Design and evaluation of drug delivery vehicles

Caroline Palm Apergi
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Caroline Palm Apergi
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To Jannis, η αγάπη μου
This thesis is based on the following publications, referred to in the text by the following Roman numerals:


A crucial aspect of drug delivery is efficient transport to the site of action. Thus, there is a need to design and evaluate new delivery vehicles. In this thesis two delivery vehicles, cell-penetrating peptides and bacterial ghosts, were evaluated. The understanding of the internalization and degradation kinetics of cell-penetrating peptides is important for the practical aspects of cargo delivery since peptides have a notorious reputation of being rapidly degraded. If the cell-penetrating peptide remains intact inside the cellular environment, there is a possibility that the peptide-cargo conjugate leaks back to the extracellular environment. However, if it is degraded outside the cell, the cargo will never be delivered. In order to improve uptake efficiency and to be able to foresee side effects, the translocation mechanism needs to be fully elucidated.

Data gathered from the first two papers led to the proposal of a new mechanism involved in cell-penetrating peptide uptake: the membrane repair response, a resealing mechanism rapidly patching up broken membranes. This mechanism could explain the divergence in perception concerning the uptake pathways. Furthermore a new assay to produce the second delivery vehicle, bacterial ghosts, was developed based on data from the cell-penetrating peptide investigations. Bacterial ghosts are dead bacteria devoid of cytoplasmic contents but still retaining their structural and morphological characteristics, after protein E lysis of the bacterial cell membrane. By using a cell-penetrating peptide with antimicrobial effects, a new rapid peptide-based strategy to produce ghosts was developed and the capability to deliver plasmid DNA into the cell for expression was evaluated.
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<tbody>
<tr>
<td>AAK</td>
<td>Adaptor-associated kinase</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AM</td>
<td>Acetoxyethylmethylester</td>
</tr>
<tr>
<td>AMP</td>
<td>Antimicrobial peptides</td>
</tr>
<tr>
<td>AP2</td>
<td>Adaptor protein 2</td>
</tr>
<tr>
<td>ARF</td>
<td>ADP-ribosylation factor</td>
</tr>
<tr>
<td>Axh</td>
<td>Aminohexanoic acid</td>
</tr>
<tr>
<td>Calu-3</td>
<td>Caucasian lung adenocarcinoma</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Calcium/calmodulin-dependent kinase II</td>
</tr>
<tr>
<td>CCV</td>
<td>Clathrin coated vesicle</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CME</td>
<td>Clathrin-mediated endocytosis</td>
</tr>
<tr>
<td>CPPs</td>
<td>Cell-penetrating peptides</td>
</tr>
<tr>
<td>CTB</td>
<td>Cholera Toxin B</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DCC</td>
<td>Dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DiBAC$_3$(4)</td>
<td>bis-(1,3-dibarbituric acid)-trimethine oxanol</td>
</tr>
<tr>
<td>DIC</td>
<td>Diisopropylcarbodiimide</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>Fl</td>
<td>Fluorescence</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorter</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosyl-phosphatidylinositol</td>
</tr>
<tr>
<td>HA2</td>
<td>Hemagglutinin peptide 2</td>
</tr>
<tr>
<td>HIV-1</td>
<td>Human immunodeficiency virus type 1</td>
</tr>
<tr>
<td>HOBr</td>
<td>N-Hydroxybenzotriazole</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>HS</td>
<td>Heparan sulfate</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan sulfate proteoglycan</td>
</tr>
<tr>
<td>hCT</td>
<td>Human calcitonin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LAP</td>
<td>Leucine aminopeptidase</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAP</td>
<td>Model amphipathic peptide</td>
</tr>
<tr>
<td>MBHA</td>
<td>4-Methylbenzhydrylamine</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby Canine Kidney</td>
</tr>
<tr>
<td>MFS</td>
<td>Major facilitator superfamily</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MRR</td>
<td>Membrane repair response</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
</tr>
<tr>
<td>Omp</td>
<td>Outer membrane proteins</td>
</tr>
<tr>
<td>Pen</td>
<td>Penetratin</td>
</tr>
<tr>
<td>PG</td>
<td>Proteoglycan</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3 kinase</td>
</tr>
<tr>
<td>PTD</td>
<td>Protein transduction domain</td>
</tr>
<tr>
<td>pVEC</td>
<td>Vascular endothelial cadherin peptide</td>
</tr>
<tr>
<td>S9</td>
<td>Spodoptera frugiperda</td>
</tr>
<tr>
<td>SNAP</td>
<td>Synaptosomal-associated protein</td>
</tr>
<tr>
<td>SPPS</td>
<td>Solid phase peptide synthesis</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>TAP</td>
<td>Transporter associated with antigen processing</td>
</tr>
<tr>
<td>TAT</td>
<td>Trans-activator of transcription</td>
</tr>
<tr>
<td>t-Boc</td>
<td>tert-Butyloxycarbonyl</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TLR-4</td>
<td>Toll-like-receptor-4</td>
</tr>
<tr>
<td>TOP</td>
<td>Thimet oligopeptidase</td>
</tr>
<tr>
<td>TP10</td>
<td>Transportan 10</td>
</tr>
<tr>
<td>TPPII</td>
<td>Tripeptidyl peptidase II</td>
</tr>
</tbody>
</table>
**Introduction**

One of the major obstacles in drug delivery today, is for the drug to reach inside the cell to exert its biological effect. The difficulty lies in crossing the hydrophobic plasma membrane since many drug candidates are hydrophilic. Thus, there is a great need to design drug delivery vehicles, able to cross the protective plasma membrane, without inducing toxicity. Twenty years ago, it was shown that some proteins were able to internalize into cells. From these proteins, a relatively new group of delivery vehicles, named cell-penetrating peptides (CPPs), has evolved over the last fifteen years. Even though these drug delivery vehicles have been shown to deliver different cargo into cells, such as drugs and oligonucleotides, both *in vivo* and *in vitro*, the mechanism of entry has not been fully elucidated. Another delivery system, with inherent adjuvant properties, is nonliving bacteria known as bacterial ghosts, devoid of cytoplasmic contents but still retaining the native morphology. These features make them an excellent tool in drug delivery and DNA vaccination.

The focus of the thesis is to evaluate CPPs as transporters, to better understand how these peptides are able to traverse the plasma membrane, not only by themselves but also conjugated to cargo. The degradation kinetics of CPPs, both inside and outside the cell, was investigated both in mammalian and non-mammalian cells, in order to better understand the mechanism of entry, determine the influence of membrane composition on uptake, and to compare them to antimicrobial peptides. Shorter peptides were designed from proteins known to internalize into cells, to explore if the new peptides might have translocation ability. The new peptides, together with CPPs, were analyzed to investigate if a membrane repair response (MRR) was induced in cells during translocation, which could explain how CPPs translocate across the plasma membrane into the cytoplasm. By taking advantage of the results from the first paper in the thesis, CPPs were used to produce another delivery vehicle, bacterial ghosts, to investigate if the peptide-produced ghosts were able to deliver plasmids into cancer cells.
The cell membrane

The cytoplasm of both prokaryotes and eukaryotes, are surrounded by a plasma membrane, protecting it from the extracellular environment. The membrane is a requirement for all living organisms for many reasons and its structure was first described as a fluid mosaic model by Singer in 1972\(^1\). Membranes are not only needed to maintain intracellular compartments in a highly ordered state but also for acquiring nutrients and eliminating wastes. Independent of cell type, all membranes consist of a phospholipid bilayer, about 5-10 nm thick\(^2,3\). The protein, sugar, and lipid composition can vary and determines the function of the cell. The phospholipid itself consists of a hydrophilic polar head and a hydrophobic tail. These amphipathic properties result in the formation of a bilayer. Inside the bilayer, the lipids are shielded from the aqueous surrounding, in a hydrophobic interior approximately 4 nm thick\(^2\). The bilayer can be divided into two leaflets and the major driving force for the formation of the bilayer, is hydrophobic interactions between the fatty acyl chains, and hydrogen bonding and electrostatic interactions between polar head groups and water molecules, stabilizing the bilayer. Due to the thermal motion, phospholipids and glycolipids can rotate freely around their own axes and diffuse several micrometers per second laterally, within the membrane leaflet. Most importantly, the plasma membrane has to respond to extracellular changes and transfer external stimuli to the cell. Thus, the membrane has an important role in cell function and the membrane composition of the analyzed cells will be described below.

