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Translocation of cell-penetrating peptides across biological membranes and interactions with plasma membrane constituents

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Translocation of cell-penetrating peptides across biological membranes and interactions with plasma membrane constituents

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Press

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LIST OF ORIGINAL PUBLICATIONS

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

I Säälik, P., Niinep, A., **Pae, J**., Hansen, M., Lubenets, D., Langel, Ü., Pooga, M. (2011). Penetration without cells: membrane translocation of cell-penetrating peptides in the model giant plasma membrane vesicles. Journal of Controlled Release, 153(2):117–125.

II **Pae, J**., Säälik, P., Liivamägi, L., Lubenets, D., Arukuusk, P., Langel, Ü., Pooga, M. (2014). Translocation of cell-penetrating peptides across the plasma membrane is controlled by cholesterol and microenvironment created by membranous proteins. Journal of Controlled Release. 192: 103–113.

III **Pae, J**., Liivamägi, L., Lubenets, D., Arukuusk, P., Langel, Ü., Pooga, M. (2016). Glycosaminoglycans are required for translocation of amphipathic cell-penetrating peptides across membranes. BBA – Biomembranes 1858(8):1860–1867.

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My contribution to the articles referred to in this study is following:

Paper I performed part of the confocal microscopy experiments, participated in writing of the paper

Paper II designed and performed the experiments, analyzed the data and wrote the paper

Paper III designed the study and performed majority of the experiments, analyzed the data and wrote the paper

ABBREVIATIONS

Arg_9	Nona-arginine
CHO	Chinese Hamster Ovary
CLSM	Confocal laser scanning microscopy
CPP	Cell-penetrating peptide
CS	Chondroitin sulfate
DS	Dermatan sulfate
DTT	Dithiothreitol
FA	Formaldehyde
FACS	Fluorescence-activated cell sorting
GAG	Glycosaminoglycan
GAG^{neg}	Glycosaminoglycan deficient
GPI	Glycosylphosphatidylinositol
GPMV	Giant plasma membrane vesicle
GUV	Giant unilamellar vesicle
HA	Hyaluronic acid
HP	Heparin
HS	Heparan sulfate
HS^{neg}	Heparan sulfate deficient
HSPG	Heparan sulfate proteoglycan
KS	Keratan sulfate
L_d	Liquid-disordered
L_o	Liquid-ordered
LUV	Large unilamellar vesicle
MAP	Model amphipathic peptide
MCD	Methyl-β-cyclodextrin
NEM	N-ethylmaleimide
pAntp	Penetratin
PFA	Paraformaldehyde
$PI(4,5)P_2$	Phosphatidylinositol-4,5-bisphosphate
pTat	Tat peptide
TP	Transportan
TP10	Transportan10
WFA	*Wisteria floribunda* agglutinin
WGA	Wheat germ agglutinin
WT	Wild-type

INTRODUCTION

Introduction of a vast number of biomolecules with high therapeutic potential into experimental medical and clinical practice is hampered by the poor plasma membrane permeability of these macromolecules. To overcome this major bottleneck, development of cellular delivery vectors has increasingly been in the focus of biomedical research.

One class of very promising delivery tools is cell-penetrating peptides (CPPs), short amino acid sequences that are capable of efficient cellular transport of various bioactive macromolecules. The mechanisms and pathways of cellular entry of CPPs have been intensely studied in last two decades, and while it is widely acknowledged that these peptides internalize using different modes of endocytosis, the second internalization route – direct translocation through the plasma- or endosomal membrane, has remained elusive so far. Furthermore, despite the extensive research, the understanding of the exact role of specific cell surface molecules mediating the direct translocation of CPPs across membranes is still controversial.

The detailed identification of the molecular interactions with the plasma membrane constituents and more detailed knowledge about the exact internalization mechanism of CPPs would enable the design and efficient usage of these cellular delivery vehicles. Thus, the main purpose of this thesis was to pinpoint the specific binding partners of CPPs on the cell surface and the membrane dynamics that enable direct translocation through the cellular membranes. The CPPs studied in this thesis are widely used representatives of the cationic, arginine-rich (nona-arginine and Tat peptide) and amphipathic (transportan, TP10, penetratin, MAP) peptide classes, which have proven to be efficient transporters for various macromolecular cargoes.

The constant flow of cellular processes in living cells, including endocytosis, impedes assessment of direct translocation of CPPs across biomembranes. To overcome this obstacle, we, for the first time utilized in our studies simple and a highly relevant model membrane system – giant plasma membrane vesicles (GPMVs), that maintain the compositional complexity of biological membranes, but are devoid of endocytosis. By using GPMVs we demonstrate the role of several plasma membrane constitutes, as well as reveal the specific membrane composition, essential for the translocation. More precisely, we show that direct translocation of arginine-rich CPPs is mediated by protein component of the plasma membrane, and the internalization of these peptides can be increased via induction of ceramide in the membrane of GPMVs; while the membrane penetration of amphipathic CPPs is strongly dependent on the lipid packing and takes place at more dynamic membrane regions. Finally, we show that glycosaminoglycans are crucial for the internalization of amphipathic peptides, while these cell surface polysaccharides have little influence on the direct crossing of arginine-rich CPPs through the membrane.

The current study provides an overview of the cell surface constituents that are required for the direct translocation of CPPs across membranes and analyses the lipid components of the membrane that facilitate or interfere with the cellular entry of CPPs.

1. LITERATURE OVERVIEW

1.1 Cellular delivery systems

A wide range of bioactive molecules, including potentially efficient drugs and gene therapeutics, are not applicable in clinical practice because of the poor translocation into cells through the protective lipid bilayer. Intracellular carrier systems have a vital role in overcoming this limiting step. The field of cellular delivery systems has therefore undergone an increasing and rapid progress over the past decades. A vast number of different chemical and physical methods and carriers for cellular transport have been developed for biomedical research, including electroporation, viral vectors, liposomes, microspheres, nanoparticles and polymers, microinjection, etc. However, the field still faces many challenges and the use of carrier systems is often hampered by their cellular toxicity, low efficacy and immunogenicity.

In past three decades, peptide-based delivery vehicles have emerged as a powerful platform for the intracellular delivery. These amino acid sequences, capable of cellular transport, are called peptide transduction domains or simply cell-penetrating peptides (CPPs). CPPs are defined as *"relatively short peptides, 4–40 amino acids, with the ability to gain access to the cell interior by means of different mechanisms, mainly including endocytosis, and/or with the capacity to promote the intracellular effects by these peptides themselves, or by the delivered covalently or noncovalently conjugated bioactive cargoes."* (Langel 2015).

Over the years, CPPs have been used to deliver numerous bioactive payloads that include peptides and proteins, oligonucleotides, plasmid DNA and silencing RNA, small-molecule drugs (reviewed in Meade and Dowdy 2007; Lehto *et al.* 2012; Vasconcelos *et al.* 2013; Ramsey and Flynn 2015). By omitting fundamental shortcomings of many delivery systems, CPPs with a relatively low or no cytotoxicity (Saar *et al.* 2005; Tünnemann *et al.* 2008; Suhorutšenko *et al.* 2011) and highly efficient delivery rates, were quickly recognized as powerful tools for the intracellular delivery of macromolecules that possess high biomedical and biotechnological potential.

1.2 Cell-penetrating peptides

First evidence that certain amino acid sequences are able to penetrate the plasma membrane emerged in 1988, when it was discovered that Tat protein from human immunodeficiency virus (HIV-1) is taken up by cells and can *trans*-activate the viral promoter (Frankel and Pabo 1988; Green and Loewenstein 1988). Few years later, another discovery confirmed the phenomenon of translocating proteins when the group of A. Prochiantz showed that a homeodomain of the Antennapedia protein from *Drosophila* is able to penetrate the membrane of neurons (Joliot *et al.* 1991). The research field of CPPs was established in 1994, when the same group discovered that the whole sequence of homeodo-

main of Antennapedia is not necessary for the penetration and a 16 amino acid long peptide sequence is required for the translocation through the membrane (Derossi *et al.* 1994). Shortly after that, similar results were obtained with truncated versions of Tat protein (Vives *et al.* 1997; Schwarze *et al.* 1999). In 1998, a chimeric peptide transportan was designed, which induced peptide nucleic acid transport into the cells (Pooga *et al.* 1998). Since these discoveries were made this field of research has expanded rapidly and new CPPs have been designed to use these peptides as transport vectors for the therapeutic and biotechnologically important molecules. Large numbers of new CPPs have been found and to date, over 1850 peptides have cell-penetrating properties according to CPP database, gathered from research papers and published patents (Agrawal *et al.* 2016). CPPs can vary greatly in their length and amino acid sequence, causing major variations in their physicochemical properties. However, CPPs still hold some common features, such as relatively short length and most of them contain cationic amino acids; and are therefore positively charged at the physiological pH. Some CPPs are amphipathic, containing hydrophobic and hydrophilic amino acids (Zorko and Langel 2005; Ziegler 2008).

In clinical setting it is critical that transport vectors that bind the cargo molecules would transport the bioactive molecules into the cells with high efficiency and specificity without causing any side effects. CPPs have kept up with the fast pace of medical demands and the field of CPPs have been expanded tremendously, as CPPs have been modified or new ones designed for improved cellular uptake (reviewed in Wang *et al.* 2014; Kurrikoff *et al.* 2016). Furthermore, CPPs have been incorporated into nanoparticles, liposomes and conjugated with other chemical moieties (Mäe *et al.* 2009; Biswas *et al.* 2013; Kurrikoff *et al.* 2016) for the enhanced drug delivery and longer retention time. CPPs are also meeting the needs for the development of intelligent drug delivery systems that can be specifically targeted (Myrberg *et al.* 2008; Svensen *et al.* 2012; Teesalu *et al.* 2013).

1.2.1 Classification of CPPs

A high and still growing number of CPPs makes their classification a difficult task. However, two main approaches to classifying CPPs can be distinguished. The first and historical categorization is based on CPPs origin, dividing the peptides into three main classes: protein-derived (e.g Tat peptide), synthetic (e.g oligoarginine) and chimeric (e.g. transportan) peptides (Pooga and Langel 2015). The second and more common approach for grouping CPPs is based on the physicochemical properties of the peptides, which divides them into three major classes: cationic, amphipathic and hydrophobic (Milletti 2012). The first classification gives a good overview of the CPPs origin, but the second approach provides more information about the characteristics of the CPPs, and is therefore often preferred. CPPs used in this thesis, their origin and division into classes are presented in Table 1.

Table 1. Examples of cell-penetrating peptides

Peptide	Sequence	Origin	Ref.
Cationic			
Oligo-arginine	$(R)_n$; n=6–12	*Synthetic* Based on Tat peptide	(Mitchell *et al.* 2000)
Tat peptide	GRKKRRQRRRPPQ	*Protein-derived* HIV-1 Tat Protein (48–60)	(Vives *et al.* 1997)
Primary amphipathic			
Transportan	GWTLNSAGYLLGKI NLKALAALAKKIL	*Chimeric* Galanin/Mastoparan	(Pooga *et al.* 1998)
TP10	AGYLLGKINLKALAA LAKKIL	*Chimeric* Transportan analogue	(Soomets *et al.* 2000)
Secondary amhipathic			
Penetratin	RQIKIWFQNRRMKW KK	*Protein-derived* Antennapedia homeodomain (43–58)	(Derossi *et al.* 1994)
MAP	KLALKLALKALKAA LKLA	*Synthetic* Model Amphipathic Peptide	(Oehlke *et al.* 1998)

1.2.1.1 Cationic cell-penetrating peptides

The majority of CPPs hold a net positive charge at physiological conditions (Milletti 2012), which stems from the abundance of cationic amino acids in the sequence of CPPs. Among positively charged amino acids like histidine, lysine, ornithine and arginine, the latter is by far the most common amino acid in the sequence of CPPs. For this reason, the class of cationic cell-penetrating peptides is sometimes also referred to as arginine-rich CPPs. In fact, arginine has shown to be the most efficient for inducing internalization among all the positively charged amino acids (Mitchell *et al.* 2000; Tünnemann *et al.* 2008; Åmand *et al.* 2008). Furthermore, peptides composed of only arginines have proven to be highly potent transporters, while poly-lysine, -ornithine and -histidine have poor cellular uptake (Mitchell *et al.* 2000; Tünnemann *et al.* 2008). The number of arginines is also an important parameter for efficient transduction. Polyarginines composed of less than 6 amino acids result in low internalization efficacies. Although longer sequences have better cellular uptake (less than 20 arginines), these have shown to be more cytotoxic (Mitchell *et al.* 2000; Futaki *et al.* 2001) and have elevated absorption to serum proteins (Kosuge *et al.* 2008), which in turn prevents the internalization. Therefore, the optimal number of arginine

residues has been considered to be between 8 and 12 (Futaki *et al.* 2001; Kosuge *et al.* 2008). Although cationic nature of arginine favours electrostatic interactions with negatively charged components of biological membranes, the positive charge is not the only parameter that can be attributed to the higher degree of internalization of arginine-containing CPPs, since the rest of the basic amino acids internalize in a much lower extent. Thus, the key property of arginine that determines the increased cellular uptake is its guanidinium group, which can form two hydrogen bonds with anionic compounds, like phosphates, sulfates and carboxylates, promoting the interactions with the membrane components; whereas lysine can only form one hydrogen bond (Mitchell *et al.* 2000; Rothbard *et al.* 2004). It has been also proposed that lysine and arginine both induce negative curvature in membranes through electrostatic interactions, but the guanidinium group can also generate membrane positive curvature through multidentate bonds with lipid molecules. This results in the formation of saddle-splay curvature that was suggested to be the basis for the higher transduction ability of arginine-containing peptides (Mishra *et al.* 2011).

Other well-known and thoroughly studied arginine-rich CPPs are various amino acid sequences from the Tat protein (Vives *et al.* 1997). Tat is a transcription factor that facilitates HIV-1 replication; and by testing many sequences from the range of amino acids, which are responsible for the translocation of the protein, it was found that out of the tested peptides, the amino acid sequence 48–60 (GRKKRRQRRRPPQ) internalized most efficiently (Vives *et al.* 1997). Furthermore, this sequence also contains nuclear localization signal at the C-terminus (Ruben *et al.* 1989), which explains the high accumulation of the peptide into the nucleus (Vives *et al.* 1997).

1.2.1.2 Amphipathic cell-penetrating peptides

Some CPPs have distinguishable hydrophilic and hydrophobic regions and are therefore classified as amphipathic. This group can be further subcategorized as primary and secondary amphipathic CPPs (Ziegler 2008). Cationic and hydrophobic amino acids in primary amphipathic CPPs are ordered in a way that there is a clear separation between the respective hydrophilic and hydrophobic part in the structure of the peptide. The first region favours dissolution of the peptide in water and binding to the negatively charged groups in the membrane. The hydrophobic part facilitates interactions with the lipids (Deshayes *et al.* 2004b; Fernandez-Carneado *et al.* 2004). One well-known primary amphipathic CPP is a chimeric peptide transportan (TP). TP is a 27 amino acid long peptide, containing 12 amino acids from the N-terminus of the neuropeptide galanin, which is conjugated through a lysine to 14 amino acids long wasp venom toxin peptide mastoparan (Pooga *et al.* 1998). Another well-known CPP belonging to this group is transportan analogue TP10, which has the same sequence as TP, but lacks 6 amino acids from the N-terminus (Soomets *et al.* 2000). By further modifying TP10, the next generation of efficient CPPs, PepFects and NickFects, have been created (Mäe *et al.* 2009; Oskolkov *et al.* 2011; Arukuusk *et al.*

2013a). Based on studies with artificial membranes, TP and TP10 both adopt an α-helical structure to a various degree (Soomets *et al.* 2000; Barany-Wallje *et al.* 2004). This amphipathic helix, as well as tyrosine moiety in the peptide sequence, was proposed to be necessary for the interactions and cellular uptake of TP and its analogues (Soomets *et al.* 2000).

