peptides that are efficiently taken up by eukaryotic cells and are able to transport numerous cargo molecules into intracellular environment. Because of this remarkable property, they have gained much attention as transport vehicles for biologically active compounds that *per se* are cell impermeable and, therefore, would not be suitable for therapeutic purposes. However, attachment of these payloads to CPPs makes them ulti-

mately functional for biomedical approaches and research. Moreover, application of CPPs has less side-effects and drawbacks as compared to the other "classical" methods used to facilitate the uptake of cell-impermeable molecules. Namely, CPPs are homogeneously taken up by all cell types studied so far, they exhibit less cytotoxicity and no immunogenicity and, more importantly, CPPs can be used *in vivo*.

After the discovery of first CPPs, the researchers have put much effort into this field to find more efficient transport peptides with fewer side-effects (*e.g.* lower cytotoxicity or greater resistance to degradation) and/or demonstrating specific targeting ability to certain tissues or organs. As a result, already over a hundred peptides possessing cell-penetrating properties have been identified at present. CPPs are usually short, 5–40 amino acid long sequences that often contain basic amino acids in their sequence. However, many of them display different primary structures and chemical properties, making it rather complicated to divide CPPs into subgroups or families. One possibility is to divide them according to their origin to:

a) protein-derived CPPs, such as Tat-peptide (Vivés et al., 1997), penetratin (Derossi et al., 1994), and pVEC (Elmquist et al., 2001);
b) chimeric CPPs, such as transportans (Pooga et al., 1998; Soomets et al., 2000) and $S4_{13}$-PV (Hariton-Gazal et al., 2002);
c) entirely synthetic or designed CPPs, such as model amphipathic peptide (MAP) (Oehlke et al., 1998) and oligoarginines (Futaki et al., 2001; Mitchell et al., 2000).

However, this classification does not give any clues about their chemical or physical properties, interaction with the plasma membrane, internalization efficiency, or toxicity. Therefore, quite recently, André Ziegler (Ziegler, 2008) proposed a new classification system for CPPs that is based on the peptide sequence and different peptide-lipid binding affinities. According to this, CPPs are categorized to primary amphipathic, secondary amphipathic, and non-amphipathic peptides.

1.2.1. Primary amphipathic peptides

Like the name implies, primary amphipathic CPPs consist of cationic and hydrophobic segments that reside sequentially in the primary structure of the peptide. Their interaction with the membrane lipids is governed by hydrophobic interactions rather than electrostatic ones, since they are able to bind with both anionic and neutral lipid membranes with a high affinity (Ziegler, 2008). In general, primary amphipathic CPPs penetrate deeper into the lipid bilayer (Deshayes et al., 2004); however, they do not span the membrane in a pore like manner unless a transmembrane potential is present (Deshayes et al., 2006).

CPPs that can be categorized under this group are, for example, transportans. Transportan (TP) is a 27 amino acid long chimeric peptide. It consists of a seg-

ment of neuropeptide galanin (first 13 amino acids of TP) and a segment of the wasp venom mastoparan that are linked via a lysine-residue (Pooga et al., 1998). The lysine linker was incorporated to enable attachment of different reporter groups (*e.g.* biotin and fluorophores) to the peptide. Even though TP is efficiently taken up by cells via receptor-independent pathway, it also exhibits an inhibitory effect on the basal GTPase activity (Zorko et al., 1998) that might become an obstacle in the utilization of TP as a carrier peptide. Therefore, several deletion analogues of transportan were designed to find a peptide without the above-mentioned side-effect but with similar translocation efficiency (Soomets et al., 2000). This resulted in the discovery of TP10, a truncated version of TP that lacks six amino acids from the N-terminus. Nevertheless, it shows comparable uptake levels with TP with lower cytotoxic effects. In the presence of membrane lipids, the mastoparan part of transportan adopts an α-helical conformation, while the galanin part stays more unstructured (Barany-Wallje et al., 2004).

The model amphipathic peptide (MAP, also known as KLAL) is a synthetic peptide that is composed of lysine, alanine, and leucine (Steiner et al., 1991). As a classical amphipathic peptide, it adopts an α-helical conformation where hydrophobic amino acids localize on one side and basic amino acids on the opposite side. Originally it was thought that amphipathic nature of MAP is crucial for the cellular uptake of the peptide (Oehlke et al., 1998); however, later studies revealed that non-amphipathic analogues are also taken up by cells (Scheller et al., 2000). It has been demonstrated that MAP is able to enter the cells both via energy-dependent and -independent mechanisms (Oehlke et al., 1998; Oehlke et al., 2004). Although MAP rapidly internalizes into cells and efficiently delivers cargo molecules, it also exhibits profound toxicity; namely, MAP induces membrane leakage already at 1 μM concentration (Hällbrink et al., 2001). Even though Ziegler has categorized MAP under secondary amphipathic peptides, we would rather place it under primary amphipathic ones. First, secondary amphipathic CPPs acquire their secondary structure upon binding to negatively charged membranes. However, MAP adopts its helical conformation already in the "membrane-mimicking" solvent, and the helical content is similar in the presence of both neutral and negatively charged lipids (Dathe et al., 1996). Secondly, the insertion of secondary amphipathic CPPs into the lipid bilayer is not pronounced. MAP peptide, however, inserts deeply into lipid layers, and most surprisingly, the membrane-disturbing effect of MAP significantly increases with reduction of the negative surface charge of the liposomes (Dathe et al., 1996; Erbe et al., 2009). And finally, primary amphipathic CPPs induce membrane leakage already at low micromolar concentration that is also consistent with MAP peptide.

1.2.2. Secondary amphipathic peptides

Secondary amphipathic CPPs expose their amphipathic nature after a change in their secondary structure upon binding to the membrane lipids or cell surface sugars. Therefore, when a peptide adopts either α-helical conformation or β-strand structure (Futaki et al., 2001; Lamaziere et al., 2007; Oehlke et al., 1997), basic amino acids and hydrophobic amino acids are displayed on the opposite sides. Furthermore, these CPPs do not bind efficiently to neutral membrane lipids (Ghibaudi et al., 2005; Magzoub et al., 2001); however, the affinity increases by several orders of magnitude with the anionic lipid content of the membrane (Ziegler, 2008). As was mentioned above, secondary amphipathic CPPs are not buried deep inside the lipid bilayer (Persson et al., 2004; Persson et al., 2003).

By far the most studied secondary amphipathic CPP is penetratin. It is 16 amino acid long CPP that corresponds to the third helix of the *Drosophila* Antennapedia homeodomain (Derossi et al., 1994). Basic amino acids are important for the efficient cellular uptake of penetratin, since the substitution of any of these residues with an uncharged alanin results in a decreased uptake of the peptide (Drin et al., 2001; Fischer et al., 2000). Hydrophobic amino acids are also essential, since the substitution of tryptophans with phenylalanines reduces the translocation efficiency drastically (Dom et al., 2003). It has been demonstrated that penetratin can adopt an α-helical conformation, or a β-structure, or stay unstructured. Thus, conformational changes in the secondary structure of penetratin highly depend on the concentration of the peptide and the membrane lipid composition (Bellet-Amalric et al., 2000; Derossi et al., 1994; Drin et al., 2001; Magzoub et al., 2002; Maniti et al., 2010).

pVEC is a CPP derived from the cell adhesion molecule of murine vascular endothelial cadherin. It corresponds to the hydrophobic segment of 5 amino acids from the transmembrane region and a hydrophilic segment of 13 amino acids from the cytosolic tail of the protein. The amphipathic nature of pVEC has been suggested to be essential for the cellular uptake of the peptide (Elmquist et al., 2001).

The $S4_{13}$-PV peptide is a chimeric CPP that contains a cell-penetrating sequence of 13 amino acids derived from Dermaseptin S4 peptide and the nuclear localization signal (NLS) of the Simian Virus 40 large T antigen (Hariton-Gazal et al., 2002). In addition to the wild-type $S4_{13}$-PV, analogues with reversed NLS and scrambled sequence are known. Upon binding to negatively charged lipids, the wild-type $S4_{13}$-PV and its analogue with reversed NLS undergo significant conformational changes that are consistent with the formation of helical structures. However, the conformational changes of the scrambled peptide were significantly less pronounced (Mano et al., 2007; Mano et al., 2006).

1.2.3. Non-amphipathic peptides

Non-amphipathic CPPs contain a high number of cationic amino acids in their sequence and, consequently, do not exhibit any amphipathicity in their primary or secondary structure. These peptides do not bind to lipids (Magzoub et al., 2001), but rather float on or are only superficially absorbed to the membranes (Goncalves et al., 2005; Ziegler et al., 2003; Tiriveedhi and Butko, 2007). In addition, non-amphipathic CPPs do not change their conformation in the presence of membrane lipids, and they do not penetrate into lipid monolayers at physiologically relevant surface pressures (Ziegler et al., 2003). As such, they are not able to translocate across lipid membranes at biologically relevant concentrations (1–10 μM) (Krämer and Wunderli-Allenspach, 2003; Lamaziere et al., 2007; Ziegler et al., 2003; Tiriveedhi and Butko, 2007).

Highly cationic Tat-peptide is derived from Tat protein that is a human immunodeficiency virus (HIV) transcription factor (Vivés et al., 1997). After the discovery of translocation properties of the full-length protein (Frankel and Pabo, 1988; Green and Loewenstein, 1988), shorter sequences were synthesized to define the most efficiently internalizing peptide. This resulted in Tat(48–60) that is currently known as Tat-peptide (Vivés et al., 1997). Even though Tat-peptide is one of the most studied non-amphipathic CPPs, the mechanism(s) by which it is taken up into cells is still a great puzzle. It appears that Tat-peptide is able to exploit all possible internalization pathways available, including clathrin-dependent endocytosis (Richard et al., 2005; Richard et al., 2003), caveolin-dependent endocytosis (Ferrari et al., 2003; Fittipaldi et al., 2003), and macropinocytosis (Al Soraj et al., 2012; Wadia et al., 2004), in addition to receptor- and energy-independent mechanisms (Futaki et al., 2001; Ziegler et al., 2005).

Since highly cationic CPPs (penetratin and Tat-peptide) were amongst the first CPPs discovered, basic amino acids were considered to be essential for the cellular uptake. Therefore, several homopolymers composed of arginine, lysine, ornithine, or histidine were synthesized and their translocation abilities were compared (Futaki et al., 2001; Mitchell et al., 2000; Wender et al., 2000). Amongst the studied polymers, polyarginines demonstrated the highest internalization efficiency that can be attributed to the guanidinium group of the arginine side-chain, since it can form multidentate hydrogen bonds with the phosphates of lipid head-groups and facilitate the adaptive translocation of the peptide (Rothbard et al., 2005). However, the length of oligoarginines plays a role also in their internalization capacity. Namely, oligomers composed of less than six arginines are poor translocators, 6–9 arginines are most efficiently taken up by cells, and the longer analogues already exhibit cytotoxic side-effects (Mitchell et al., 2000).

1.3. Internalization mechanisms of cell-penetrating peptides

The uptake pathways by which CPPs or their constructs with cargo molecules gain access into cells are by far more complex than was originally thought. A number of recent studies have demonstrated the change between different cellular distribution patterns of CPPs in response to the shift of experimental conditions, like the concentration of CPP, temperature, and the size of cargo. Therefore, currently, a growing number of studies support the view that CPPs enter cells by using different mechanisms that act in parallel, and for most CPPs probably more than one uptake pathway exists.

1.3.1. Crossing the plasma membrane

After the discovery of CPPs, the prevailing route of internalization of these peptides was considered to be a receptor- and energy-independent penetration across the membrane bilayer. However, around a decade later it was discovered that fixation of cells may lead to the redistribution of CPPs and, therefore, re-evaluation of the uptake mechanisms was enforced (Richard et al., 2003). This, in turn, has led to the generally accepted belief that CPPs mostly exploit different types of endocytic pathways to enter cells. Nevertheless, it has been demonstrated by several groups that under certain conditions at least a fraction of CPPs is able to enter cells via direct translocation. For instance, the uptake mechanisms of non-amphipathic CPPs has been reported to change from endocytic pathway to direct translocation upon raising the concentration of the peptide or by lowering the incubation temperature (Fretz et al., 2007; Watkins et al., 2009). A change to non-endocytic uptake pathway at higher peptide concentrations has also been confirmed for secondary amphipathic $S4_{13}$-PV (Mano et al., 2005). However, the direct translocation of CPPs might become hampered by the addition of a larger cargo molecule; namely, Tünnemann and collegues (Tünnemann et al., 2006) demonstrated that attachment of a cargo molecule (>50 amino acids) to Tat-peptide changed the internalization mechanism of the peptide from a direct translocation to an endocytic uptake.

The ability of CPPs to translocate across lipid bilayers is also supported by several biophysical studies demonstrating that some CPPs interact with lipids and, as a result, are embedded deeper into the membrane. For example, MAP peptide that adopts an α-helical conformation, interacts not only with the lipid head-groups, but also with the hydrophobic core of the membranes. Namely, MAP is able to intercalate between the lipid side-chains under a 60° degree angle (Erbe et al., 2009). In addition, wild-type $S4_{13}$-PV and its analogue with reversed NLS are partially able to adopt an α-helical conformation and insert deeper into the lipid bilayer that is considered to be essential for the non-endocytic uptake of these CPPs (Mano et al., 2007; Mano et al., 2006).

1.3.2. Endocytosis

Endocytosis is a process by which eukaryotic cells take up macromolecules and fluids from the surrounding environment by forming relatively small membrane-enclosed sacs termed endocytic vesicles that pinch off from the plasma membrane and are subsequently targeted to specific sub-cellular destinations. Besides being a fundamental pathway for the uptake of nutrients, it also plays a central role in the regulation of cell surface receptors, controls several signaling cascades, and is important for cell division and motility (Doherty and McMahon, 2009; Hoeller et al., 2005). In addition, some bacteria and viruses are able to take advantage of this route of uptake to gain access into cells (Dimitrov, 2004; Gruenberg and van der Goot, 2006; Mercer et al., 2010; Steele-Mortimer et al., 2000).

Endocytosis is classically divided into phagocytosis and pinocytosis. The first one is typical for specialized cells and is mainly used for the uptake of pathogens, dead cells, and cell debris. The second one, however, is distinctive to almost all eukaryotic cells, and can be further divided into several pathways depending on the proteins and lipids involved in the vesicle formation stadium.

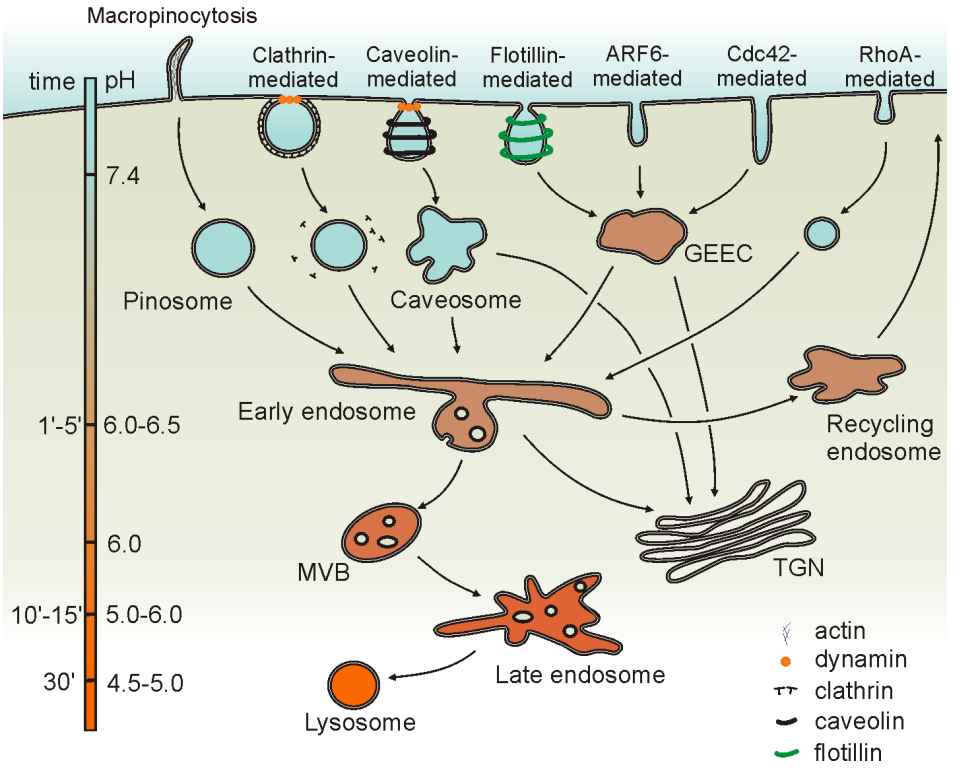

Figure 1. Acknowledged pinocytotic pathways and intracellular trafficking of endocytosed material in mammalian cells. GEEC – glycosylphosphatidylinositol-anchored protein enriched early endosomal compartment, MVB – multivesicular body, TGN – trans-Golgi network.

1.3.2.1. Clathrin-mediated endocytosis

Clathrin-mediated endocytosis is by far the most studied receptor-dependent endocytosis that is initiated by the binding of a ligand to the trans-membrane receptor molecule on the cell surface. This association process triggers the formation of coated pits on the cytosolic side of the plasma membrane. The "coat" is assembled by recruitment of different proteins, where clathrin is the main unit. Clathrin is a protein that has a three-legged structure (triskelion) composed of three clathrin heavy chains and three light chains (Heuser and Keen, 1988; Smith et al., 1998). When these triskelions assemble, they form a curved lattice under the plasma membrane that eventually drives the invagination of anchored membrane and the formation of clathrin-coated pits. Even though clathrin is able to spontaneously assemble into "cages" *in vitro* (Ford et al., 2002), under physiological conditions this process is mediated by recruitment of specific adaptor and accessory proteins (Brodsky et al., 2001; Ehrlich et al., 2004; Schmid, 1997), because clathrin itself does not interact directly with the membrane. In addition, adaptor proteins (*e.g.* AP-2) serve as recognition sites for different sorting signals and cargoes (Traub, 2009).

The final step in the vesicle formation process is its fission from the cell surface. This process is mediated and regulated by a protein from GTPase family – dynamin – that assembles into an helical collar around the neck of the forming vesicle and irreversibly detaches it from the plasma membrane (Herzog et al., 1977; Johnson, 1972). GTPase activity of dynamin is triggered by the interaction with phosphoinositides and phosphatidylinositol-4,5-bisphosphate (Barylko et al., 1998; Zheng et al., 1996; Tuma et al., 1993) and as a result vesicles, usually with 100–150 nm in diameter, pinch off. Once the vesicle is detached, the clathrin-coat rapidly disassembles enabling the clathrin to recycle back to the plasma membrane while the vesicle is further trafficked to endocytic compartments.

Several viral pathogens target the receptors of clathrin-mediated endocytosis and, therefore, are taken up by this pathway. Studies describing the uptake of pathogens, both viruses and bacteria, have also shed light on the flexibility in the regulation mechanisms of clathrin-coated vesicle formation. More precisely, virus particles more often induce the formation of clathrin-coated pits at the site of binding to the plasma membrane rather than entering via preassembled one (Marsh and Helenius, 2006). In addition, *L. monocytogenes*, a microorganism many times larger than a normal endocytic cargo, invades cells via clathrin-mediated endocytosis (Veiga and Cossart, 2005).

Since CPPs can interact with cell surface heparan sulfate proteoglycans (HSPG), it is believed that, as a consequence, they are internalized through clathrin-mediated endocytosis, because binding of ligands to surface sugars (such as HSPG) triggers their clustering and uptake by this type of endocytosis (Richard et al., 2005). In addition, several other studies have demonstrated that CPPs or CPPs with their payloads are able to exploit this pathway to enter the cells (Padari et al., 2005; Rinne et al., 2007; Vendeville et al., 2004).