Prokaryotes

The plasma membrane of most prokaryotes usually has higher protein content than eukaryotes, due to the many functions that need to be performed in bacteria. Sterols like cholesterol are often not present in prokaryotic membranes. Instead hopanoids, a pentacyclic sterol-like molecule, synthesized from the same steroid precursor, is found in prokaryotic membranes (Ourisson87). Outside the cytoplasmic plasma membrane lies the periplasmic space, surrounded by a complex cell wall of peptidoglycan, together forming the cell envelope. There are two types of bacterium that differ in the structural composition of the membrane, gram-negative and gram-positive bacteria. Gram-positive bacteria, have a plasma membrane surrounded by a thin periplasmic space, followed by a single 20-80 nm thick peptidoglycan layer, where acidic polysaccharides, such as teichoic acid, give the membrane a net negative charge (Figure 1)\(^4\).
Peptidoglycan is a giant polymer composed of glycan strands consisting of alternating N-acetylglucosamine and N-acetylmuramic acid, cross-linked by short peptides, referred to as stem peptides. The stem peptides comprise amino acids of both D and L configurations, shielding the peptidoglycan from most peptidases. In comparison to gram-positive bacteria, the peptidoglycan layer of gram-negative bacteria, is approximately 2-7 nm and surrounded by an outer membrane (Figure 2). The peptidoglycan layer and the outer membrane, are firmly linked by a membrane protein named Braun’s lipoprotein. In addition, the outer part of the lipid bilayer, in the outer membrane, is composed of lipopolysaccharides (LPSs), giving the membrane a negative net charge. These LPS molecules are composed of three parts: lipid A, comprising two glucosamine units and long fatty tails, the core polysaccharide and an O side chain extending from the polysaccharide that can be rapidly changed, to avoid detection by the immune system. Thus, the cell envelope of gram-negative bacteria is much more complex than the cell envelope of gram-positive bacteria.

Figure 1. Structure of the gram-positive cell envelope. The plasma membrane is composed of phospholipids (brown) with hydrophobic tails (yellow area) and comprises several proteins (blue). The periplasmic space (turquoise) is situated between the peptidoglycan layer (pink) and the plasma membrane. Anionic saccharides, such as teichoic acids (grey), protrude from the plasma membrane through the peptidoglycan layer.
Figure 2. Structure of the gram-negative cell envelope. The inner and outer plasma membranes are composed of phospholipids (brown) with hydrophobic tails (yellow area) and comprise several proteins (blue) with different functions. The periplasmic space (turquoise), together with the peptidoglycan layer (pink), is situated between the inner and outer plasma membranes. The outer leaflet of the outer membrane consists of lipid A (green), and not phospholipids. Linked to lipid A, is a core polysaccharide, with an extending O side chain (grey), together forming the LPS.

Eukaryotes

Prokaryotes and eukaryotes differ in several structural features. Eukaryotes have membrane bound organelles and membrane enclosed nuclei, whereas the size of prokaryotes is often the same as chloroplasts or mitochondria. The much more structurally organized eukaryotes have only one lipid bilayer and most of them do not have a cell wall. One exception is yeast, where the plasma membrane is surrounded by a cell wall of chitin. The chitin layer comprises repetitive units of glucose, N-acetylglucosamine and mannose residues that function to maintain the structure and rigidity of the cell\(^9\),\(^10\). Solutes of lower mass than 600 Da, can diffuse freely through the cell wall, whereas the plasma membrane forms a relatively impermeable barrier for hydrophilic molecules\(^11\). Nevertheless, the plasma membrane of the yeast \textit{S. cerevisiae} resembles the membrane of higher eukaryotes, with respect to lipid composition. Like prokaryotes, specialized proteins mediate the uptake
and secretion of solutes, in and out of the cell. However, the lipid composition of the plasma membrane and organelle membrane is distinct. Therefore special trafficking of lipids from internal membranes, especially to the endoplasmic reticulum (ER) and Golgi, is required. Nearly all cells, from invertebrates, to humans, have the capability to produce glycoproteins, such as proteoglycans (PGs). The PGs are composed of a core protein, covalently linked to one or several polysaccharide chains. A major constituent of the extracellular matrix, are the heparan sulfate PGs (HSPGs), comprising repetitive disaccharide units of variable sugars. Under physiological conditions, the polysaccharide chains, or glycosaminoglycans (GAGs), are anionic due to the alternating N-acetylated or N-sulfated glucosamine and uronic acid units. In vivo, the GAG side chains are approximately 50-150 disaccharide unit repeats long, resulting in great structural diversity. The HSPGs can be divided into three subgroups: the membrane spanning PGs, the glycophasphatidylinositol (GPI)-linked PGs, and the secreted extracellular matrix PGs. Other variably sulfated sugars can be found in the polysaccharide chains of PGs, such as chondroitin sulfate, dermatan sulfate, and heparin, closely related to HSPGs.

Membrane transport

An enormous number of channels and transporter proteins have been evolved by nature to control the translocation of molecules across the plasma membrane and internal membranes. Some systems can be found in both prokaryotes and eukaryotes e.g. ATP-binding cassette (ABC) transporters. Other systems are specific for only eukaryotes, such as endocytosis, which can not be found in prokaryotes. A brief introduction will follow, covering the main transporter systems of prokaryotes and eukaryotes.

Prokaryotic transporter systems

The term passive diffusion refers to concentration-dependent movement of molecules, from one compartment to another, without requiring metabolic energy. Prokaryotes, do not depend on passive diffusion since the concentration of most nutrients outside the cell, is lower than inside the cell. Thus, nutrients need to be transported against the concentration gradient via a carrier protein, a process requiring energy. Three main transporters are engaged by bacteria: 1) primary transporters, such as ABC transporters that use ATP to drive substrates across membranes, 2) secondary transporters, that use the free energy from the primary transporters, to translocate substrates, 3) group translocation systems, which modify the substrates chemically during translocation.
The largest family of protein transport systems belonging to the group of primary transporters, is the ABC transporters, present in both prokaryotes and eukaryotes. ABC transporters can be divided into three categories; importers, exporters, and a group not involved in transport but DNA repair. A common organization is found in importers and exporters, which are composed of two hydrophobic membrane spanning or integral membrane domains, forming a pore in the membrane. On the cytosolic side of the membrane, two hydrophilic domains carrying the ABC, and two nucleotide binding domains, peripherally associated with the integral membrane domains, can be found. ABC importers are dependent on special binding proteins, located in the periplasmic space of gram-negative bacteria. In gram-positive bacteria, they are predominantly lipoproteins, attached to the cytoplasmic membrane. However, they can be fused to the membrane transporter itself, in some gram-positive organisms. The first barrier, in translocation into the cytoplasm of gram-negative bacteria, is the outer membrane. Molecules below 650 Da, can pass through the nonspecific outer membrane proteins (Omp), belonging to the class of porin proteins, e.g. OmpA in Escherichia coli. However, specialized porins take over, when the size of the substrate exceeds the limit of the generalized porins. After translocation through porins to the periplasm, binding proteins in the periplasm can bind and transport many different substrates, such as oligosaccharides and inorganic ions but most importantly, amino acids and peptides.

The second largest family of transporters belonging to the family of secondary transporters, is the major facilitator superfamily (MFS) that covers about 25% of all prokaryotic transport proteins. However, in some gram-positive bacteria, MFS is more prevalent than ABC transporters. There are three distinct mechanisms coupled to the MFS; uniporters, symporters, and antiporters that transfer a tremendous variety of molecules such as sugars, drugs and peptides. Thus, there are several transporter systems able to translocate peptides in an energy dependent manner. Some transporters, e.g. the ABC transporter OppA in Lactococcus lactis, can handle peptides up to 18 amino acids in length. However, most of the MFS and ABC transporters can only translocate shorter peptides, less than 10 amino acids.

Eukaryotic transporter systems

Small molecules, such as saccharides, amino acids, and short peptides, can translocate across the membrane via protein transport systems, as mentioned above. However, larger macromolecules have to be taken up by membrane invagination or protrusion, forming a vesicle that can be pinched off from the membrane, in order to enter the cell. This uptake process, called endocytosis, is a characteristic of eukaryotic cells and can be divided into two subgroups: phagocytosis and pinocytosis. Phagocytosis occurs mostly in cells involved in innate immunity, whereas pinocytosis, is found in most cells and
can be divided into 4 categories: macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis (Figure 3). An introduction to the different forms of endocytosis will be given below.

**Phagocytosis**

Phagocytosis involves uptake of many large particles, *e.g.*, pathogens or apoptotic cells and is in most cases performed by specialized cells, such as monocytes and macrophages. Other cells known to have evolved the receptor-mediated, actin-driven process of phagocytosis, are epithelial and endothelial cells. However, it should be noted that large particles and macromolecules, can be internalized by cells via macropinocytosis. When a particle has bound a receptor on the plasma membrane, transient actin rearrangement is required for the membrane to engulf the particle. It is a highly regulated process, involving signaling cascades mediated by Rho-family GTPases, where Rac and Cdc42 are activated, inducing the formation of protrusions on the cell surface that zipper up around the particle and engulf it.