Secondary amphipathic peptides do not have distinctive hydrophilic and hydrophobic segments; however, these peptides acquire their amphipathic nature upon association with the membrane components, when the peptide adopts α-helical or β-strand secondary structure, where charged polar amino acids are located on one side and non-polar amino acids on another side (Fernandez-Carneado *et al.* 2004; Ziegler 2008). The most widely known and thoroughly studied secondary amphipathic CPP is a 16 amino acid long penetratin; and as discussed above, it is derived from the third helix of *Drosophila* Antennapedia homeodomain of the homeoprotein (Derossi *et al.* 1994). Although penetratin is often classified as cationic, it acquires α-helical structure in the presence of negatively charged lipids (Alves *et al.* 2008; Maniti *et al.* 2010), a common characteristic of secondary amphipathic peptides; whereas in the presence of neutral lipids, no defined secondary structure could be observed (Maniti *et al.* 2010). The prototypic representative of secondary amphipathic peptides is an 18 amino acid long MAP (model amphipathic peptide) (Oehlke *et al.* 1998). Upon interacting with the membrane, MAP forms a perfect α-helical structure, resulting from the specific order of positively charged amino acid lysine and hydrophobic amino acids leucine and alanine. Due to these amino acids, this peptide is also often called KLAK.

1.2.1.3 Hydrophobic cell-penetrating peptides

Only a few members of CPPs fall into this category. The representatives of this CPP class consist mostly of hydrophobic amino acids and contain only a low number of cationic or even anionic amino acids. For example, Pep-7 is mostly hydrophobic, but also contains few anionic amino acids in its sequence, yielding the net negative charge –2 (Gao *et al.* 2002).

1.3 Mechanisms of CPP internalization

Endocytosis is the primary cellular internalization pathway for the majority of macromolecules. Therefore, the early understanding that CPPs avoid endocytosis and can directly penetrate through the membrane was rather unconventional. However, not long after their discovery, it was demonstrated that CPPs internalize also via different types of endocytic routes; and by now it is well established that the cellular uptake of CPPs involves both pathways, endocytosis and direct translocation. The cellular internalization route depends strongly on the peptide sequence, its physicochemical properties (Tünnemann *et al.* 2006; Jiao *et al.* 2009), target cell or tissue type (Tünnemann *et al.* 2006; Duchardt *et*

al. 2007; Jiao *et al.* 2009), peptide concentration (Duchardt *et al.* 2007; Kosuge *et al.* 2008; Jiao *et al.* 2009; Melikov *et al.* 2015), size and physicochemical nature of the cargo, attached to the peptide (Erazo-Oliveras *et al.* 2012).

1.3.1 Direct translocation

Early research in the field of CPPs revealed that these peptides can directly penetrate the cellular membrane and the internalization does not require cellular energy, and therefore it is independent of endocytosis. Although, since then, a great number of studies have demonstrated that the cellular uptake of CPPs proceeds mostly via different endocytosis pathways, it is also well established by now that the unique internalization pathway of CPPs – direct translocation through the membrane is frequently utilized. This unconventional internalization of CPPs via direct penetration raises the question, how CPPs can directly surpass the protective lipid bilayer, while other macromolecules gain access to cells via endocytosis. There is a great volume of studies describing the penetration mechanism, but several controversies still exist. An overview of some of the most well-appreciated mechanisms and models of direct translocation will be presented below.

Inverted micelle model

The first model proposed to explain the mechanism of CPP translocation was the inverted micelle model suggested for the internalization of penetratin to cells (Derossi *et al.* 1996). According to this model, the electrostatic interactions between the CPP and negatively charged membrane components facilitate the accumulation of the peptide to the plasma membrane. This leads to invaginations, which in turn will be covered with lipids, resulting in the formation of a micelle, where the peptide localizes inside the hydrophilic cavity. The peptide trapped in the micelle destabilises the membrane and is subsequently released into the cytoplasm (Figure 1A) (Derossi *et al.* 1996). Later, it was shown that penetratin can induce non-lamellar lipid structures in model membranes and as in the study discussed above, the formation of inverted-micelle like structures was reasoned to be the method for direct translocation through the lipid bilayer (Alves *et al.* 2008). Other arginine-rich CPPs, such as pTat and Arg_9, but also hydrophobic amino acid-containing R6/W3, have been demonstrated to operate through the same mechanism, supporting the concept that direct penetration can include the formation of inverted micelle-like structures (Afonin *et al.* 2006; Swiecicki *et al.* 2014).

Pore formation

Many CPPs share some common features with antimicrobial peptides, such as positive charge, amphipathicity and relatively short sequence (Melo *et al.* 2009). Therefore, several internalization mechanisms, such as pore formation that are attributed to antimicrobial peptides, have been also proposed as the direct trans-

location modes of CPPs. According to a barrel-stave pore, the peptide inserts into the membrane perpendicularly and forms a helix, with the hydrophobic amino acids facing the membrane. Hydrophilic amino acid residues form the internal part of the pore, giving rise to a structure that resembles a barrel (Figure 1B) (Lundberg and Langel 2003).

The second proposed model is a toroidal pore, where similarly to barrel-stave, the peptide inserts into the membrane in a perpendicular orientation. Interactions between the peptide and phospholipid head groups result in the induction of the membrane curvature and the formation of a pore, where lipid head groups partially line the lumen of the pore (Figure 1C). A modification of this model is a disordered toroidal pore, where similar structures form, but the insertion of the peptide into the membrane is more stochastic (Melo *et al.* 2009).

According to the carpet model, cationic CPPs interact with negatively charged phospholipids and CPPs cover the membrane by orienting themselves in parallel to the bilayer, similarly to a carpet. At higher peptide concentrations, although no permanent pore is formed, the plasma membrane is disrupted, which results in the displacement of the phospholipids and leads to the internalization of peptides (Figure 1D) (Lundberg and Langel 2003).

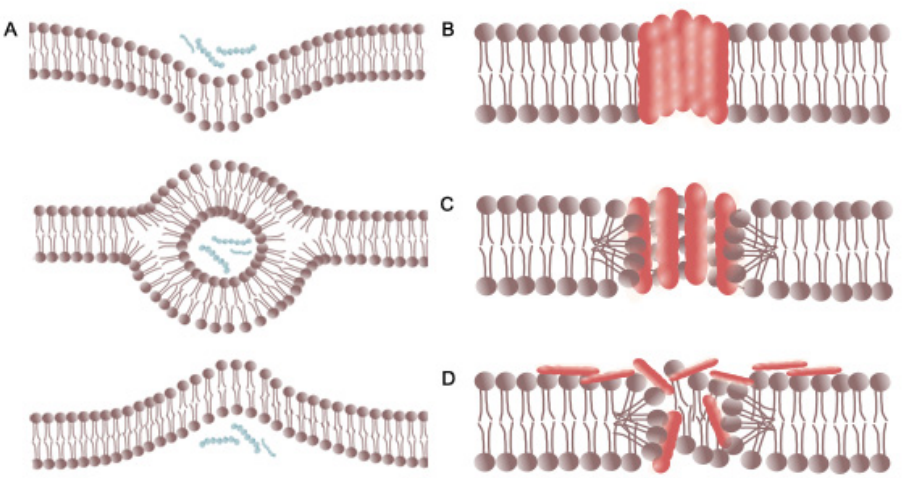


Figure 1. Translocation models of CPPs. A. Inverted micelle model. The peptide accumulates to the plasma membrane leading to invaginations, which will be covered with lipids. This results in the formation of a micelle, where the peptide localizes inside the hydrophilic cavity. The peptide trapped in the micelle destabilises the membrane and is subsequently released into the cytoplasm **B.** Barrel-stave pore. The peptide inserts into the membrane perpendicularly and forms a barrel-like structure **C.** Toroidal pore. The peptide inserts into the membrane in a perpendicular orientation. Peptide induces membrane curvature and the formation of a pore, where lipid head groups partially line the lumen of the pore **D.** Carpet model. Peptides cover the membrane by orienting themselves in parallel to the bilayer, similarly to a carpet. At higher peptide concentrations, the membrane is disrupted, which results in the displacement of the phospholipids and internalization of the peptides.

Although none of the above-mentioned models has a real factual proof as membrane permeabilization mechanisms for CPPs, numerous studies, predominantly with model lipid bilayers, suggest that pore formation is indeed one of the penetration mechanisms. Formation of a pore is usually anticipated when the internalization of the peptide into liposomes is accompanied by an influx or efflux of dye or dextran molecules of various sizes. By using these tracer molecules, it was demonstrated that Tat peptide can form pores of nanometer-size (Ciobanasu *et al.* 2010). However, a relatively high concentration of anionic or negative curvature-inducing lipids was required for induction of pores and vesicle disruption. A few other studies have proposed that the induction of membrane curvature precedes pore formation by Tat peptide (Mishra *et al.* 2008; Mishra *et al.* 2011). The formation of pore-like structure has been also suggested as the internalization mechanism for Pep-1 and MPG peptide (Deshayes *et al.* 2004a; Deshayes *et al.* 2006). Both peptides adopt α-helical and β-sheet secondary structures in the presence of lipids, respectively. These secondary structures are commonly found in the polypeptide chains in transmembrane proteins, which traverse the lipid bilayer as a single or multiple α-helix/helices or as a β-sheet that form barrel-like structures (Alberts *et al.* 2013); and similarly could favour the interactions of CPPs with the membrane. Furthermore, the majority of these polypeptides in transmembrane proteins form an α-helix, where approximately 20 amino acids are required to cross the cell membrane (Alberts *et al.* 2013). If to draw parallels, only some CPPs, such as transportan and TP10, could theoretically span through the membrane in a similar fashion. Furthermore, both transportans are amphipathic and adopt the respective secondary structure similarly to some antimicrobial peptides that are prone to form pores in the membrane (Melo *et al.* 2009). This concept is supported by several studies, which proposed that the internalization of TP10 was accompanied by the formation of pores (Anko *et al.* 2012; Islam *et al.* 2014). Carpet-like membrane destabilization upon penetration of TP10 into cells has been also observed (Yandek *et al.* 2007). However, in general, carpet-like internalization would require high local concentration of the peptide (Shai and Oren 2001), which might not reflect the real situation *in vivo*. Similarly, the probability of the formation of the permanent barrel-stove pore has been proposed to be rather low, as for macromolecule passage, at least 20 peptides are needed for the formation of the "ring" that would have to be stable (Wimley 2015). In light of this view, if one of the aforementioned models would apply, the most probable scenario would include the formation of the (disordered) toroidal pore-like temporal membrane destabilization, as recently suggested by Wimley (Wimley 2015).

Destabilization and reorganization of lipid bilayers

Besides the aforementioned models, many other mechanisms have been proposed for CPP penetration that include destabilization and reorganization of membranes. For example, dodeca-arginine was demonstrated to internalize via induction of particle-like multilamellar structures (Hirose *et al.* 2012). Similarly,

nona-arginine and amphipathic peptide RW9 were shown to provoke the formation of multilamellar membrane particles on the surface of the plasma membrane spheres (Maniti *et al.* 2014). Furthermore, according to the same study, the membrane permeabilization of two amphipathic CPPs, RW9 and RW16, included segregation of membrane`s lipid phases; a similar penetration mechanism was observed earlier in the case of penetratin (Lamaziere *et al.* 2010).

Internalization from boundary regions between lipid phases

Research into the membrane composition-dependent translocation suggests that CPPs might internalize via the boundary regions of either peptide-induced or innate lipid phases of membranes. In the case of the first mode, the internalization of a nona-arginine peptide was shown to induce the formation of ceramide-enriched regions in the plasma membrane. The penetration of nona-arginine into cells was proposed to take place at the boundary regions of those "rafts" and the rest of the membrane, at so-called nucleation zones (Verdurmen *et al.* 2010). Similarly, nucleation zones have been previously described as translocation sites into cells also for nona-arginine and Tat peptide at higher concentrations (Duchardt *et al.* 2007). Peptide-induced lateral lipid phase separation and resulting defect lines in the interface region between different domains were suggested to be also favourable sites for translocation of S4(13)-PV peptide (Cardoso *et al.* 2012). Furthermore, CPP-induced lipid segregation and internalization from the membrane "defect" regions with higher fluidity has been suggested for several other peptides as well (Joanne *et al.* 2009). The lipid "fault lines" could indeed present favourable sites for the reorganization of lipids and subsequent penetration, as lipid phase boundary regions could lower the energy that is needed for the charged peptides to cross the membrane.

Membrane tubulation and physical endocytosis

Studies with model membrane systems demonstrate that some CPPs can also induce membrane tubulation and invagination termed as "physical endocytosis" (Lamaziere *et al.* 2007; Lamaziere *et al.* 2008; Maniti *et al.* 2012). For example, penetratin can induce negative curvature in model membranes (Lamaziere *et al.* 2008). However, by using vesicles with different lipid composition, which mimic membranes with a varying degree of lipid-packing density, penetratin was able to induce membrane curvature and tubulation only in vesicles that had higher membrane fluidity. Analogous results were also obtained with plasma membrane spheres, which by their constitution resemble plasma membrane very closely (Maniti *et al.* 2012). Furthermore, several other studies have demonstrated the formation of CPP-induced tubular structures (Lamaziere *et al.* 2007; Lamaziere *et al.* 2010; Anko *et al.* 2012) and consecutive tubule rolling (Maniti *et al.* 2014).

Lamaziere *et al.* systematically analysed the membrane activity of several basic and amphipathic CPPs in relation to their length, amphipathicity and total charge. Their study reveals that cationic non-amphipathic CPPs induce membrane aggregation, tubulation and negative curvature in the membrane, while am-

phipathic CPPs induce membrane deformations (pores), the formation of tubular structures, and generation of small vesicles (physical endocytosis). Cationic CPPs and shorter amphipathic peptides, in contrary, could not induce pores (Lamaziere *et al.* 2007).

1.3.2 Endocytosis

Endocytosis is the "digestive" system of the cell that involves two types of basic uptake modes: phagocytosis and pinocytosis. The former is responsible for taking up large particles into cells and the latter facilitates the ingestion of smaller molecules and fluid. Phagocytosis occurs only in specific cells like macrophages, while pinocytosis is common for all eukaryotic cells (Alberts *et al.* 2013). Extracellular fluid and macromolecules, including CPPs, are transported into cells via different routes of pinocytosis, which are classified as macropinocytosis, clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis, and caveolae- and clathrin-independent endocytosis (Kumari *et al.* 2010).