1.3.2.2. Caveolin-mediated endocytosis

Caveolins are 21 kDa transmembrane proteins that are anchored into the lipid bilayer from the cytosolic side by a hydrophobic sequence, and as a result form a hairpin loop with both amino- and carboxy-terminal regions facing the cytoplasm (Dupree et al., 1993; Pelkmans and Helenius, 2002). Caveolin-1 is responsible for the structural formation of caveolae (from Latin, little caves) (Pelkmans and Zerial, 2005) that are defined as 50–80 nm large flask shaped invaginations (Palade, 1953; Yamada, 1955). Therefore, caveolins are referred to as the membrane coat proteins for caveolae. Recently, it was found that a cytoplasmic protein called cavin-1, in parallel with caveolin-1, is required for the formation and function of caveolae (Hill et al., 2008; Liu and Pilch, 2008). Since caveolins reside in highly ordered lipid microdomains called lipid rafts (Tagawa et al., 2005), caveolae are also abundant in glycosphingolipids, lipid-anchored proteins, and cholesterol.

Caveolin-mediated endocytic pathway is involved in many biological processes, such as lipid regulation, cell signaling, vesicular transport (Anderson, 1998; Kurzchalia and Parton, 1999; Pelkmans and Helenius, 2002; Rothberg et al., 1992), and most prominently in transcytosis (Frank et al., 2009; Tuma and Hubbard, 2003). It has also been suggested that caveolae can act as membrane tension regulators (Raucher and Sheetz, 1999; Sens and Turner, 2006). Furthermore, several toxins and viruses (*e.g.* tetanus, cholera, Simian Virus 40) enter the cells via this pathway (Anderson et al., 1996; Herreros et al., 2001; Shogomori and Futerman, 2001; Stang et al., 1997).

Despite the fact that caveolae are associated with several biological functions, there is scarce information available about the specific receptors, and the mechanism by which cargo molecules are concentrated in caveloae is not understood. However, it has been demonstrated that CPP-cargo constructs, such as protein complexes with Tat-peptide or transportans, are taken up by cells via caveolin-mediated endocytosis (Ferrari et al., 2003; Fittipaldi et al., 2003; Säälik et al., 2009), and that this route contributes up to 60–70% of uptake in the case of transportans (Säälik et al., 2009).

1.3.2.3. Macropinocytosis

Macropinocytosis is an actin driven endocytic pathway that was for many years thought to occur via non-selective membrane uptake (McNeil, 1984). However, macropinocytosis is now defined as a highly coordinated and dynamic process that involves membrane ruffling and subsequent engulfment of a large quantity of extracellular fluids by the formation of large uncoated vesicles (usually 200 nm – 2 μm in size) called macropinosomes (Bar-Sagi and Feramisco, 1986; Dowrick et al., 1993; Swanson and Watts, 1995). Depending on the cell type and activation pathway, plasma membrane ruffling can lead to the formation of different types of membrane outgrowths. More precisely, lamellipodia-like

protrusions (Matin et al., 2002; Mercer and Helenius, 2009), circular cup-shaped ruffles (Araki et al., 2000; Dowrick et al., 1993; Orth and McNiven, 2006), and large plasma membrane extrusions called blebs (Mercer and Helenius, 2008; Mercer and Helenius, 2009) are all found to be able to induce micropinosomes once they "fall back" onto the cell surface. However, only a small fraction of these "collapsing" events lead to the formation of endocytic vesicles.

The formation of membrane outgrowths can be stimulated with the activation of growth factor receptors (Dowrick et al., 1993; Hewlett et al., 1994; Kerr and Teasdale, 2009), apoptotic bodies, necrotic cells, and pathogens, such as bacteria and viruses (Ammendolia et al., 2004; Kerr and Teasdale, 2009; Mercer and Helenius, 2008; Mercer and Helenius, 2009). The process itself is driven by actin polymerization that is mediated by small family of Rho GTPases, such as Rac1 that is activated already in the early steps of macropinocytosis (Ridley et al., 1992; Swanson, 2008). In addition to activation of Rac1 upon binding of epidermal growth factor to its receptor, generation of phosphoinositide 4,5-bisphosphate is initiated that, in turn, leads to the activation of several downstream proteins resulting in the modulation of actin polymerization (Kerr and Teasdale, 2009; Lee and Knecht, 2002; Mercer and Helenius, 2009; Wang et al., 2008; Vidricaire and Tremblay, 2007). Furthermore, CtBP1/BARS (C-termnal-binding protein-1/brefeldin A ribosylation substrate) proteins localize to the region and affect macropinosome membrane closure in a phosphorylation-dependent manner (Liberali et al., 2008).

Like with the other endocytic pathways, once the pinocytotic vesicle is formed, it has to be detached from the plasma membrane. In case the micropinocytosis occurs by lamellipodia-like and circular ruffles, the fission process is regulated by dynamin (Doherty and McMahon, 2008; Herzog et al., 1977). However, bleb-associated macropinocytosis is found to be dynamin independent (Mercer and Helenius, 2008).

Uptake of CPP-protein conjugates has been demonstrated to be sensitive to specific inhibitors and regulators of macropinocytosis (Al Soraj et al., 2012; Wadia et al., 2004), thus, implying the involvement of macropinocytosis in the internalization of these constructs. Furthermore, the ability of CPPs to activate Rac1, and the subsequent remodeling of actin cytoskeleton by CPPs or their constructs with cargo, has been confirmed repeatedly (Gerbal-Chaloin et al., 2007; Imamura et al., 2011; Nakase et al., 2004).

1.3.2.4. Other clathrin-independent carrier pathways

Besides the classical endocytic pathways, the discovery of other uptake mechanisms mediated by vesicular or tubular carriers has expanded drastically. For instance, currently, we can distinguish flotillin-, RhoA, Cdc42-, and ARF6-mediated uptake pathways. Most probably, however, other clathrin- and caveolin-independent pathways also exist and are yet to be discovered.

Flotillins (also known as reggies) are structurally similar to caveolins. Namely, they are palmitoylated proteins that adopt a hairpin-like conformation in the lipid bilayer (Stuermer, 2010). In addition to structural similarities, flotillins are recognized as markers of lipid rafts because they reside in detergent insoluble membrane fractions. Since caveolins are also confined into lipid rafts, it was found that flotillins localize into caveolae-like structures (Blanchet et al., 2008; Frick et al., 2007; Glebov et al., 2006; Morrow and Parton, 2005). However, flotillins associate with these structures only once they are formed (Kirkham et al., 2008) inducing the formation of membrane microdomains that subsequently internalize GPI-anchored proteins and fluid phase cargos (Frick et al., 2007; Glebov et al., 2006).

Cdc42 (cell division cycle 42) is a protein that belongs to the Ras superfamily of GTP-binding proteins and, therefore, regulates actin cytoskeleton that is important for several cellular mechanisms, such as control of growth, differentiation, and apoptosis of cells. Cdc42-mediated endocytosis pathway is associated with the formation of tubular invaginations (30–50 nm wide and 200–600 nm long) on the plasma membrane (Kirkham et al., 2005) that is initiated by the clustering of GPI-anchored proteins that interact with Cdc42 and are subsequently internalized (Mayor and Riezman, 2004). In addition, GTPase GRAF1 (GTPase regulator associated with focal adhesion kinase-1) was recently shown to also localize in tubular endocytic structures involved in the uptake of glycosphingolipids, GPI-linked proteins, etc (Doherty and Lundmark, 2009; Lundmark et al., 2008).

ARF6 (ADP-ribosylation factor 6) is a member of the ARF family of six small GTPases that are expressed in all eukaryotic cells (Donaldson, 2003). The role of ARF6 in clathrin-independent endocytosis is not fully understood yet, however, this pathway is morphologically associated with tubular rather than vesicular structures (Caplan et al., 2002; Massol et al., 2005). ARF6 is a protein that most likely regulates membrane curvature; more specifically, binding of ARF6 to GTP depends on membrane-curvature that, in turn, generates even more profound curvature. Because ARF6 has a BAR (Bin/Amphiphysin/Rsv) domain that is able to sense changes in membrane curvature (Frost et al., 2009; Masuda and Mochizuki, 2010; Qualmann et al., 2011; Suetsugu et al., 2010), the membrane budding is facilitated by the BAR domain that inserts into the lipid bilayer (Lundmark et al., 2008).

RhoA also belongs to the Ras homolog gene family (Ridley, 1997), and its main function is to control actin cytoskeleton dynamics. To be exact, it regulates gene expression, actin filament reorganization, determination of the cell shape, and proliferation (Hsu et al., 2003; Zhang and Zheng, 1998). First implications for RhoA-mediated endocytic uptake mechanisms came from the discovery that the receptor for interleukin-2 is internalized via this pathway (Lamaze et al., 2001). Later it was demonstrated to control the uptake of ricin (Dalton et al., 2006) and Baculovirus (Grafahrend et al., 2008). However, it is

not known whether RhoA is required for the sorting of the receptors into the endocytic vesicles or during the whole endocytic process itself.

The role of these pathways in the uptake of CPPs has not been thoroughly elucidated because of the lack of specific markers and inhibitors that would help to unambiguously identify clathrin- and caveolin-independent endocytosis. However, the ability of CPPs to trigger the remodeling of the actin cytoskeleton (Al Soraj et al., 2012; Nakase et al., 2004) might not be confined only to macropinocytosis, since regulation of actin dynamics is also involved in Cdc42-, ARF6-, and RhoA-mediated endocytosis.

1.3.2.5. Endocytic compartments

After the formed vesicles pinch of from the cell surface, they are targeted into complex machinery of endocytic compartments that continuously sort and interchange their content by fusion with each other or by communicating via trafficking vesicles. This process is spatiotemporally segregated and highly dynamic, generally resulting in receptor recycling and/or cargo degradation.

The first sorting station is the early endosomal compartment that consists of large vesicles (300–400 nm diameter) and cisternae regions with slender tubules (60–80 nm diameter). Endocytic vesicles reach here within 1–5 min after they are pinched off from the plasma membrane. Early endosomes are rich in GTPase Rab5 and phoshatidylinositol-3-phosphate, and target their content (i) back to the plasma membrane either directly or via recycling endosomes, (ii) to trans-Golgi network (TGN), or (iii) to lysosomes for degradation (Haas et al., 2005; Marshansky and Futai, 2008; Rybin et al., 1996).

The multivesicular parts of early endosomes lead to the formation of multivesicular bodies (MVBs). Specifically, MVBs are believed to evolve from the early endosomal compartment by forming intraluminal vesicles from the limiting membrane of the compartment (Eden et al., 2009; Gruenberg and Stenmark, 2004; Hurley et al., 2010; van der Goot and Gruenberg, 2006) eventually leading to the formation of globular MVB that acidify their lumen to about pH 5.5.

MVBs deliver their content to late endosomes that are structures also rich in tubules, intraluminal vesicles, and effector molecule Rab7 (Pfeffer, 2001). These regions have different protein and lipid composition to further assist the sorting of the internalized material (Gruenberg, 2003; Russell et al., 2006). Eventually, late endosomes deliver their content to lysosomes that are the final station of the endocytic pathway. The material designated for degradation reaches lysosomes in about 30 min from its entry into cells. Lysosomes are acidic organelles (pH 4.5–5.0) that contain a high number of soluble lysosomal hydrolases with each having a specific substrate (Saftig and Klumperman, 2009).

The above discussed trafficking routes are conventional for all endocytic pathways. However, some exceptions have been described, especially in the early steps of trafficking. In case of caveolin-mediated endocytosis, the

caveolae first fuse with an organelle called the caveosome. This rosette-like structure is rich in caveolin-1, and has a lumen with neutral pH (Nichols, 2002; Pelkmans et al., 2001; Pietiainen et al., 2004). From there, the material is further targeted to the early endosomes, TGN, or endoplasmatic reticulum (Pelkmans et al., 2004). In case of flotillin-, Cdc42-, and ARF6-mediated endocytosis, the vesicles are first transported to a specific organelle termed the GPI-enriched early endosomal compartment (GEEC), where GPI-anchored proteins are recycled back to the plasma membrane (Sabharanjak et al., 2002). However, rest of the content may be directed to the early endosomes or to the Golgi network (Doherty and Lundmark, 2009).

Membrane proteins that are confined into recycling endosomes are usually transported back to the plasma membrane (Mellman, 1996). The effector molecules that are associated with the recycling endosomes are Rab4 and Rab11 (Pfeffer, 2001); however, Rab4-positive vesicles facilitate rather quick recycling events, whereas Rab11-containing endosomes localize into the perinuclear region and, therefore, contribute to a slower recycling pathway (Sheff et al., 1999).

1.4. Membrane repair response

Organisms are often exposed to all possible stimuli and, in some cases, to challenging surroundings that may lead to injuries and wounding. Even otherwise normal biological processes, such as muscle contraction (McNeil and Khakee, 1992), can lead to disruption of the plasma membrane. Fortunately, all organisms studied so far – protists (Szubinska, 1971), yeast (Levin, 2005), bacteria (Hambleton, 1971), and multicellular organisms (Los et al., 2011) – are able to deal with these unexpected downsides to a certain extent by initiating healing processes. Proficient self-repair is apparent in all levels of biological organization including cells, tissues, organs, and organisms. In spite of the level, wound healing process addresses several aspects during the repair: the injury site is patched up, redundant material is degraded or expelled, the material lost as a result of wounding is regenerated, and finally, all of the evidence of the damage is removed.

Characterization and analysis of single-cell wound repair response did not gain much research interest until relatively recently (McNeil and Steinhardt, 2003). McNeil with colleagues showed that the plasma membrane repair is a an universal response in different mammalian cell types and that resealing of the wound is an active cellular process rather than a passive reorganization of membrane lipids as it was generally believed (McNeil et al., 2003).

The membrane repair response (MRR) is initiated by the influx of calcium ions into cells. Under normal conditions, the cytosolic Ca^{2+} concentration is maintained low (micromolar range) by the bidirectional ion transport across the plasma membrane, however, extracellular Ca^{2+} concentrations are much higher

(millimolar range). Therefore, in case of injury, the barrier function of the plasma membrane is breached and the cell interior is exposed to the high external Ca^{2+} levels that can be harmful, or even toxic to the cells. Influx of Ca^{2+} triggers rapid fusion of intracellular vesicles with each other forming a large membranous "patch" that can be used to seal the disruption site (McNeil et al., 2000). This prevents the cell from being killed as a result of either continuous influx of Ca^{2+}, or the efflux of essential cytoplasmic components.

Originally, it was proposed that lysosomes are the major, if not the only, membranous organelles that can be used for resealing the plasma membrane (Jaiswal et al., 2002; Reddy et al., 2001). However, it is reasonable to assume that most of the membranous compartments near the injury site could be exploited (Bement et al., 2007; Borgonovo et al., 2002; Mellgren, 2010; Shen et al., 2005). Though, the exact mechanisms by which the fusion events are governed are poorly defined so far. This process probably involves SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) (Steinhardt et al., 1994) and synaptotagmin (Shen et al., 2005), since it is sensitive to toxins that target these proteins. In addition, annexins (McNeil et al., 2006) and other proteins (Bansal et al., 2003; Cai et al., 2009) are potential regulators of membrane fusion events.

The MRR is assisted by a transient, local disassembly of the cortical actin network beneath the injury site that most probably facilitates local exocytosis (Godin et al., 2011; Miyake et al., 2001). However, the mechanisms by which the local actin disassembly is controlled, is not well characterized yet.

In addition, the replacement of lipids originating from the intracellular compartments with the intrinsic plasma membrane ones is not fully understood, however, it might involve microtubule-dependent membrane trafficking. Even though wounding initially triggers local, calcium-dependent depolymerization of microtubules, the microtubules polymerize and are transported back to the disruption site within ~1 min of wounding. As a result, the site of injury is surrounded by a radial array of microtubules (Mandato and Bement, 2003; Togo, 2006). Thereafter, the Golgi apparatus-derived vesicles are transported along the microtubules to the wound region (Togo, 2006), and as these are thought to be responsible for replenishing the plasma membrane lipids and proteins during constitutive exocytosis (Jaiswal et al., 2009), this might also be the case during the plasma membrane repair.

After the damaged region of the cell surface is patched up, the integrity of the actin cytoskeleton beneath it needs to be restored. This is probably facilitated by the accumulation of F-actin and myosin-2 to the wound region (Bement et al., 1999; Godin et al., 2011; Mandato and Bement, 2001; Miyake et al., 2001). The assembly F-actin and myosin-2 into a purse string around the injury is directed by RhoA and Cdc42. Specifically, active RhoA directs myosin-2 accumulation right at the edge of the wound, whereas Cdc42 directs dynamic actin assembly several micrometers further, leading to the formation of concentric rings around the wound (Benink and Bement, 2005; Vaughan et al.,

2011). The closure of a purse string (Bement and Capco, 1991; Bement et al., 1999) pulls unwounded plasma membrane inward, facilitating membrane repair.

As a final step, the patching membrane must be removed to restore the initial state of the plasma membrane. The elimination might be accomplished by either endocytosis or expulsion of the plasma membrane extensions. It has been demonstrated that besides local exocytosis, injury triggers also local endocytosis (Bi et al., 1995; Idone et al., 2008; Keefe et al., 2005). In addition, wounding can trigger extensive formation of blebs and thin tubular extensions (Babiychuk et al., 2009; Babiychuk et al., 2011; Geuskens and Tencer, 1979; Morgan et al., 1987). However, this is highly hypothetical, since the activation of endocytosis was demonstrated in the studies with pore-forming proteins (*e.g* cytolytic perforin and bacterial toxin streptolysin), and this process might contribute to the removal of transmembrane pores that must be eliminated from the cell surface for cells to survive. Furthermore, the formation of plasma membrane blebs at the injury sites might be a particular defense mechanism as well. Namely, a bleb acts as a trap that blocks the excessive influx of Ca^{2+}, since the bleb "neck" is plugged by calcium-sensitive proteins until the damaged plasma membrane regions are repaired (Babiychuk et al., 2011).

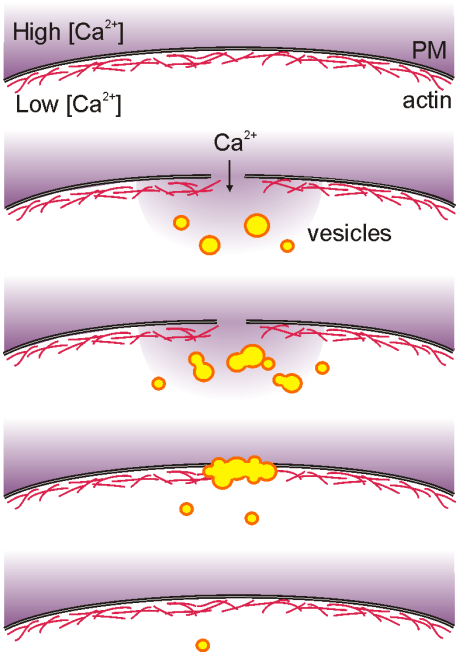

Figure 2. Membrane repair response in single cells. Membrane damage allows the influx of calcium ions down its concentration gradient into the cell activating depolymerization of the cortical actin cytoskeleton. At the same time intracellular vesicles rush to the site of injury and fuse with each other to form a "patch" that is used to plug the wound region, followed by the restoration of cortical actin cytoskeleton. PM – plasma membrane.