**Macropinocytosis**

Several characteristics are shared between macropinocytosis and phagocytosis. They are distinct from other pinocytic pathways, in that much larger vesicles are formed, which lack coating and can be several micrometers in diameter. Both pathways are also morphologically more heterogeneous and induce protrusions, rather than invaginations of the plasma membrane. However, one distinguishing feature is that the protrusions in macropinocytosis, collapse onto the plasma membrane, forming a vesicle that can be pinched off from the membrane, in comparison to phagocytosis, where two protrusions come together like a zipper to form a vesicle. There are little or no receptors involved in macropinocytosis. Instead, macropinosomes form spontaneously from cell surface ruffles, or in response to growth-factor-receptor stimulation. Ruffles are sheet-like extensions of the cell surface that most of the time, recede into the cytoplasm, without forming vesicles and only sometimes, lead to the formation of macropinosomes. There are, however, cell lines with constitutive macropinocytosis, such as dendritic cells but in most cell lines, it is transiently induced.
In fact, two bacterial pathogens, *Salmonella typhimurium* and *Legionella pneumophila*, stimulate cell-ruffling and enter cells via macropinocytosis. The route of macropinosomes, do not depend on dynamin, unlike clathrin- and caveolae-mediated endocytosis and differs from other pinocytic pathways in that the vesicles do not reach the early/late endosomes or lysosomes. Although much has happened since Warren Lewis first described macropinocytosis, as waving sheets in 1931, there are still many questions to be answered about the intracellular destination of macropinosomes.

**Clathrin-mediated endocytosis**

The major route for nutrient uptake and receptor recycling, is clathrin-mediated endocytosis (CME), which occurs constitutively in all mammalian cells (Figure 3). Proteins, e.g. the cholesterol transporter low-density lipoprotein and the iron transporter transferrin, are classical exploiters of CME. The fundamental aspects of clathrin-coated pit formation, were first described in 1964, after visualization in electron microscopy. CME is a multistep process that begins with the assembly of soluble constituents, such as clathrin, adaptors, and accessory proteins of the coat, to site in the membrane where the vesicle will form around the cargo, *i.e.* the bud site.
Vesicle budding and package of cargo into vesicles, are triggered by interacting subunits of the coat complex, forming the coated vesicles as the emerging bud detaches from the membrane. Coat assembly may be enough to promote vesicle detachment in vitro, but in vivo the packaging requires other regulatory factors, like the dynamin family of GTPases. The major coat proteins for clathrin-coated vesicles, are two oligomeric complexes, clathrin and adaptor protein 2 (AP2). Clathrin is composed of three light chains and three heavy chains, coming together as a heterohexameric three-legged structure, named triskelion. Under non-physiologic conditions, the triseklion self-assembles into cages. However, the recruitment and assembly of clathrin on the plasma membrane into a polygonal lattice, surrounding the endocytic vesicles, are coordinated by adaptor proteins such as AP2. The invagination of the plasma membrane is thought to be driven by the assembly of the clathrin lattice, to facilitate vesicle formation. CME begins with the recruitment of AP2 complexes, to protease-sensitive sites on the plasma membrane, followed by binding to tyrosine-based sorting motifs on the cytoplasmic domain of the receptor, to coordinate clathrin assembly and concentration into coated pits. When the coat is assembled and pinched off from the plasma membrane, coat proteins are disassembled by the help of ATPases, releasing the coat proteins for the cycle to begin again. Evidence has been presented, indicating that CME is regulated via phosphorylation by adaptor-associated kinase 1 (AAK1), a binding partner of AP2 that phosphorylates AP2. Together with an unidentified phosphatase, facilitating AP2 recruitment to sorting signals, AAK1 was shown to regulate CME via a phosphorylation/dephosphorylation cycle. Clathrin also plays a regulatory role, by modulating the activity of AP2 complexes, through activation of AAK1. Moreover, AP2 has been shown to have a more prominent role in cargo recruitment than previously believed. Thus, more information is needed to fully understand the mechanistic order in which CME occurs and the exact assignments for each protein involved.

Caveolae-mediated endocytosis

In the 1950s, caveolae were identified as flask shaped invaginations of the plasma membrane, approximately 50-80 nm in diameter (Figure 3). Although present in many cell lines, they are especially abundant in endothelial cells, where they are involved in endocytosis and transcytosis of blood components. Caveolae are subdomains of lipid rafts, or plasma membrane microdomains, rich in cholesterol and sphingolipids. Caveolae-mediated endocytosis is believed to be involved in intracellular cholesterol trafficking and homeostasis but since caveolin null mice show no cholesterol imbalance at the plasma membrane, it is believed not to be the only mechanism. Caveolin, cholesterol and dynamin are known regulators of caveolae-mediated endocytosis, as well as regulators of the actin cytoskeleton. The shape and structural organization of caveolae is conferred by caveolin, a
dimeric protein that binds cholesterol and inserts as a loop into the inner leaflet of the plasma membrane, followed by self-association to form a caveolin coated vesicle. There are three types of caveolin; caveolin-1, 2 and 3, where caveolin-1 is a scaffolding protein that oligomerizes at the plasma membrane and is essential for the formation of caveolar vesicles. Cells that do not express caveolin, are absent of caveolae. However, by reintroducing it, the function is restored. Caveolin-2 is also involved in caveolae formation but is not essential, whereas caveolin-3 is specifically expressed in muscle cells. The internalization via caveolae-mediated endocytosis is dependent on dynamin and actin rearrangement, and uptake can be blocked by overexpressing dominant negative mutants of dynamin or disassembling the actin cytoskeleton. Even though caveolae are highly immobile at the plasma membrane, caveolin-1 mobility can be increased upon activation, by e.g. the simian virus 40 (SV40) that uses a caveolae-mediated pathway to enter cells and reach the smooth ER. However, in caveolin-1 knockout mice, the SV40 exploits a caveolin-independent pathway to internalize cells, indicating that if one endocytic route is blocked, other pathways may be utilized. This is supported by another toxin, cholera toxin B (CTB) that is internalized by cells via caveolae and CME, as well as dynamin-dependent and independent raft pathways. The small caveolar vesicles are internalized slowly, with a half-life longer than 20 minutes and similarly to macropinocytosis and phagocytosis, caveolae-mediated endocytosis is triggered by the cargo itself. Nevertheless, it seems to be highly regulated and more investigations are needed to fully understand the mechanism.

Clathrin- and caveolae-independent endocytosis

A variety of nutrients, signaling receptors and pathogens use clathrin- and caveolae-independent endocytosis to enter cells (Figure 3). Pathogens known to be internalized by cells via caveolae or CME, e.g. SV40 and CTB, can alternatively use this clathrin- and caveolae-independent pathway. Cholesterol rich microdomains on the plasma membrane referred to as rafts, can diffuse freely in the plasma membrane and are distinct from caveolae-mediated endocytosis and CME, in that they lack caveolae and clathrin. It is a constitutive internalization mechanism for the interleukin 2 (IL-2) receptor and GPI-anchored proteins. Even though dynamin mutants have been used to inhibit the uptake of IL-2, as well as caveolae and CME, they have failed to inhibit fluid-phase uptake, indicating that there are other unidentified pinocytic pathways. One new pathway independent of clathrin, caveolae, and dynamin, was shown to involve flotillin-1. However, recently it was found that a clathrin- and caveolae-independent but dynamin- and flotillin-dependent route for PG-bound ligands, efficiently transported endocytic vesicles to late endosomes. The PG-dependent pathway was distinct from CME, in that it did not require phosphatidylinositol-3 kinase (PI3K) dependent sorting from early endosomes or microtubule-dependent
transport, to reach the late endosomes. There is also evidence for lipid-based endocytosis in yeast since the yeast sterol, ergosterol, has a higher tendency to form lipid rafts than cholesterol\textsuperscript{10,65}. In summary, the clathrin- and caveolae-independent pathway may constitute a specialized high capacity endocytic pathway for both lipids and fluids, thus, the relationship of this pathway to other forms of endocytosis remains elusive.

Membrane repair response

Although endocytosis and exocytosis allow macromolecules to enter and exit cells via vesicular transport systems, without compromising the plasma membrane, not all cellular import can be explained by this system\textsuperscript{66}. Disruption of the plasma membrane, in multicellular organisms, is not unusual. Especially in mammalian cells, \textit{e.g.} skeletal or cardiac muscle cells\textsuperscript{67,68}, where the disruption frequency is directly correlated to the level of physical activity\textsuperscript{69}. Thus, the plasma membrane constantly needs to be repaired.