Macropinocytosis is considered to be largely a non-selective endocytic route, through which bigger particles that are attached to the plasma membrane, as well as membrane and fluid with solutes are ingested (Swanson 2008). The induction of macropinocytosis is regulated through signalling and is induced upon stimuli. The exact regulation, however, has remained elusive so far, but has been shown to be dependent on actin and cholesterol (Doherty and McMahon 2009). This pathway usually entails membrane protrusions and ruffling, resulting in the formation of vesicles that can be up to a few μm-s in size (Swanson 2008). A large and growing body of literature, describing CPP endocytic entry, has considered macropinocytosis as one of the prime pathways for CPP-cargo uptake (Nakase *et al.* 2004; Wadia *et al.* 2004; Kaplan *et al.* 2005; Duchardt *et al.* 2007; Nakase *et al.* 2007). Furthermore, there is very little evidence that CPP-cargo complex would interact with plasma membrane receptors, which would often induce an internalization pathway of higher specificity.

CME is typically triggered by the binding of a ligand molecule to its receptor and a wide variety of different cargo molecules utilize this pathway (Doherty and McMahon 2009). After the initiation of CME, the uptake of material and the vesicle formation is mediated by the clathrin coat. The size of formed vesicles depends on the properties of the cargo and they are markedly smaller than macropinosomes, usually not exceeding 200 nm in diameter (McMahon and Boucrot 2011). In parallel with macropinocytosis, CME has been often shown to mediate the cellular uptake of CPP-cargo constructs (Richard *et al.* 2005; Hassane *et al.* 2011). Moreover, CME often serves as a complementary pathway to macropinocytosis (Petrescu *et al.* 2009), which supports the notion that the internalization of CPPs is not restricted to one endocytic pathway, but rather involves several mechanisms, which might function in parallel.

Caveolin/caveolae-mediated endocytosis appears to be a rather infrequent pathway of cellular uptake harnessed by CPPs. The reason might lie in the fact

that this endocytic route regulates many specific cell-signalling cascades, which are typically activated by a ligand binding to its receptor. The formed vesicles are relatively small, approximately 120 nm of diameter (Kumari *et al.* 2010), and thus, could limit the uptake of bigger CPP-cargo complexes. Nevertheless, caveolin-mediated endocytosis has been shown to mediate the cellular uptake of full-length Tat protein and Tat peptide conjugated to a protein cargo (Ferrari *et al.* 2003; Fittipaldi *et al.* 2003; Säälik *et al.* 2009), as well as transportan and TP10 (Säälik *et al.* 2009). However, the contribution of this particular pathway to the uptake of CPP-cargo was shown to be moderate; and as stated before, alternative routes are probably exploited in parallel (Säälik *et al.* 2009).

Several other endocytosis pathways have been discovered recently, which are grouped as clathrin- and caveolin-independent endocytosis, including CLIC/GEEC-type endocytosis, Arf6 dependent and flotillin-dependent endocytosis (Doherty and McMahon 2009; McMahon and Boucrot 2011). However, these have not shown to contribute to the internalization of CPPs, perhaps because of the insufficient understanding of these cellular transport routes compared to what is known about the aforementioned endocytic pathways.

In general, one common endocytic route cannot be attributed to all CPPs, since the utilized pathway depends on several factors. Furthermore, the internalization can involve many concurrently acting pathways, and if one is down-regulated, CPPs can swiftly switch to another internalization mode (Duchardt *et al.* 2007).

1.3.2.1 Endosomal escape

Ingested material, including CPPs, end up in early endosomes, where it is further directed to either recycling route for externalization or to degradation pathway. In order to exert its biological effect, CPP-cargo construct has to escape from the endosomes to avoid these pathways. In principle, two possible scenarios exist to achieve that: CPPs have to either translocate through the endosomal membrane or rupture it. Translocation seems a more plausible explanation, as membrane rupture would cause cytotoxic effects, which are usually not observed. Regardless of the endosomal escape mechanism, one of the key questions is why the escape from endosomes occurs, while the plasma membrane remains impenetrable. One explanation for this divergence stems in the different composition of the endosomal and plasma membrane. In line with this, higher content of anionic lipids has been shown to promote the interactions with the membrane and subsequent translocation of CPPs (Ciobanasu *et al.* 2010). Since the membrane of endosomes contains more anionic lipids, this could result in the increased endosomal escape (van Meer *et al.* 2008; Erazo-Oliveras *et al.* 2012). Moreover, some CPPs are known to change conformation in the presence of anionic lipids (Alves *et al.* 2008). A change in the conformation that is induced upon interaction with anionic lipids in the membrane of endosomes could facilitate the subsequent escape. Another explanation proposed for the

preferred penetration through the endosomal membrane as opposed to the plasma membrane, is that the electrostatic interactions between the cell surface proteoglycans and CPPs interfere with the association of the peptide with lipids. However, in endosomes, proteoglycans are gradually hydrolysed, as endosomes mature, and CPPs can interact with lipids, leading to their penetration through the endosomal membrane (Fuchs and Raines 2004). A higher peptide to lipid ratio could be the major determinant for the escape of CPPs from the endosomes, since the local peptide concentration is much higher in endosomal vesicles compared with the plasma membrane, and this might trigger liberation of CPPs from endosomes. This view is supported by a recent study where endosomal membrane-mimicking vesicles were used to study the endosomal escape of various arginine-rich cyclic peptides. Remarkably, the translocation efficacy of CPPs was dependent on the endosomal membrane-binding affinity (Qian *et al.* 2016). Furthermore, the cyclic peptide was shown to penetrate through the membrane without causing a remarkable efflux of the reporter dye trapped in "endosomes", indicating that the escape of CPPs from these vesicles did not induce membrane rupture or formation of permanent pores.

Several different strategies have been implemented to enhance the efficiency of cargo delivery by CPPs by increasing the endosomal escape while avoiding the damage to the plasma membrane. One approach is to harness the antimicrobial peptides that destabilize the membrane of endosomes, which was recently used to increase the efficacy of Tat-mediated plasmid DNA transport into cells (Salomone *et al.* 2013). The authors suggested that during the endosomal trafficking, the complex of plasmid DNA with CPP is partially dissociated, which results in the release of the antimicrobial peptide that might destabilize the endosomal membrane. Another common strategy exploits fusion peptides from influenza virus hemagglutinin. Upon acidification of endosomes, the pH sensitive fusion peptide destabilizes the limiting membrane and facilitates the escape of cargo molecules into the cytosol. Thus, the exploitation of the pH differences between the endosome lumen and the plasma membrane avoids the damage to the latter. This method was efficiently utilized for co-incubation of protein cargo with the Tat-fusion peptide conjugate, which markedly increased the endosomal release (Wadia *et al.* 2004). In the same study, a lysosomotropic agent chloroquine was used to increase the endosomal release; however, it led to a very high cytotoxicity. Chloroquine inhibits acidification of endosomes and induces swelling and subsequent rupture of endosomal vesicles. This property of chloroquine was utilized in the design of some of the PepFect family peptides, introducing a modification with its analogue – trifluoromethylquinoline. This moiety has been very potent in facilitating the cargo escape from the endosomes and thereby augmenting its biological effect (Andaloussi *et al.* 2011; Lindberg *et al.* 2013). Furthermore, all the CPPs in the PepFect and NickFect family are chemically modified with a stearyl group, which increases the lipophilicity of the peptides and membrane-destabilizing properties (Mäe *et al.* 2009; Oskolkov *et al.* 2011).

1.4 Plasma membrane and its constituents

1.4.1 Glycocalyx

Cell surface is covered with carbohydrates that form a network called glycocalyx, and this "cell coat" mainly consists of glycans. Glycans are carbohydrates that can be assembled from different combinations of monosaccharides fucose, galactose, glucose, N-acetylgalactosamine, N-acetylglucosamine, glucuronic acid, iduronic acid, mannose, sialic acid and xylose (reviewed in Moremen *et al.* 2012). These structural variations give rise to a vast collection of glycans that can be further modified with the phosphate, sulfate, or acetyl esters (Bertozzi and Rabuka 2009), which broadens their diversity even more. Glycans are found in the composition of glycolipids, glycoproteins, and proteoglycans.

Glycolipids are lipids that contain one or more monosaccharide(s) in their head group. The most abundant glycolipids in the eukaryotic cell membrane are glycosphingolipids, where glycan group is attached via glucose or galactose to the terminal primary hydroxyl group of the lipid moiety ceramide (Varki and Sharon 2009). Glycosylphosphatidylinositol (GPI) is a distinct type of glycolipid, which is added to some proteins to anchor these to the plasma membrane. Gangliosides form another family of glycolipids that contain one or more sialic acid residues, which gives the lipid a negative charge. Glycolipids localize exclusively on the outer layer of the plasma membrane, glycan part facing the cell exterior (Alberts *et al.* 2013). The carbohydrate content in glycolipids is relatively modest, usually less than 15 sugar units (Alberts *et al.* 2013). Moderate carbohydrate content applies also to glycoproteins, which mainly consist of a protein core and bear a low number of glycans. The carbohydrate content is highest in proteoglycans (PG) that are composed of long linear branches of glycosaminoglycans (GAGs) attached to a core protein. PGs can be secreted into the extracellular matrix (e.g. aggrecan family), stored in secretory granules (e.g. serglycin) or attached to the plasma membrane. Membrane-bound PGs can be linked to the lipid bilayer via GPI anchor (glypican family) or by its hydrophobic transmembrane domain (e.g. syndecan family) (Esko *et al.* 2009). Although PGs can also contain O- and N-glycans that are typically attached to glycoproteins, the vast majority of carbohydrates on PGs are bulky GAG chains (Esko *et al.* 2009). There is a huge heterogeneity within proteoglycans, resulting from various types of core proteins, but also from the diverse number, length, and sulfation levels of GAGs attached to PGs (Esko *et al.* 2009).

1.4.1.1 Glycosaminoglycans

Glycosaminoglycans (GAGs) are negatively charged linear polysaccharides that consist of disaccharide repeating units. These units are composed of uronic acid (glucuronic acid or iduronic acid) or galactose and an amino sugar (either N-acetylglucosamine or N-acetylgalactosamine). This structural variety is the basis for the different types of GAGs that include hyaluronic acid, heparin and

heparan sulfate, chondroitin sulfate, dermatan sulfate and keratan sulfate. GAGs can be found as a free complex or covalently attached to a protein, forming PGs (Esko *et al.* 2009). Glycosaminoglycans form large structures, the molecular weight ranging usually between 1–2,000 kDa (Coccheri and Mannello 2014); and are highly negatively charged due to the uronic acids in their backbone and extensive sulfation (Ernst *et al.* 1995). The repeating structural units of different classes of glycosaminoglycans and the average number of disaccharides in one chain is presented in Table 2.

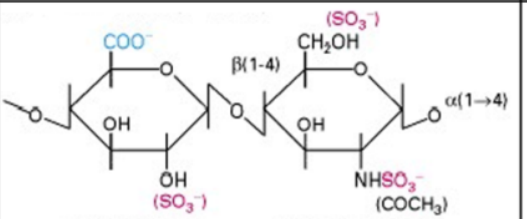
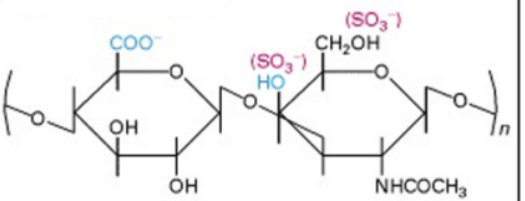
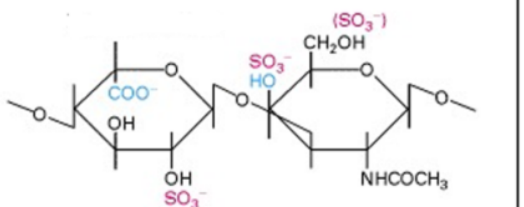
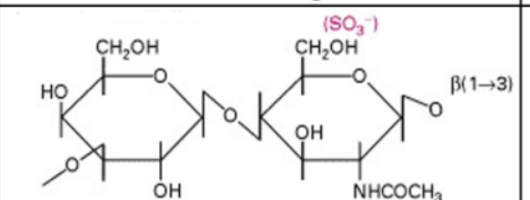
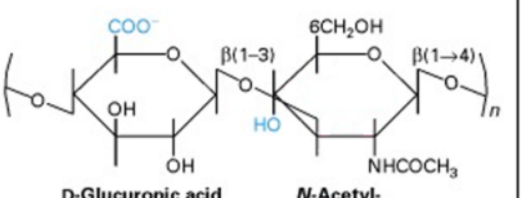
Heparan sulfate (HS) is widely found all over the body and it is produced by virtually all cell types (Esko *et al.* 2009). *Heparin* (HP) is structurally analogous to HS, although HP is synthesized only in specific cells (mostly primary mast cells). Both, HS and HP, consist of repeating units of glucuronic acid and N-acetylglucosamine (Esko *et al.* 2009). HS and HP chains can be long, the molecular weight ranging from 10–70 kDa and 7–20 kDa, respectively; and are usually heavily modified with 0- and N-sulfation. However, heparin has even higher degree of sulfation compared with heparan sulfate (Esko *et al.* 2009). HS is abundantly found in the composition of various PGs, namely heparan sulfate proteoglycans (HSPGs). HSPGs act as (co)receptors and mediate various vital cellular processes, such as signalling and transport, to name a few (Sarrazin *et al.* 2011). One of the examples of HSPGs is the syndecan family. The members of this HSPGs family carry only HS chains (syndecan-2 and syndecan-4), or also chondroitin sulfates (syndecan-1 and syndecan-3) (Esko *et al.* 2009).

Chondroitin sulfate (CS) is the most abundant GAG in the body, found in cartilage, bones, cornea, etc. The members of this class of polysaccharides are involved in various physiological functions, such as wound healing, infections, cell division; and also participate in different signalling pathways (Coccheri and Mannello 2014). CS form long and heavy chains (15 to 70 kDa) consisting of repeating disaccharide units of N-acetylgalactosamine and glucuronic acid (Ernst *et al.* 1995). The degree of sulfation can vary greatly, as well as the position of the sulfate group; the latter giving rise to CS subclasses: chondroitin sulfate A, C, D and E (Coccheri and Mannello 2014).

Dermatan sulfate (DS), also known as chondroitin sulfate B, is structurally similar to CS, containing N-acetylgalactosamine. However, instead of glucuronic acid, DS consist of a varying degree of iduronic acid. Similarly to the other GAGs, the position of the sulfate group can vary. DS chains are usually shorter (15 to 55 kDa), forming smaller PGs that are widely spread in skin (Ernst *et al.* 1995; Coccheri and Mannello 2014).

Keratan sulfate (KS) is composed of N-acetylglucosamine, and unlike other GAGs, KS contains galactose instead of uronic acid. The molecular weight of the KS chains usually varies between 2–20 kDa; and almost all glucosamine and some galactose units contain sulfate groups (Ernst *et al.* 1995). KS is widely found in bones, cartilage and nerve tissue (Coccheri and Mannello 2014).

Table 2. The structural units of glycosaminoglycans (adapted from Lodish *et al.* 2000)

Glycos-aminoglycan	Uronic acid/ galactose	Hexosamine	Typical No. of disaccharides
Heparin/ heparan sulfate			15-30
Chondroitin sulfate			< 250
Dermatan sulfate			< 250
Keratan sulfate			20-40
Hyaluronic acid			< 50 000

Hyaluronic acid (HA), also called hyaluronan, consists of N-acetylglucosamine and glucuronic acid. Unlike other GAGs, which are synthesised in the Golgi complex, HA is formed by integral membrane proteins at the plasma membrane. Another property that differentiates HA from the aforementioned GAGs is the lack of sulfate groups. However, HA is still negatively charged due to the presence of glucuronic acid (Hascall and Esko 2009). Furthermore, HA is not covalently linked to PGs, but instead forms massive polysaccharide complex, in which a single chain can consist of 25 000 repeating disaccharide units (Alberts *et al.* 2013). Long HA chains are binding sites for proteoglycans, which in turn are linked with other GAGs. Hyaluronan can be found in various concentrations in extracellular matrix everywhere in the body (Hascall and Esko 2009).