2. AIMS OF THE STUDY

Cell-penetrating peptides have gained much attention because of their high efficiency to deliver otherwise impermeable cargo molecules into cellular tissues. Even though it is recognized that the uptake mode might change after the addition of a cargo molecule to the CPP, it is of high importance to understand the behavior of the peptides in cellular environment that will contribute to our current understanding of how CPPs cross biological membranes and enter cells. Therefore, to tackle the complex puzzle of CPP-membrane interactions, potential membrane destabilization, and downstream responses, we have mostly focused on the internalization pathways of CPPs alone.

More specifically our goals were:

- ✓ to characterize novel sequences derived from perforin, granulysin, and granzyme B proteins and compare their cell-penetrating properties with MAP and penetratin. In addition, to investigate whether the internalization of the CPPs into cells induces disturbances in the plasma membrane sufficient enough to cause the influx of calcium, triggers the exocytosis of lysosomes, and activates membrane repair response (Paper I)
- ✓ to provide ultra-structural insights into the uptake of a cell-penetrating peptide $S4_{13}$-PV and its analogues with reversed NLS and scrambled sequence. Specifically, to describe their association with the extracellular matrix and plasma membrane, the involvement of anionic glycosaminoglycans in these particular interactions and, consequently, their uptake pathways and localization in cells (Paper II)
- ✓ to characterize the processes accompanying the cellular translocation of different representatives of the CPP families. In particular, to assess the changes in the intracellular calcium levels, triggering of the membrane repair response, and the ability of cells to overcome the stress caused by the elevated cytosolic calcium concentrations in a more detailed manner (Paper III).

3. METHODOLOGICAL CONSIDERATIONS

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

3.1. Cell cultures

Throughout this study we have conducted experiments with four different cell lines. HeLa cells derived from human cervical adenocarcinoma were isolated in 1951 and, since then, this is the the most widely used cell type in scientific research. Therefore, these cells have been employed in the field of CPP research numerous times as well, giving us a possibility to compare our results with the work of other groups but, moreover, to complement existing findings. We have performed experiments with HeLa cells in all the papers included in this thesis (Paper I–III).

Wild-type Chinese hamster ovary cells (CHO-K1), in parallel with HeLa cells, were used in Paper I to assess whether CPPs exhibit analogous effects in different adherent cell lines. In addition, a wild-type CHO and a CHO mutant cell line (CHO pgsA-745) were included in Paper II. CHO pgsA-745 cells have a defect in the enzyme xylosyltransferase and, therefore, are not capable of expressing glycosaminoglycans (GAG) on the cell surface. It is believed that GAGs are the first interaction partners for CPPs with the plasma membrane and, because of this, we were able to evaluate how and if the lack of GAGs on the cell surface impacts CPP-membrane interactions and, consequently, the uptake pathway.

Another cell line of human origin, Jurkat cells derived from human T lymphocytes, was used in parallel with HeLa cells in Paper III. Due to morphological and physiological differences between cell types, and because cellular susceptibility to injury and disruptions in the plasma membrane is largely determined by cell physiology and might therefore lead to different calcium influx patterns, we wanted to compare adherent cell lines with the ones growing in suspension.

3.2. Cell-penetrating peptides and constructs with cargo

In Paper I, we synthesized 15–17 amino acid long peptides derived from perforin, granulysin, and granzyme B. These proteins are natively involved in the human defense mechanism and are released by natural killer cells and cytotoxic T lymphocytes to protect the organism against viruses, bacteria, and cancer. At the same time, these proteins share some common characteristics with CPPs in their cellular uptake mode. Therefore, we aimed to test whether

the sequences responsible for the internalization of the whole protein could be employed as novel CPPs. To assess their cell-penetrating properties, we used two well-known CPPs for comparison. Specifically, MAP peptide was chosen because it is known to exhibit membrane disturbing activity and high cytotoxicity, and secondly, penetratin as a representative of a different CPP family with considerably lower toxic effects was also included.

In Paper II, we studied the internalization of wild-type $S4_{13}$-PV and its analogues with reversed NLS and scrambled sequence to understand the differences responsible for their dissimilar uptake efficiency.

In Paper III, we characterized the processes accompanying the cellular translocation of seven CPPs representing different CPP families: MAP and transportans (TP and its shorter analogue TP10) as highly efficient transport vectors that possess amphipathic characteristics and may cause membrane leakage; penetratin and pVEC that are less membrane-active and exhibit amphipathic properties only under certain conditions; and finally, Tat-peptide and nona-arginine that consist of a high number of arginines and are unstructured in solution were studied.

Even though in this thesis we have mainly focused on the internalization mechanism(s) of CPPs, it is acknowledged that the uptake pathway and, therefore, also cytotoxicity might change after the addition of a cargo molecule to the CPP. Different strategies have been used for conjugation of a payload to the peptide; for example, expression of a CPP-fusion protein in bacterial systems, or linkage by disulfide bond that will be cleaved in the reducing environment of the cytoplasm enabling the release of a cargo from the transport vector. However, the easiest and most convenient way is a non-covalent complexation of a cargo to a carrier peptide by simple co-incubation. This type of interaction can only occur in case of high affinity between the two molecules, such as binding of biotin to avidin. Avidin is a ~67 kDa tetrameric egg white glycoprotein with each monomer having one biotin binding pocket. Because avidin has a total of four potential biotin binding sites, we co-incubated biotinylated CPPs with avidin with 3:1 molar ratio. Under this condition, the protein would be semi-saturated and there is no free peptide in the solution to compete with the uptake of CPP-avidin complexes.

Table 1. Names and sequences of cell-penetrating peptides included in this thesis

Peptide	Sequence
pPrF 82–98 (pPrF82)	QRHVTRAKVSSTEAVAR-NH_2
pPrF 338–354 (pPrF338)	ALRRALSQYLTDRARWR-NH_2
pGrL 68–82 (pGrL)	NAATRVCRTGRSRWR-NH_2
pGrB 89–104 (pGrB)	IMLLQLERKAKRTRAV-NH_2
$S4_{13}$-PV wild-type peptide ($S4_{13}$-PVwt)	ALWKTLLKKVLKAPKKKRKVC-NH_2
$S4_{13}$-PV with reversed NLS ($S4_{13}$-PVrev)	ALWKTLLKKVLKAVKRKKKPC-NH_2
$S4_{13}$-PV with scrambled sequence ($S4_{13}$-PVscr)	KTLKVAKWLKKAKPLRKLVKC-NH_2
Model amphipathic peptide (MAP)	KLALKLALKALKAALKLA-NH_2
Transportan (TP)	GWTLNSAGYLLGKINLKALAALAKKIL-NH_2
TP10	AGYLLGKINLKALAALAKKIL-NH_2
pVEC	LLIILRRRIRKQAHAHSK-NH_2
Penetratin	RQIKIWFQNRRMKWKK-NH_2
Tat-peptide	GRKKRRQRRRPQ-NH_2
Nona-arginine	RRRRRRRRR-NH_2

3.3. Calcium indicators

Calcium ions are involved in many cellular processes and, therefore, numerous methods have been developed to measure Ca^{2+} concentrations in sub-cellular compartments and to analyze the changes in intracellular Ca^{2+} levels. Unfortunately, like with a large amount of scientific questions, there is no ideal technique to measure Ca^{2+} concentrations in cells. In our studies we have used different techniques to analyze changes in intracellular Ca^{2+} concentration, and taken advantage of two different Ca^{2+} indicators – Fura-2-AM (Paper I) and Fluo-4-AM (Paper III).

Fura-2 is a widely harnessed ratiometric fluorescent reporter that binds to free calcium ions, thus, allowing accurate measurement of intracellular calcium concentrations. Even though most of the calcium indicators are cell impermeable, we took advantage of the acetoxymethyl (AM) ester forms available nowadays. This type of modification gives the indicator the ability to passively diffuse across cellular membranes, and once inside the cells, the esterases remove the AM groups from the probe resulting in a cell-impermeant calcium sensitive dye. However, Fura-2 redistributes relatively easily to different sub-cellular compartments unequally and is prone to interact with proteins leading to possible misinterpretation of the data. Another limitation of Fura-2 is that this probe is not suited for experiments with confocal laser scanning microscopy (CLSM) and fluorescence activated cell sorting (FACS),

because it is difficult to alter excitation wavelength rapidly enough using this type of instrumentation.

Mainly because of the above mentioned drawbacks, we employed a nonratiometric Fluo-4 in our later studies (Paper III). In addition, we lowered the dye loading temperature from 37 °C to room temperature to achieve a uniformly localizing probe, because it became evident that, in addition to cytoplasmic and nuclear distribution, at higher loading temperatures this indicator was to a great extent also accumulating in endosomal vesicles.

3.4. Internalization of cell-penetrating peptides

To study the uptake of CPPs or their constructs with cargo molecules, several techniques can be applied. Flow cytometry enables quantification of the amount of the fluorescently labeled peptides in live cells; however, it is not able to discriminate the intracellular signal from the label bound to the outer leaflet of the plasma membrane. To eliminate the signal of peptide associated with the extracellular side of the membrane, we used trypsin and trypan blue treatments. The first one should cleave non-internalized CPPs from the cell surface, however, the peptides might aggregate and/or be buried into the lipid bilayer making them hardly accessible to enzymatic digestion. Therefore, trypan blue was used to quench the residual fluorescence signal.

3.4.1. Transmission electron microscopy

To visualize the uptake and localization of the fluorescently labeled CPPs in cells, we have applied fluorescence microscopy. However, this was merely used for the overall estimation whether the CPPs exploit mainly endocytic pathways or are able to diffusely stain the cytoplasm. Nevertheless, a diffuse staining detected by CLSM does not give us information whether the CPPs were able to directly cross the barrier of the plasma membrane or were liberated from the endocytic vesicles after their internalization and, therefore, gained access to the cytoplasm of the cells.

To get a more detailed insight into this matter and to characterize the uptake of CPPs on the ultra-structural level, we took advantage of the conventional transmission electron microscopy. Electron microscopy is not an extensively applied technique in the field of CPP research so far. On the other hand, electron microscopy is a powerful tool and provides resolution necessary for an unambiguous distinction whether CPPs associate first with the extracellular matrix or directly with the plasma membrane, what is the size and shape of the loci of initial contacts of CPPs with cells, which type of vesicles mediate the uptake, where are CPPs confined in relation to sub-cellular compartments, and the like.

For electron microscopy, the cells were incubated with CPPs labeled with a 1.4 nm Nanogold tag, fixed with 2.5% glutaraldehyde in cacodylate buffer and, thereafter, the Nanogold label on the peptide was magnified by silver

enhancement to enable detection of the particles. Finally, the specimen was processed for flat embedding into the epoxy resin for the best preservation of the morphology of cellular ultra-structure, and ultrathin sections were cut in parallel to the growth substrate.

To analyze whether the CPPs associate preferentially with the glycosidic component of the cell surface, we used staining with ruthenium red, which was added to fixative to reveal cell surface regions rich in mycopolysaccharides. In addition, cells were treated with hyaluronidase to remove the hyaluronan strands from the cell surface. In order to assess the importance of plasma membrane fluidity and the role of classical endocytotic pathways, we performed experiments at 10 ºC, since the vesicular uptake should be blocked below 18 ºC. In parallel, we suppressed endocytosis by inhibiting the dynamin activity by pre-treating the cells with the drug dynasore.

3.5. Visualization of the membrane repair response

For the studies of the MRR activation, we first had to work out and optimize a fixation protocol that would not interfere with the integrity of the plasma membrane enabling us to detect the integral lysosomal protein (LAMP-2 – lysosome-associated membrane protein 2) on the cell surface to where, under normal conditions, it is not targeted. Therefore, we used fixation with 3% paraformaldehyde in a phosphate buffer for 30 min at the room temperature. This method avoids permeabilization of the cells in case the plasma membrane integrity is not challenged, however, if incubation with CPPs or CPP-cargo constructs induces destabilization of the lipid bilayer then a small quantity of antibodies against LAMP-2 is able to diffuse deeper into cells and visualize also a fraction of lysosomes inside the cells. Nevertheless, the pool of lysosomes inside the cells is considerably bigger, and the fixation and permeabilization of cells with methanol enabled the detection of the total amount of LAMP-2 in cells that was used as a positive control.

Furthermore, after the fixation and staining of cells with fluorescently labeled antibodies, it became evident that two different patterns of LAMP-2 occur – cell surface LAMP-2 staining and lysosomal LAMP-2 staining. The first one is visualized as a “network” of small punctuate staining on the plasma membrane; on the other hand, lysosomes are marked as much bigger structures mostly closer to the perinuclear region. In addition, it should be mentioned that from the whole population ~10% of the cells are always weaker and/or damaged *per se* and, therefore, have a less intact plasma membrane and exhibit more prominent staining of the cell center. This phenomenon was included in the figure representing the MRR of CPPs in Paper I (figure 6 in Paper I), and might therefore lead to minor misinterpretation of the data presented. However, we have corrected this in Paper III, where we did not incorporate the figures demonstrating this type of “background” (figure 3 in Paper III).

4. RESULTS AND DISCUSSION

4.1. Destabilization of the plasma membrane by cell-penetrating peptides activates the membrane repair response (Paper I)

Three proteins involved in target cell apoptosis – perforin, granulysin, and granzymes – share some common features in the uptake mode of cell-penetrating peptides (Bird et al., 2005; Ernst et al., 2000; Keefe et al., 2005). Moreover, perforin is known to form small pores in the plasma membrane that in turn induces the influx of calcium ions into cells and, subsequently, activates a membrane repair response (MRR) (Keefe et al., 2005). Since some CPPs are able to directly penetrate across the plasma membrane (Fretz et al., 2007; Tünnemann et al., 2006) and massive accumulation of CPPs on the cell surface might destabilize the lipid membrane, we hypothesized that internalization of CPPs might also induce the influx of Ca^{2+} and trigger the MRR.

4.1.1. Interaction of novel cell-penetrating sequences with the cell surface and internalization into cells

It has been previously demonstrated that shorter cationic sequences from perforin, granulysin, and granzyme B contribute to the cytotoxicity and/or uptake of the respective proteins (Bird et al., 2005; Li et al., 2005; Liu et al., 1995). Therefore, the sequences of peptides were taken from the full-length proteins and synthesized to assess their cell-penetrating properties and propensity to destabilize cellular membranes. The uptake efficiency of four novel peptides (pPrF82, pPrF338, pGrB, and pGrL) was analyzed by FACS and compared with two well-known CPPs (MAP and penetratin). All studied peptides were taken up by cells, although the new peptides were not as efficient as penetratin or MAP. CLSM results confirmed the internalization of the novel CPPs, which localized in endosomes that were visible as distinct punctuate structures, and also diffusely in the cytoplasm.

To characterize the association of pGrB (peptide derived from granzyme B) with the plasma membrane and its distribution inside the cells in detail, we employed electron microscopy. Incubation of HeLa cells with the nanogold-tagged (1.4 nm) peptide revealed that pGrB associated with the cell surface preferentially at protrusions. The interaction of the peptide with the plasma membrane induced the formation of spherical structures with an electron-dense background, which contained several peptide molecules per cluster. At low concentration (0.5 μM), the peptide assemblies were able to insert into the plasma membrane and translocate into the cortical cytoplasm but not deeper inside the cells. However, at higher peptide concentration (2.5 μM), pGrB particles accumulated on the cell surface, and massive vesicular translocation

into the cortical cytoplasm was detected. In addition, the peptide seemed to interfere with the ordered packing of the membrane phospholipids because the plasma membrane became less distinct as visualized by electron microscopy.

4.1.2. Uptake of cell-penetrating peptides induces the influx of calcium ions

Our electron microscopy data implicated that the peptides induce changes in the membrane morphology and, therefore, we wanted to further assess whether the studied peptides are able to induce the influx of calcium ions. The cells were loaded with Ca^{2+} indicator Fura-2, followed by the treatment with peptides for 5 or 30 min at different concentrations. During the first 5 min, a transient and concentration-dependent influx of Ca^{2+} was observed with penetratin and pGrB in HeLa cells, although the Ca^{2+} level did not return to the basal level. MAP induced a continuous influx of Ca^{2+} that was sustained throughout the measurement; however, the influx of Ca^{2+} was not as high as with the ionophore ionomycin that was used as a positive control. The Ca^{2+} influx was not only concentration dependent but also followed the uptake efficiency of the peptide. Namely, MAP induced an influx of Ca^{2+} at lower peptide concentrations than penetratin, and penetratin at lower concentrations than pGrB.

4.1.3. Cell-penetrating peptides trigger lysosomal exocytosis and membrane repair response

Influx of Ca^{2+} into the cells activates several processes, including lysosomal exocytosis and the MRR. To quantify the lysosomal exocytosis caused by the CPPs, the influence of penetratin and MAP on the activity of the lysosomal protein β-hexosaminidase in cell supernatant was assessed. An increase in the enzyme activity could be deteced already at 100 nM concentrations of penetratin and MAP after the incubation of HeLa or CHO-K1 cells with these CPPs for 5 min. The β-hexosaminidase activity was concentration-dependent and, as expected, MAP was more potent in promoting lysosomal exocytosis. Analogously with other forms of exocytosis, lysosomes are subject to a so-called "kiss-and-run" events, which after stronger stimulation leads to the complete fusion of vesicles with the plasma membrane (Jaiswal et al., 2002; Zhang et al., 2007). Our results showed that the increase in intracellular Ca^{2+} levels caused by the peptides was well correlated with the higher β-hexosaminidase activity in the extracellular medium, indicating that influx of Ca^{2+} could be responsible for the stimulated β-hexosaminidase release.

To assess whether the extracellular Ca^{2+} concentration affects the efflux of β-hexosaminidase, cells were incubated in a buffer containing peptides and 5 mM calcium. Under these conditions, extracellular enzyme activity was significantly increased after treatment of penetratin, both in HeLa and CHO-K1 cells.

Even though increased β-hexosaminidase activity was detected with MAP in CHO-K1 cells, no significant increase over the basal level could be detected with this CPP in HeLa cells. However, the fraction of lysosomes available for the plasma membrane docking is not known and these discrepancies might be attributed to the differences between cell types. Specifically, more membrane-active MAP peptide might have facilitated the maximum fusion of lysosomes in HeLa cells already at normal concentration of the extracellular calcium.

In addition, if the peptides are able to cause a Ca^{2+} influx sufficient enough to trigger the MRR, lysosomes should be translocated to the plasma membrane. The fusion of the lysosomal membranes with the plasma membrane can be detected by the exposure of the lysosomal protein LAMP-2 on the cell surface. First, we confirmed that the full-length protein perforin was able to induce the activation of the MRR in our experimental set-up in a concentration-dependent manner. After that, we analyzed if the CPPs are also able to exert analogous effects. Our results with fluorescence microscopy demonstrated that MAP, peptide known to interfere with the plasma membrane integrity at low concentrations (Hällbrink et al., 2001), stimulated targeting of LAMP-2 to the plasma membrane in some cells even at 0.5 μM concentration. Raising the concentration of MAP to 1 μM led to the accumulation of the lysosomal protein to the cell surface in all cells in the population. Analogously to perforin, higher concentrations of MAP induced the translocation of LAMP-2 to the plasma membrane to a lesser extent and exhibited a more pronounced staining of the cell center. This effect is probably observed because of the substantial influx of Ca^{2+} that becomes toxic to cells. Therefore, cells die before they are able to reseal the damaged plasma membrane regions and, since the plasma membrane has lost its barrier function, antibodies against LAMP-2 visualize also lysosomes inside the cells, in addition to the LAMP-2 exposed on the cell surface.

Penetratin that is considered less toxic to cells, induced the appearance of LAMP-2 on the plasma membrane of cells at markedly higher concentrations. Translocation of lysosomes remained undetectable up to 3 μM and was negligible at 5 μM; however, starting from 10 μM concentration, the activation of the MRR was observed. Because the influx of calcium ions triggers the resealing of the injured plasma membrane within seconds, this would explain why no leakage of cytoplasmic contents after the uptake of CPPs is detected, even though there appear to be membrane disturbances caused by the peptides.