One of the first studies to investigate cell membrane resealing \textit{in vivo}, used water-soluble markers, such as dextran labeled with fluorescein. It was found that gut cells, wounded by mechanical forces, were able to reseal disruptions of the plasma membrane\textsuperscript{70}. However, resealing occurs in all cell types with injured plasma membrane and the membrane repair process allows the cells to survive injury\textsuperscript{71}. Due to the high extracellular calcium concentration, calcium ions leak into the cell when the plasma membrane is injured and trigger a MRR mechanism. The MRR induces mobilization of intracellular vesicles, to the disrupted site on the plasma membrane, to donate their membrane and patch up the broken membrane (Figure 4). The resealing is an active and complex structural modification, with endomembranes as the primary building block, together with cytoskeletal and membrane fusion proteins as catalysts\textsuperscript{71}.

The MRR has been compared to neurotransmitter release since block of calcium/calmodulin-dependent kinase II (CaMKII), a regulator of kinesin and exocytic vesicles at the synapses, block both processes\textsuperscript{72}. Moreover, in embryos, exocytosis and membrane resealing was blocked by neurotoxins that selectively cleaved synaptobrevin, syntaxin, and synaptosomal-associated protein-25 (SNAP-25), from the SNARE complex proteins. By undocking cortical vesicles, resealing was blocked, indicating that internal membranes are required and that a SNARE-like complex is important for resealing, by membrane docking\textsuperscript{73}. It is believed that after injury the local calcium level rises and activates CaMKII, which phosphorylates synapsin I, thus releasing vesicles from actin filaments to be used for resealing\textsuperscript{74}.
Figure 4. MRR mechanism. Due to the disruption calcium ions can leak into the cell and trigger a MRR mechanism which induces the mobilization of intracellular vesicles to donate their membrane and patch up the broken membrane. Lysosomes are usually the main donor of membrane, resulting in the luminal side of the lysosome being exposed to the extracellular part of the plasma membrane.

This is followed by a kinesin driven delivery of vesicles, to the site of disruption, where vesicle docking and fusion is mediated by the SNARE proteins. MRR driven exocytosis was visualized in confocal microscopy during resealing upon wounding with laser and was shown to be calcium-regulated, as a rapid burst of localized calcium-regulated exocytosis was detected. The involvement of kinesin and myosin was further investigated by blocking them with a kinesin antibody or with a myosin ATPase inhibitor, respectively. Different temporal phases of exocytosis were found, where the block of kinesin inhibited the slow phase and block of myosin inhibited both the slow and fast phase. The blockage of CaMKII resulted in inhibition of both phases, consistent with disruption of a myosin-actin-dependent step of vesicle recruitment. Thus, kinesin and myosin motors may mediate two sequential transport steps that recruit vesicles to the release sites of calcium-regulated exocytosis. Furthermore it was shown that calpain was required for the MRR as the calpain inhibitor, calpeptin, calpain knock-outs, or the highly specific calpain inhibitor protein, calpastatin, decreased survival during plasma membrane disruption. Together, these results indicate that
calcium influx during membrane disruption activates calpain, which is necessary for remodeling of the cortical cytoskeleton, at the site of injury and serves as a protector of the plasma membrane, against mechanical damage.

**Target cell apoptosis**

The intracellular environment inside the body is constantly monitored by an internal surveillance system that recognizes injury or defects of cells. Programmed cell death, apoptosis, is an innate mechanism, enabling organisms to eliminate defected cells during e.g. development or the response of the immune system to viral infection\(^80\)\(^81\). When cells are infected with pathogens or transformed into tumor cells, a group of key players in the immune response, the killer lymphocytes, recognize the defected cells and eliminate them. Natural killer (NK) cells and cytotoxic T cells (CTL) belong to the group of killer lymphocytes and are involved in adapted and innate immunity. They both act by the same mechanism, although triggered by different stimuli\(^82\). Two pathways can be utilized by CTL and NK cells, the granule exocytosis pathway (Figure 5), which is the dominant pathway in eliminating virus infected cells, or the involvement of cell-surface death receptors.

*Figure 5.* Simplified schematic of the mechanism during target cell apoptosis. Granules are exocytosed from CTL and NK cells, containing proteins such as perforin, granzymes and granulysin. Inside the cytoplasm, granulysin and granzymes induce apoptosis.
Moreover, human NK cells are distinct from CTLs, in that they constitutively express perforin and granzymes, whereas CTLs require pre-activation by dendritic cells. Thus, NK cells are important in the early stages of infection, until the T-cell response is fully activated. The exocytosed granules are secretory vesicles stored in killer lymphocytes, containing several proteins such as perforin, a pore-forming protein and granzymes, a group of serine proteases that activates the caspase pathway during target cells apoptosis. Other proteins found in granules are chemokines and granulysin, a cytolytic protein against microbes and tumors cells. Following interaction with the target cell, granular proteins are exocytosed from the CTL, together with structural elements, such as chondroitin 4-sulfate PGs, to form a lattice to which the toxins bind through electrostatic interactions. The basic granzymes, were shown to be non-covalently bound to the negatively charged granule PG serglycin, which has a protein backbone of alternating serine-glycine residues, modified by side-chains of chondroitin 4-sulfate GAGs. Perforin has also been proposed to bind GAGs but not as strongly as granzymes, due to the lower amount of basic residues. Well inside the cytoplasm, apoptosis is induced by granzyme A and granzyme B via caspase-independent or dependent pathway, respectively.

**Perforin**

Perforin is synthesized in the rough ER inside CTL and NK cells, followed by transportation through the Golgi apparatus, where it is post-translationally modified and packed into lysosomal-like cytoplasmic granules. Its expression is regulated during lymphocyte differentiation, by receptor activation signals and cytokines. Upon plasma membrane interaction, perforin can produce pores up to 20 nm in diameter. Perforin has a calcium-dependent membrane binding domain that plays a crucial role in the first step of the membranolytic activity of perforin. Its activity is exerted in the extracellular environment and require >100 μM calcium to bind efficiently to the target membrane. The low calcium affinity, probably protects the cytotoxic cells from autolysis during synthesis of perforin and the low granular pH protonates an aspartic residue in perforin, preventing calcium and membrane binding during exocytosis. However, the pore-forming ability of perforin has been questioned, since little or no leakage of cytoplasmic contents has been detected at physiological concentrations. Therefore two hypotheses has formed: 1) perforin induces pores in the plasma membrane of the cell, enabling granzymes to diffuse freely into the cytoplasm of the target cell and induce apoptosis, or 2) perforin is endocytosed together with granzymes and disrupts the endosomes, thus releasing them into the cytoplasm of the target cell. One study proposed that at physiologically relevant concentrations, perforin induced the MRR that resealed the broken plasma membrane rapidly, after the delivery of granzymes. By triggering the MRR, membrane...
disturbances caused by perforin were resealed, supporting the first hypoth-
esis. Thus, no leakage of endogenous molecules could be detected. Neverthe-
less, there are studies still claiming that perforin lysed the membrane of en-
dosomes and that perforin is not required for granzyme-induced apoptosis.85

Granzymes

There are several groups within the family of granzymes. In humans and
mice, granzyme A and B, are most abundant. This group of highly specific
serine proteases, are processed either on route to, or in granules, from inac-
tive pro-enzymes into active enzymes by cathepsin C82. In addition to induc-
ing apoptosis by cleaving caspases, granzymes have been proposed to have
other functions, as the presence of elevated numbers of circulating gran-
zymes in various inflammatory processes and cleavage of extracellular sub-
strates, suggest involvement in e.g. virus and tumor rejection95. It was pre-
viously shown that granzyme B reduced the growth of adherent tumor cell
lines by preventing their adherence to extracellular matrix proteins96. Inside
the granules, granzymes are bound to the chondroitin 4-sulfate PG, sergly-
cin, as mentioned above and due to the low pH they are inactive97. After exocytosis granzymes are proposed to undergo electrostatic exchange, from
serglycin to highly sulfated cell surface PGs98. Two cationic sequences were
identified as important in uptake since replacing arginines within the se-
quence by alanines, inhibited the uptake, corroborating the exchange of
chondroitin 4-sulfate on serglycin, to anionic components on the plasma
membrane99. In addition, the mutations and treatment with heparin reduced
the cytotoxicity. Two uptake models were proposed; receptor-dependent
endocytosis and nonselective adsorptive pinocytosis. However, the first
study did not find perforin to be involved in uptake, whereas the second
study found the granzyme internalization to be perforin-dependent. Nonethe-
less, serglycin of granules and PGs on the plasma membrane seem to be
involved in uptake. The question if perforin is involved, and how, remains
 unanswered but a majority of the recent investigations find that perforin at
least enhances granzyme-dependent apoptosis81,82,84,92.