1.4.1.2 Glycans and CPPs

Cell surface is heavily decorated with glycans, which perform various physiological functions (Varki and Lowe 2009). Given their abundance, the internalization of CPPs is likely to involve interactions with glycans as a first step. A considerable amount of literature has been published on the role of glycans in CPP transduction, where the involvement of GAGs in the internalization of CPPs has been studied the most intensively. GAGs form huge complex structures and are highly negatively charged. Since the majority of CPPs are cationic, strong electrostatic interactions between them are highly probable. Indeed, high affinity of CPPs towards GAGs has been demonstrated in many reports (Fuchs and Raines 2004; Ziegler and Seelig 2004; Goncalves *et al.* 2005). Furthermore, the higher degree of GAG sulfation, and thus higher negative charge, was shown to correlate with the binding efficacy of the Tat peptide to GAGs (Ziegler and Seelig 2004). In addition, treating cells with agents that block GAG sulfation resulted in the decreased cellular uptake of Tat peptide (Sandgren *et al.* 2002; Letoha *et al.* 2010), octa-arginine and penetratin (Letoha *et al.* 2010). Moreover, numerous studies conducted with the cell lines that lack GAGs or only HS have demonstrated that internalization efficacy of arginine-rich CPPs, such as Tat peptide, oligoarginines and penetratin, is markedly lower into HS-deficient cells; and even more so in the case of cells that lack all GAGs (Console *et al.* 2003; Fuchs and Raines 2004; Richard *et al.* 2005; Nakase *et al.* 2007). According to one study, the uptake of octa-arginine was less dependent on HSPGs compared with the Tat peptide (Nakase *et al.* 2007). It was proposed that this resulted from the higher charge density of octa-arginine, which enabled interactions with other GAGs when HSPGs were lacking; as opposed to Tat peptide that could only interact with HSPGs, which had higher sulphuric acid content, compared with the rest of the GAGs (Nakase *et al.* 2007). It has been also pointed out that CPP concentration influences peptide-GAG interactions. At lower concentrations, the internalization efficacy into GAG-deficient cells was similar to that of wild-type cells, while at higher concentrations, the accumulation into a HS-deficient cell line was considerably lower (Kosuge *et al.*

2008). Furthermore, the presence or lack of GAGs influenced also the concentration at which the peptide "switches" from endocytosis to translocation. It was shown that the absence of GAGs increased the concentration that was needed for inducing translocation, that otherwise replaced endocytosis already at lower concentrations (Kosuge *et al.* 2008).

Syndecans, a family of HSPGs, has been reported to mediate the cellular uptake of arginine-rich CPPs. Syndecans are transmembrane PGs that carry either HS or HS/CS chains. A significant increase in the uptake of octa-arginine and Tat-peptide was detected into cells that overexpressed syndecan-2 (Nakase *et al.* 2007). Similar results were obtained in another study where cells expressing specific syndecan variants were utilized to demonstrate that all syndecans, but particularly syndecan-4, mediate the entry of octa-arginine, Tat peptide and penetratin into cells (Letoha *et al.* 2010). Furthermore, it was emphasized that HS chains have a crucial role in binding to syndecans, and cytosolic co-localization of these with the peptides was detected. These results were corroborated by a recent study demonstrating that the internalization of octa-arginine via clathrin-mediated endocytosis was induced by syndecan-4 (Kawaguchi *et al.* 2016).

Several recent studies investigating the GAG-dependent uptake of CPPs have uncovered the CPPs ability to cluster these molecules. HSPG clustering by pTat was observed when multiple pTat molecules were conjugated to quantum dots. Cross-linking of these PGs resulted in the initiation of a signalling cascade that induced the uptake of CPPs via macropinocytosis. These effects were not detected when a quantum dot was linked to only two pTat molecules (Imamura *et al.* 2011). It was further demonstrated that eight pTat molecules were necessary so that each individual nanoparticle would induce the uptake by cross-linking HSPGs (Suzuki *et al.* 2013). These results indicate that a certain number of peptide molecules is needed for GAG-clustering and the induction of endocytosis. GAG-clustering ability has been also demonstrated for amphipathic peptides, TP10 analogues (Verdurmen *et al.* 2013). However, it did not result in enhanced uptake, although enzymatic removal of HS or competitive binding resulted in reduced cellular uptake. It was further demonstrated that arginine residues and peptide cyclization are beneficial for cross-linking HS, which results in the increased uptake efficacy (Wallbrecher *et al.* 2014). It was also proposed that peptides with the lower binding stoichiometry can cross-link HS more efficiently, which in turn leads to the clustering of PGs at the cell membrane and higher cellular uptake, while strong binding reduces the capacity to cross-link HS in such a manner that would lead to peptide internalization. Similarly, the transduction of WR9 peptide has been previously demonstrated to depend on the ability of the peptide to cluster GAGs (Ziegler and Seelig 2011). According to the another study, tryptophan-rich CPP was shown to adopt β-strand secondary structure upon interacting with GAGs, which led to the formation of more stable GAG aggregates and increased internalization efficacy (Bechara *et al.* 2013). It was further demonstrated that the internalization of tryptophan-containing peptides, penetratin and R_6W_3, was even more enhanced

when the formation of ceramide in the plasma membrane was induced. The formation of ceramide was proposed to mediate GAG clustering, which in turn benefits for the cellular entry of these peptides; confirming the importance of GAG cross-linking in the internalization of CPPs (Bechara *et al.* 2015). Furthermore, it has been also proposed that the stability of the CPP-induced clusters determines the subsequent internalization pathway, as it was demonstrated that penetratin forms more stable clusters than Tat peptide and induces endocytosis, while Tat peptide may dissociate from GAGs and eventually translocate (Rullo *et al.* 2011).

Despite a large number of studies demonstrating the prime role of GAGs in CPP internalization, contradictory results have been obtained several times. For instance, Tat peptide was shown to internalize independently of HSPGs (Silhol *et al.* 2002). Similarly, internalization efficacy of Tat peptide was not impaired neither by enzymatic removal of HS or sialic acids, nor was the uptake efficiency reduced into cells deficient in those glycans (Gump *et al.* 2010). These results were corroborated by another study demonstrating that the cellular uptake of penetratin was equally efficient into wild-type, GAG- and sialic acid-deficient cell lines (Alves *et al.* 2011). However, these results were obtained only at low peptide concentrations, while higher concentrations resulted in reduced uptake into GAG-deficient cells and interestingly, increased internalization efficacy into sialic acid-deficient cells. Also, it has been suggested that GAGs can facilitate the cellular uptake of CPPs to some extent, but these polysaccharides are not the main binding partners, as studies with GAG-deficient cell lines or degradation of these carbohydrates by enzymes never abolished the uptake completely (Brock 2014). Nevertheless, based on numerous studies, the indispensable role of GAGs as mediators of cellular uptake of CPPs cannot be refuted.

1.4.2 Plasma membrane proteins as receptors for CPPs

Plasma membrane is heavily loaded with proteins, which count for about 50% of the membrane mass. Proteins carry numerous vital functions such as cell communication, adhesion and trans-membrane transport, including the uptake of the majority of macromolecules (Alberts *et al.* 2013). The involvement of proteins in the internalization of CPPs has been seldom demonstrated. Indications of protein-facilitated CPP uptake started to emerge when it was demonstrated that degradation of proteins from the cell surface blocked the macropinocytotic internalization of pTat and cargo protein (Gump *et al.* 2010). After that, only a few recent studies have revealed the role of membrane receptors in the transduction of some CPPs. For instance, the internalization of a peptide composed of 12 arginines was shown to be mediated by a chemokine receptor CXCR4 (Tanaka *et al.* 2012). Interestingly, the receptor did not participate in the internalization of the shorter oligoarginine (R8) or Tat peptide. According to another recent study, the cellular entry of nanoparticles coated with Tat peptide

required a transmembrane receptor neuropilin-1 and heparan sulfates (Pang *et al.* 2015). Similarly, neuropilin-1 has been previously reported to regulate the cellular uptake of tumor-penetrating peptides (Teesalu *et al.* 2009; Sugahara *et al.* 2015). Recently, scavenger receptors were found to mediate the uptake of the second-generation CPPs, PepFects and NickFects. However, the uptake via scavenger receptors is not induced by the peptide itself, but requires the net negative charge of the CPP-oligonucleotide (Ezzat *et al.* 2012; Lindberg *et al.* 2013; Lindberg *et al.* 2015) or CPP-plasmid DNA nano complexes (Arukuusk *et al.* 2013b; Veiman *et al.* 2013).

Although there have been rare implications of direct protein-CPP interactions, the role of a specific receptor has yet to be discovered. Furthermore, perhaps membrane proteins play a role in the internalization of CPPs indirectly by affecting the membrane composition or fluidity.

1.4.3 Lipids and sterols

Cells are surrounded with a lipid bilayer, which together with proteins and carbohydrates form a protective barrier around the cell. Lipid bilayer consists of thousands of different types of lipids. Each lipid molecule is amphipathic, containing a polar hydrophilic head and two hydrophobic hydrocarbon tails. The length of the fatty acid tail can vary, but it is usually between 14 and 24 carbon atoms. The eukaryotic plasma membrane is predominantly composed of glycerophospholipids, which contain a phosphate-carrying diacylglycerol linked to various head groups. A neutral lipid phosphatidylcholine constitutes over half of the lipids in the eukaryotic membrane. Another major lipid found in the plasma membrane is sphingomyelin, a lipid with a ceramide in its hydrophobic backbone. Similarly to phosphatidylcholine, sphingomyelin does not hold a net charge. Both lipids localize predominantly in the outer leaflet of the plasma membrane together with the carbohydrate-containing glycolipids; whereas the inner leaflet consists mainly of phosphatidylethanolamine and of a smaller amount of phosphatidylserine (PS). In addition, phosphatidylinositol localizes in minor quantities in the cytosolic part of the membrane, and together with PS they generate the negative charge of the inner leaflet of the plasma membrane due to their anionic head groups (reviewed in van Meer *et al.* 2008).

The majority of CPPs are positively charged, which favours the electrostatic interactions with the negatively charged lipids. However, anionic lipid content in the plasma membrane is rather small, around 10%. Furthermore, only around 2% of negatively charged lipids localize in the extracellular leaflet, decreasing the probability that the cellular uptake of CPPs is mainly mediated by anionic lipids. Nevertheless, the impact of anionic lipids cannot be neglected, since in some medical conditions negatively charged PS can be exposed on the cell surface (Zwaal *et al.* 2005), which favours the interactions with cationic CPPs. Furthermore, as suggested previously, interactions with negatively charged lipids may come into focus when CPPs are trapped in endosomes, since the late

endosome membrane normally contains more negatively charged lipids (Erazo-Oliveras *et al.* 2012). Indeed, the central role of negatively charged PS in the membrane interaction and the subsequent internalization has been demonstrated for several occasions. Studies with model membrane systems demonstrated that anionic phosphatidylserine is essential for the translocation of Tat peptide (Ciobanasu *et al.* 2010). The finding was complemented by a study according to which the accumulation efficacy of arginine-rich CPPs was proportional to the anionic phospholipid content in the vesicle membrane and the lipid flip-flop caused by the peptide internalization (Swiecicki *et al.* 2014). Furthermore, the relocation of PS during CPPs internalization has been demonstrated also in the cell culture, where PS signal was detected at the peptide influx sites (Hirose *et al.* 2012).

In general, the internalization of amphipathic CPPs, such as transportan, does potentially involve hydrophobic but not electrostatic interactions, since these peptides bind to neutral and anionic lipids with the similar affinity (Magzoub *et al.* 2001). Nevertheless, since these peptides contain also cationic amino acids, electrostatic interactions have been also shown to be involved in the insertion of the peptide into the membrane (Anko *et al.* 2012). According to the same study, PepFect3 and PepFect6, interact more avidly with the neutral membranes compared with the parent peptide TP10 due to the stearyl moiety, indicating that interactions are predominantly hydrophobic.

Besides phospholipids, membranes of animal cells are enriched with cholesterol, which constitutes around 20% of the lipids by weight (Alberts *et al.* 2013). By localizing in-between phospholipids in both membrane leaflets, cholesterol affects the membrane fluidity, making the membrane more rigid and less permeable (Alberts *et al.* 2013). For this reason, cholesterol potentially has an inhibitory effect on the direct penetration of CPPs. This view is supported by a study showing that the high cholesterol content prevents the insertion of transportan into the lipid membrane. Furthermore, peptide-membrane interactions were shown to take place in the more fluid and cholesterol-poor membrane regions (Arsov *et al.* 2008). In light of these results, penetration of CPP through the membrane can happen more easily in endosomes, as cholesterol content in the late endosomal membrane is much lower compared with the plasma membrane (van Meer *et al.* 2008). Furthermore, depletion of cholesterol from the plasma membrane has been shown to promote the cellular uptake of octa-arginine (Fretz *et al.* 2007) and also penetratin, although at higher concentrations (Bechara *et al.* 2015). On the other hand, as cholesterol is one of the main lipid raft components, its depletion has been shown to decrease the internalization of some CPPs, indicating that these peptides internalize via raft-dependent endocytosis (Kaplan *et al.* 2005).

In addition to cholesterol, sphingolipids influence the membrane rigidity. One of the major sphingolipids in mammalian cells is sphingomyelin, which is localized mainly in the extracellular leaflet of the plasma membrane (van Meer *et al.* 2008; Alberts *et al.* 2013). Sphingomyelin is hydrolysed by sphingomyelinase into ceramide, a bioactive lipid that regulates several cell signalling pathways

(Grassmé *et al.* 2007; Hannun and Obeid 2008). Furthermore, ceramide induces lipid reorganization, including displacement of cholesterol and destabilization of lipid ordered phases (Megha and London 2004; Yu *et al.* 2005), and has also been proposed to induce membrane curvature in model membranes (Holopainen *et al.* 2000). Moreover, similarly to cholesterol, ceramide can form rigid platforms in membranes (Megha and London 2004). Recent studies have reported the role of ceramide in the internalization of CPPs. The borderlines between CPP-induced ceramide-rich regions and fluid membrane domains have been proposed to be the sites for translocation of arginine-rich CPPs (Verdurmen *et al.* 2010). The indirect role of ceramide was demonstrated for the cellular entry of penetratin, where ceramide formation promoted GAG-clustering, leading to higher uptake (Bechara *et al.* 2015).