4.1.4. Extracellular calcium concentration affects the uptake efficiency of cell-penetrating peptides

Since the influx of calcium ions was detected after the treatment of cells with CPPs, it was important to investigate whether the concentration of Ca^{2+} in the medium surrounding the cells affected the uptake of peptides. If the peptides

activate the resealing of the plasma membrane, the uptake of CPPs should most probably increase if no Ca^{2+} is present outside the cells because the MRR cannot be activated. Moreover, if the Ca^{2+} concentration is increased in the extracellular environment, it might lead to a diminished internalization of CPPs because of a more efficient plasma membrane repair under these conditions.

Based on this hypothesis, we incubated the cells with fluorescently labeled peptides in buffers with different Ca^{2+} concentrations (0–5 mM calcium concentration) and analyzed the cell lysates by high-performance liquid chromatography. Penetratin and pGrB were more efficiently taken up by cells when incubated in a buffer without Ca^{2+}, which was expected because there was no Ca^{2+} to activate the MRR. In the presence of excess Ca^{2+}, the uptake of these peptides was significantly reduced in a concentration-dependent manner. However, it should be mentioned that the decrease in the internalization efficiency of CPPs could also be a consequence of the increased concentration of the positive calcium ions in the extracellular environment that compete with CPPs for electrostatic interactions with the proteoglycans. Furthermore, it has also been shown that extracellular calcium ions can increase the stability of membranes (Kim and Southard, 1999) and, therefore, hamper the uptake of CPPs. Nevertheless, uptake of MAP was not affected by the concentration of Ca^{2+} in the surrounding medium.

4.2. Efficient uptake of $S4_{13}$-PV peptides by cells is facilitated by the formation of nanoparticle-like structures (Paper II)

In paper II, we focused on the characterization of the membrane interactions, the subsequent uptake pathways, and the intracellular localization of $S4_{13}$-PV peptides. $S4_{13}$-PVwt and $S4_{13}$-PVrev are shown to be efficiently taken up by cells through an apparently rapid receptor- and energy-independent process and, less efficiently, through clathrin-mediated endocytosis (Mano et al., 2006; Mano et al., 2005). In addition, biophysical experiments have revealed that $S4_{13}$-PV peptide acquires an helical conformation upon interaction with target membranes and is partially inserted into the hydrophobic environment of the lipid bilayer (Mano et al., 2007). However, these properties are not fully shared by its analogue with a scrambled sequence ($S4_{13}$-PVscr).

4.2.1. Interaction of $S4_{13}$-PV peptides with the plasma membrane

Incubation of HeLa cells with different concentrations of $S4_{13}$-PVwt-nanogold conjugates (0.1–10 μM peptide concentration) led to the assembly of peptides into spherical structures that accumulated on the plasma membrane. These

clusters were detectable by electron microscopy after already 10 min of addition of 0.1 μM peptide to the cells. However, at this low concentration the peptide-containing structures were small and contained only a few gold particles per cluster. Raising the peptide concentration (0.5–1 μM) led to the assembly of structures that had a regular shape and a relatively uniform size of about 80 nm reminiscent of the nanoparticles (diameter 79.8 ± 16.4 nm). The assembly of the wild-type peptide into nanoparticles was rather prevailing, but still a small fraction of peptides bound to the extracellular matrix without the formation of these distinct electron-dense spherical structures and, at higher peptide concentrations (>1 μM), assembly into bigger aggregates was also evident.

$S4_{13}$-PV peptide with the reversed NLS was able to form nanoparticle-like structures as well; however, these were moderately bigger and more variable in size and shape. Namely, quantitative analysis revealed that the average size of the particles formed by $S4_{13}$-PVrev after 30 min incubation with 1 μM peptide was about 142 nm (diameter 142.6 ± 22.7 nm). In contrast, we found that the peptide with the scrambled sequence was not able to assemble into regular nanoparticle-like structures like detected with the wild-type and the reverse NLS peptides. $S4_{13}$-PVscr typically formed bigger irregular aggregates on the surface of cells indicating that not only amino acid composition, but also their position within the peptide influences the mode of interaction with the plasma membrane.

The nanoparticle-like structures of the wild-type and the reversed NLS peptides were formed only in the presence of cell membranes, since the incubation of peptides in the cell culture medium with or without serum did not lead to the assembly of this type of particles. In addition, we ruled out the possibility that these spherical assemblies were formed because of the clustering of the Nanogold tag, since Nanogold itself was not able to associate with the cell surface and, furthermore, no aggregation of the gold particles was detected. These results clearly indicate that a direct interaction with the cell surface or a specific environment is essential for the formation of the peptide nanoparticles.

4.2.2. Cell surface glycosaminoglycans play a role in the formation of peptide nanoparticles

The electron microscopy results demonstrated that the peptide nanoparticles had an electron-dense background. First, taking into account the fact that binding of the polycationic compounds to the HSPGs is able to induce the clustering and the concentration of the ligand to the cell surface as dense aggregates before their internalization (Ziegler et al., 2005; Ziegler and Seelig, 2008); and secondly, because our experiments showed that after the staining of the extracellular matrix with ruthenium red, a polycationic cytochemical dye for mucopolysaccharides, the electron density of $S4_{13}$-PV particles' background was markedly increased, we wanted to further assess the incorporation of the

GAGs into the CPP nanoparticles. Therefore, we analyzed the association of $S4_{13}$-PVwt with the cell surface of wild-type (CHO-K1) and mutant CHO pgsA-745 cells that are defective in GAG biosynthesis (Esko 1985) and lack specific GAGs on the core protein of proteoglycans. In wild-type CHO cells, the peptide accumulated extensively to the plasma membrane of cells and formed nanoparticles with similar size and shape as detected in HeLa cells. Surprisingly, these particles were also present at the surface of CHO pgsA-745, however, the amount of the peptide particles associating with the plasma membrane was markedly reduced in comparison to the wild-type CHO. Thus, the formation of nanoparticles is not only dependent on the presence of GAGs (e.g. heparan sulfate) on the cell surface.

Furthermore, the treatment of the mutant CHO cells with hyaluronidase, an enzyme that specifically cleaves glycosidic bonds in hyaluronic acid and thereby removes the hyaluronan strands from the cell surface, almost abolished the formation of $S4_{13}$-PV nanoparticles. Even though a negligible fraction of the nanoparticles were still able to assemble on the cell surface and internalize into the cells, this data suggest that the glycosidic component is required for the formation of the peptide nanoparticles and that hyaluronic acid containing proteoglycans might contribute to this phenomenon.

4.2.3. $S4_{13}$-PV nanoparticles interfere with the ordered packing of the plasma membrane and enter the cells

The $S4_{13}$-PVwt and $S4_{13}$-PVrev peptides preferentially associated with specific areas of the plasma membrane and upon a longer incubation, the nanoparticles continued to accumulate to these particular regions. Accumulation of the peptides interfered with the ordered packing of the lipid bilayer making the distinct ultra-structure hardly detectable by electron microscopy. In parallel with the interaction of the $S4_{13}$-PVwt particles with the plasma membrane and the destabilization of the lipid bilayer, these particles inserted into the cortical cytoplasm and induced the formation of small vesicles (diameter 50–100 nm). Our electron microscopy results indicate that the formation of the small and regular peptide nanoparticles was in good correlation with their ability to enter cells. Namely, the peptides forming regular structures ($S4_{13}$-PVwt and $S4_{13}$-PVrev) are more efficiently taken up by cells compared to $S4_{13}$-PVscr that forms large irregular aggregates on the cell surface. In addition, the plasma membrane of cells becomes less distinct upon interaction with $S4_{13}$-PVwt or $S4_{13}$-PVrev but not with $S4_{13}$-PVscr. These observations could explain earlier reports (Mano et al., 2007; Mano et al., 2006) demonstrating that the cellular uptake of the scrambled peptide was significantly less efficient than the internalization of $S4_{13}$-PVwt and $S4_{13}$-PVrev.

After the peptide was taken up by cells, the nanoparticles were found in rosette-like structures that morphologically resemble to caveosomes and, at later

time points, they also resided in MVBs. MVBs are usually destined to fuse with lysosomes and eventually the content of these endosomes is degraded in the acidic environment. However, the peptide nanoparticles retained their regular organization within the endosomes for at least 4 hours and in some cases these particles were relatively intact even after 24 hours. These data suggest that the degradation of the nanoparticles is inhibited or drastically slowed down that might be a consequence of the strong association of the cationic peptides with the anionic GAGs. Moreover, continuous uptake of $S4_{13}$-PV peptides during 24 hours impaired the membrane integrity of the peptide-containing vesicles and resulted in the escape of peptides into the cytosol, indicating that the high local concentration of the peptide is able to destabilize also cellular membranes.

4.2.4 Cellular uptake of $S4_{13}$-PV peptides is hampered by dynamin inhibition and low temperature, but does not abolish the vesicular uptake of $S4_{13}$-PV

Since the uptake of $S4_{13}$-PV peptides involves the formation of small vesicles and the peptide nanoparticles are also found inside endocytic compartments, such as caveosomes and MVBs, it is possible that these peptides exploit well-know endocytic pathways to gain entry into cells. Therefore, we examined the effect of dynasore on the translocation efficiency of $S4_{13}$-PVwt. Dynasore is an inhibitor of the dynamin GTPase activity that blocks the formation and fission of vesicles in most endocytic pathways (Macia et al., 2006). Inhibition of the dynamin activity decreased the uptake of $S4_{13}$-PVwt, however, the peptide nanoparticles were still able to assemble, insert into the plasma membrane, and induce the formation of small vesicles.

Next, we analyzed the impact of lowering the incubation temperature below 18 °C that decreases the plasma membrane fluidity and inhibits endocytosis. At low temperature (10 °C), despite the markedly decreased amount of surface-bound peptides, the vesicular uptake of $S4_{13}$-PVwt and both of its analogues was significantly reduced but not completely blocked. A small fraction of the peptide was found in small vesicles or bigger vesicular structures, which located in the close proximity of the plasma membrane but not deeper in the cells. We can conclude that endocytic pathways are involved in the internalization of the $S4_{13}$-PV peptides, however, dynamin is not necessarily required for the fission of these vesicles.

4.2.5. $S4_{13}$-PV peptides activate the membrane repair response at higher peptide concentrations than required for the efficient cellular entry of the peptide

In paper I, we demonstrated that the accumulation of some CPPs on the cell surface induces disturbances in the ordered packing of the lipid bilayer and,

consequently, the plasma membrane becomes less distinct (Palm-Apergi et al., 2009). These particular peptides were also able to trigger the membrane resealing mechanism called membrane repair response (MRR). Since our electron microscopy results revealed that the association of the $S4_{13}$-PVwt and the $S4_{13}$-PVrev particles with the cell surface complicates the detection of the plasma membrane at the accumulation sites, we further analyzed the ability of the $S4_{13}$-PV peptides to activate the MRR. The fluorescence microscopy results demonstrated that $S4_{13}$-PV peptides induced the MRR, but this mechanism was activated at higher peptide concentrations than necessary for the efficient uptake of $S4_{13}$-PVwt and $S4_{13}$-PVscr. We also observed that the appearance of the lysosomal protein LAMP-2 on the plasma membrane correlated with the size and heterogeneity of the $S4_{13}$-PV nanoparticles. Namely, the $S4_{13}$-PV with the reversed NLS that formed bigger and more heterogeneous nanoparticles (diameter ~142 nm) triggered the MRR at lower peptide concentration compared to the wild-type peptide that formed a homogeneous population of small regular particles (diameter ~80 nm). Furthermore, the $S4_{13}$-PV with the scrambled sequence assembled on the cell surface into large irregular aggregates and associated with the cells without disturbing the integrity of the lipid bilayer, and as expected, induced the activation the MRR in cells at 2–3 fold higher peptide concentration than its analogues.

4.3. Amphipathic cell-penetrating peptides induce the influx of calcium ions and activate several downstream responses (Paper III)

In paper III, we mainly focused on the detailed analysis of the seven representatives of the different CPP families regarding their propensity to induce the influx of extracellular calcium ions into cells and the activation of downstream responses, such as blebbing of the plasma membrane, lysosomal exocytosis, and MRR. In addition, a new aspect – the ability of the cells to deal with the elevated intracellular Ca^{2+} levels – was thoroughly assessed.

4.3.1. Primary amphipathic cell-penetrating peptides induce calcium influx in cells starting from low peptide concentrations

To evaluate whether the studied CPPs induce membrane disturbances and a subsequent influx of Ca^{2+} into cells, we employed two different cell lines and methods. First, we performed the intracellular Ca^{2+} measurements with Jurkat cells using a fluorescence plate reader. This gave us a possibility to quantitatively compare the increase of Ca^{2+} induced by the CPPs to the effect of ionophore – ionomycin – that was used as a positive control. Secondly, in order

to analyze the dynamics of Ca^{2+} concentration in single cells as well as on a subcellular level, we performed the experiments in HeLa cells applying CLSM.

Our results with fluorometer revealed that MAP was the most membrane-active peptide, inducing the Ca^{2+} influx into Jurkat cells in a concentration- and time-dependent manner. In our experimental set-up, the first effects were detected starting from 0.5 μM peptide concentration after 5 min incubation, and the highest elevation of Ca^{2+} signal (~85% as compared to ionomycin) was induced with 5 μM MAP after 30 min incubation.

TP was less membrane-active and first effects in Jurkat cells were detected starting from 3 μM TP after 5 min incubation. The TP-induced Ca^{2+} influx was continuous at lower peptide concentrations; however, 5 μM TP elevated the Ca^{2+} concentration up to 60% where a plateau seemed to be reached. A shorter analog of transportan, TP10, induced a detectable Ca^{2+} influx at even higher concentrations than TP. Namely, 3–5 μM TP10 caused a 35% increase after 30 min incubation, and the Ca^{2+} level reached up to 50% of the maximum signal at a 10 μM concentration.

Our results with CLSM demonstrated that the observed changes at lower peptide concentrations were a result of a substantial Ca^{2+} influx into a subpopulation of cells rather than a minor uniform increase in the whole cell population. However, the increase in the concentration of the CPPs led to the elevation of intracellular Ca^{2+} concentration, both in the cytoplasm and nucleus, in the whole population of cells.

In addition, the incubation of HeLa cells with the primary amphipathic CPPs induced occasional blebbing of the plasma membrane that was dependent on both the concentration of used CPP and time. For example, 3 μM MAP triggered blebbing of the plasma membrane after 30 min incubation, however, treatment of cells with 5 μM MAP led to the formation of blebs already after 8 min. In general, the plasma membrane blebbing was observed substantially faster at higher peptide concentrations with all the primary amphipathic CPPs tested.

Blebbing of the plasma membrane has been observed during physical and chemical stress, and is usually preceded by an elevation of the intracellular Ca^{2+} that has to rise over ~ 300 nM concentration (Smith et al., 1991). Our results imply that, in fact, a certain intracellular Ca^{2+} concentration has to be reached and retained before the depolymerization of the actin cortex takes place and, as a consequence, big stable blebs of the plasma membrane form.

4.3.2. Secondary amphipathic cell-penetrating peptides induce calcium influx in cells starting from higher peptide concentrations

In contrast, penetratin and pVEC triggered the influx of Ca^{2+} into Jurkat cells at markedly higher concentrations than primary amphipathic CPPs, and their

effects were observed starting from 20 μM concentrations after 5 min treatment. Surprisingly, a prolonged incubation did not increase the Ca^{2+} influx and the detected fluorescence signals remained at the comparable levels for 30 min after the addition of these CPPs. In analogy with the results obtained in Jurkat cells, incubation of HeLa cells with penetratin and pVEC exerted similar effects. An increase in the Ca^{2+} levels and, thus, a diffuse staining of the cytoplasm and nucleus with the calcium indicator in approximately 15% of cells was detected after 5 min starting from 20 μM concentrations. A longer incubation (30 min) with these peptides did not have an additional effect on the Ca^{2+} levels in the cells.

In case of pVEC and penetratin, the formation of big stable blebs was not detected during a 30-min incubation even at the highest used concentration (20 μM), suggesting that although the intracellular Ca^{2+} concentration was increased, its level did not reach the threshold necessary for the depolymerization of the cortical actin and the disruption of interactions between the plasma membrane and the cytoskeleton.

4.3.3. Non-amphipathic cell-penetrating peptides do not induce calcium influx in cells

The arginine-rich peptides, Tat-peptide and nonaarginine, did not influence the intracellular Ca^{2+} levels in Jurkat cells even at very high peptide concentrations. The fluorescence of the calcium indicator remained at the basal levels throughout the measurement in the used concentration range from 5 to 50 μM. In addition, CLSM results corroborated that Tat-peptide and nonaarginine did not affect the intracellular Ca^{2+} concentration in HeLa cells. Even 30 min after the addition of 50 μM peptides, no increase in the fluorescence signal was observed. Moreover, since these CPPs did not modulate the intracellular Ca^{2+} concentrations in Jurkat and HeLa cells in our experiments, blebbing of the plasma membrane was not detected. It has been shown that Tat-peptide and nonaarginine are unstructured in solution and, therefore, less prone to stably associate with membranes (Ziegler et al., 2003). This might explain why they do not induce the Ca^{2+} influx into cells even at very high concentrations.

4.3.4. Calcium influx induced by cell-penetrating peptides triggers lysosomal exocytosis and the plasma membrane repair response

To corroborate the fusion of lysosomes with the plasma membrane and the release of their content, we measured the efflux of the lysosomal enzyme β-hexosaminidase into the extracellular medium. Among the studied CPPs, MAP was the most potent in inducing the release of the lysosomal content followed by TP, TP10, penetratin, and pVEC. On the other hand, Tat-peptide and

nonaarginine, did not increase the activity of β-hexosaminidase in the extracellular environment.

In order to further characterize the effect of CPPs on the integrity of the plasma membrane, we analyzed their potency to trigger the MRR. We have previously demonstrated that MAP induced the exposure of LAMP-2 on the plasma membrane at low concentrations (even below 1 μM), whereas with penetratin a significant effect was detected starting from 20 μM concentration (Palm-Apergi et al., 2009). In this study, the plasma MRR was in very good correlation with the cytosolic Ca^{2+} concentration induced by the CPPs. The fluorescence microscopy experiments demonstrated that 1 μM TP did not induce a Ca^{2+} influx, nor did it trigger the MRR. On the other hand, raising the concentration to 3 μM or 5 μM induced the appearance of LAMP-2 on the plasma membrane in approximately 30% or 80% of cells in the observed population, respectively. The pVEC peptide, in contrast, stimulated the translocation of LAMP-2 to the cell surface in approximately 20–30% of cells at 20 μM concentration. Our results demonstrate that the targeting of lysosomes to the cell periphery and the associated fusion events, as well as the release of β-hexosaminidase, were triggered by the primary amphipathic CPPs (MAP, TP, TP10) at markedly lower peptide concentrations compared to the secondary amphipathic ones (pVEC and penetratin). In concordance with the Ca^{2+} influx assays, Tat-peptide and nonaarginine did not induce the fusion of lysosomes with the plasma membrane or the release of β-hexosaminidase from the cells.

4.3.5. Coupling of a cargo protein to cell-penetrating peptides decreases the membrane interfering activity of the peptides

It has been suggested that the attachment of a cargo molecule to the CPP may alter the uptake mode of the CPP (El-Andaloussi et al., 2007) and its cytotoxic effects. Therefore, we assessed the induction of the MRR by amphipathic CPPs after complexation with a ~67 kDa cargo protein avidin.