Granulysin

As a member of the saposin-like family of lipid binding proteins, granulysin
is a lytic molecule active against e.g. tumor cells and microbes100. During
target cell apoptosis two protein products of 15 and 9 kDa are detected in
CTL and NK cells100,101. The proteins are exocytosed together with perforin
and granzymes. Recombinant 9 kDa granulysin was shown to activate cas-
pase-9 to induce apoptosis in nucleated cells but not red blood cells102. How-
ever, shorter peptides of 9 kDa granulysin lyse erythrocytes and the hemolys-
is was shown to depend on specific ion channels, as blockage of them inhi-
bit lysis\textsuperscript{103}. Peptides corresponding to the N or C terminus are not lytic, whereas peptides from the central region lyse bacteria, human cells and synthetic liposomes. The replacement of arginines by glutamine also reduces the lysis\textsuperscript{104}. Upon binding to the cell surface, the intracellular calcium level in human T cell tumor Jurkat increase\textsuperscript{102}. Furthermore, granulysin treatment damage mitochondria, resulting in loss of electrostatic potential, release of cytochrome c and apoptosis-inducing factor\textsuperscript{105}. It was shown that both cytosolic and mitochondrial calcium levels rise, whereas potassium levels decrease and, that this is required for granulysin-induced apoptosis since cell death is prevented by calcium and potassium blockers\textsuperscript{106}.

**Cell-penetrating peptides as drug delivery vehicles**

The greatest hindrance in drug delivery of hydrophilic drugs today, is to cross the hydrophobic plasma membrane, in order to reach the cytoplasm or nucleus, to exert their therapeutic effect. Several transporters in the plasma membrane are able to internalize smaller molecules but for larger macromolecules such as peptides, proteins, and oligonucleotides, some kind of delivery vehicle is needed to deliver the cargo. Carriers have been developed for delivery of different cargoes \textit{e.g.} cationic lipids\textsuperscript{107}, nanotubes\textsuperscript{108} and polymers\textsuperscript{109}. Two classes of delivery vehicles are assessed in this thesis namely, CPPs (Table 1) and bacterial ghosts.

Twenty years ago, during the development of an activity assay for a protein from the human immunodeficiency virus type 1 (HIV-1), the trans-activator of transcription (Tat) protein, was found to be internalized by tissue culture cells\textsuperscript{110}. It localized to the nucleus and if lysosomotropic agents as chloroquine were added, its activity increased dramatically.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Origin</th>
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<tr>
<td>Penetratin\textsuperscript{111}</td>
<td>RQIKIWFQNRRMKWKK</td>
<td>Protein derived</td>
</tr>
<tr>
<td>Tat(48–60)\textsuperscript{112}</td>
<td>GRKKRRQRRRRPPQ</td>
<td>Protein derived</td>
</tr>
<tr>
<td>pVEC\textsuperscript{113}</td>
<td>LLLLRRRIRKQAHASK</td>
<td>Protein derived</td>
</tr>
<tr>
<td>bPrP\textsuperscript{114}</td>
<td>MVKSIGSWI10LVLFVAMWSDVGLCKKRPKP</td>
<td>Protein derived</td>
</tr>
<tr>
<td>MPG\textsuperscript{115}</td>
<td>GALFLGFLGAAGSTMGAWSQPPKKRKV</td>
<td>Chimera</td>
</tr>
<tr>
<td>Pep-1\textsuperscript{116}</td>
<td>KETWWETWEWSQPKKRKV</td>
<td>Chimera</td>
</tr>
<tr>
<td>Transportan\textsuperscript{117}</td>
<td>GWTLNSAGYLLGKINLKALALAKKIL</td>
<td>Chimera</td>
</tr>
<tr>
<td>TP10\textsuperscript{118}</td>
<td>AGYLLGKINLKALALAKKIL</td>
<td>Chimera</td>
</tr>
<tr>
<td>MAP\textsuperscript{119}</td>
<td>KLALKLALKAALKLA</td>
<td>Non-protein derived</td>
</tr>
<tr>
<td>Poly Arginine\textsuperscript{120}</td>
<td>(R\textsubscript{n})</td>
<td>Non-protein derived</td>
</tr>
</tbody>
</table>
In another laboratory, chemically synthesized Tat, together with two shorter analogs of 21 and 41 amino acids with retained activity, was shown to enter cells rapidly\(^{121}\). About the same time, it was found that a 60 amino acids long polypeptide from another transcription factor, corresponding to the Antennapedia gene homeobox sequence in *Drosophila*, internalized into cells\(^{122}\). Some years later it was shown that amino acids 48-60 of Tat\(^{112}\) and 16 amino acids from the third helix of the Antennapedia protein, named penetratin\(^{111}\), were sufficient for translocation. These two peptides, Tat (48-60) and penetratin, initiated the field of CPPs which have expanded greatly over the last two decades.

**Structural features**

Several attempts have been made to divide CPPs into different structural categories *i.e.* cationic, amphipathic, chimeric. However, it has proven to be difficult due to the high degree of overlap between the different categories. Nevertheless, there is one feature that seems to be more important than others; basic residues in the peptide sequence, due to the ability of arginines and lysines to form hydrogen bonds with phosphate, carboxylate and sulfate anions\(^{120,123,124}\). Classical examples of CPPs containing arginines are penetratin, Tat (48-60) and polyarginine\(^{120,125}\). However, there are several peptides without arginines *e.g.* MAP\(^{119}\), transportan\(^{117}\) and TP10\(^{118}\), containing lysines that are also able to form hydrogen bonds, although not bidentate bonds as arginines. The fact that both Tat (48-60) and penetratin originate from transcription factors, suggests that there are natural sequences within some proteins, enabling them to translocate across plasma membranes of cells, in order to exert their effect. Other CPPs, *e.g.* MAP and polyarginines, are not derived from proteins, although a high sequence resemblance with certain proteins can be found. Some CPPs even originate from toxins, such as cro-tamine\(^{126}\) originating from the venom of rattlesnake, and transportan and TP10, which are chimeras of mastoparan from the wasp venom and the neuropeptide galanin\(^{117,118}\). Furthermore, CPPs are in many ways similar to antimicrobial peptides and a comparison will be described in an upcoming section. Thus, the origin of the CPPs varies greatly and some of the structural investigations are summarized below.

Many modifications and analyses of existing CPPs have been done in an attempt to improve uptake efficiency and understand more about the uptake mechanism. Introduction of a negative net charge, as well as reduction of the amino acid sequence of MAP, by more than two amino acids, abolishes its translocation\(^{127}\). On the contrary a shorter analog of transportan, TP10, retains its activity despite the truncation\(^{118}\). In the presence of phospholipid vesicles, transportan shows 60% helical structure and penetratin a β-sheet like structure, whereas both peptides have random coiled structures in aqueous solution\(^{128}\). Another study showed that penetratin has an amphipath-
ic helical structure in SDS micelles and that the indole group of tryptophan inserts into micelles and is important for uptake\textsuperscript{129}. Structure-activity relationship studies of truncated or mutated penetratin revealed that shorter sequences are be taken up, although not as efficiently as penetratin and replacing the basic residues by alanines, reduces the uptake, whereas substitution of hydrophobic residues with alanine is almost unnoticeable\textsuperscript{130}.

Extensive studies and modifications have been performed on several analogs of Tat (48-60) and polyarginine. Unfortunately, it is difficult to compare data from Tat studies since the analyzed sequence, referred to as Tat, varies greatly in amino acid composition. Trucation of the 9-mer Tat (49-57), by one amino acid from either terminus, is sufficient to reduce the uptake by 80\% and additional truncation, reduces the uptake even further\textsuperscript{131,132}. Taken together, the results indicate that arginine residues are more important for the uptake of Tat (49-57) than lysine residues, as both nonaarginine and Tat (49-57) were superior to nonlysine. After the discovery that the backbone stereochemistry of the polyarginines is not necessary for uptake, a cavalcade of non-peptide transporters has been designed. One potent non-peptide transporter is the R-ahx-R, compromising a single ahx acid spacer between consecutive arginines, indicating that higher flexibility increases uptake. A comparative study of transporters coupled to cargo corroborated that (R-ahx-R)\textsubscript{4} is the most efficient carrier out of Tat (48-60) and R9\textsuperscript{133}.

Many structural and conformational analyses have been performed with the MPG peptide. MPG is a chimeric peptide derived from HIV-1 gp41 protein and the nuclear localization sequence (NLS) from SV40 large T antigen\textsuperscript{115,134,135}. Its uptake is endocytosis-independent with or without cargo and by replacing a lysine to a serine in the NLS sequence, targeting of the CPP is changed from the nucleus to the cytoplasm, respectively\textsuperscript{136}. Both MPG and its analog insert spontaneously into phospholipids membranes and strongly disrupt lipid organization of the monolayers\textsuperscript{137}.