1.4.3.1 Lipid rafts

The lipid raft concept was proposed in 1997 as a hypothesis that lipids, cholesterol and proteins form nanoscale platforms in the plasma membrane that are involved in the membrane transport and signalling (Simons and Ikonen 1997). Lipid rafts or membrane microdomains are formed from cholesterol, glycosylated sphingolipids, lipids with saturated acyl chains and certain saturated lipid-anchored or transmembrane proteins (Hancock 2006; Sezgin *et al.* 2012). It is thought that these rafts are submicroscopic and highly dynamic, associating and disassociating continuously (Sengupta *et al.* 2008; Lingwood and Simons 2010; Simons and Gerl 2010). In physiological conditions, rafts have been almost impossible to detect without any external influence, such as cross-linking (Simons and Gerl 2010) and are therefore usually examined in artificial or plasma membrane-derived vesicles (Baumgart *et al.* 2007; Lingwood *et al.* 2008; Simons and Gerl 2010). Not long ago, a first cell-based study provided direct evidence of plasma membrane rafts (Owen *et al.* 2012). Furthermore, most of the membrane of cells was shown to be highly ordered, and only about 24% of the cell surface is occupied by the disordered lipid domains. However, because of their dynamic properties, and thus, complicated analysis there is a poor understanding about their properties and regulation. Therefore it is not clear whether membrane rafts facilitate or interfere with the internalization of CPPs.

1.5 Model membrane systems and plasma membrane vesicles

1.5.1 Model membrane systems

The plasma membrane of a eukaryotic cell is very complex and dynamic, which complicates the detailed analysis of the interactions between specific membrane constituents and CPPs. Furthermore, the continuous flow of endocytosis and exocytosis makes it difficult to distinguish whether CPPs internalize via

endocytosis, translocation or both. Therefore, simplified model membrane systems have become invaluable tools for studying the interactions of CPPs with the membrane and its constituents. Moreover, these lipid models enable to focus solely on cellular energy-independent internalization mechanisms (Anko *et al.* 2012; Maniti *et al.* 2012; Katayama *et al.* 2013).

Although a variety of different cell-free models are available, including lipid monolayers, supported lipid bilayers and vesicles of different composition and size, the most frequently used artificial membranes to study CPPs are large- and giant unilamellar vesicles (LUVs and GUVs). More natural systems that are used for CPP studies are giant plasma membrane vesicles (GPMVs) and plasma membrane spheres (PMS), which mimic the native plasma membrane of cells most closely.

1.5.1.1 Artificial membrane systems

Artificial model membrane systems, such as large and giant unilamellar vesicles, can be prepared by using several methods that include hydration of lipid films, electroformation, the fusion of smaller vesicles, etc. (Walde *et al.* 2010). Lipids that are used to form the vesicles are present in biological membranes, such as POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) and others. These vesicles can be supplemented with cholesterol and sphingomyelin (Walde *et al.* 2010) or even proteins (Chan and Boxer 2007). LUVs have usually diameters of about 100 nm or less, while GUVs` diameter may range from 1 up to 100 of micrometers (Chan and Boxer 2007; Walde *et al.* 2010). LUVs are utilized less often than GUVs for studying CPPs, because of their smaller size and therefore more difficult analysis by conventional microscopy. Defined composition of GUVs and LUVs makes them good models to study the role of individual membrane constituents under controlled conditions. However, on the other hand, these models are severely limited in mimicking the complexity of a native lipid membrane.

1.5.1.2 Giant plasma membrane vesicles

GUVs, LUVs and other artificial membrane systems can be very useful, but are often too simplified to imitate the complexity of the native plasma membrane. Plasma membrane spheres (PMS) and giant plasma membrane vesicles (GPMVs), derived from the cell membrane, mimic the native plasma membrane the most. PMS are obtained using a hypotonic buffer that induces cell swelling and leads to the formation of vesicles (Lingwood *et al.* 2008). Similarly to PMS, GPMVs are derived from the plasma membrane and are therefore nearly identical to the latter (Keller *et al.* 2009). These giant vesicles are released from the membrane via the process termed blebbing. Blebbing is a very common phenomenon, as it is required for cytokinesis, cell migration, apoptosis and it is

one of the defence mechanisms of an injured cell (Charras *et al.* 2008; Babiychuk *et al.* 2011). During blebbing, the plasma membrane is dissociated from the actin cortex and that part of the membrane will bud outward. This can lead to a vesicle formation, but usually blebs are retracted. The formation of these kinds of blebs can be also evoked by treatment of the cells with various chemicals. The blebs induced by chemicals are released from the membrane and cannot retract. Two basic protocols are mainly used for inducing GPMVs and both of these involve treatment of cells with a buffer that is rich in calcium ions. The first method evokes blebs using a buffer supplemented with minute amounts of dithiothreitol (DTT) and formaldehyde (FA) (Scott 1976; Holowka and Baird 1983). DTT affects the proteins by reducing disulfide bridges between cysteine residues, while formaldehyde cross-links and modifies sulfhydryl and amino groups. In the second method DTT and FA are substituted with N-ethylmaleimide (NEM). NEM forms irreversible covalent bonds with sulfhydryl moieties in proteins, and blocks these groups. Unlike FA, NEM does not cross-link sulfhydryl groups, and is therefore considered to be a somewhat milder treatment for vesicle formation compared with the DTT/FA method. Chemical treatment and modification of proteins trigger an influx of calcium ions into the cells. Since calcium regulates a vast number of processes in cells, it is difficult to pinpoint, which of these are actually influenced by elevated Ca^{2+} in the cytosol that result in the formation of vesicles. However, it is known that an increased concentration of calcium ions is essentially the key to the formation of GPMVs (Keller *et al.* 2009). It is suggested that elevated concentration of calcium in cell cytosol activates Ca^{2+} dependent enzymes - phospholipase C and scramblases. The latter induces lipid flip-flop, and at high calcium concentrations, it destroys the plasma membrane asymmetry, while phospholipase C degrades phosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$]. $PI(4,5)P_2$-rich microdomains concentrate proteins that anchor actin cytoskeleton to the plasma membrane; and degradation of $PI(4,5)P_2$ leads to membrane destabilization. This triggers the budding of the plasma membrane and the intracellular pressure induces an influx of the cytoplasm into the forming vesicle, which can lead to the release of the GPMVs from cells (Keller *et al.* 2009) (Figure 2).

Although, GPMVs that are formed using chemicals are very similar to the native plasma membrane of cells, there are still a few factors to be considered when using this model membrane system. The major changes in the membrane of GPMVs are the covalent modifications – cross-linking and blocking of the sulfhydryl groups by the chemicals that are used to induce the vesicles. Covalent modifications with DTT and FA treatment can lead to the relocation of some proteins from the raft membrane phase (Levental *et al.* 2010). The alternative method for the preparation of vesicles with NEM that avoids cross-linking of proteins is therefore considered to be more “natural” (Levental *et al.* 2011). The second difference between cells and GPMVs is the partial loss of the membrane asymmetry that includes translocation of phosphatidylserine to the outer leaflet

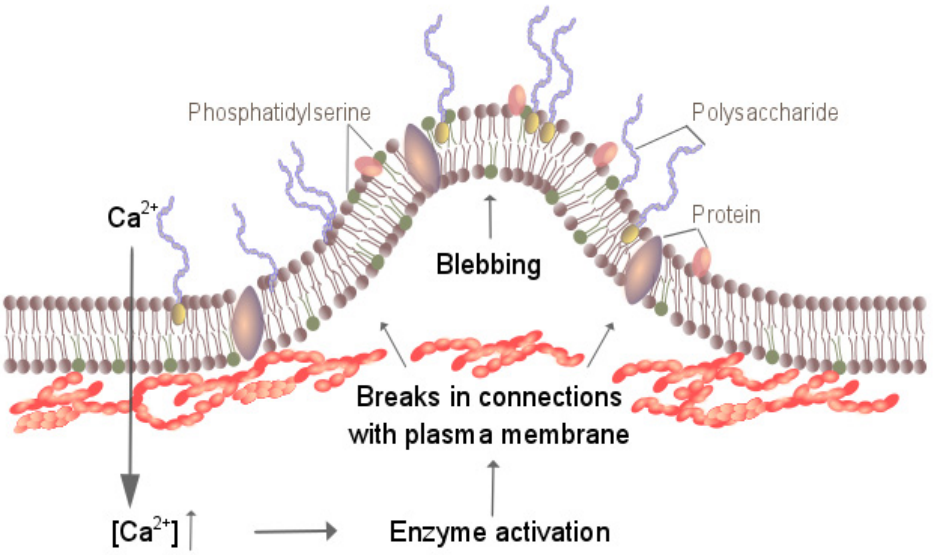


Figure 2. The potential mechanism of giant plasma membrane vesicle formation. Chemical treatment and modification of proteins trigger an influx of calcium ions into the cells. Elevated Ca^{2+} in the cytosol activates Ca^{2+} dependent enzymes, which induce lipid flip-flop and destroy the plasma membrane asymmetry. The activation of calcium-dependent enzymes leads also to the degradation of connections between the actin cytoskeleton and the plasma membrane. This triggers the budding of the plasma membrane and the release of vesicles.

of the membrane. Phosphatidylserine is found in the intracellular leaflet of the plasma membrane in live cells and it is exposed on the extracellular side only in certain physiological conditions, including apoptosis (Keller *et al.* 2009). Phosphatidylserine has a negative charge and therefore interactions with positively charged CPPs and consecutive effect to the internalization cannot be ruled out. Despite these limitations, the high similarity with the plasma membrane provides several advantages of GPMVs, compared with other model membrane systems. Because GPMVs are derived from the cells, the lipids, proteins and polysaccharides contained in these structures originate from natural plasma membrane (Scott 1976; Scott *et al.* 1979; Bauer *et al.* 2009). The interior of the vesicle is filled with the cytoplasm and it contains RNA (Åmand *et al.* 2011), however, GPMVs lack organelles and actin filaments (Baumgart *et al.* 2007; Keller *et al.* 2009). Thus, GPMVs lack cellular energy-driven processes, including endocytosis. The absence of endocytosis is a great advantage of this system over cells, because it allows to only focus on translocation of CPPs without employing endocytosis inhibitors, which could have nonspecific effects on the internalization of the peptides. Moreover, unlike cells with a continuous flow of different processes, (e.g. changes in membrane composition, vesicle trafficking, active interactions, signalling networks, enzyme activity), the composition of GPMVs remains

constant, providing the opportunity to study the interactions and internalization mechanisms of CPPs under stable experimental conditions.

1.5.1.3 Liquid ordered- and disordered membrane phases

Under certain conditions, lipid nanodomains in GPMV membrane coalesce and form two segregated membrane phases: liquid-disordered (L_d) and liquid-ordered (L_o) lipid phase (Baumgart *et al.* 2007; Sengupta *et al.* 2008). The latter is considered to resemble membrane rafts of the cells (Hancock 2006; Simons and Gerl 2010). Similarly to the membrane rafts, tightly packed L_o phase is rich in cholesterol and sphingolipids, contains phospholipids with long saturated acyl chains; and it is therefore often referred to as raft phase also in GPMVs (Hancock 2006; Sengupta *et al.* 2008; Levental *et al.* 2010). In contrast, less densely packed and more dynamic L_d phase mainly consists of unsaturated glycerophospholipids (Sengupta *et al.* 2008; Levental *et al.* 2011). Similar lipid domain separation can be induced also in GUVs, if the lipid mixture contains sterols and/or saturated lipids, especially sphingolipids (Sezgin *et al.* 2012). The segregated lipid domains in GPMVs can be specifically tagged with probes, for example with cholera toxin subunit B, which binds to the ganglioside GM1 that is a typical resident of the raft phase (Baumgart *et al.* 2007; Sengupta *et al.* 2008); and annexin V, which binds to phosphatidylserine in the L_d domain (Johnson *et al.* 2010). Laurdan is also often used, and can be utilized to analyze the extent of lipid packing (Kaiser *et al.* 2009). Segregation of phases in GPMVs is determined by the temperature – at 10 °C, almost all DTT/FA-induced vesicles undergo phase separation, whereas increased temperatures lead to the decreased L_o/L_d phase coexistence (Levental *et al.* 2009). The average miscibility transition temperature and the abundance of the phases are strongly dependent on the cholesterol content of the vesicles. Modulation of the cholesterol amount in GPMVs revealed that the higher concentration of cholesterol led to the disappearance of disordered lipid phase and decreased miscibility transition temperatures. The opposite results were obtained when the cholesterol was depleted from GPMVs (Levental *et al.* 2009). It was recently shown that temperature of phase separation depends also on the density of the cells – GPMVs formed from densely plated cells can have lower phase transition temperatures. The lower phase transition temperature also characterizes vesicles isolated from cells that have been serum-starved, or from cells that are under-going apoptosis (Gray *et al.* 2015). The phase separation temperature is also dependent on the induction method of the vesicles. Coexisting lipid phases can be detected around 18 °C if vesicles are induced by DTT and paraformaldehyde (PFA), while the domains in NEM vesicles were shown to be smaller and phase segregation took place around 10 °C (Levental *et al.* 2011). The higher temperature for the segregation of lipid phases is caused by combined activity of DTT and PFA and does not occur when the chemicals are used alone. The cross-linking and reducing activity of PFA and DTT, respectively, do not elevate the phase separation temperature and it is not caused by the protein

depalmitoylation by DTT. Although PFA, which is a cross-linking agent, could be expected to increase the order in vesicles, the enhanced domain stability was strongly dependent on the DTT concentration (Levental *et al.* 2011). In terms of phase separation, the vesicles induced by NEM treatment are considered to be more "natural" and reflective of the plasma membrane of mammalian cells. In phase-separated GPMVs, the majority of proteins localize in the same lipid microdomain than in living cells (Baumgart *et al.* 2007; Sengupta *et al.* 2008; Johnson *et al.* 2010). For example, GPI-anchored proteins localize preferentially into the L_o phase in GPMVs (Baumgart *et al.* 2007; Sengupta *et al.* 2008) and many transmembrane proteins were found to reside in L_d lipid phase (Sengupta *et al.* 2008). Furthermore, it was shown that palmitoylation is required for transmembrane proteins to localize into raft-phase (Levental *et al.* 2010).

2. AIMS OF THE STUDY

CPPs internalize into cells via different modes of endocytosis and direct translocation through the cell membrane. While endocytic pathways have been well described in the cellular uptake of CPPs, the energy-independent direct crossing of the plasma membrane has still remained elusive. Furthermore, the understanding of the exact role of the cell surface molecules mediating the direct translocation is still controversial; and it is not fully understood how the membrane composition affects the translocation through the plasma- and endosomal membrane. Our aim was to focus on the direct translocation of CPPs by using a model membrane system that is devoid of endocytosis, but closely resembles the plasma membrane by its composition. The main goal of this thesis was to provide information about membrane lipid dynamics and the molecular partners which facilitate the penetration of several widely known CPPs through the biological membranes.

The specific objectives of the current study were:

- to define the membrane subdomains that favour or inhibit the direct cellular entry of CPPs; more precisely, to analyse the impact of cholesterol and ceramide on the translocation of CPPs across biomembranes (Paper I and II).
- to assess whether plasma membrane proteins are required for the penetration of CPPs through the biological membranes (Paper II).
- to analyse the role of cell surface glycans, in particular, glycosaminoglycans, in the translocation process of CPPs (Paper III).

3. METHODOLOGICAL CONSIDERATIONS

Detailed information about the methods used in the studies can be found in the included articles and only a brief rationale for chosen methodological approaches and summary of the relevant theoretical background is presented here.