Our results with CLSM showed that MAP-avidin and TP-avidin complexes were both less potent in the induction of the MRR compared to the respective peptides alone. For example, the MAP-avidin complexes triggered the MRR only in a few cells at 1 μM peptide concentration; however, the exposure of LAMP-2 on the plasma membrane was detectable in more than 80% of the cells after the incubation with 5 μM MAP-avidin complexes. Since similar effects with MAP alone were detected with ~2-fold lower peptide concentrations (Palm-Apergi et al., 2009), we can conclude that the attachment of avidin to a biotinylated CPP drastically reduces the membrane disturbing activity of the peptide. An analogous effect where coupling of a cargo protein reduced the CPPs' toxicity has been observed earlier (Barany-Wallje et al., 2007).

4.3.6. Cells can overcome the stress caused by the uptake of cell-penetrating peptides

Significant elevation of the cytoplasmic Ca^{2+} is toxic to the cells; however, the activation of different damage control mechanisms might help the cells to restrain the uncontrolled influx of Ca^{2+} ions and to restore the ionic homeostasis. To assess this hypothesis, the viability of Jurkat cells was quantified by flow cytometry using a double-staining with annexin V and DAPI 24 hours after the treatment with peptides. As expected, MAP was the most toxic peptide and induced apoptosis or necrosis of cells in a concentration-dependent manner. Transportans were considerably less toxic to cells; and, penetratin induced apoptotic or necrotic events only in a small population of cells at the highest used concentration (20 μM). The viability of the cells stayed at a comparable level with the untreated cells after incubation with 20 μM pVEC, 50 μM Tat-peptide, and 50 μM nonaarginine.

To assess the barrier function of the plasma membrane of HeLa cells, we analyzed the uptake of DAPI to the cells after the challenge with the highest concentrations of TP. After a 30-min incubation with 5 μM TP only a few cells were permeable to DAPI and with 10 μM TP ~20% of cells became permeable to DAPI. Taken together, these results demonstrate that Jurkat and HeLa cells are able to replace the damaged plasma membrane regions and overcome the overload of cytosolic Ca^{2+} to a considerable extent. This is in good correlation with the earlier studies with the bacterial toxin streptolysin O which showed that the MRR functions effectively if the intracellular Ca^{2+} concentration stays between 5–10 μM (Babiychuk et al., 2009).

SUMMARY

The plasma membrane of cells forms an essential barrier between the extra- and intracellular milieu that, amongst many other functions, protects cells from the detrimental external influences. At the same time, the barrier function of the membrane is the major obstacle in therapeutics and biotechnology, since many promising bioactive compounds are not able to traverse lipid bilayers. To overcome this drawback, a class of specific transport vectors that gain access into the cell interior and mediate the transport of otherwise impermeable molecules – cell-penetrating peptides – can be utilized. Even though their potential as efficient carrier peptides has been demonstrated repeatedly, the uptake mechanisms of CPPs are still not explicitly specified. It is known that certain CPPs are able to destabilize the plasma membrane; however, it is not clear how cells deal with the disturbances of the cell surface. Therefore, we focused on the internalization pathways of CPPs; more precisely, we characterized the association of CPPs with the cell surface and the subsequent uptake on the ultra-structural level by transmission electron microscopy. The potential membrane destabilization effects of CPPs were assessed by the measurement of intracellular calcium concentrations with a fluorescence plate reader. In addition, sub-cellular calcium dynamics and downstream responses (e.g. lysosomal exocytosis and membrane repair response) were studied by confocal laser scanning microscopy.

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

1. Novel CPP sequences derived from proteins perforin, granulysin, and granzyme B are all taken up by cells, although the new peptides are not as efficient as penetratin or MAP (Paper I)
2. pGrB and $S4_{13}$-PV peptides induce changes in morphology of the plasma membrane, since they interfere with the ordered packing of the membrane phospholipids (Paper I and II)
3. Membrane-active CPPs induce disturbances in the plasma membrane sufficient enough to cause the influx of calcium ions, triggering the exocytosis of lysosomes, and activating the membrane repair response (Paper I–III)
4. Wild-type $S4_{13}$-PV peptide and its analogue with reversed NLS form nanoparticle-like structures, and efficiently enter the cells via a vesicular pathway that is partially dynamin-independent and is not abolished by low temperature (Paper II)
5. Glycosidic component of the cell surface is required for the formation of $S4_{13}$-PV peptide nanoparticles, and the hyaluronic acid containing proteoglycans might contribute to this phenomenon (Paper II)
6. $S4_{13}$-PV peptides induce the membrane repair response; however, this mechanism is activated at higher peptide concentrations than necessary for the efficient uptake of $S4_{13}$-PVwt and $S4_{13}$-PVscr (Paper II)

7. Based on the interaction with the plasma membrane, the induction of the calcium influx, and downstream responses, three subgroups of CPPs can be distinguished (Paper III):
   a. primary amphipathic CPPs that affect the plasma membrane integrity, induce influx of calcium, and activate downstream responses starting from low micromolar concentrations;
   b. secondary amphipathic CPPs that induce calcium influx in cells starting from higher peptide concentrations than necessary for their efficient internalization (≥10 μM);
   c. non-amphipathic CPPs that do not evoke any changes at relevant concentrations (<50 μM)
8. Influx of calcium ions induced by CPPs triggers the plasma membrane repair response that helps cells to overcome the stress caused by the uptake of CPPs (Paper III)
9. Coupling of a cargo protein to CPPs decreases the membrane interfering activity of peptides (Paper III)

SUMMARY IN ESTONIAN

Plasmamembraani barjääri ületamine: amfipaatsete rakku penetreeruvate peptiidide sisenemine põhjustab kaltsiumi sissevoolu ja rakuliste vastuste aktiveerimise

Rakkudesse penetreeruvad peptiidid (RPP-d) on lühikesed (5–40 aminohappe pikkused), loodusliku või sünteetilise päritoluga spetsiifilised transportpeptiidid, mis hõlbustavad märkimisväärselt bioaktiivsete molekulide sisenemist rakkudesse. Selliste vektorpeptiididega on rakkudesse viidud väga erinevaid lastmolekule (näiteks oligonukleotiide, peptiide, valke, halvasti imenduvaid ravimeid, nanopartikleid jne) ning positiivseid tulemusi on saadud nii *in vitro* kui ka *in vivo* katsetes. RPP-sid kirjeldati algselt kui ühendeid, mis võivad liikuda läbi plasmamembraani raku sisemusse sõltumata endotsütoosist ja teistest raku energiat nõudvatest protsessidest. Praeguseks on jõutud arusaamale, et RPP-de sisenemise mehhanism(id) on tegelikkuses palju rohkem raku füsioloogilistest teguritest tulenev protsess ning täpne mehhanism(id) ja paiknemine rakus võib sõltuda peptiidi ja lastmolekuli füüsikalis-keemilistest omadustest, kontsentratsioonist, rakutüübist ning mitmetest teistest teguritest. RPP-d ei kasuta rakkudesse sisenemiseks plasmamembraanil olevaid retseptorvalke, vaid seonduvad rakupinnal negatiivselt laetud plasmamembraani komponentidega, eelistades arvatavasti proteoglükaane. Sellest tulenevalt peetakse sisenemise seisukohalt oluliseks eelkõige peptiidide positiivset laengut. Kuigi viimase paari aasta jooksul on tõendust leidnud endotsütoosi suurem osatähtsus peptiidide/peptiid-lastmolekuli rakku sisenemisel, on samas näidatud, et kõrgemal kontsentratsioonil paikneb peptiid difuusselt tsütoplasmas ja on võimeline rakkudesse sisenema otse läbi plasmamembraani. Antud peptiidide vähene toksilisus, ühtlane sisenemise saagis ja suhteliselt suur efektiivsus lubavad pidada RPP-dega vahendatud transporti perspektiivseks alternatiiviks elektroporeerimisele, lipofektsioonile ja teistele invasiivsetele meetoditele, mida on seniajani kasutatud makromolekulide rakudesse sisestamiseks.

Teadaolevalt „sukelduvad" mõned RPP-d osaliselt rakumembraani, mis võib kaasa aidata nende RPP-de efektiivsemale sisenemisele. Samas võib selline omadus põhjustada plasmamembraani häiritusi, mis võib osutuda rakkudele kahjulikuks. Nimelt on raku väliskeskkonnas kaltsiumioonide kontsentratsioon kõrge ja tsütosoolis madal ning membraani ebanormaalse läbilaskvuse korral on see tasakaal häiritud, mis viib omakorda raku surmani kui kaltsiumi rakusisest taset ei saada kontrolli alla. Sellest tulenevalt oli käesoleva töö põhieesmärk uurida, kas RPP-de kontsentreerumine ja seondumine rakumembraanile ning järgnev sisenemine võib põhjustada kaltsiumi sissevoolu rakkudesse ja aktiveerida teisi kaltsiumi ioonide vahendatud protsesse.

Esimeses artiklis sünteesisime potentsiaalseid uusi RPP-sid, mis pärinevad inimese kaitsemehhanismis osalevatest valkudest (perforiin, granulüsiin ja gransüüm B), et võrrelda nende sisenemisefektiivsust teadaolevate RPP-dega

(penetratiin ja MAP). Uued RPP-d olid küll võimelised rakkudesse sisenema, kuid ei olnud nii efektiivsed kui tuntud RPP-d. Samas näitasid fluorestsentsmikroskoopia tulemused, et uudsed RPP-d paiknesid difuusselt tsütoplasmas, mis viitab nende otsesele membraani läbimisele. Sellest tulenevalt uurisime me nende RPP-de seondumist raku pinnaga ultrastruktuuri tasemel kasutades elektronmikroskoopiat. Saadud tulemused kinnitasid, et RPP-d põhjustavad rakumembraani lipiidses kaksikkihis häiritusi ning selle tagajärjel on plasmamembraani raske detekteerida. Järgmiseks tegime me kindlaks, et RPP-de poolt esile kutsutud membraani häiritused on erineva ulatusega, kuna rakusisese kaltsiumi taseme tõus sõltus kasutatud peptiidist, selle kontsentratsioonist ja rakutüübist. Kõige enam mõjutas rakke MAP peptiid, mis põhjustas märkimisväärset kaltsiumi kontsentratsiooni tõusu juba esimeste sekunditega väga madala peptiidi kontsentratsiooni korral. Lisaks näitasime, et kaltsiumi sissevool aktiveerib ka rakulised mehhanismid nagu lüsosoomide eksotsütoos ja membraani parandamise vastus (MPV). MPV tagajärjel mobiliseeritakse kiiresti rakusisesed vesiikulid kahjustatud plasmamembraani vahetusse lähedusse ning neid vesiikuleid kasutades sulgetakse vigastatud raku membraan, et peatada kahjulike ainete sissevool rakkudesse. Seega RPP-de põhjustatud häiritusi saab rakkude tasandil elimineerida, mis on väga oluline aspekt RPP-de kui transportvektorite välja töötamisel.

Teises artiklis tahtsime välja selgitada, miks on metsik-tüüpi $S4_{13}$-PV peptiidi ($S4_{13}$-PVwt) ja selle pööratud tuumalokalisatsioonisignaali järjestusega analoogi ($S4_{13}$-PVrev) rakkudesse sisenemise efektiivsus palju parem kui $S4_{13}$-PV segatud järjestusega analoogil ($S4_{13}$-PVscr). Kasutades elektronmikroskoopiat näitasime, et $S4_{13}$-PVwt ja $S4_{13}$-PVrev moodustavad raku membraaniga seondudes nanopartiklite sarnaseid sfäärilisi struktuure, mis sisenevad efektiivselt rakkudesse põhiliselt endotsütoosi teel. $S4_{13}$-PVscr ei olnud võimeline sellisteks nanopartikliteks assambleeruma ning moodustas raku pinnal hoopis suuri ja ebakorrapäraseid agregaate. RPP-de poolt kasutatud endotsütoosirada sarnanes morfoloogiliste näitajate põhjal kaveoliin-sõltuvale endotsütoosile, kuid see oli osaliselt dünamiin-sõltumatu kuna dünamiini inhibeerimine ei blokeerinud täielikult RPP-de sisenemist rakkudesse. Lisaks vesikulaarsel teel sisenemisele põhjustasid $S4_{13}$-PVwt ja $S4_{13}$-PVrev rakumembraani hägustumist, mistõttu uurisime järgmiseks MPV indutseerimist. Saadud tulemused näitasid selget seost nanopartiklite moodustumise ja rakumembraani häirituste ulatuse vahel. Nimelt $S4_{13}$-PVwt ja $S4_{13}$-PVrev moodustatud nanopartiklid aktiveerisid MPV palju madalamal kontsentratsioonil kui ebaregulaarsed agregaadid, mida moodustas $S4_{13}$-PVscr. Samas sisenevad $S4_{13}$-PVwt ja $S4_{13}$-PVrev efektiivselt rakkudesse juba madalamatel kontsentratsioonidel kui on vajalik MPV indutseerimiseks.

Esimestest töödest tuli välja, et RPP-d on võimelised põhjustama plasmamembraani häiritusi ning tõstavad rakusisese kaltsiumi taset, kuid see sõltub kasutatud peptiidist ja selle kontsentratsioonist. Seetõttu me keskendusime laiaulatuslikule RPP-de klassifitseerimisele nende omaduste põhjal. Kolmandas

artiklis on kaasatud seitse RPP-d erinevatest peptiidiperekondadest – kunstlikud peptiidid MAP ja oligoarginiin, kimäärsed peptiidid transportaan ja selle lühem analoog TP10 ning valkudest pärinevad peptiidid penetratiin, pVEC ja Tat-peptiid. Ainsad uuritud RPP-d, mis ei põhjustanud kaltsiumi sissevoolu ja rakulisi vastuseid olid oligoarginiin ja Tat-peptiid. Ülejäänutest olid vähem membraani häiritusi põhjustavad penetratiin ja pVEC ning kõige membraani destabiliseerivateks osutusid TP, TP10 ja MAP. Saadud tulemused on väga heas korrelatsioonis nende füüsikalis-keemiliste omadustega – primaarsed amfipaatsed peptiidid (MAP, TP, TP10) mõjutavad märkimisväärselt plasmamembraani juba väga madalatel peptiidi kontsentratsioonidel, sekundaarsed amfipaatsed peptiidid (penetratiin ja pVEC) põhjustavad plasmamembraani häiritusi ainult kõrgel peptiidi kontsentratsioonil ning mitte-amfipaatsed peptiidid (Tat-peptiid ja oligoarginiin) ei mõjuta rakumembraani. Uurides kaltsiumi poolt aktiveeritavaid protsesse tuli välja, et suhteliselt kõrge rakusisese kaltsiumi taseme juures aktiveeritakse rakumembraani „mullitamine", mida olid võimelised esile kutsuma ainult primaarselt amfipaatsed RPP-d. Samuti oli MPV aktiveerimine väga heas korrelatsioonis rakusisese kaltsiumi taseme tõusuga ning selle mehhanismi käivitamine aitab rakkudel üle saada amfipaatsete RPP-de sisenemisega kaasnevast stressist.

Kokkuvõttes näitavad käesoleva töö tulemused, et osade RPP-de sisenemine rakkudesse põhjustab plasmamembraani häiritusi või katkemisi. Samas erinevate perekondade RPP-d destabiliseerivad rakumembraani erineval määral, mistõttu rakusisese kaltsiumi taseme tõus sõltub kasutatud peptiidist ja selle kontsentratsioonist. Kaltsiumi ioonide sisenemine aktiveerib omakorda rakulised vastused, mis aitavad rakkudel plasmamembraani häiritustega toime tulla ning seetõttu on ka tugevalt membraani mõjutavate RPP-de kasutamine transportvektoritena tulevikus võimalik.

REFERENCES

Al Soraj, M., He, L., Peynshaert, K., Cousaert, J., Vercauteren, D., Braeckmans, K., De Smedt, S. C., and Jones, A. T. (2012). siRNA and pharmacological inhibition of endocytic pathways to characterize the differential role of macropinocytosis and the actin cytoskeleton on cellular uptake of dextran and cationic cell penetrating peptides octaarginine (R8) and HIV-Tat. J Control Release *161*, 132–141.

Ammendolia, M. G., Bertuccini, L., Minelli, F., Meschini, S., and Baldassarri, L. (2004). A Sphingomonas bacterium interacting with epithelial cells. Res Microbiol *155*, 636–646.

Anderson, H. A., Chen, Y., and Norkin, L. C. (1996). Bound simian virus 40 translocates to caveolin-enriched membrane domains, and its entry is inhibited by drugs that selectively disrupt caveolae. Mol Biol Cell *7*, 1825–1834.

Anderson, R. G. (1998). The caveolae membrane system. Annu Rev Biochem *67*, 199–225.

Araki, N., Hatae, T., Yamada, T., and Hirohashi, S. (2000). Actinin-4 is preferentially involved in circular ruffling and macropinocytosis in mouse macrophages: analysis by fluorescence ratio imaging. J Cell Sci *113 (Pt 18)*, 3329–3340.

Babiychuk, E. B., Monastyrskaya, K., Potez, S., and Draeger, A. (2009). Intracellular Ca(2+) operates a switch between repair and lysis of streptolysin O-perforated cells. Cell Death Differ *16*, 1126–1134.

Babiychuk, E. B., Monastyrskaya, K., Potez, S., and Draeger, A. (2011). Blebbing confers resistance against cell lysis. Cell Death Differ *18*, 80–89.

Bansal, D., Miyake, K., Vogel, S. S., Groh, S., Chen, C. C., Williamson, R., McNeil, P. L., and Campbell, K. P. (2003). Defective membrane repair in dysferlin-deficient muscular dystrophy. Nature *423*, 168–172.

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

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

Bar-Sagi, D., and Feramisco, J. R. (1986). Induction of membrane ruffling and fluid-phase pinocytosis in quiescent fibroblasts by ras proteins. Science *233*, 1061–1068.

Barylko, B., Binns, D., Lin, K. M., Atkinson, M. A., Jameson, D. M., Yin, H. L., and Albanesi, J. P. (1998). Synergistic activation of dynamin GTPase by Grb2 and phosphoinositides. J Biol Chem *273*, 3791–3797.

Bellet-Amalric, E., Blaudez, D., Desbat, B., Graner, F., Gauthier, F., and Renault, A. (2000). Interaction of the third helix of Antennapedia homeodomain and a phospholipid monolayer, studied by ellipsometry and PM-IRRAS at the air-water interface. Biochim Biophys Acta *1467*, 131–143.

Bement, W. M., and Capco, D. G. (1991). Analysis of inducible contractile rings suggests a role for protein kinase C in embryonic cytokinesis and wound healing. Cell Motil Cytoskeleton *20*, 145–157.

Bement, W. M., Mandato, C. A., and Kirsch, M. N. (1999). Wound-induced assembly and closure of an actomyosin purse string in Xenopus oocytes. Curr Biol *9*, 579–587.

Bement, W. M., Yu, H. Y., Burkel, B. M., Vaughan, E. M., and Clark, A. G. (2007). Rehabilitation and the single cell. Curr Opin Cell Biol *19*, 95–100.

Benink, H. A., and Bement, W. M. (2005). Concentric zones of active RhoA and Cdc42 around single cell wounds. J Cell Biol *168*, 429–439.

Bi, G. Q., Alderton, J. M., and Steinhardt, R. A. (1995). Calcium-regulated exocytosis is required for cell membrane resealing. J Cell Biol *131*, 1747–1758.

Bird, C. H., Sun, J., Ung, K., Karambalis, D., Whisstock, J. C., Trapani, J. A., and Bird, P. I. (2005). Cationic sites on granzyme B contribute to cytotoxicity by promoting its uptake into target cells. Mol Cell Biol *25*, 7854–7867.