Mechanisms of uptake

Two decades ago the internalization of the Tat protein was believed to depend on endocytosis\textsuperscript{138}. Nevertheless, shorter peptides of Tat and Antennapedia, were interpreted to be independent of metabolic energy since uptake still occurred at 4 °C\textsuperscript{111,112} and several direct penetration mechanisms were proposed\textit{e.g.} the inverted micelle\textsuperscript{139} and sinking raft models\textsuperscript{140} (Figure 6). Two uptake mechanisms for antimicrobial peptides (AMPs), namely the barrel-stave and carpet model, described in the following section (Figure 7), were also proposed as potential mechanisms for CPP uptake. However, the direct penetration mechanism had to be reevaluated since it was discovered that fixation of cells caused artifacts\textsuperscript{141} and several well-characterized CPPs were shown to enter cells by different forms of endocytosis.
Figure 6. Proposed mechanisms for CPP uptake. The inverted micelle model proposes that following peptide binding, the membrane is destabilized by tryptophan and the formation of a micelle is induced\textsuperscript{139}. In the sinking raft model, the peptide helices aggregate parallel to the surface, forming a “raft” that sinks into the outer bilayer leaflet\textsuperscript{142}. The hydrophobic residues remain in contact with the lipids, as the helices sink deeper, while the hydrophilic residues form a hole, similarly to the barrel-stave model.

Therefore the debate on translocation is still ongoing and highly active. Nonetheless, the general opinion is that the first step of translocation involves hydrogen bonding between guanidinium/amino groups with PGs on the plasma membrane, although it is not crucial\textsuperscript{143,144}. Several endocytosis routes have been suggested as major uptake pathways, e.g. macropinocytosis\textsuperscript{124,145,146}, CME\textsuperscript{147} and caveolae-mediated endocytosis\textsuperscript{148-150}. It has also been suggested that different forms of endocytosis function simultaneously and by blocking one pathway, other pathways become more active\textsuperscript{151,152}, which may explain ambiguous results in the application of endocytosis inhibitors. Our investigations together with several other studies, suggest that there are two uptake pathways involved in CPP uptake; endocytosis and direct translocation through the plasma membrane (Paper I, II, III). One study with D and L enantiomers of octaarginine, showed that the uptake is concentration-dependent and that the CPPs use two pathways; endocytosis at low concentrations and direct penetration at high concentrations\textsuperscript{153}. Another study with Tat (47-57) indicated that at least two functionally distinct uptake mechanisms are involved and that the uptake is dependent on the size of the cargo\textsuperscript{154}. The direct translocation mechanism is supported by a molecular dynamics simulation, indicating that arginine residues of Tat (47-57) insert into the membrane, lowering the free energy by binding phospholipids and carrying them along through a transient pore, as they insert into the membrane\textsuperscript{155}.
Another CPP, Pep-1 change conformation upon increasing concentration, from non-structured to helical and the helical conformation is also found in contact with phospholipids\textsuperscript{116,156}. It was proposed that the peptide induces formation of a transient transmembrane pore-like structure and that the conformational change occurs upon association with the plasma membrane, independently of cargo presence. Moreover, the first step in the internalization of the MPG peptide is also independent of cargo binding and involve Rac-1 GTPase-dependent remodeling of the actin network\textsuperscript{157}. By binding GAGs, MPG induces local membrane disturbances and actin rearrangement that lead to uptake either by membrane fusion or endocytosis. An analytical study found two kinetic models describing peptide-induced membrane leakage of two prion-protein-derived peptides and melittin, a peptide from the honey bee, as one pore-forming model and second membrane destabilizing/perturbing model\textsuperscript{158}.

In summary, there are several studies indicating that endocytosis are not the only uptake pathway and by changing only a single amino acid, the destination of the CPP can be changed\textsuperscript{136}.

**Cargo delivery**

The capacity of CPPs to deliver cargo molecules into the cell and induce a biological response, has opened up new possibilities in drug delivery research\textsuperscript{159-161}. In addition, the CPP alone, can induce biological effects, as shown with a shorter sequence from the ADP-ribosylation factor (ARF) protein that translocates into cells efficiently and induce apoptosis\textsuperscript{162}. The two main strategies in CPP delivery are: 1) to couple the cargo covalently to the CPP or 2) to bind the cargo electrostatically to the CPP.

The first strategy most often involves disulfide bridges that are used to couple the cargo covalently to the peptide, as in the delivery of oligonucleotides to induce splice-switching both \textit{in vivo}\textsuperscript{163} and \textit{in vitro}\textsuperscript{164}. The peptide-cargo conjugate can also be coupled via a native peptide bond. However, this will most probably affect the binding of the cargo to the target negatively, due to steric hindrance. Inside the cell, the disulfide bridge is reduced by glutathione and the cargo is released. To be able to quantitatively determine how much cargo is internalized, a screening system was developed based on the conversion of luciferin in the cytoplasm of cells, from an inactive to an active state\textsuperscript{165,166}. Thus, successful delivery into the cytoplasm is ascertained. Another study used a Tat-Cre (48-57) fusion peptide to investigate both the biological effect and the translocation mechanism. The fusion peptide was found to be internalized via lipid raft-dependent macropinocytosis and by adding a sequence from the influenza virus protein, hemagglutinin (HA2), known to disrupt endosomes upon acidification, the biological effect was enhanced\textsuperscript{167}. Similarly, another study took advantage of the endosomal acidification by inserting histidines into the sequence of penetratin, in an attempt.
to construct a lysosomal disrupting peptide$^{168}$. The new peptide, named EB1, was found to be more efficient in cargo delivery than penetratin. Importantly, the luciferin assay, the splice correction assay, and the Tat-Cre (48-57) fusion peptide, eliminate the risk of fixation artifacts. In addition, the covalent strategy is advantageous since it is possible to measure the effective concentration of the construct. However, it includes several purification steps in which a certain amount of material will be lost.

The second strategy used for delivery of oligonucleotides and proteins, is coincubation, where the peptide binds the cargo electrostatically and/or by hydrophobic interactions$^{166,169}$. This method does not require covalent coupling or purification of the constructs and is therefore time-saving and results in minor loss of material. CPPs have even been conjugated to already established carriers, such as liposomes, to improve the efficiency of the delivery vehicle even further$^{170,171}$. In summary, numerous investigations have been performed to analyze internalization and biological functions of CPPs. However, due to the high quantity of studies, further information on the diversity of cargo delivery by CPPs can be found in several excellent reviews$^{172-175}$.

Stability of cell-penetrating peptides

After proteins are processed in the proteasome, nearly all generated peptides are rapidly cleaved into amino acids, either in the cytoplasm or nucleus$^{176}$. Today, there are only a few known cytoplasmic peptidases, e.g. tripeptidyl peptidase II (TPPII)$^{177}$, leucine aminopeptidase (LAP)$^{178}$, and thimet oligopeptidase (TOP)$^{179}$ that are able to degrade peptides with different specificities. Almost independently of sequence, aminopeptidases degrade peptides in the cytoplasm within seconds$^{180,181}$. LAP represents one of the more specific peptidases, cleaving single hydrophobic amino acids from the N terminus. Peptides comprising more than 15 amino acids are trimmed by TPPII, a serine protease removing tripeptides from the N terminus but also exhibiting low endopeptidase activity$^{182}$. Sequences of 6-17 amino acids are preferentially cleaved by the zinc-containing metallopeptidase, TOP, in addition to TPPII, by endoproteolytic cleavage that can be inhibited by phenanthroline$^{176}$. TOP is a highly conserved enzyme and a homolog can be found in both bacteria$^{183}$ and yeast$^{184}$. Furthermore, peptides in the cytosol can be transferred to the ER by a transporter associated with antigen processing (TAP), belonging to the family of ABC transporters, to be further degraded by ER-aminopeptidases$^{185,186}$. There may even be a competition between cytosolic peptidases and TAP, in binding of peptides that may result in delayed degradation, if peptides are bound to cytoplasmic/nuclear chaperons$^{187}$. Although TOP belongs to the family of matrix metalloproteinase (MMP), it is predominantly situated in the cytoplasm. Otherwise most MMPs are capable of degrading all constituents of the extracellular matrix and members of this family are believed to be zinc proteinases$^{188-190}$. MMPs
are either secreted from the cell or anchored to the plasma membrane and up to date, 24 different vertebrate MMPs have been identified. There are several other groups of transmembrane extracellular proteases, such as the β-secretase and a disintegrin and metalloproteinase (ADAM) families, which can be transmembrane or secreted and play important roles in cell adhesion, migration, proteolysis and signaling. 