3.1 Cell-penetrating peptides

Throughout the studies, 6 widely known CPPs were examined, which varied in their sequence and physicochemical properties. The features of the peptides are described in more detail in the literature overview, and their sequence and classification are presented in Table 1. Briefly, nona-arginine (Arg_9) and Tat peptide (pTat) belong to a class of cationic arginine-rich CPPs, which were among the first CPPs discovered and are still the most frequently used (Vives *et al.* 1997; Mitchell *et al.* 2000). Primary amphipathic CPPs, transportan (TP) and its truncated analogue TP10, were among the first chimeric peptides designed as cellular delivery vectors (Pooga *et al.* 1998; Soomets *et al.* 2000). A well-studied penetratin (pAntp) and a model amphipathic peptide, MAP were chosen to represent the secondary amphipathic CPPs. All six peptides were used in Paper I to study their direct translocation through the biological membrane and their lipid-phase preference. Dependence of the peptide internalization on the membrane composition and the specific role of cholesterol was analysed in Paper II. Two representatives of amphipathic (TP and TP10), and two arginine-rich CPPs (Arg_9 and pTat) were chosen for further studies to examine the mechanisms and specific membrane components that are involved in the direct translocation (Paper II and Paper III).

3.2 Cell cultures

Altogether, five cell lines were used to induce giant plasma membrane vesicles, which were utilized in the experiments described in this thesis. Rat basophilic leukemia RBL-2H3 (RBL) cells were used in experiments conducted in the Paper I and II. RBL cells were chosen based on the initial protocol for preparation of the vesicles (Holowka and Baird 1983). Furthermore, the detachment of the vesicles from the plasma membrane of these cells is more efficient than in other cell lines, including human cervical carcinoma cell line HeLa, which was used as a control in the Paper I.

Additionally, three different types of Chinese hamster ovary (CHO) cell lines were utilized. The wild-type CHO-K1 cells (WT) were used in paper II and III. In paper II, GPMVs were derived from CHO cells but not from RBL cells in order to study the impact of ceramide on the direct translocation of CPPs, because RBL cells contain a small amount of ceramide in the plasma membrane. In paper III, CHO glycosaminoglycan (GAG) mutant cell lines were utilized to

study the effects of different GAGs in the direct translocation process of CPPs. For this purpose, GAG-deficient CHO pgsA-745 cells (GAG^{neg}) and heparan sulfate-deficient CHO pgsD-677 cells (HS^{neg}) were employed. CHO pgsA-745 cells are deficient in xylosyltransferase, which is a key enzyme in the synthesis of proteoglycans, and therefore these cells lack GAGs on the cell surface (Esko *et al.* 1985). However, CHO pgsA-745 cells still contain hyaluronic acid on the plasma membrane, because it is synthesized at the cell membrane (Prehm 1983). CHO pgsD-677 lack only HS, due to a defect in N-acetylglucosaminyltransferase and glucuronyltransferase, which are required for heparan sulfate synthesis. However, chondroitin sulfates (CS) are three-fold overexpressed in HS^{neg} cells compared with the WT CHO cell line (Lidholt *et al.* 1992).

3.3 Giant plasma membrane vesicles

The plasma membrane of the eukaryotic cell is highly complex and dynamic, and it is continuously involved in various processes, which also cause changes in the composition of the plasma membrane. Thus, studying the specific interactions and membrane composition in the translocation of CPPs using cells is complicated. Furthermore, in order to examine the direct translocation in the cell culture, endocytosis has to be blocked either by low temperature or a set of endocytosis inhibitors; which both can have a substantial effect on the membrane interactions of CPPs. To overcome these major drawbacks, many model membrane systems have been introduced, such as giant unilamellar vesicles. However, since they contain only a few types of lipids and usually no protein or carbohydrates, these systems can be overly simplified to study the internalization of CPPs. Therefore, throughout the studies, we have used cell-derived giant plasma membrane vesicles (GPMVs), which have the advantages of the model membranes, such as the stable composition and lack of endocytosis, allowing us to focus solely on the translocation mechanism. At the same time their membrane composition resembles closely that of a native plasma membrane of cells. Furthermore, in GPMVs, the lipid phases segregate, which allowed us to examine the role of the lipid "rafts" in the translocation of CPPs.

Giant plasma membrane vesicles (GPMVs) were prepared by using two standard protocols (Scott 1976; Holowka and Baird 1983; Baumgart *et al.* 2007). According to the first method, the cells are treated with a calcium-rich buffer supplemented with dithiothreitol (DTT) and formaldehyde (FA) (Holowka and Baird 1983). The second method also includes treating the cells with calcium-rich buffer; however, DTT/FA are substituted with N-ethylmaleimide (NEM) (Holowka and Baird 1983). Initially, in Paper I, we used DTT/FA for the preparation of the vesicles. Protocol with NEM was introduced later (Paper II), after the discovery that DTT/FA method could cause crosslinking of proteins and NEM was considered a somewhat milder inducer of blebbing (Levental *et al.* 2010). However, since we could not prepare a sufficient number of GPMVs from CHO cells by NEM treatment, we returned to DTT/FA for inducing

vesicles from CHO cells in paper II and paper III. The integrity of the vesicles was verified by incubating GPMVs with the peptides that are classified as non-CPPs: Perforin (82–96) (QRHVTRAKVSSTEAVAR, charge: +3) and a peptide from C-terminus of β1 adrenergic receptor, CSSLDEPGRGGFSSESKV, charge: −1); as well as with polysaccharide dextran (Mw 2–3 kDa), none of which was able to penetrate the membrane of GPMVs.

3.4 Visualization and quantification of the direct translocation by confocal microscopy and flow cytometry

Confocal laser scanning microscopy (CLSM) was used in all three papers to analyse the interactions of CPPs with the membrane of vesicles, as well as to quantify the accumulation of the peptides inside the GPMVs. Although confocal microscopy provides a good estimate of the internalization of CPPs, fluorescence-activated cell sorting (FACS) was used in parallel in order to acquire quantitative data from a high number of individual GPMVs. Furthermore, FACS enables to examine the kinetics of the interactions and internalization of CPPs immediately after addition of CPPs into medium containing GPMVs.

3.4.1 Studying the direct translocation of CPPs into GPMVs and preferential accumulation in membrane subdomains

Throughout the studies, we examined the accumulation efficacies of the CPPs by using peptides labelled with fluorescein, and recording images at different confocal planes to obtain images at different levels of the vesicles by CLSM.

In order to assess the formation of pores in the membrane of GPMVs during the translocation of CPPs, the efflux of the peptides was examined. First, the vesicles were immobilized on the glass coverslip and loaded with CPPs, then unbound peptides were removed, and the efflux of CPPs from GPMVs was estimated by the decrease in fluorescence intensity by confocal microscopy.

At lower temperatures (below 10 °C), the membrane of the vesicles undergoes lipid phase separation in the majority of GPMVs, where the rigid liquid-ordered (L_o) and more dynamic liquid-disordered (L_d) membrane phase segregate. These membrane domains were visualised with fluorescently labelled cholera toxin subunit B, which binds to the ganglioside GM1 that localizes in the liquid-ordered phase (Baumgart *et al.* 2007; Sengupta *et al.* 2008); and annexin V, which binds to the phosphatidylserine in the L_d domain (Johnson *et al.* 2010).

3.4.2 Studying the role of specific membrane constituents in the direct translocation of CPPs across membranes

In the paper I, we discovered that besides efficient accumulation into the lumen of the vesicles, amphipathic CPPs accumulated in the L_d regions of the membrane that has low cholesterol concentration (Simons and Gerl 2010). Thus, in Paper II, we further investigated whether the internalization of the CPPs is influenced by the cholesterol concentration in the membrane of vesicles. For this purpose, we depleted cholesterol from the membrane of GPMVs with methyl-β-cyclodextrin (MCD) to increase the proportion of L_d domains in GPMVs. We also reversed the situation by enhancing the cholesterol concentration in the membrane of vesicles using MCD-cholesterol complex (Watkins *et al.* 2009), increasing the proportion of rigid and raft-like membrane. The respective decrease and increase in the cholesterol concentration was estimated using a fluorescent macrolide, filipin, and analysed by FACS.

Similarly to cholesterol, ceramide can generate highly ordered platforms in membranes (Megha and London 2004). Therefore, in Paper II, we proceeded by introducing ceramide in the membrane of GPMVs hydrolysing sphingomyelin into ceramide with sphingomyelinase. The anti-ceramide antibody was used for corroborating the emergence of ceramide-rich regions in the membrane of GPMVs. In Paper II, we also assessed the potential role of proteins in penetration of CPPs across the limiting membrane of GPMVs. Thus, we degraded the membrane proteins on the surface of GPMVs with trypsin and analyzed the internalization efficacy of CPPs by confocal microscopy.

In paper III, we continued with the identification of the membrane constituents that facilitate the direct translocation of CPPs across membranes and focused on the role of specific cell surface polysaccharides, namely glycosaminoglycans (GAGs). For this purpose, GAG- and heparan sulfate-deficient cell lines were utilized for the preparation of GPMVs and the yield of CPP accumulation was examined by CLSM and FACS analysis. To confirm the results, the particular GAGs were degraded by treating the vesicles with hyaluronidase VIII, heparinase III or chondroitinase ABC that remove hyaluronic acid, heparan sulfates; chondroitin and dermatan sulfates, respectively. The effectiveness of the removal of these GAGs upon enzyme treatment was confirmed by using fluorescently labelled carbohydrate-binding proteins – lectins. More specifically, we applied wheat germ agglutinin (WGA), that binds to *N*-acetyl-glucosamine residues, which are present in heparan sulfates, hyaluronic acids, keratan sulfates and sialic acids. *Wisteria floribunda* agglutinin (WFA) was harnessed for quantification of N-acetylgalactosamine residues, the constituent of chondroitin and dermatan sulfates. *AutoQuant X*3 was used for the quantitative analysis of the confocal microscopy images of the internalized CPPs in enzyme-treated vesicles, as well as to measure the labelling of the membranes with lectins.

4. RESULTS AND DISCUSSION

Direct translocation of CPPs across the plasma and/or endosomal membrane has been intensely studied since the discovery of CPPs. However, a detailed molecular understanding of the exact translocation mechanism and the potential role of specific binding partners have remained elusive for several reasons. Firstly, the direct translocation can be studied in cell cultures only when endocytosis is blocked either by the pharmacological inhibitors, RNA interference or by low temperature. All of these factors may affect cellular processes and the properties of the plasma membrane. Additionally, low temperature can lead to the undervaluation of the internalization efficacies of CPPs, resulting from the decreased membrane dynamics that hinders the translocation. Although several model membrane systems have been used to overcome these limitations these systems fail to recapitulate the complexity of the native plasma membrane due to the presence of only a few lipid species in such membranes. Therefore, in order to study the mechanisms of direct penetration of CPPs, as well as the concomitant interactions with the membrane constituents, we utilized giant plasma membrane vesicles (GPMVs) that are devoid of endocytosis, and at the same time, closely resemble the native plasma membrane by its composition. By using GPMVs, we examined the interaction of CPPs with the membranes of variable composition to gain a detailed understanding of the specific molecular partners that are essential for the direct translocation of a number of widely known representatives of the cationic and amphipathic CPPs.

4.1 CPPs translocate into the lumen of giant plasma membrane vesicles at low and high temperature (Paper I)

We first assessed the translocation efficacy of the representatives of cationic (Arg_9 and pTat) and amphipathic (pAntp, TP, TP10 and MAP) CPPs. All studied peptides were able to penetrate the membrane and also accumulated into the lumen of vesicles at room temperature. We also studied the internalization efficacy at low temperature that decreased the accumulation markedly. The inhibitory effect of the lower temperature is probably resulting from the fact that membrane becomes more solid or gel-like, which reduces the membrane penetration of CPPs.

When we examined the accumulation of CPPs into the vesicles, a distinct internalization pattern emerged for the two classes of CPPs: the signal of cationic CPPs was detected only from the interior of the vesicles, while all amphipathic CPPs also concentrated into the membrane of GPMVs. Thus, there is a clear correlation between the amphipathicity and the membrane affinity of CPPs.

Another interesting finding was that after incubation of the vesicles with penetratin, we detected the appearance of smaller vesicles budding out from GPMVs derived from RBL cells. Similar effects have been later described when

the plasma membrane spheres were used: nona-arginine and amphipathic RW9 induced outward budding of the membrane and the amphipathic peptide showed stronger bud-inducing activity (Maniti *et al.* 2014).

To gain further insights into the internalization kinetics of the CPPs, we analysed the interactions of CPPs with GPMVs at different time points and concentrations (0.01–1 μM). Arginine-rich CPPs interacted with GPMVs within few minutes, followed by continuous accumulation, and reaching a plateau in 1h. Similar internalization profile was detected at all used CPP concentrations, and also at both, low (around 4 °C) and ambient temperature. However, the accumulation efficacy into the vesicles at low temperature was markedly decreased compared with the room- and physiological temperature, as also noted above. On the contrary to Arg_9 and pTat, the association of transportan with vesicles was more rapid, reaching the saturation plateau almost instantly at all concentrations. Furthermore, the incubation temperature had seemingly no impact on the translocation of TP.

To obtain a better understanding of the actual mechanism behind the direct translocation, we further examined the pore-forming capability of the peptides. We reasoned that if the internalization would include the formation of a pore, we could detect the efflux of the peptide from the vesicles. However, no substantial leakage of the peptide could be observed, indicating that studied CPPs do not induce pores, at least not the permanent ones.

4.2 Amphipathic CPPs preferentially accumulate into the lipid disordered membrane phase (Paper I)

At low temperature, the membrane of GPMVs phase-segregates into liquid-disordered (L_d) and liquid-ordered (L_o) lipid phase (Baumgart *et al.* 2007; Sengupta *et al.* 2008). The latter is thought to resemble membrane rafts of the cells (Hancock 2006; Simons and Gerl 2010), which are cholesterol- and sphingolipid-rich and contain phospholipids with long saturated acyl chains (Hancock 2006; Sengupta *et al.* 2008; Simons and Gerl 2010). On the contrary, L_d phase mainly consists of unsaturated glycerophospholipids and is thus more dynamic (Sengupta *et al.* 2008; Levental *et al.* 2011). We labelled the respective liquid phases with the cholera toxin B subunit and annexin V. Upon incubation of these phase markers with CPPs, all amphipathic peptides co-localized with annexin V into the L_d phase, demonstrating that these peptides have a higher affinity towards more fluid membrane regions. No accumulation of the peptides was seen in the L_o domain, indicating that these "raft" like regions are not preferable binding sites for amphipathic CPPs. It is worth mentioning that during the formation of GPMVs a fraction of phosphatidylserine (PS) relocates into the outer leaflet of the vesicles, while in intact cells, it is predominantly localized in the intracellular leaflet of the plasma membrane. PS is a negatively charged lipid that has been shown to promote the internalization of CPPs (Ciobanasu *et al.* 2010). Furthermore, it localizes in the disordered membrane phase, and

therefore could be a potential binding partner for CPPs. However, we did not find a correlation between the intensity of annexin-labelling (the concentration of PS) and accumulation of CPPs in GPMVs (unpublished data). Probably the presence of PS does not induce localization of amphipathic CPPs to L_d regions of GPMVs.

Consistent with our findings, the binding of penetratin and its analogues to the model lipid membranes was markedly decreased when the limiting membrane of vesicles was enriched with cholesterol, which induces higher "order" in the membrane (Christiaens *et al.* 2002). Similarly, by using artificial liposomes with different degree of lipid packing, it was demonstrated that Cys-TP induced lipid rearrangements and membrane permeabilization in vesicles with more dynamic membrane, whereas in liposomes with higher rigidity and cholesterol content the reorganization of lipids did not occur (Arsov *et al.* 2008). Based on published and our own results, it is possible to conclude that the fluidity of the membrane greatly affects the membrane activity of amphipathic CPPs.