Blanchet, M. H., Le Good, J. A., Mesnard, D., Oorschot, V., Baflast, S., Minchiotti, G., Klumperman, J., and Constam, D. B. (2008). Cripto recruits Furin and PACE4 and controls Nodal trafficking during proteolytic maturation. Embo J *27*, 2580–2591.

Borgonovo, B., Cocucci, E., Racchetti, G., Podini, P., Bachi, A., and Meldolesi, J. (2002). Regulated exocytosis: a novel, widely expressed system. Nat Cell Biol *4*, 955–962.

Brodsky, F. M., Chen, C. Y., Knuehl, C., Towler, M. C., and Wakeham, D. E. (2001). Biological basket weaving: formation and function of clathrin-coated vesicles. Annu Rev Cell Dev Biol *17*, 517–568.

Cai, C., Masumiya, H., Weisleder, N., Matsuda, N., Nishi, M., Hwang, M., Ko, J. K., Lin, P., Thornton, A., Zhao, X.*, et al.* (2009). MG53 nucleates assembly of cell membrane repair machinery. Nat Cell Biol *11*, 56–64.

Caplan, S., Naslavsky, N., Hartnell, L. M., Lodge, R., Polishchuk, R. S., Donaldson, J. G., and Bonifacino, J. S. (2002). A tubular EHD1-containing compartment involved in the recycling of major histocompatibility complex class I molecules to the plasma membrane. Embo J *21*, 2557–2567.

Dalton, P. D., Lleixa Calvet, J., Mourran, A., Klee, D., and Moller, M. (2006). Melt electrospinning of poly-(ethylene glycol-block-epsilon-caprolactone). Biotechnol J *1*, 998–1006.

Dathe, M., Schumann, M., Wieprecht, T., Winkler, A., Beyermann, M., Krause, E., Matsuzaki, K., Murase, O., and Bienert, M. (1996). Peptide helicity and membrane surface charge modulate the balance of electrostatic and hydrophobic interactions with lipid bilayers and biological membranes. Biochemistry *35*, 12612–12622.

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

Deshayes, S., Plenat, T., Aldrian-Herrada, G., Divita, G., Le Grimellec, C., and Heitz, F. (2004). Primary amphipathic cell-penetrating peptides: structural requirements and interactions with model membranes. Biochemistry *43*, 7698–7706.

Deshayes, S., Plenat, T., Charnet, P., Divita, G., Molle, G., and Heitz, F. (2006). Formation of transmembrane ionic channels of primary amphipathic cell-penetrating peptides. Consequences on the mechanism of cell penetration. Biochim Biophys Acta *1758*, 1846–1851.

Dimitrov, D. S. (2004). Virus entry: molecular mechanisms and biomedical applications. Nat Rev Microbiol *2*, 109–122.

Doherty, G. J., and Lundmark, R. (2009). GRAF1-dependent endocytosis. Biochem Soc Trans *37*, 1061–1065.

Doherty, G. J., and McMahon, H. T. (2008). Mediation, modulation, and consequences of membrane-cytoskeleton interactions. Annu Rev Biophys *37*, 65–95.

Doherty, G. J., and McMahon, H. T. (2009). Mechanisms of endocytosis. Annu Rev Biochem *78*, 857–902.

Dom, J., Shaw-Jackson, C., Matis, C., Bouffioux, O., Picard, J., Prochiantz, A., Mingeot-Leclercq, M.-P., Brasseur, R., and Rezsohazy, R. (2003). Cellular uptake of Antennapedia Penetratin peptides is a two-step process in which phase transfer precedes a tryptophan-dependent translocation. Nucl Acids Res *31*, 556–561.

Donaldson, J. G. (2003). Multiple roles for Arf6: sorting, structuring, and signaling at the plasma membrane. J Biol Chem *278*, 41573–41576.

Dowrick, P., Kenworthy, P., McCann, B., and Warn, R. (1993). Circular ruffle formation and closure lead to macropinocytosis in hepatocyte growth factor/scatter factor-treated cells. Eur J Cell Biol *61*, 44–53.

Drin, G., Mazel, M., Clair, P., Mathieu, D., Kaczorek, M., and Temsamani, J. (2001). Physico-chemical requirements for cellular uptake of pAntp peptide. Role of lipid-binding affinity. Eur J Biochem *268*, 1304–1314.

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

Eden, E. R., White, I. J., and Futter, C. E. (2009). Down-regulation of epidermal growth factor receptor signalling within multivesicular bodies. Biochem Soc Trans *37*, 173-177.

Ehrlich, M., Boll, W., Van Oijen, A., Hariharan, R., Chandran, K., Nibert, M. L., and Kirchhausen, T. (2004). Endocytosis by random initiation and stabilization of clathrin-coated pits. Cell *118*, 591–605.

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

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

Erbe, A., Kerth, A., Dathe, M., and Blume, A. (2009). Interactions of KLA amphipathic model peptides with lipid monolayers. Chembiochem *10*, 2884–2892.

Ernst, W. A., Thoma-Uszynski, S., Teitelbaum, R., Ko, C., Hanson, D. A., Clayberger, C., Krensky, A. M., Leippe, M., Bloom, B. R., Ganz, T., and Modlin, R. L. (2000). Granulysin, a T cell product, kills bacteria by altering membrane permeability. J Immunol *165*, 7102–7108.

Esko, J. D., Stewart, T. E., and Taylor, W. H. (1985). Animal cell mutants defective in glycosaminoglycan biosynthesis. Proc Natl Acad Sci U S A 82,3197-3201.

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

Fischer, P. M., Zhelev, N. Z., Wang, S., Melville, J. E., Fåhraeus, R., and Lane, D. P. (2000). Structure-activity relationship of truncated and substituted analogues of the intracellular delivery vector Penetratin. J Pept Res *55*, 163–172.

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

Ford, M. G., Mills, I. G., Peter, B. J., Vallis, Y., Praefcke, G. J., Evans, P. R., and McMahon, H. T. (2002). Curvature of clathrin-coated pits driven by epsin. Nature *419*, 361–366.

Frank, P. G., Pavlides, S., and Lisanti, M. P. (2009). Caveolae and transcytosis in endothelial cells: role in atherosclerosis. Cell Tissue Res *335*, 41–47.

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

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

Frick, M., Bright, N. A., Riento, K., Bray, A., Merrified, C., and Nichols, B. J. (2007). Coassembly of flotillins induces formation of membrane microdomains, membrane curvature, and vesicle budding. Curr Biol *17*, 1151–1156.

Frost, A., Unger, V. M., and De Camilli, P. (2009). The BAR domain superfamily: membrane-molding macromolecules. Cell *137*, 191–196.

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

Gerbal-Chaloin, S., Gondeau, C., Aldrian-Herrada, G., Heitz, F., Gauthier-Rouviere, C., and Divita, G. (2007). First step of the cell-penetrating peptide mechanism involves Rac1 GTPase-dependent actin-network remodelling. Biol Cell *99*, 223–238.

Geuskens, M., and Tencer, R. (1979). An ultrastructural study of the effects of wheat germ agglutinin (WGA) on cell cortex organization during the first cleavage of Xenopus laevis eggs. II. Cortical wound healing. J Cell Sci *37*, 59–67.

Ghibaudi, E., Boscolo, B., Inserra, G., Laurenti, E., Traversa, S., Barbero, L., and Ferrari, R. P. (2005). The interaction of the cell-penetrating peptide penetratin with heparin, heparansulfates and phospholipid vesicles investigated by ESR spectroscopy. J Pept Sci *11*, 401–409.

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

Godin, L. M., Vergen, J., Prakash, Y. S., Pagano, R. E., and Hubmayr, R. D. (2011). Spatiotemporal dynamics of actin remodeling and endomembrane trafficking in alveolar epithelial type I cell wound healing. Am J Physiol Lung Cell Mol Physiol *300*, L615–623.

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

Grafahrend, D., Calvet, J. L., Klinkhammer, K., Salber, J., Dalton, P. D., Moller, M., and Klee, D. (2008). Control of protein adsorption on functionalized electrospun fibers. Biotechnol Bioeng *101*, 609–621.

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

Gruenberg, J. (2003). Lipids in endocytic membrane transport and sorting. Curr Opin Cell Biol *15*, 382–388.

Gruenberg, J., and Stenmark, H. (2004). The biogenesis of multivesicular endosomes. Nat Rev Mol Cell Biol *5*, 317–323.

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

Haas, A. K., Fuchs, E., Kopajtich, R., and Barr, F. A. (2005). A GTPase-activating protein controls Rab5 function in endocytic trafficking. Nat Cell Biol *7*, 887–893.

Hambleton, P. (1971). Repair of wall damage in Escherichia coli recovered from an aerosol. J Gen Microbiol *69*, 81–88.

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

Herreros, J., Ng, T., and Schiavo, G. (2001). Lipid rafts act as specialized domains for tetanus toxin binding and internalization into neurons. Mol Biol Cell *12*, 2947–2960.

Herzog, M., Gerard, D., Hirth, L., and Laustriat, G. (1977). Natural fluorescence properties of brome mosaic virus protein. Biochim Biophys Acta *493*, 167–177.

Heuser, J. E., and Keen, J. (1988). Deep-etch visualization of proteins involved in clathrin assembly. J Cell Biol *107*, 877–886.

Hewlett, L. J., Prescott, A. R., and Watts, C. (1994). The coated pit and macropinocytic pathways serve distinct endosome populations. J Cell Biol *124*, 689–703.

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

Hoeller, D., Volarevic, S., and Dikic, I. (2005). Compartmentalization of growth factor receptor signalling. Curr Opin Cell Biol *17*, 107–111.

Hsu, M. J., Lee, S. S., Lee, S. T., and Lin, W. W. (2003). Signaling mechanisms of enhanced neutrophil phagocytosis and chemotaxis by the polysaccharide purified from Ganoderma lucidum. Br J Pharmacol *139*, 289–298.

Hurley, J. H., Boura, E., Carlson, L. A., and Rozycki, B. (2010). Membrane budding. Cell *143*, 875–887.

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

Idone, V., Tam, C., Goss, J. W., Toomre, D., Pypaert, M., and Andrews, N. W. (2008). Repair of injured plasma membrane by rapid Ca2+-dependent endocytosis. J Cell Biol *180*, 905–914.

Imamura, J., Suzuki, Y., Gonda, K., Roy, C. N., Gatanaga, H., Ohuchi, N., and Higuchi, H. (2011). Single particle tracking confirms that multivalent Tat protein transduction domain-induced heparan sulfate proteoglycan cross-linkage activates Rac1 for internalization. J Biol Chem *286*, 10581–10592.

Jaiswal, J. K., Andrews, N. W., and Simon, S. M. (2002). Membrane proximal lysosomes are the major vesicles responsible for calcium-dependent exocytosis in nonsecretory cells. J Cell Biol *159*, 625–635.

Jaiswal, J. K., Rivera, V. M., and Simon, S. M. (2009). Exocytosis of post-Golgi vesicles is regulated by components of the endocytic machinery. Cell *137*, 1308–1319.

Johnson, M. H. (1972). The distribution of a blastokinin-like uterine protein studied by immune fluorescence. Fertil Steril *23*, 929–939.

Keefe, D., Shi, L., Feske, S., Massol, R., Navarro, F., Kirchhausen, T., and Lieberman, J. (2005). Perforin triggers a plasma membrane-repair response that facilitates CTL induction of apoptosis. Immunity *23*, 249–262.

Kerr, M. C., and Teasdale, R. D. (2009). Defining macropinocytosis. Traffic *10*, 364–371.

Kim, J. S., and Southard, J. H. (1999). Membrane stabilizing effects of calcium and taxol during the cold storage of isolated rat hepatocytes. Transplantation *68*, 938–943.

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

Kirkham, M., Nixon, S. J., Howes, M. T., Abi-Rached, L., Wakeham, D. E., Hanzal-Bayer, M., Ferguson, C., Hill, M. M., Fernandez-Rojo, M., Brown, D. A.*, et al.* (2008). Evolutionary analysis and molecular dissection of caveola biogenesis. J Cell Sci *121*, 2075–2086.

Krämer, S. D., and Wunderli-Allenspach, H. (2003). No entry for TAT(44–57) into liposomes and intact MDCK cells: novel approach to study membrane permeation of cell-penetrating peptides. Biochim Biophys Acta *1609*, 161–169.

Kurzchalia, T. V., and Parton, R. G. (1999). Membrane microdomains and caveolae. Curr Opin Cell Biol *11*, 424–431.

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

Lamaziere, A., Burlina, F., Wolf, C., Chassaing, G., Trugnan, G., and Ayala-Sanmartin, J. (2007). Non-metabolic membrane tubulation and permeability induced by bioactive peptides. PLoS One *2*, e201.

Lee, E., and Knecht, D. A. (2002). Visualization of actin dynamics during macropinocytosis and exocytosis. Traffic *3*, 186–192.

Levin, D. E. (2005). Cell wall integrity signaling in Saccharomyces cerevisiae. Microbiol Mol Biol Rev *69*, 262–291.

Li, Q., Dong, C., Deng, A., Katsumata, M., Nakadai, A., Kawada, T., Okada, S., Clayberger, C., and Krensky, A. M. (2005). Hemolysis of erythrocytes by granulysin-derived peptides but not by granulysin. Antimicrob Agents Chemother *49*, 388–397.

Liberali, P., Kakkonen, E., Turacchio, G., Valente, C., Spaar, A., Perinetti, G., Bockmann, R. A., Corda, D., Colanzi, A., Marjomaki, V., and Luini, A. (2008). The closure of Pak1-dependent macropinosomes requires the phosphorylation of CtBP1/BARS. Embo J *27*, 970–981.

Liu, C. C., Walsh, C. M., and Young, J. D. (1995). Perforin: structure and function. Immunol Today *16*, 194–201.

Liu, L., and Pilch, P. F. (2008). A critical role of cavin (polymerase I and transcript release factor) in caveolae formation and organization. J Biol Chem *283*, 4314–4322.

Los, F. C., Kao, C. Y., Smitham, J., McDonald, K. L., Ha, C., Peixoto, C. A., and Aroian, R. V. (2011). RAB-5- and RAB-11-dependent vesicle-trafficking pathways are required for plasma membrane repair after attack by bacterial pore-forming toxin. Cell Host Microbe *9*, 147–157.

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

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

Magzoub, M., Eriksson, L. E., and Graslund, A. (2002). Conformational states of the cell-penetrating peptide penetratin when interacting with phospholipid vesicles:

effects of surface charge and peptide concentration. Biochim Biophys Acta *1563*, 53–63.
Magzoub, M., Kilk, K., Eriksson, L. E., Langel, Ü., and Gräslund, A. (2001). Interaction and structure induction of cell-penetrating peptides in the presence of phospholipid vesicles. Biochim Biophys Acta *1512*, 77–89.
Mandato, C. A., and Bement, W. M. (2001). Contraction and polymerization cooperate to assemble and close actomyosin rings around Xenopus oocyte wounds. J Cell Biol *154*, 785–797.
Mandato, C. A., and Bement, W. M. (2003). Actomyosin transports microtubules and microtubules control actomyosin recruitment during Xenopus oocyte wound healing. Curr Biol *13*, 1096–1105.
Maniti, O., Alves, I., Trugnan, G., and Ayala-Sanmartin, J. (2010). Distinct behaviour of the homeodomain derived cell penetrating peptide penetratin in interaction with different phospholipids. PLoS One *5*, e15819.
Mano, M., Henriques, A., Paiva, A., Prieto, M., Gavilanes, F., Simoes, S., and de Lima, M. C. (2007). Interaction of $S4_{13}$-PV cell penetrating peptide with model membranes: relevance to peptide translocation across biological membranes. J Pept Sci *13*, 301–313.
Mano, M., Henriques, A., Paiva, A., Prieto, M., Gavilanes, F., Simoes, S., and Pedroso de Lima, M. C. (2006). Cellular uptake of $S4_{13}$-PV peptide occurs upon conformational changes induced by peptide-membrane interactions. Biochim Biophys Acta *1758*, 336–346.
Mano, M., Teodosio, C., Paiva, A., Simoes, S., and Pedroso de Lima, M. C. (2005). On the mechanisms of the internalization of S4(13)-PV cell-penetrating peptide. Biochem J *390*, 603–612.
Marsh, M., and Helenius, A. (2006). Virus entry: open sesame. Cell *124*, 729–740.
Marshansky, V., and Futai, M. (2008). The V-type H+-ATPase in vesicular trafficking: targeting, regulation and function. Curr Opin Cell Biol *20*, 415–426.
Massol, R. H., Larsen, J. E., and Kirchhausen, T. (2005). Possible role of deep tubular invaginations of the plasma membrane in MHC-I trafficking. Exp Cell Res *306*, 142–149.
Masuda, M., and Mochizuki, N. (2010). Structural characteristics of BAR domain superfamily to sculpt the membrane. Semin Cell Dev Biol *21*, 391–398.
Matin, M. M., Andrews, P. W., and Hornby, D. P. (2002). Multidimensional differential display via ion-pair reversed-phase denaturing high-performance liquid chromatography. Anal Biochem *304*, 47–54.
Mayor, S., and Riezman, H. (2004). Sorting GPI-anchored proteins. Nat Rev Mol Cell Biol *5*, 110–120.
McNeil, A. K., Rescher, U., Gerke, V., and McNeil, P. L. (2006). Requirement for annexin A1 in plasma membrane repair. J Biol Chem *281*, 35202–35207.
McNeil, P. L. (1984). Mechanisms of nutritive endocytosis. III. A freeze-fracture study of phagocytosis by digestive cells of Chlorohydra. Tissue Cell *16*, 519–533.
McNeil, P. L., and Khakee, R. (1992). Disruptions of muscle fiber plasma membranes. Role in exercise-induced damage. Am J Pathol *140*, 1097–1109.
McNeil, P. L., Miyake, K., and Vogel, S. S. (2003). The endomembrane requirement for cell surface repair. Proc Natl Acad Sci U S A *100*, 4592–4597.
McNeil, P. L., and Steinhardt, R. A. (2003). Plasma membrane disruption: repair, prevention, adaptation. Annu Rev Cell Dev Biol *19*, 697–731.

McNeil, P. L., Vogel, S. S., Miyake, K., and Terasaki, M. (2000). Patching plasma membrane disruptions with cytoplasmic membrane. J Cell Sci *113 (Pt 11)*, 1891–1902.

Mellgren, R. L. (2010). A plasma membrane wound proteome: reversible externalization of intracellular proteins following reparable mechanical damage. J Biol Chem *285*, 36597–36607.

Mellman, I. (1996). Endocytosis and molecular sorting. Annu Rev Cell Dev Biol *12*, 575–625.

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

Mercer, J., and Helenius, A. (2009). Virus entry by macropinocytosis. Nat Cell Biol *11*, 510–520.

Mercer, J., Schelhaas, M., and Helenius, A. (2010). Virus entry by endocytosis. Annu Rev Biochem *79*, 803–833.

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

Miyake, K., McNeil, P. L., Suzuki, K., Tsunoda, R., and Sugai, N. (2001). An actin barrier to resealing. J Cell Sci *114*, 3487–3494.

Morgan, B. P., Dankert, J. R., and Esser, A. F. (1987). Recovery of human neutrophils from complement attack: removal of the membrane attack complex by endocytosis and exocytosis. J Immunol *138*, 246–253.

Morrow, I. C., and Parton, R. G. (2005). Flotillins and the PHB domain protein family: rafts, worms and anaesthetics. Traffic *6*, 725–740.

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

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

Oehlke, J., Krause, E., Wiesner, B., Beyermann, M., and Bienert, M. (1997). Extensive cellular uptake into endothelial cells of an amphipathic beta- sheet forming peptide. FEBS Lett *415*, 196–199.