A crucial aspect of drug delivery is, of course, efficient transport of the pharmaceuticals to the site of action, but also the following clearance of the transporting moiety from the cell. The CPP needs to translocate its cargo before it is extracellularly degraded, although, inside the cell it has to be eliminated, in order not to cause toxicity. In addition, if the CPPs remain intact in the cellular environment, there is a possibility that the peptide-cargo conjugate leaks back to the extracellular environment. The understanding of the internalization and degradation kinetics of CPPs is important for the practical aspects of cargo delivery. In uptake experiments, with incubation time points of one hour or longer, the peptide needs to be stable outside the membrane and not degraded within that time frame. Hence, evaluation of peptide stability is an important parameter. These studies are also essential in order to achieve a better understanding of the mechanism by which CPPs pass through membranes and enter cells. Recently, it was shown that the main mechanism by which CPP-cargo accumulate into the cellular interior, is by proteolytic processing of the CPP-cargo conjugate, into membrane impermeable products. Thus, it is important to characterize the degradation kinetics and products of CPPs, in order to investigate how they relate to cargo-delivery efficiency.

A few studies have investigated the metabolic stability and/or the pattern of degradation of CPPs. Enzymatic degradation of pVEC and its all-D analog was investigated in buffer containing physiological concentration of trypsin or carboxypeptidase A and B and the half-lives were found to be 10.5 and 44.6 min in respective buffer sample. Another study investigating transportan, TP10 and penetratin in contact with Caco-2 cells, found that the stability of the peptides was in the order of transportan>TP10>penetratin. Moreover, a comparative study of Tat (47-57), penetratin and several human calcitonin (hCT) analogs in MDCK (Madin-Darby Canine Kidney), Calu-3 (Caucasian, lung, adenocarcinoma) and TR146 cells, showed the levels of proteolytic activity varies highly among cell lines and that the extracellular half-life of the individual peptides depend on cell type and cell density. In an attempt to increase CPP stability, two amino acids were replaced with N-methylphenylalanine or D-phenylalanine in hCT(9-32) and the proteolytic resistance in human blood plasma and HEK 293T cell culture supernatants was found to increase. However, these studies did not include intracellular degradation and how it affects the uptake. A similar study investigated the possibility to increase the stability of the peptide by incorporating non-α-amino acids into CPPs coupled to oligonucleotides. Both intra- and extracel-
Cell-penetrating peptides versus antimicrobial peptides

AMPs serve as natural antibiotics and can be found in all organisms including humans and plants. In vitro and in vivo studies have illustrated the importance of AMPs in innate immunity. Similarly to CPPs, the high diversity of AMPs makes it difficult to categorize them, except on structural features. Several structural characteristics are shared between AMPs and CPPs, e.g. the high degree of cationic amino acids in combination with hydrophobic amino acids. Still, most AMPs are characterized by high amphipathicity that often results in helical conformations. Many AMPs have random coil structure in solution but adopt helical conformation upon binding to the membrane, such as cecropin and magainin, originating from the silk moth and the African clawed frog, respectively. It is believed that the net positive charge of the AMPs facilitates binding to the negatively charged LPS on gram-negative bacteria and teichoic acid on gram-positive bacteria. Proposed uptake mechanisms have been e.g. the carpet and barrel-stave model (Figure 7). Although all AMPs are derived from larger precursors, the diversity of peptides is such that the same peptide sequence seldom is found in two different species. However, there are exceptions, e.g. buforin II discovered in the stomach of the Asian toad Bufo bufo gargarizans.

![Barrel-stave and Carpet Models](image)

*Figure 7. Proposed mechanisms of AMP uptake. The barrel-stave model is a classical transmembrane pore, where amphipathic α-helices form bundles. The hydrophobic residues interact with the lipid membrane and the hydrophilic residues form a water-filled pore. In the carpet model, peptides adsorb to the membrane until a critical concentration, where the peptides act like a detergent, causing disintegration of the membrane.*
There are also differences in how the peptides kill the bacteria \textit{i.e.} some AMPs permeabilize the bacterial membrane, others target intracellular compounds and some utilize both procedures\textsuperscript{203,210,211}.

Recently, we showed that CPPs translocate across non-mammalian cells and act as AMPs against gram-positive and gram-negative bacteria (Paper I). Similarly, an analog of the CPP Pep-1, is antimicrobial against gram-positive and negative bacteria already at 2 \( \mu \text{M} \), whereas no toxicity is found in human erythrocytes even at 200 \( \mu \text{M} \)\textsuperscript{212}. AMPs like magainin 2 and lactoferricin B, have been shown to translocate across the bacterial membrane and reside in the cytoplasm\textsuperscript{213}. Furthermore, analogs of magainin 2 and buforin 2, internalize mammalian HeLa cells and the buforin analog is able to deliver green fluorescent protein (GFP) in a comparative study with TAT (47-57)\textsuperscript{214}. Although the peptides internalize cells, the respective mechanisms seem to be distinct. Another AMP belonging to the class of cathelicidins, LL-37 is able to transfer extracellular DNA plasmids to the nuclear compartment of mammalian cells\textsuperscript{215}. LL-37 expression is known to be induced by bacterial infections resulting in efficient lysis of the microbes. Interestingly, when LL-37 is incubated with plasmid DNA at concentrations lethal to bacteria but without cytotoxicity to mammalian cells, the plasmid DNA is transferred and expressed in the nucleus. The uptake was found to be caveolae-independent raft-mediated endocytosis, dependent on PGs. In addition, LL-37 protects the DNA from serum nuclease degradation. Taken together, there are a great number of studies illustrating the similarities between CPPs and AMPs. However, due to previous proposals of non-endocytic uptake for AMPs\textsuperscript{206}, together with the opinion that CPPs do not enter cells by membrane disturbing pathways \textit{i.e.} direct penetration, the similarities have not been well-accepted.

**Bacterial ghosts as drug delivery vectors**

A major breakthrough in the field of vaccinology is the use of naked DNA for immunization. However, DNA vaccines today are restrained by low immunogenicity and the requirement of high doses of plasmids. An explanation for the poor immunogenicity may be that antigens are not delivered in the context of an optimal danger signal and since the immune system rather recognizes injury, than foreign entities, it is not activated. Thus, proper activation of antigen presenting cells can only occur by pathologically altered cells\textsuperscript{216}. A new field exploring the ability of dead but morphologically intact bacteria, known as bacterial ghosts, to act as potential delivery vehicles of DNA, has emerged\textsuperscript{217,218}. Bacterial ghosts are considered to be a new and exciting non-viral tool in gene delivery and vaccination since they efficiently target antigen-presenting cells and other eukaryotic cells. The ghosts are produced by protein E-mediated lysis of gram-negative bacteria, carrying the
plasmid encoding lysis gene E of bacteriophage PhiX174\textsuperscript{219,220}. The lytic activity of the membrane protein comprising 91 amino acids, has been localized to 29 amino acids in the N terminus and is believed to be positioned in the membrane spanning region of the protein\textsuperscript{220,221}. The protein induces a transmembrane tunnel in the bacteria by fusing the inner and outer membrane, releasing the cytoplasmic contents from the bacteria, leaving it empty but structurally intact\textsuperscript{222}. Thus, the surface structures and antigenic features, including bioadhesive properties of the natural cell, remain unaltered. Lysis of bacteria has also been shown to depend on the rate of gene E translation and on the bacterial growth phase\textsuperscript{223}. The protein E lysis is a time consuming process since the bacteria have to be loaded with the lysis plasmid in order to be lysed and then reloaded with new plasmids or DNA of interest.

As macrophages localize to sites of inflammation and tumors, as well as adhere to endothelium and transmigrate to the focus of injury, they have been proposed as cellular delivery vehicles for adoptive immunotherapy. Applications as gene-dependent enzyme prodrug therapy and expression of cytokines for the stimulation of macrophage tumoricidal activity, have been proposed\textsuperscript{224}. Thus, increased targeting of macrophages by DNA delivery vehicles and expression of transgenes in macrophages is needed. Bacterial ghosts are known to efficiently target antigen presenting cells as well as other eukaryotic cells\textsuperscript{225-228}. Transfection of macrophages has otherwise proven to be difficult due to the low size limit and efficiency of transfection agents. Thus, bacterial ghosts are potential delivery vehicles of DNA as they efficiently internalize macrophages.