4.3 Cholesterol inhibits the internalization of CPPs (Paper II)

Since amphipathic CPPs preferentially accumulated into the liquid-disordered membrane regions, we reasoned that the phase preference could be the result of the higher fluidity of the L_d domain; and on the contrary, tighter packing of the L_o phase abolishes the interactions. One of the main factors triggering the formation of ordered or "raft" phase is cholesterol, which could modulate the penetration of CPPs by packing lipids in membranes "more densely". In order to examine the role of membrane fluidity and the effect of cholesterol in particular, we removed the latter from the membrane of GPMVs, which led to about four-fold decrease in the signal of cholesterol-binding macrolide filipin. The decrease in the cholesterol content promoted the internalization of Arg_9, pTat and pAntp predominantly into the small population of vesicles, that otherwise were not as prone for the uptake of CPP as compared with the majority of the vesicles. On the other hand, the accumulation of transportans and MAP was seemingly unaffected by the lower degree of cholesterol. In order to gain further insights into the membrane fluidity-dependent internalization, we proceeded by reversing the experiment and added cholesterol into the membrane of the vesicles. We detected almost a two-fold increase in the filipin staining, confirming that the membrane of GPMVs was enriched with cholesterol. The addition of cholesterol resulted in a slight increase in the number of vesicles that contained Arg_9, pTat and pAntp at a very low level. Probably the high proportion of densely packed membrane subdomains does not abolish the translocation of Arg_9, pTat and pAntp into vesicles, but these subdomains are not the preferred areas for the internalization of cationic CPPs. The greatest impact of cholesterol on the translocation efficacy was detected in the case of transportans and MAP, which

showed a strong decrease in the accumulation into cholesterol-rich GPMVs. Thus, the results suggest that more rigid membrane areas interfere with the translocation of these peptides. Moreover, in untreated GPMVs the interaction is rapid, reaching the saturation plateau within 5 minutes; but in cholesterol-enriched vesicles, we detected slower and gradual interaction with transportans and MAP. These results are in a good agreement with those described above, where we observed the preferential accumulation of the peptides into more dynamic membrane regions. Based on lipid phase preference and negative influence of cholesterol, it is feasible that the internalization of the studied peptides, especially amphipathic CPPs, takes place preferentially at the less rigid and more dynamic disordered membrane subdomains.

4.4 Ceramide promotes the translocation of arginine-rich CPPs and inhibits the internalization of amphipathic CPPs (Paper II)

Since cholesterol had a substantial effect on the internalization ability of CPPs, we wanted to further assess the impact of membrane rigidity. Similarly to cholesterol, ceramide can form highly ordered platforms in biomembranes (Megha and London 2004). We proceeded by analyzing the accumulation efficacy of two representatives of arginine-rich, Arg_9 and pTat, and amphipathic, TP and TP10, CPPs into ceramide-rich GPMVs. Corroborating our previous results, we observed nearly a 50% decrease in the internalization efficacy of both amphipathic CPPs into ceramide-rich GPMVs, confirming that the translocation of TP and TP10 is strongly dependent on membrane rigidity and the cellular entry is likely to take place at the sites of the plasma membrane that have higher fluidity.

On the contrary, both arginine-rich CPPs displayed an increased membrane permeabilization into ceramide-rich vesicles. Prior to generation of ceramide in the membrane of GPMVs, the internalization of cationic CPPs is rather heterogeneous, and the accumulation of peptide greatly varies between vesicles. After ceramide formation, the number of vesicles that poorly internalized CPPs diminished. Thus, the results imply that ceramide is not a prerequisite for the translocation of arginine-rich CPPs across membranes, but it can greatly improve the membrane penetration of these peptides. Corroborating to our findings, Verdurmen *et al.* demonstrated that the borderlines between the ceramide-rich regions and fluid membrane domains are preferable sites for the translocation of arginine-rich CPPs (Verdurmen *et al.* 2010).

4.5 Plasma membrane proteins facilitate the internalization of arginine-rich CPPs (Paper II)

The plasma membrane is heavily decorated with proteins that mediate a great number of cellular processes, including transport of macromolecules. Therefore, we next focused on the role of proteins in the direct translocation of CPPs. Upon degradation of proteins from the surface of GPMVs' membrane, the accumulation of arginine-rich CPPs into vesicles was almost completely abolished, while the internalization of TP and TP10 was only slightly affected. The publications, showing the pivotal role of membrane receptors in the cellular entry of CPPs, have only recently begun to emerge (Tanaka *et al.* 2012; Pang *et al.* 2015). However, association with the receptor usually triggers the endocytic uptake, but not direct penetration (Tanaka *et al.* 2012; Pang *et al.* 2015). Nevertheless, protein components can also affect the translocation indirectly, e.g affecting the membrane organization, or contributing to the formation of membrane lipid rafts (reviewed in Hancock 2006). Protein-dependent lipid raft stability is supported by our results, showing that after degradation of membrane proteins, lipid phases do not segregate so well even at low temperatures, indicating permanent changes in the membrane microenvironment (unpublished data). This indirect effect of proteins on CPPs internalization can be explained by the membrane packing defects at the borderlines of raft regions and the rest of the membrane. Defects in the membrane organization could create the favourable environment for the direct translocation of CPPs, which also explains the increased penetration through the membrane that is rich in ceramide rafts. These "sites" might also decrease the energy that is needed for the penetration of highly charged molecule through the membrane. In addition, since the plasma membrane proteins are also capable of inducing curvature in the membrane (reviewed in Zimmerberg and Kozlov 2006), another possible explanation is that the translocation of the studied arginine-rich CPPs takes place due to the protein-induced membrane curvature. The detailed understanding of the exact role of proteins, and perhaps the involvement of a possible receptor, requires further studies.

4.6 Glycosaminogycans mediate the direct translocation of amphipathic CPPs (Paper III)

Before the cellular uptake of CPPs, the peptides are encountered by the long branches of highly negatively charged glycosaminoglycans (GAGs). Since the majority of the CPPs are positively charged, interactions with GAGs are very likely to occur. Although numerous studies have investigated the involvement of GAGs in the cellular uptake of CPPs, the results have remained contradictory. In order to assess whether GAGs are involved in the direct penetration of CPPs, we utilized heparan sulfate- (HS^{neg}) and GAG-deficient (GAG^{neg}) CHO cell lines to prepare the GPMVs. To our surprise, accumulation of arginine-rich

CPPs into all three types of vesicles remained similar, suggesting that despite of the expected high affinity towards GAGs, these polysaccharides are not involved in the direct translocation of arginine-rich CPPs through the membrane. In contrast, we detected almost 50 % decrease in the accumulation of TP into GAG-deficient vesicles. Similar results were obtained with TP10, although translocation of the peptide into GPMVs was affected to a slightly lower extent. The accumulation rate of all four peptides into HS^{neg} vesicles was comparable to the translocation efficacy of WT GPMVs. Although in heparan sulfate-negative cell line, chondroitin sulfates are overexpressed at three times higher level, which could compensate the lack of HS, the obtained results still show only a negligible role of HS in the translocation of studied CPPs. Furthermore, although we show here that the direct translocation of amphipathic CPPs into GPMVs is strongly dependent on the presence of GAGs, still, in their absence, the accumulation of peptides into vesicles was not abolished completely. This could be resulting from the fact that GAG^{neg} vesicles still contain hyaluronic acid polymers on their surface, which could also interact with CPPs and mediate their translocation. To confirm the results, we specifically degraded particular GAGs from the membrane of the WT GPMVs using treatment with selective glycosidases. In concordance with our results obtained with GAG- and HS-deficient vesicles, the internalization into vesicles that lacked specifically HS, CS and HA was equally efficient in the case of both arginine-rich CPPs, further indicating that the direct translocation is not specifically mediated by any of these GAGs. Also, membrane-crossing of both amphipathic CPPs was greatly affected by the removal of HA confirming our previously obtained results. However, the internalization of the amphipathic CPPs into vesicles where HS and CS were degraded was not hindered, implying that the translocation is not specifically facilitated by those GAGs. Translocation of amphipathic CPPs is not specifically mediated by HA either, as the internalization was decreased in GAG^{neg} cells, despite the presence of HA. On the other hand, the uptake was almost completely abolished when HA was degraded on the surface of GPMVs, which most likely results from the fact that with the removal of HA, other GAGs that are attached to HA are simultaneously removed. Thus, these results suggest that the interaction of amphipathic CPPs is not facilitated by a specific type of GAG, but rather the overall quantity of these polysaccharides is essential for their translocation.

Numerous articles have reported on the key role of GAGs, especially HS, in the cellular uptake of highly cationic CPPs, such as Tat peptide and oligoarginines (Console *et al.* 2003; Fuchs and Raines 2004; Richard *et al.* 2005; Nakase *et al.* 2007). Fewer studies have focused on the importance of GAGs in the internalization of amphipathic peptides. As cationic CPPs carry a high positive charge and therefore have a strong affinity towards negatively charged GAGs, the logical conclusion is that the stronger electrostatic interactions are responsible for the more efficient internalization. In light of our results, this view is overly simplified, which is also supported by the studies showing that high affinity does not always correlate with the better cellular uptake of CPPs (Jiao *et al.* 2009; Verdurmen *et al.* 2013). Furthermore, hydrophobic interactions are highly

frequent between glycans and polypeptides, and can also involve aromatic residues and alkyl side chains of amino acids (Cummings and Esko 2009). The role of hydrophobic amino acids in GAG-binding and subsequent internalization was also recently demonstrated for CPPs (Bechara *et al.* 2013). Nevertheless, the surface charge is an important parameter of glycosaminoglycans in binding to polypeptides (Varki *et al.* 2009). Furthermore, GAG-binding amino acid sequences in protein always contain positively charged amino acids, arginine and lysine (Esko and Linhardt 2009).

Although we demonstrate here that the role of GAGs in the internalization of Arg_9 and pTat is less significant, the involvement of glycans and also GAGs cannot be excluded altogether, since other glycans may compensate the lack of GAGs, in particular, when the arginine content in CPPs is higher. This assumption was confirmed by a study demonstrating that when some GAGs are missing the internalization of the peptide with the highest arginine content is least affected (Åmand *et al.* 2012). This view is also supported by an earlier report, where Nakase *et al.* proposed that if HS is missing, pTat that has lower charge density than nona-arginine cannot bind to other GAGs, which have lower sulfate content compared with HS. Octa-arginine, in contrary, can compensate the absence of HS by associating with other GAGs and internalize even though HS are missing (Nakase *et al.* 2007). However, these findings were described for the endocytic uptake and might not apply for the direct translocation, since we did not observe differences in the GAG-dependent accumulation of Arg_9 and pTat in GPMVs. Indeed, the consensus view of GAG-dependent cellular uptake of CPPs seems to support the notion that GAGs, and in particular heparan sulfate proteoglycans (HSPGs), mediate the endocytic internalization of several CPPs. This is not unexpected, as PGs can act as receptors at induction of endocytosis for the clearance of bound ligands (Esko *et al.* 2009).

4.7 Transportan co-localizes in the membrane subdomains enriched with glycans (Paper III)

Interestingly, using co-incubation of WGA and TP, we detected that these molecules co-localize in the same subdomains in the membrane of GPMVs. As WGA binds to sialic acids and to the *N*-acetylglucosamine residues, which are present in heparan sulfates, hyaluronic acids and keratan sulfates, TP probably also interacts with these glycans. Furthermore, after addition of TP to the suspension of GPMVs, the WGA nanoclusters assembled into microdomains in the membrane of vesicles. In principle, there are two plausible explanations for the appearance of TP-induced WGA microdomains. The first involves the reorganization in the membrane of vesicles by TP, which in turn induces the relocalization of GAGs. On the other hand, TP can cross-link GAGs and by this induce reorganization of lipids and formation of bigger GAG clusters. Both scenarios are substantiated by results published earlier: the first demonstrate reorganization of lipids by CPPs in the membrane of artificial vesicles and

plasma membrane spheres (Alves *et al.* 2009; Maniti *et al.* 2014). Later reports have demonstrated clustering of GAGs by various CPPs, including amphipathic peptides (Ziegler and Seelig 2011; Verdurmen *et al.* 2013). However, GAG-clustering does not always correlate with the increased internalization efficacy of CPPs (Verdurmen *et al.* 2013). Although, in cells, such micro-scale lipid rearrangements are hindered by the actin cortex and are thus, unlikely to occur, the membrane reorganization during the direct translocation can still take place at a smaller scale.

4.8 Discussion

Taken together, the findings of this research provide novel insights into the translocation mechanism of CPPs across membranes and provide information about the putative partner molecules that facilitate efficient internalization. We first identified that all studied CPPs were able to penetrate and accumulate in the lumen of GPMVs. However, further investigations led to a division of studied CPPs into separate classes, based on their interactions with the membrane constituents and internalization. The first group comprises arginine-rich pTat and Arg_9 that belong to a class of cationic-CPPs, whereas amphipathic CPPs, TP, TP10, MAP form the second group. Correlating well with its sequence – containing arginines, but also hydrophobic amino acids; pAntp falls in-between those two classes based on membrane interactions.