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

Oehlke, J., Wallukat, G., Wolf, Y., Ehrlich, A., Wiesner, B., Berger, H., and Bienert, M. (2004). Enhancement of intracellular concentration and biological activity of PNA after conjugation with a cell-penetrating synthetic model peptide. European Journal of Biochemistry *271*, 3043–3049.

Orth, J. D., and McNiven, M. A. (2006). Get off my back! Rapid receptor internalization through circular dorsal ruffles. Cancer Res *66*, 11094–11096.

Padari, K., Säälik, P., Hansen, M., Koppel, K., Raid, R., Langel, Ü., and Pooga, M. (2005). Cell transduction pathways of transportans. Bioconjug Chem *16*, 1399–1410.

Palade, G. E. (1953). An electron microscope study of the mitochondrial structure. J Histochem Cytochem *1*, 188–211.

Palm-Apergi, C., Lorents, A., Padari, K., Pooga, M., and Hällbrink, M. (2009). The membrane repair response masks membrane disturbances caused by cell-penetrating peptide uptake. Faseb J *23*, 214–223.

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

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

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

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

Persson, D., Thoren, P. E., Esbjorner, E. K., Goksor, M., Lincoln, P., and Norden, B. (2004). Vesicle size-dependent translocation of penetratin analogs across lipid membranes. Biochim Biophys Acta *1665*, 142–155.

Persson, D., Thorén, P. E., Herner, M., Lincoln, P., and Nordén, B. (2003). Application of a novel analysis to measure the binding of the membrane-translocating peptide penetratin to negatively charged liposomes. Biochemistry *42*, 421–429.

Pfeffer, S. R. (2001). Rab GTPases: specifying and deciphering organelle identity and function. Trends Cell Biol *11*, 487–491.

Pietiainen, V., Marjomaki, V., Upla, P., Pelkmans, L., Helenius, A., and Hyypia, T. (2004). Echovirus 1 endocytosis into caveosomes requires lipid rafts, dynamin II, and signaling events. Mol Biol Cell *15*, 4911–4925.

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

Qualmann, B., Koch, D., and Kessels, M. M. (2011). Let's go bananas: revisiting the endocytic BAR code. Embo J *30*, 3501–3515.

Raucher, D., and Sheetz, M. P. (1999). Characteristics of a membrane reservoir buffering membrane tension. Biophys J *77*, 1992–2002.

Reddy, A., Caler, E. V., and Andrews, N. W. (2001). Plasma membrane repair is mediated by Ca(2+)-regulated exocytosis of lysosomes. Cell *106*, 157–169.

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

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

Ridley, A. J. (1997). Signalling by Rho family proteins. Biochem Soc Trans *25*, 1005–1010.

Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D., and Hall, A. (1992). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell *70*, 401–410.

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

Rothbard, J. B., Jessop, T. C., and Wender, P. A. (2005). Adaptive translocation: the role of hydrogen bonding and membrane potential in the uptake of guanidinium-rich transporters into cells. Adv Drug Deliv Rev *57*, 495–504.

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

Russell, M. R., Nickerson, D. P., and Odorizzi, G. (2006). Molecular mechanisms of late endosome morphology, identity and sorting. Curr Opin Cell Biol *18*, 422–428.

Rybin, V., Ullrich, O., Rubino, M., Alexandrov, K., Simon, I., Seabra, M. C., Goody, R., and Zerial, M. (1996). GTPase activity of Rab5 acts as a timer for endocytic membrane fusion. Nature *383*, 266–269.

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

Saftig, P., and Klumperman, J. (2009). Lysosome biogenesis and lysosomal membrane proteins: trafficking meets function. Nat Rev Mol Cell Biol *10*, 623–635.

Scheller, A., Wiesner, B., Melzig, M., Bienert, M., and Oehlke, J. (2000). Evidence for an amphipathicity independent cellular uptake of amphipathic cell-penetrating peptides. Eur J Biochem *267*, 6043–6050.

Schmid, S. L. (1997). Clathrin-coated vesicle formation and protein sorting: an integrated process. Annu Rev Biochem *66*, 511–548.

Sens, P., and Turner, M. S. (2006). Budded membrane microdomains as tension regulators. Phys Rev E Stat Nonlin Soft Matter Phys *73*, 031918.

Sheff, D. R., Daro, E. A., Hull, M., and Mellman, I. (1999). The receptor recycling pathway contains two distinct populations of early endosomes with different sorting functions. J Cell Biol *145*, 123–139.

Shen, S. S., Tucker, W. C., Chapman, E. R., and Steinhardt, R. A. (2005). Molecular regulation of membrane resealing in 3T3 fibroblasts. J Biol Chem *280*, 1652–1660.

Shogomori, H., and Futerman, A. H. (2001). Cholera toxin is found in detergent-insoluble rafts/domains at the cell surface of hippocampal neurons but is internalized via a raft-independent mechanism. J Biol Chem *276*, 9182–9188.

Smith, C. J., Grigorieff, N., and Pearse, B. M. (1998). Clathrin coats at 21 A resolution: a cellular assembly designed to recycle multiple membrane receptors. Embo J *17*, 4943–4953.

Smith, M. W., Phelps, P. C., and Trump, B. F. (1991). Cytosolic Ca2+ deregulation and blebbing after HgCl2 injury to cultured rabbit proximal tubule cells as determined by digital imaging microscopy. Proc Natl Acad Sci U S A *88*, 4926–4930.

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

Szubinska, B. (1971). "New membrane" formation in Amoeba proteus upon injury of individual cells. Electron microscope observations. J Cell Biol *49*, 747–772.

Stang, E., Kartenbeck, J., and Parton, R. G. (1997). Major histocompatibility complex class I molecules mediate association of SV40 with caveolae. Mol Biol Cell *8*, 47–57.

Steele-Mortimer, O., Knodler, L. A., and Finlay, B. B. (2000). Poisons, ruffles and rockets: bacterial pathogens and the host cell cytoskeleton. Traffic *1*, 107–118.

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

Steinhardt, R. A., Bi, G., and Alderton, J. M. (1994). Cell membrane resealing by a vesicular mechanism similar to neurotransmitter release. Science *263*, 390–393.

Stuermer, C. A. (2010). The reggie/flotillin connection to growth. Trends Cell Biol *20*, 6–13.

Suetsugu, S., Toyooka, K., and Senju, Y. (2010). Subcellular membrane curvature mediated by the BAR domain superfamily proteins. Semin Cell Dev Biol *21*, 340–349.

Swanson, J. A. (2008). Shaping cups into phagosomes and macropinosomes. Nat Rev Mol Cell Biol *9*, 639–649.

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

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

Zhang, B., and Zheng, Y. (1998). Regulation of RhoA GTP hydrolysis by the GTPase-activating proteins p190, p50RhoGAP, Bcr, and 3BP-1. Biochemistry *37*, 5249–5257.

Zhang, Z., Chen, G., Zhou, W., Song, A., Xu, T., Luo, Q., Wang, W., Gu, X. S., and Duan, S. (2007). Regulated ATP release from astrocytes through lysosome exocytosis. Nat Cell Biol *9*, 945–953.

Zheng, J., Cahill, S. M., Lemmon, M. A., Fushman, D., Schlessinger, J., and Cowburn, D. (1996). Identification of the binding site for acidic phospholipids on the pH domain of dynamin: implications for stimulation of GTPase activity. J Mol Biol *255*, 14–21.

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

Ziegler, A., Blatter, X. L., Seelig, A., and Seelig, J. (2003). Protein transduction domains of HIV-1 and SIV TAT interact with charged lipid vesicles. Binding mechanism and thermodynamic analysis. Biochemistry *42*, 9185–9194.

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

Ziegler, A., and Seelig, J. (2008). Binding and clustering of glycosaminoglycans: a common property of mono- and multivalent cell-penetrating compounds. Biophys J *94*, 2142–2149.

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

Tagawa, A., Mezzacasa, A., Hayer, A., Longatti, A., Pelkmans, L., and Helenius, A. (2005). Assembly and trafficking of caveolar domains in the cell: caveolae as stable, cargo-triggered, vesicular transporters. J Cell Biol *170*, 769–779.

Tiriveedhi, V., and Butko, P. (2007). A fluorescence spectroscopy study on the interactions of the TAT-PTD peptide with model lipid membranes. Biochemistry *46*, 3888–3895.

Togo, T. (2006). Disruption of the plasma membrane stimulates rearrangement of microtubules and lipid traffic toward the wound site. J Cell Sci *119*, 2780–2786.

Traub, L. M. (2009). Tickets to ride: selecting cargo for clathrin-regulated internalization. Nat Rev Mol Cell Biol *10*, 583–596.

Tuma, P. L., and Hubbard, A. L. (2003). Transcytosis: crossing cellular barriers. Physiol Rev *83*, 871–932.

Tuma, P. L., Stachniak, M. C., and Collins, C. A. (1993). Activation of dynamin GTPase by acidic phospholipids and endogenous rat brain vesicles. J Biol Chem *268*, 17240–17246.

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

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

van der Goot, F. G., and Gruenberg, J. (2006). Intra-endosomal membrane traffic. Trends Cell Biol *16*, 514–521.

Wang, J. H., Wells, C., and Wu, L. (2008). Macropinocytosis and cytoskeleton contribute to dendritic cell-mediated HIV-1 transmission to CD4+ T cells. Virology *381*, 143–154.

Watkins, C. L., Schmaljohann, D., Futaki, S., and Jones, A. T. (2009). Low concentration thresholds of plasma membranes for rapid energy-independent translocation of a cell-penetrating peptide. Biochem J *420*, 179–189.

Vaughan, E. M., Miller, A. L., Yu, H. Y., and Bement, W. M. (2011). Control of local Rho GTPase crosstalk by Abr. Curr Biol *21*, 270–277.

Veiga, E., and Cossart, P. (2005). Listeria hijacks the clathrin-dependent endocytic machinery to invade mammalian cells. Nat Cell Biol *7*, 894–900.

Wender, P. A., Mitchell, D. J., Pattabiraman, K., Pelkey, E. T., Steinman, L., and Rothbard, J. B. (2000). The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters. Proc Natl Acad Sci USA *97*, 13003–13008.

Vendeville, A., Rayne, F., Bonhoure, A., Bettache, N., Montcourrier, P., and Beaumelle, B. (2004). HIV-1 Tat enters T cells using coated pits before translocating from acidified endosomes and eliciting biological responses. Mol Biol Cell *15*, 2347–2360.

Vidricaire, G., and Tremblay, M. J. (2007). A clathrin, caveolae, and dynamin-independent endocytic pathway requiring free membrane cholesterol drives HIV-1 internalization and infection in polarized trophoblastic cells. J Mol Biol *368*, 1267–1283.

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

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

ACKNOWLEDGMENTS

The main part of this work has been carried out at the Institute of Molecular and Cell Biology, University of Tartu, and I would like to thank all the people (in and outside the institute) who have helped and supported me during all the years of my studies.

I am truly happy that around 8 years ago I accidentally met Prof. Margus Pooga in the corridor of cell biology that, in turn, ended with me diving into the world of cell-penetrating peptides. So, first and foremost, I want to thank my supervisor for being a great mentor, for his patience and accepting my nocturnal work "days" and, especially, for letting me evolve in my own pace, and disappear from time to time to reload my batteries.

I am very grateful for all my former and current lab-mates, but especially Helin, Kärt, Pille, Külliki, and Kaida – all the years spent alongside you have been absolutely wonderful. Helin, even though we come from really different worlds, I am so happy for your 14+ years of friendship (from Clueless & Speed sessions to serious scientific discussions, from your chocolate drawer to our badminton battles). Kärt, every time I think of your crazy ideas it makes me laugh, even our countless hours in the dark basement with "flying" chemicals. Pille, your spicy jokes and management of our espresso stash has made my workdays much enjoyable. Külliki & Morten, I want to thank you for waking me up for so many times for good food and *fika*-time, and for Eddie Izzard (no dissertation, no PhD!). Also, other colleagues around the corridors – Signe, Sulev I., Toivo, Tiiu, Viljar, Lilian, Daima, Tõnis, Caroline, Anneli Kukk, etc – thank you for your help and advice whenever I needed it.

Special thanks goes to Liis Andresen – for your CDR magic (saving my nerves and much more), and for your companionship during our cultural discoveries over the past years. Also, my other course mates and semi-course mates – Ott, Marko, Kuldar, Vallo, Paula, Jaanis – it has been as much fun as bizarre with you guys.

I want to thank my (extended) family for their encouragement and support, especially my sister, Vambola, and uncle Miša. I want to thank Rannar for always being there when I needed it; you have helped me so many times that I would need another long chapter to express my gratitude. I would also include here Geili & Kalev – thank you for our legendary Sundays at Pikk 2 with fruitful philosophical discussions and fried potatoes, for always being my safety net, for your stress-relief-sessions in Pärnu since you moved away, and much-much more. Thanks to you I'm even closer to that boat;)

And last but not least, I want to thank my melomaniac friends (especially Allan, Merilin, and S.Ärk), thanks to whom I've discovered new favourites to dance along the dark corridors at the institute trying not to fall asleep and, amongst other things, write this thesis. The hardest part is over now:)

PUBLICATIONS

CURRICULUM VITAE

Annely Lorents

Date of Birth:	01.11.1982
Nationality:	Estonian
Contact:	University of Tartu, Institute of Molecular and Cell Biology, 23 Riia Street, 51010, Tartu, Estonia
E-mail:	anny@ut.ee

Educational and professional employment

1998–2001	Pärnu Co-Educational Gymnasium (graduated with honors)
2001–2005	University of Tartu, BSc in gene technology (specialty of transgenic technology)
2005–2007	University of Tartu, MSc in gene technology (specialty of transgenic technology)
2007–…	University of Tartu, Ph.D student at the Institute of Molecular and Cell Biology
2010	University of Tartu, Institute of Molecular and Cell Biology, researcher

Special Courses

2011	EMBO conference "Dynamic Endosomes: Mechanisms Controlling Endocytosis", Chania, Greece
2011	GRC conference "Calcium Signaling", Colby College, USA
2011	Conference "Peptide Vectors and Delivery of Therapeutics", Tallinn, Estonia
2011	EMBO/FEBS joint lecture course "Protein Transport Systems", Santa Margherita di Pula, Italy
2010	Carrying out a project "Cell-penetrating peptide-membrane interactions: a biophysical approach" at the University of Coimbra, Portugal
2010	EPS symposium "Cell Penetrating Peptides", Copenhagen, Denmark
2009	IUBMB & FAOMBM congress "Biomolecules for Quality of Life", Shanghai, China
2009	GRC conference "Calcium Signaling", Lucca (Barga), Italy
2008	"Electron microscopy & cell fractionation course", University of Helsinki, Finland
2008	FEBS YSF & congress "Biochemistry of Cell Regulation", Athens, Greece

2008	Nordic microscopy society meeting SCANDEM, Lyngby, Denmark
2007	FEBS/EMBO workshop "Endocytic Systems: Mechanism & Function", Villars-sur-Ollon, Switzerland
2007	FEBS congress "Molecular Machines", Vienna, Austria
2006	FEBS Special Meeting "Cellular Signalling", Cavtat, Croatia

Professional organization

Since 2008	Member of Scandem Nordic Microscopy Society
Since 2006	Member of Estonian Biochemical Society

List of publications

1. **Lorents, A.**, Kodavali, P. K., Oskolkov, N., Langel, Ü., Hällbrink, M., and Pooga, M. (2012) Cell-penetrating Peptides Split into Two Groups Based on Modulation of Intracellular Calcium Concentration. J Biol Chem 287, 16880–16889
2. Cardoso, A. M., Trabulo, S., Cardoso, A. L., **Lorents, A.**, Morais, C. M., Gomes, P., Nunes, C., Lucio, M., Reis, S., Padari, K., Pooga, M., Pedroso de Lima, M. C., and Jurado, A. S. (2012) S4(13)-PV cell-penetrating peptide induces physical and morphological changes in membrane-mimetic lipid systems and cell membranes: implications for cell internalization. Biochim Biophys Acta 1818, 877–888
3. Padari, K., **Lorents, A.**, Jokitalo, E., and Pooga, M. (2011) Insight into cell-entry mechanisms of CPPs by electron microscopy. Methods Mol Biol 683, 181–193
4. Padari, K., Koppel, K., **Lorents, A.**, Hällbrink, M., Mano, M., Pedroso de Lima, M. C., and Pooga, M. (2010) S4(13)-PV cell-penetrating peptide forms nanoparticle-like structures to gain entry into cells. Bioconjug Chem 21, 774–783
5. Säälik, P., Padari, K., Niinep, A., **Lorents, A.**, Hansen, M., Jokitalo, E., Langel, Ü., and Pooga, M. (2009) Protein delivery with transportans is mediated by caveolae rather than flotillin-dependent pathways. Bioconjug Chem 20, 877–887
6. Palm-Apergi, C., **Lorents, A.,** Padari, K., Pooga, M., and Hällbrink, M. (2009) The membrane repair response masks membrane disturbances caused by cell-penetrating peptide uptake. FASEB J 23, 214-223
7. Koppel, K., Padari, K., **Lorents, A.**, Mano, M., Raid, R., de Lima, M.C., and Pooga, M. (2009). Interaction of S4(13)-PV peptide with plasma membrane and cell entry. An electron microscopy view. Ed. by Lankinen, H. in Peptides 2008, 394–395

CURRICULUM VITAE

Annely Lorents

Sünniaeg:	01.11.1982
Kodakondsus:	Eesti
Kontaktandmed:	Molekulaar- ja rakubioloogia instituut, Tartu Ülikool, Riia 23, 51010, Tartu, Eesti
E-mail:	anny@ut.ee

Haridus ja erialane teenistuskäik

1998–2001	Pärnu Ühisgümnaasium (hõbemedal)
2001–2005	Tartu Ülikool, BSc geenitehnoloogias (transgeense tehnoloogia eriala)
2005–2007	Tartu Ülikool, MSc geenitehnoloogias (transgeense tehnoloogia eriala)
2007–…	Tartu Ülikool, Molekulaar- ja rakubioloogia instituut, doktoriõpingud geenitehnoloogia erialal
2010	Tartu Ülikool, Molekulaar- ja rakubioloogia instituut, teadur

Erialane enesetäiendus

2011	EMBO konverents "*Dynamic Endosomes: Mechanisms Controlling Endocytosis*", Chania, Kreeka
2011	GRC konverents "*Calcium Signaling*", Colby Kolledž, USA
2011	Konverents "*Peptide Vectors and Delivery of Therapeutics*", Tallinn, Eesti
2011	EMBO/FEBS'i ühendkursus "*Protein Transport Systems*", Santa Margherita di Pula, Itaalia
2010	Projekti "*Cell-penetrating peptide-membrane interactions: a biophysical approach*" läbi viimine Coimbra Ülikoolis, Portugalis
2010	EPS'i sümpoosion "*Cell Penetrating Peptides*", Kopenhaagen, Taani
2009	IUBMB & FAOMBM kongress "*Biomolecules for Quality of Life*", Shanghai, Hiina
2009	GRC konverents "*Calcium Signaling*", Lucca (Barga), Itaalia
2008	Elektronmikroskoopia ja raku fraktsioniseerimise kursus, Helsingi Ülikool, Soome
2008	FEBS'i noorte teadlaste foorum ning kongress "*Biochemistry of Cell Regulation*", Ateena, Kreeka

2008	Põhjamaade Mikroskoopiaühingu konverents SCANDEM, Lyngby, Taani
2007	FEBS/EMBO kursus "*Endocytic Systems: Mechanism & Function*", Villars-sur-Ollon, Šveits
2007	FEBS'i kongress "*Molecular Machines*", Viin, Austria
2006	FEBS'i konverents "*Cellular Signalling*", Cavtat, Horvaatia

Teadusorganisatsioonid

Alates 2008	Põhjamaade Mikroskoopiaühingu Scandem liige
Alates 2006	Eesti Biokeemia Seltsi liige

Teaduspublikatsioonid

1. **Lorents, A.**, Kodavali, P. K., Oskolkov, N., Langel, Ü., Hällbrink, M., and Pooga, M. (2012) Cell-penetrating Peptides Split into Two Groups Based on Modulation of Intracellular Calcium Concentration. J Biol Chem 287, 16880–16889
2. Cardoso, A. M., Trabulo, S., Cardoso, A. L., **Lorents, A.**, Morais, C. M., Gomes, P., Nunes, C., Lucio, M., Reis, S., Padari, K., Pooga, M., Pedroso de Lima, M. C., and Jurado, A. S. (2012) S4(13)-PV cell-penetrating peptide induces physical and morphological changes in membrane-mimetic lipid systems and cell membranes: implications for cell internalization. Biochim Biophys Acta 1818, 877–888
3. Padari, K., **Lorents, A.**, Jokitalo, E., and Pooga, M. (2011) Insight into cell-entry mechanisms of CPPs by electron microscopy. Methods Mol Biol 683, 181–193
4. Padari, K., Koppel, K., **Lorents, A.**, Hällbrink, M., Mano, M., Pedroso de Lima, M. C., and Pooga, M. (2010) S4(13)-PV cell-penetrating peptide forms nanoparticle-like structures to gain entry into cells. Bioconjug Chem 21, 774–783
5. Säälik, P., Padari, K., Niinep, A., **Lorents, A.**, Hansen, M., Jokitalo, E., Langel, Ü., and Pooga, M. (2009) Protein delivery with transportans is mediated by caveolae rather than flotillin-dependent pathways. Bioconjug Chem 20, 877–887
6. Palm-Apergi, C., **Lorents, A.,** Padari, K., Pooga, M., and Hällbrink, M. (2009) The membrane repair response masks membrane disturbances caused by cell-penetrating peptide uptake. FASEB J 23, 214-223
7. Koppel, K., Padari, K., **Lorents, A.**, Mano, M., Raid, R., de Lima, M.C., and Pooga, M. (2009). Interaction of S4(13)-PV peptide with plasma membrane and cell entry. An electron microscopy view. Ed. by Lankinen, H. in Peptides 2008, 394–395

DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS

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

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

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

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

82. **Ivar Ilves.** Virus-cell interactions in the replication cycle of bovine papillomavirus type 1. Tartu, 2003. 89 p.
83. **Andres Männik.** Design and characterization of a novel vector system based on the stable replicator of bovine papillomavirus type 1. Tartu, 2003. 109 p.
84. **Ivika Ostonen.** Fine root structure, dynamics and proportion in net primary production of Norway spruce forest ecosystem in relation to site conditions. Tartu, 2003. 158 p.
85. **Gudrun Veldre.** Somatic status of 12–15-year-old Tartu schoolchildren. Tartu, 2003. 199 p.
86. **Ülo Väli.** The greater spotted eagle *Aquila clanga* and the lesser spotted eagle *A. pomarina*: taxonomy, phylogeography and ecology. Tartu, 2004. 159 p.
87. **Aare Abroi.** The determinants for the native activities of the bovine papillomavirus type 1 E2 protein are separable. Tartu, 2004. 135 p.
88. **Tiina Kahre.** Cystic fibrosis in Estonia. Tartu, 2004. 116 p.
89. **Helen Orav-Kotta.** Habitat choice and feeding activity of benthic suspension feeders and mesograzers in the northern Baltic Sea. Tartu, 2004. 117 p.
90. **Maarja Öpik.** Diversity of arbuscular mycorrhizal fungi in the roots of perennial plants and their effect on plant performance. Tartu, 2004. 175 p.
91. **Kadri Tali.** Species structure of *Neotinea ustulata*. Tartu, 2004. 109 p.
92. **Kristiina Tambets.** Towards the understanding of post-glacial spread of human mitochondrial DNA haplogroups in Europe and beyond: a phylogeographic approach. Tartu, 2004. 163 p.
93. **Arvi Jõers.** Regulation of p53-dependent transcription. Tartu, 2004. 103 p.
94. **Lilian Kadaja.** Studies on modulation of the activity of tumor suppressor protein p53. Tartu, 2004. 103 p.
95. **Jaak Truu.** Oil shale industry wastewater: impact on river microbial community and possibilities for bioremediation. Tartu, 2004. 128 p.
96. **Maire Peters.** Natural horizontal transfer of the *pheBA* operon. Tartu, 2004. 105 p.
97. **Ülo Maiväli.** Studies on the structure-function relationship of the bacterial ribosome. Tartu, 2004. 130 p.
98. **Merit Otsus.** Plant community regeneration and species diversity in dry calcareous grasslands. Tartu, 2004. 103 p.
99. **Mikk Heidemaa.** Systematic studies on sawflies of the genera *Dolerus, Empria,* and *Caliroa* (Hymenoptera: Tenthredinidae). Tartu, 2004. 167 p.
100. **Ilmar Tõnno.** The impact of nitrogen and phosphorus concentration and N/P ratio on cyanobacterial dominance and N_2 fixation in some Estonian lakes. Tartu, 2004. 111 p.
101. **Lauri Saks.** Immune function, parasites, and carotenoid-based ornaments in greenfinches. Tartu, 2004. 144 p.
102. **Siiri Rootsi.** Human Y-chromosomal variation in European populations. Tartu, 2004. 142 p.

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

123. **Priit Jõers.** Studies of the mitochondrial helicase Hmi1p in *Candida albicans* and *Saccharomyces cerevisia*. Tartu, 2006. 113 p.
124. **Kersti Lilleväli.** Gata3 and Gata2 in inner ear development. Tartu, 2007. 123 p.
125. **Kai Rünk.** Comparative ecology of three fern species: *Dryopteris carthusiana* (Vill.) H.P. Fuchs, *D. expansa* (C. Presl) Fraser-Jenkins & Jermy and *D. dilatata* (Hoffm.) A. Gray (Dryopteridaceae). Tartu, 2007. 143 p.
126. **Aveliina Helm.** Formation and persistence of dry grassland diversity: role of human history and landscape structure. Tartu, 2007. 89 p.
127. **Leho Tedersoo.** Ectomycorrhizal fungi: diversity and community structure in Estonia, Seychelles and Australia. Tartu, 2007. 233 p.
128. **Marko Mägi.** The habitat-related variation of reproductive performance of great tits in a deciduous-coniferous forest mosaic: looking for causes and consequences. Tartu, 2007. 135 p.
129. **Valeria Lulla.** Replication strategies and applications of Semliki Forest virus. Tartu, 2007. 109 p.
130. **Ülle Reier**. Estonian threatened vascular plant species: causes of rarity and conservation. Tartu, 2007. 79 p.
131. **Inga Jüriado**. Diversity of lichen species in Estonia: influence of regional and local factors. Tartu, 2007. 171 p.
132. **Tatjana Krama.** Mobbing behaviour in birds: costs and reciprocity based cooperation. Tartu, 2007. 112 p.
133. **Signe Saumaa.** The role of DNA mismatch repair and oxidative DNA damage defense systems in avoidance of stationary phase mutations in *Pseudomonas putida.* Tartu, 2007. 172 p.
134. **Reedik Mägi**. The linkage disequilibrium and the selection of genetic markers for association studies in european populations. Tartu, 2007. 96 p.
135. **Priit Kilgas.** Blood parameters as indicators of physiological condition and skeletal development in great tits (*Parus major*): natural variation and application in the reproductive ecology of birds. Tartu, 2007. 129 p.
136. **Anu Albert**. The role of water salinity in structuring eastern Baltic coastal fish communities. Tartu, 2007. 95 p.
137. **Kärt Padari.** Protein transduction mechanisms of transportans. Tartu, 2008. 128 p.
138. **Siiri-Lii Sandre.** Selective forces on larval colouration in a moth. Tartu, 2008. 125 p.
139. **Ülle Jõgar.** Conservation and restoration of semi-natural floodplain meadows and their rare plant species. Tartu, 2008. 99 p.
140. **Lauri Laanisto.** Macroecological approach in vegetation science: generality of ecological relationships at the global scale. Tartu, 2008. 133 p.
141. **Reidar Andreson**. Methods and software for predicting PCR failure rate in large genomes. Tartu, 2008. 105 p.
142. **Birgot Paavel.** Bio-optical properties of turbid lakes. Tartu, 2008. 175 p.

143. **Kaire Torn.** Distribution and ecology of charophytes in the Baltic Sea. Tartu, 2008, 98 p.
144. **Vladimir Vimberg.** Peptide mediated macrolide resistance. Tartu, 2008, 190 p.
145. **Daima Örd.** Studies on the stress-inducible pseudokinase TRB3, a novel inhibitor of transcription factor ATF4. Tartu, 2008, 108 p.
146. **Lauri Saag.** Taxonomic and ecologic problems in the genus *Lepraria* (*Stereocaulaceae*, lichenised *Ascomycota*). Tartu, 2008, 175 p.
147. **Ulvi Karu.** Antioxidant protection, carotenoids and coccidians in greenfinches – assessment of the costs of immune activation and mechanisms of parasite resistance in a passerine with carotenoid-based ornaments. Tartu, 2008, 124 p.
148. **Jaanus Remm.** Tree-cavities in forests: density, characteristics and occupancy by animals. Tartu, 2008, 128 p.
149. **Epp Moks.** Tapeworm parasites *Echinococcus multilocularis* and *E. granulosus* in Estonia: phylogenetic relationships and occurrence in wild carnivores and ungulates. Tartu, 2008, 82 p.
150. **Eve Eensalu.** Acclimation of stomatal structure and function in tree canopy: effect of light and CO_2 concentration. Tartu, 2008, 108 p.
151. **Janne Pullat**. Design, functionlization and application of an *in situ* synthesized oligonucleotide microarray. Tartu, 2008, 108 p.
152. **Marta Putrinš.** Responses of *Pseudomonas putida* to phenol-induced metabolic and stress signals. Tartu, 2008, 142 p.
153. **Marina Semtšenko.** Plant root behaviour: responses to neighbours and physical obstructions. Tartu, 2008, 106 p.
154. **Marge Starast.** Influence of cultivation techniques on productivity and fruit quality of some *Vaccinium* and *Rubus* taxa. Tartu, 2008, 154 p.
155. **Age Tats.** Sequence motifs influencing the efficiency of translation. Tartu, 2009, 104 p.
156. **Radi Tegova.** The role of specialized DNA polymerases in mutagenesis in *Pseudomonas putida.* Tartu, 2009, 124 p.
157. **Tsipe Aavik.** Plant species richness, composition and functional trait pattern in agricultural landscapes – the role of land use intensity and landscape structure. Tartu, 2008, 112 p.
158. **Kaja Kiiver.** Semliki forest virus based vectors and cell lines for studying the replication and interactions of alphaviruses and hepaciviruses. Tartu, 2009, 104 p.
159. **Meelis Kadaja.** Papillomavirus Replication Machinery Induces Genomic Instability in its Host Cell. Tartu, 2009, 126 p.
160. **Pille Hallast.** Human and chimpanzee Luteinizing hormone/Chorionic Gonadotropin beta (*LHB/CGB*) gene clusters: diversity and divergence of young duplicated genes. Tartu, 2009, 168 p.
161. **Ain Vellak.** Spatial and temporal aspects of plant species conservation. Tartu, 2009, 86 p.

162. **Triinu Remmel.** Body size evolution in insects with different colouration strategies: the role of predation risk. Tartu, 2009, 168 p.
163. **Jaana Salujõe.** Zooplankton as the indicator of ecological quality and fish predation in lake ecosystems. Tartu, 2009, 129 p.
164. **Ele Vahtmäe.** Mapping benthic habitat with remote sensing in optically complex coastal environments. Tartu, 2009, 109 p.
165. **Liisa Metsamaa.** Model-based assessment to improve the use of remote sensing in recognition and quantitative mapping of cyanobacteria. Tartu, 2009, 114 p.
166. **Pille Säälik.** The role of endocytosis in the protein transduction by cell-penetrating peptides. Tartu, 2009, 155 p.
167. **Lauri Peil.** Ribosome assembly factors in *Escherichia coli.* Tartu, 2009, 147 p.
168. **Lea Hallik.** Generality and specificity in light harvesting, carbon gain capacity and shade tolerance among plant functional groups. Tartu, 2009, 99 p.
169. **Mariliis Tark.** Mutagenic potential of DNA damage repair and tolerance mechanisms under starvation stress. Tartu, 2009, 191 p.
170. **Riinu Rannap.** Impacts of habitat loss and restoration on amphibian populations. Tartu, 2009, 117 p.
171. **Maarja Adojaan.** Molecular variation of HIV-1 and the use of this knowledge in vaccine development. Tartu, 2009, 95 p.
172. **Signe Altmäe.** Genomics and transcriptomics of human induced ovarian folliculogenesis. Tartu, 2010, 179 p.
173. **Triin Suvi.** Mycorrhizal fungi of native and introduced trees in the Seychelles Islands. Tartu, 2010, 107 p.
174. **Velda Lauringson.** Role of suspension feeding in a brackish-water coastal sea. Tartu, 2010, 123 p.
175. **Eero Talts.** Photosynthetic cyclic electron transport – measurement and variably proton-coupled mechanism. Tartu, 2010, 121 p.
176. **Mari Nelis.** Genetic structure of the Estonian population and genetic distance from other populations of European descent. Tartu, 2010, 97 p.
177. **Kaarel Krjutškov.** Arrayed Primer Extension-2 as a multiplex PCR-based method for nucleic acid variation analysis: method and applications. Tartu, 2010, 129 p.
178. **Egle Köster.** Morphological and genetical variation within species complexes: *Anthyllis vulneraria* s. l. and *Alchemilla vulgaris* (coll.). Tartu, 2010, 101 p.
179. **Erki Õunap.** Systematic studies on the subfamily Sterrhinae (Lepidoptera: Geometridae). Tartu, 2010, 111 p.
180. **Merike Jõesaar.** Diversity of key catabolic genes at degradation of phenol and *p*-cresol in pseudomonads. Tartu, 2010, 125 p.
181. **Kristjan Herkül.** Effects of physical disturbance and habitat-modifying species on sediment properties and benthic communities in the northern Baltic Sea. Tartu, 2010, 123 p.

182. **Arto Pulk.** Studies on bacterial ribosomes by chemical modification approaches. Tartu, 2010, 161 p.
183. **Maria Põllupüü.** Ecological relations of cladocerans in a brackish-water ecosystem. Tartu, 2010, 126 p.
184. **Toomas Silla.** Study of the segregation mechanism of the Bovine Papillomavirus Type 1. Tartu, 2010, 188 p.
185. **Gyaneshwer Chaubey.** The demographic history of India: A perspective based on genetic evidence. Tartu, 2010, 184 p.
186. **Katrin Kepp.** Genes involved in cardiovascular traits: detection of genetic variation in Estonian and Czech populations. Tartu, 2010, 164 p.
187. **Virve Sõber.** The role of biotic interactions in plant reproductive performance. Tartu, 2010, 92 p.
188. **Kersti Kangro.** The response of phytoplankton community to the changes in nutrient loading. Tartu, 2010, 144 p.
189. **Joachim M. Gerhold.** Replication and Recombination of mitochondrial DNA in Yeast. Tartu, 2010, 120 p.
190. **Helen Tammert.** Ecological role of physiological and phylogenetic diversity in aquatic bacterial communities. Tartu, 2010, 140 p.
191. **Elle Rajandu.** Factors determining plant and lichen species diversity and composition in Estonian *Calamagrostis* and *Hepatica* site type forests. Tartu, 2010, 123 p.
192. **Paula Ann Kivistik.** ColR-ColS signalling system and transposition of Tn*4652* in the adaptation of *Pseudomonas putida.* Tartu, 2010, 118 p.
193. **Siim Sõber.** Blood pressure genetics: from candidate genes to genome-wide association studies. Tartu, 2011, 120 p.
194. **Kalle Kipper.** Studies on the role of helix 69 of 23S rRNA in the factor-dependent stages of translation initiation, elongation, and termination. Tartu, 2011, 178 p.
195. **Triinu Siibak.** Effect of antibiotics on ribosome assembly is indirect. Tartu, 2011, 134 p.
196. **Tambet Tõnissoo.** Identification and molecular analysis of the role of guanine nucleotide exchange factor RIC-8 in mouse development and neural function. Tartu, 2011, 110 p.
197. **Helin Räägel.** Multiple faces of cell-penetrating peptides – their intracellular trafficking, stability and endosomal escape during protein transduction. Tartu, 2011, 161 p.
198. **Andres Jaanus.** Phytoplankton in Estonian coastal waters – variability, trends and response to environmental pressures. Tartu, 2011, 157 p.
199. **Tiit Nikopensius.** Genetic predisposition to nonsyndromic orofacial clefts. Tartu, 2011, 152 p.
200. **Signe Värv.** Studies on the mechanisms of RNA polymerase II-dependent transcription elongation. Tartu, 2011, 108 p.
201. **Kristjan Välk.** Gene expression profiling and genome-wide association studies of non-small cell lung cancer. Tartu, 2011, 98 p.

202. **Arno Põllumäe.** Spatio-temporal patterns of native and invasive zooplankton species under changing climate and eutrophication conditions. Tartu, 2011, 153 p.
203. **Egle Tammeleht.** Brown bear (*Ursus arctos*) population structure, demographic processes and variations in diet in northern Eurasia. Tartu, 2011, 143 p.
205. **Teele Jairus.** Species composition and host preference among ectomycorrhizal fungi in Australian and African ecosystems. Tartu, 2011, 106 p.
206. **Kessy Abarenkov.** PlutoF – cloud database and computing services supporting biological research. Tartu, 2011, 125 p.
207. **Marina Grigorova.** Fine-scale genetic variation of follicle-stimulating hormone beta-subunit coding gene (*FSHB*) and its association with reproductive health. Tartu, 2011, 184 p.
208. **Anu Tiitsaar.** The effects of predation risk and habitat history on butterfly communities. Tartu, 2011, 97 p.
209. **Elin Sild.** Oxidative defences in immunoecological context: validation and application of assays for nitric oxide production and oxidative burst in a wild passerine. Tartu, 2011, 105 p.
210. **Irja Saar**. The taxonomy and phylogeny of the genera *Cystoderma* and *Cystodermella* (Agaricales, Fungi). Tartu, 2012, 167 p**.**
211. **Pauli Saag.** Natural variation in plumage bacterial assemblages in two wild breeding passerines. Tartu, 2012, 113 p.
212. **Aleksei Lulla.** Alphaviral nonstructural protease and its polyprotein substrate: arrangements for the perfect marriage. Tartu, 2012, 143 p.
213. **Mari Järve.** Different genetic perspectives on human history in Europe and the Caucasus: the stories told by uniparental and autosomal markers. Tartu, 2012, 119 p.
214. **Ott Scheler**. The application of tmRNA as a marker molecule in bacterial diagnostics using microarray and biosensor technology. Tartu, 2012, 93 p.
215. **Anna Balikova**. Studies on the functions of tumor-associated mucin-like leukosialin (CD43) in human cancer cells. Tartu, 2012, 129 p.
216. **Triinu Kõressaar.** Improvement of PCR primer design for detection of prokaryotic species. Tartu, 2012, 83 p.
217. **Tuul Sepp.** Hematological health state indices of greenfinches: sources of individual variation and responses to immune system manipulation. Tartu, 2012, 117 p.
218. **Rya Ero.** Modifier view of the bacterial ribosome. Tartu, 2012, 146 p.
219. **Mohammad Bahram.** Biogeography of ectomycorrhizal fungi across different spatial scales. Tartu, 2012, 165 p.