Arginine-rich CPPs translocated efficiently into the lumen of GPMVs and unlike amphipathic CPPs, these did not accumulate in the membrane of the vesicles. The internalization efficacy of these peptides and pAntp was slightly enhanced when the membrane fluidity was increased. Furthermore, the accumulation of Arg_9 and pTat into vesicles was greatly promoted upon the induction of ceramide in the membrane of GPMVs. Ceramide can induce membrane curvature (Holopainen *et al.* 2000), and as previously proposed, the borderlines between ceramide-rich regions and the rest of the membrane are the most permeable sites (Verdurmen *et al.* 2010), both of which favour the internalization of CPPs. The membrane permeabilization of highly charged molecules is energetically disfavoured, and these membrane regions, with the defects in the arrangements of lipids, could lower the energy needed for the direct translocation. Our results also support this assumption as we demonstrated that degradation of proteins on the surface of GPMVs almost abolished the translocation of arginine-rich CPPs into the lumen of vesicles. Since proteins have a key role in organizing membrane rafts, the edges of these membrane areas can have lipid packing defects, similarly to the borderlines of ceramide-rich regions; and after the abolition of the rafts, the favourable “sites” for translocation of CPPs are also lost. The membrane-organizing effect of proteins was actually confirmed by our observation that after the degradation of proteins on the surface of GPMVs, no lipid phase segregation was detected, even at low temperatures, indicating permanent changes in membrane microenvironment. However,

we cannot rule out the potential role of a receptor protein in the membrane that facilitates the internalization of arginine-rich CPPs. Further analysis of the potential binding partners on the cell surface, revealed that GAGs are not essential for the direct translocation of arginine-rich CPPs. As noted above, unlike arginine-rich CPPs, amphipathic peptides MAP, TP, TP10 and pAntp also concentrated into the membrane of vesicles clearly implying that amphipathicity strongly contributes to the membrane affinity. Furthermore, we identified that these peptides preferentially localized into liquid-disordered membrane regions, implying that translocation might take place from these more dynamic domains. To further confirm this notion, we modified the membrane composition of the vesicles to increase the proportion of a highly ordered "state" by the addition of cholesterol. This resulted in a dramatic decrease in the internalization efficacy of amphipathic CPPs (except pAntp), indicating that these peptides translocate through more dynamic membrane regions. Confirming these results, we demonstrated that after inducing the ceramide formation in GPMVs, and thereby creating rigid lipid platforms in the membrane of vesicles, accumulation of amphipathic CPPs in vesicles was strongly inhibited. Thus, it is reasonable to conclude that membrane regions with the higher fluidity are the preferential sites for the membrane insertion and subsequent internalization of studied amphipathic CPPs. This, in turn, implies that perhaps translocation of CPPs across membranes involves hydrophobic interactions with the lipids and lipid rearrangements that can favourably take place in these more dynamic membrane regions as it has been also previously suggested (Arsov *et al.* 2008). The analysis of the composition of the native plasma membrane demonstrates that most of it is in the ordered or "raft" phase, whereas only about 25% is in a disordered state (Owen *et al.* 2012). In light of this, the major part of the plasma membrane of the cells is not suitable for the translocation of amphipathic CPPs. Nevertheless, the studied peptides are still efficient CPPs, capable of penetrating the membrane, implying that although the entry into cells can take place only from a small area of the membrane, it is still sufficient for the efficient internalization. Our further focusing on the role of membrane proteins in the translocation of these peptides led to the conclusion that amphipathic CPPs can penetrate the membrane despite deficiency of protein motifs on the surface of GPMVs. Although we detected a slight decrease in the accumulation efficacy after protein degradation from the membrane of GPMVs, it probably resulted from the fact that during trypsin treatment, some proteoglycans were also removed. These proteoglycans carry GAGs, which have a vital role in the internalization of amphipathic CPPs that we hereinafter discuss. We further focused on the role of GAGs and also on their abundance in the translocation of CPPs, rather than on the contribution of specific proteoglycan, since one proteoglycan molecule (e.g syndecan-1) can carry a highly varying number of GAG chains. Moreover, the GAGs on the same proteoglycan can have different length and position of sulfate groups, therefore possessing a vast number of possible motifs in the structure (Esko *et al.* 2009), and leading to dissimilar affinity for CPPs. Although there have been only rare implications of the GAG-dependent cellular

uptake of amphipathic CPPs, we show that the accumulation efficacy into the GAG-deficient vesicles is markedly lower, and the internalization was almost completely abolished after the enzymatic degradation hyaluronic acid polymers on GPMVs. In addition, we demonstrate that the internalization of amphipathic CPPs in not dependent on a specific GAG type, but rather the overall amount of GAGs is essential. It is plausible, that these huge branched cell surface polysaccharides facilitate the accumulation of amphipathic CPPs into the close proximity of the membrane and also help to achieve high local concentration, which is needed for the subsequent reorganization of lipids in order to translocate into vesicles/cells. Analogously, the high local concentration is a prerequisite for the membrane activity of antimicrobial peptides, which share many common features with the studied amphipathic CPPs (Melo *et al.* 2009). Thus, the high local peptide concentration provided by the GAGs is probably required for both, the antimicrobial and cell penetrating activity of the respective peptides. In support of this view, we demonstrated that TP localizes into the L_d regions of the membrane together with GAGs, and also TP might rearrange the membrane of GPMVs, by inducing the formation of big clusters of GAGs. Possibly the latter due to the electrostatic and hydrophobic "cross-linking" of GAGs by amphipathic CPPs and "pulling" these together to form microscopic L_d platforms.

SUMMARY

The plasma membrane of cells is an impermeable barrier for the macromolecules, including agents with very high therapeutic and biotechnological potential, posing a major hurdle for their application. However, this bottleneck can be overcome by harnessing carrier systems for cellular delivery. The cell-penetrating peptides (CPPs) form a class of efficient transport vehicles that represent short peptide sequences, generally consisting of 4–40 amino acids, and having the capacity to cross the cell membrane and promote the intracellular delivery of various bioactive cargoes.

Despite the remarkable progress in the general understanding, how CPPs operate on a cellular level, still the information about their specific binding partners, exact plasma membrane composition and the mechanisms of translocation through membranes have remained controversial. It is widely accepted that CPPs enter cells via different pathways of endocytosis and direct translocation through the plasma membrane. While the endocytosis mechanisms have been well described, the latter internalization mode has remained elusive so far. Therefore, in the current thesis, we focused on the role of several plasma membrane constitutes, as well as the specific membrane composition, essential for the translocation of CPPs across biomembranes.

The main results of this study are as follows:

- The representatives of cationic arginine-rich CPPs, Tat peptide and nona-arginine, and amphipathic CPPs, penetratin, TP, TP10 and MAP, can penetrate the lipid bilayer derived from the plasma membrane of cells. Amphipathic CPPs have higher affinity for the membrane compared with the arginine-rich CPPs, and associate with more dynamic membrane regions (Paper I).
- Cholesterol and tightly packed raft-like membrane microdomains inhibit the translocation of CPPs, affecting more strongly the penetration of amphipathic peptides (Paper II).
- Ceramide interferes with the internalization of amphipathic CPPs, but promotes the direct translocation of arginine-rich peptides (Paper II).
- The proteins in the membrane of GPMVs are indispensable for the translocation of arginine-rich CPPs, whereas do not markedly contribute to the penetration of amphipathic peptides into vesicles (Paper II).
- Cell surface glycosaminoglycans are essential for the membrane crossing of amphipathic CPPs. Translocation of amphipathic CPPs does not depend on a specific type of glycosaminoglycan, but rather on the overall amount of these polysaccharides. Glycosaminoglycans are not required for the penetration of arginine-rich CPPs across biomembranes (Paper III).

SUMMARY IN ESTONIAN

Rakku sisenevate peptiidide membraani läbimise mehhanismid ja interaktsioonid plasmamembraani komponentidega

Raku plasmamembraan ümbritseb ja kaitseb rakku ning on bioloogilistele molekulidele selektiivselt läbilaskev, olles ka barjääriks paljudele potentsiaalsetele terapeutilise toimega ühenditele. Enamik bioaktiivseid ühendeid on suured ja hüdrofiilsed ning seetõttu on takistatud nende rakkudesse pääsemine ja kasutamine ravimina. Et suurendada bioaktiivsete molekulide rakkudesse pääsemist, on kasutusele võetud erinevad transportsüsteemid. Ühed perspektiivsemad transportvektorid on 4–40 aminohappest koosnevad järjestused – rakku sisenevad peptiidid (RSP), mis on võimelised läbima bioloogilisi membraane ning vahendama erinevate bioaktiivsete ühendite transporti rakkudesse.

RSP-de efektiivseks kasutamiseks biotehnoloogias ja meditsiinis on oluline mõista RSP-de membraani läbimise mehhanisme raku tasemel. Arvukad uuringud on näidanud, et RSP-d liiguvad rakkude väliskeskkonnast tsütoplasmasse, kasutades erinevaid endotsütoosiradu või läbivad membraani raku energiast sõltumatult – translokatsiooni teel. Samas ei ole siiski seniajani üheselt selge, kuidas RSP-de translokatsioon molekulaarsete mehhanismide tasemel täpset aset leiab ning millised interaktsioonid RSP-de ja raku pinna erinevate komponentide vahel sisenemist soodustavad.

Seetõttu oli käesoleva töö põhieesmärkideks uurida:

- Millistest plasmamembraani piirkondadest toimub eelistatult RSP-de rakkudesse sisenemine ja kuidas mõjutavad membraani läbimist selle erinevate omadustega lipiidide mikrodomeenid ning membraani komponendid kolesterool ja tseramiid.
- Kas plasmamembraani valgud on funktsionaalselt olulised RSP-de translokeerumisel läbi bioloogilise membraani.
- Kas ja millised raku pinna glükoosaminoglükaanid on vajalikud RSP-dele membraani läbimiseks.

Rakumembraan on väga dünaamiline ja selle koostist mõjutavad katkematult toimivad rakulised protsessid, mistõttu on plasmamembraani erinevate koostisosade ja RSP-de vaheliste interaktsioonide uurimine rakkudes komplitseeritud. Lisaks toimuvad rakkudes pidevalt endotsütootilised protsessid, mis raskendavad arusaamist, kas RSP-de rakkudesse sisenemine toimub translokatsiooni, endotsütoosi või mõlema mehhanismi vahendusel. Seetõttu kasutati antud töös plasma membraani vesiikuleid (PMV-sid). PMV-d on ideaalne mudelsüsteem uurimaks translokatsiooni, kuna nendes vesiikulites on rakuenergiast sõltuvad protsessid k.a endotüstoos blokeeritud, võimaldades keskenduda vaid translokatsiooni uurimisele. Lisaks on vesiikulite membraan oma koostiselt äärmiselt sarnane rakkude plasmamembraaniga, andes võimaluse vaadelda RSP-de interaktsioone erinevate membraanikomponentidega. Kasutades PMVsid uuriti kuut tuntud RSP-d, mis esindavad arginiinirikkaid (Arg_9 ja Tat peptiid) ning amfi-

paatseid (TP, TP10, penetratiin, MAP) peptiide. Kuna PMV-des võivad membraani erinevate omadustega lipiidsed vedelfaasid temperatuurist sõltuvalt lahkneda, siis iseloomustati esmalt RSP-de interaktsioone ja membraani läbimist-lähtuvalt nendest mikropiirkondadest. Uurimuse tulemused näitasid, et amfi-paatsetel peptiididel on kõrgem afiinsus membraani suhtes võrreldes arginiini-rikaste RSP-dega ja et amfipaatsed RSP-d eelistavad seonduda dünaamilisema ning madalama korrapäraga lipiidifaasiga. Seetõttu võib eeldada, et ka nende RSP-de rakku sisenemine toimub sarnase iseloomuga membraanipiirkondade kaudu. Kuna kolesterool on oluline kõrgema korrapäraga lipiidifaaside moodustu-miseks, kus see võimaldab fosfolipiidide tihedalt kokku pakkida, siis järgnevalt hinnati kolesterooli mõju RSP-de vesiikulitesse sisenemise efektiivsusele. Tule-mustest selgus, et kolesterooli kõrgem kontsentratsioon membraanis vähendas kõikide uuritud RSP-de, ning eriti mõjusalt amfipaatsete peptiidide membraani läbimise efektiivsust. Seega võib järeldada, et kolesterooli lisamisel vesiikulite membraani suureneb selles tõenäoliselt korrastatud faasi osakaal ja RSP-dele vesiikulitesse sisenemiseks sobivate membraanipiirkondade pindala väheneb. Saadud tulemused kinnitasid meie hüpoteesi, et RSP-d liiguvad läbi membraani hõredamalt pakitud membraanipiirkondade kaudu. Lisaks kolesteroolile modu-leerib plasmamembraanis paiknev tseramiid membraani struktuuri ja omadusi, mõjutades sealhulgas ka membraani kõverust ja moodustades plasmamemb-raanis tihedalt pakitud tseramiidi parvesid. Sellest tulenevalt uuriti, kas taolised lipiidide ümberkorraldused võivad mõjutada RSP-de penetratsiooni PMV-desse. Selgus, et arginiinirikkad RSP-d (Arg_9 ja pTat) olid võimelised tseramiidirikast membraani läbima paremini, kuid amfipaatsete RSP-de (TP ja TP10) penetrat-sioon vesiikulitesse oli tugevalt takistatud. Varasemalt on näidatud, et tseramiid põhjustab membraani kõverdumist sissepoole ja see võis soodustada arginiini-rikaste RSP-de sisenemist vesiikulitesse. Kuna amfipaatsed peptiidid vajavad membraani läbimiseks rohkem dünaamilisi membraani piirkondi, siis arvata-vasti takistas nende RSP-de sisenemist vesiikulitesse eelkõige tseramiidi võime moodustada membraanis korrastatud lipiidifaase. Hiljuti näidati, et ka valgud osalevad plasmamembraani lipiidifaaside organiseerimises, mistõttu uuriti val-kude osalust RSP-de sisenemises vesiikulitesse. Meie tulemused näitasid, et arginiinirikkad RSP-d vajavad membraani läbimiseks valke, mis võivad olla kas peptiididele retseptoriks või siis moduleerivad valgud vesiikulite membraanide omadusi hõlbustades penetreerumist. Samas selgus, et amfipaatsed RSP-d mem-braani läbimiseks valgulist komponenti ei vaja. Viimasena hinnati rakuvälise maatriksi polüsahhariidide – glükoosaminoglükaanide osalust RSP-de trans-lokatsioonis läbi membraani PMV-desse. Kuna glükoosaminoglükaanid on nega-tiivselt laetud, siis arvatavasti on need esimesed plasmamembraani komponendid, millega katioonsed RSP-d interakteeruvad. Tulemused näitasid, et vastupidiselt eeldatule on glükoosaminoglükaanid olulised amfipaatsete peptiidide membraani läbimisel, kuid arginiinirikaste RSP-de penetratsioon toimub tõenäoliselt plasma-membraani polüsahhariididest sõltumatult. Seda kinnitab ka tulemus, et memb-raani korrastumata faasi, kuhu seonduvad amfipaatsed RSP-d, koondusid ka glükoosaminoglükaanid. Lisaks olid amfipaatsed RSP-d võimelised vesiikulite

membraani ümber korraldama, koondades need polüsahhariidid lipiidsetesse parvedesse.

Kokkuvõtvalt kinnitas käesolev töö, et uuritud RSP-d suudavad raku-energiast sõltumatut mehhanismi kasutades läbida plasmamembraani ilma selle barjäärifunktsiooni kahjustamata ning nende sisenemine ja interaktsioonid membraani erinevate komponentidega sõltuvad peptiidide füüsikalis-keemilistest omadustest. Selle põhjal jagunesid uuritud peptiidid kahte klassi: arginiinirikkad RSP-d, mille translokatsioonis osalevad membraani pinna valgud ning nende membraani läbimise efektiivsus suureneb membraanis tseramiidi olemasolul. Amfipaatsete peptiidide translokatsioon seevastu toimub membraani hõredamalt pakitud regioonidest ja kaasab glükoosaminoglükaanid.

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PUBLICATIONS

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Main research interests

The focus of my research is defining the plasma membrane constituents that are involved in the translocation of cell penetrating peptides (CPPs) and describing the following internalization mechanisms.

List of publications

Säälik, P., Niinep, A., **Pae, J**., Hansen, M., Lubenets, D., Langel, Ü., Pooga, M. (2011). Penetration without cells: membrane translocation of cell-penetrating peptides in the model giant plasma membrane vesicles. Journal of Controlled Release, 153(2):117–125.

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2011	Tartu Ülikooli korraldatav konverents "Peptide Vectors and Delivery of Therapeutics", Tallinn, Eesti

Teadusorganisatsioonid

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Teadustöö

Uurimistöö põhisuunaks on peptiidsete transportvektorite – rakku sisenevate peptiidide (RSP-de) translokatsiooni mehhanismide ja RSP-de ning raku pinna erinevate komponentide vaheliste interaktsioonide uurimine.

Teaduspublikatsioonid

Säälik, P., Niinep, A., **Pae, J**., Hansen, M., Lubenets, D., Langel, Ü., Pooga, M. (2011). Penetration without cells: membrane translocation of cell-penetrating peptides in the model giant plasma membrane vesicles. Journal of Controlled Release, 153(2):117–125.

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Ühiskondlik tegevus

2012–…	Rahvusvahelise noorteorganisatsiooni AEGEE/Euroopa tudengite foorum liige
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