Another application is to use bacterial ghosts for gene delivery into cancer cells. Recently, melanoma cells were shown to internalize ghosts containing DNA via phagocytosis, with much higher efficacy than the non-liposomal lipid transfection reagent Effectene\textsuperscript{217}. It was suggested that the LPS on the ghosts activated the melanoma cells through the toll-like-receptor-4 (TLR-4), which is constitutively expressed in these cells, resulting in an increased production of IL-8 and cell adhesion\textsuperscript{229}. Thus, the native function of the cell could potentially be restored by delivering genes encoding the appropriate proteins to tumor cells with known mutations. Potentially, even the immune system could be induced by delivering genes encoding cytokines that are known to activate and recruit antigen presenting cells, further activating the immune response\textsuperscript{230,231}. During the activation and development of the immune response, cytokines are crucial factors, including the response against tumors, which was shown in patients given IL-2, a T-cell growth factor\textsuperscript{232}. Even the anticancer drug doxorubicin exhibited antiproliferative activities against cancer cells after delivery by bacterial ghost \textit{in vitro}\textsuperscript{233}. Furthermore, the significance of which bacterial strain to use for each cell line was illustrated in the same study as the ghosts of \textit{M. haemolytica} were more potent delivery vectors than \textit{E. coli} in Caco-2 cells. Moreover, ghosts produced from \textit{M. haemolytica} induced maturation and activation of dendritic cells \textit{in}
vitro and β-galactosidase-specific immune response was detected after intravenous immunization of mice with dendritic cells transfected ex vivo with bacterial ghosts loaded with a β-galactosidase-coding plasmid. Thus, the combination of natural adjuvant properties together with the potential carrier function, make ghosts an interesting novel DNA delivery vehicle in DNA vaccination.
Aims of the thesis

Understanding how CPPs enter cells and what uptake pathways are involved is of outmost importance in order to produce more efficient CPPs and to be able to foresee side-effects. If these peptides are to be used in drug delivery in vivo, it is necessary to know the uptake and degradation kinetics. If not, the peptides might be degraded before they reach their target. Thus, the aim of the thesis has been to investigate how CPPs translocate across cell membranes and to evaluate the importance of the plasma membrane from different species to study how it affects uptake and degradation kinetics. Similarities between CPPs and antimicrobial peptides were investigated and used for development of new CPP applications. Moreover, the correlation between native proteins, able to translocate across cells in target cell apoptosis and shorter sequences from these proteins, was investigated and compared to CPPs, to establish the involvement of the MRR in CPP uptake. The main goals are presented below.

- Study how membrane composition in different cell lines affects uptake and investigate if CPPs are antimicrobial. (Paper I)
- Investigate degradation and uptake kinetics of structurally different CPPs in mammalian and non-mammalian. (Paper I and II)
- Elucidate uptake pathways involved in CPP internalization in both mammalian and non-mammalian cells. (Paper I-III)
- Examine if the MRR is responsible for masking membrane disturbances caused by CPP uptake. (Paper III)
- Develop a new CPP based strategy to produce bacterial ghosts. (Paper IV)
Methodological considerations

Design and evaluation of peptides

Throughout the thesis two well-known CPPs have been studied, namely MAP and penetratin, in order to be able to compare the effect of the peptides in each paper. These peptides are structurally different both in secondary structure and amino acid composition. The non-protein derived peptide MAP, is highly amphipathic and forms a perfect α-helix with one hydrophobic and one hydrophilic side. Unlike most CPPs, it contains no arginine residues but consists of repetitive sections of lysine, alanine and leucine. Penetratin on the other hand has several arginine residues and is derived from a non-mammalian transcription factor from the Antennapedia protein in Drosophila. The secondary structure of penetratin depends on the surrounding environment and it has been shown to change structure depending on the environment.

In paper I uptake and antimicrobial effects of CPPs were investigated in different non-mammalian cell lines. The idea was to compare peptides with different origins. Therefore a third peptide was introduced, pVEC, derived from vascular endothelial cadherin (VEC) which is an integral membrane protein from murine and involved in cell adhesion. The negative control in this paper was taken from the same protein to show that not any peptide can enter cells. In addition a D-analog of pVEC was studied to compare degradation and uptake kinetics. The degradation investigations of MAP and penetratin were continued in paper II, and in paper III 4 new peptides from 3 different proteins involved in target cell apoptosis; perforin, granulysin and granzyme B, were synthesized and investigated. The sequences were chosen based on their basic and hydrophobic amino acid residue contents. The novel peptides were named pPrF82, pPrF338, pGrL and pGrB (Table 2). Shorter sequences of the last two peptides were recently shown to be important in uptake and toxicity of the native proteins (Li and Bird) therefore these sequences were extended in order to make them more CPP-like. Two more peptides, previously reported to cause calcium influx and cytotoxicity, pPrF1a and the shorter version pPrF1b. However, in our hands the peptides did not display any of these features and were therefore used as negative controls (Table 2).
Synthesis (Paper I-IV)

All peptides were synthesized by solid phase peptide synthesis (SPPS)\textsuperscript{237}. The advantage of this method is that the amino acids are coupled to a solid support and not in solution, which simplifies the washing steps. Amino acids were coupled with dicyclohexylcarbodiimide (DCC) and N-hydroxybenzotriazole (HOBt). Each residue has a tert-butyloxy carbonyl (t-Boc) protected α-amino group which is easily removed by trifluoroacetic acid (TFA) before the next amino acid is coupled. After synthesis the peptide and the side-chain protecting groups are cleaved from the resin by hydrogen fluoride, an even stronger acid. Cresol scavengers are used to protect the side-chains from the newly formed carbocations, arisen from the protecting groups. The peptide is then extracted from the scavengers in a mixture of ether and 10\% acetic acid, filtered from the resin and lyophilized to remove the solvent.

5,6-Carboxyfluorescein was activated with HOBt and diisopropylcarbodiimide (DIC) and coupled to the N terminus of the peptide prior to peptide cleavage. Following cleavage, the peptides were lyophilized and purified on a C\textsubscript{18} column in reverse-phase (RP)-HPLC using a gradient of acetonitrile and water containing 0.1\% TFA. The corresponding peptides were identified by matrix-assisted laser desorption time-of-flight (MALDI-ToF) mass spectrometry. For electron microscopy performed in paper IV, cysteine was coupled to the N terminus of the peptide instead of fluorescein, followed by coupling to a 1.4 nm nanogold particle via a maleimide bond.

Table 2. Name and sequence of the synthesized peptides utilized in this thesis with their respective reference. All peptides were amidated at the C terminus.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>Penetratin\textsuperscript{111}</td>
<td>RQIKIWQNNRMKWKK</td>
</tr>
<tr>
<td>MAP\textsuperscript{119}</td>
<td>KLALKLALKALKAALKLA</td>
</tr>
<tr>
<td>pVEC\textsuperscript{113}</td>
<td>LLIILRRRIRKQAHAHKS</td>
</tr>
<tr>
<td>pVEC(28-35)\textsuperscript{235}</td>
<td>YDEEGGGE</td>
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<td>pPrF82\textsuperscript{238}</td>
<td>QRHVTRAKVSSTEAVAR</td>
</tr>
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<td>pPrF338\textsuperscript{238}</td>
<td>ALRRALSQYLTDARWR</td>
</tr>
<tr>
<td>pPrF1a\textsuperscript{236}</td>
<td>IMLLQLERKAKRTRAV</td>
</tr>
<tr>
<td>pPrF1b\textsuperscript{236}</td>
<td>NAATRVCRTGRSRWR</td>
</tr>
<tr>
<td>pGrB\textsuperscript{238}</td>
<td>PCHTAARSECKRSHKFVPGAWLAGEGVDVTSLRR</td>
</tr>
<tr>
<td>pGrL\textsuperscript{238}</td>
<td>PCHTAARSECKRSHKF</td>
</tr>
</tbody>
</table>

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Cell cultures

Non-mammalian cells (Paper I and IV)
Four non-mammalian cell lines were analyzed in paper I to investigate how important the composition of the plasma membrane is for uptake and if CPPs are able to traverse non-mammalian cell barriers. In addition to internalization, the antimicrobial effects of CPPs were analyzed. *Bacillus megaterium* was chosen to represent the class of gram-positive bacteria. It has been used in industry for over 50 years due to its high capacity to produce exoenzymes. The size of *B. megaterium* is about a hundred times that of *E. coli* and was therefore named the big beast by De Bary. It has been used as a genetic tool for cloning since it can maintain a variety of plasmids and does not express external alkaline proteases. Next *E. coli* was chosen to represent gram-negative bacteria in paper I. *E. coli* was first discovered in 1885 by the German pediatrician and bacteriologist Theodor Escherich and is widely used as a genetic tool. Moreover, in paper IV bacterial ghosts produced from *E. coli* were used as delivery vehicles of plasmids into mammalian cells. *S. cerevisiae*, representing yeast in paper I, is often used as a tool in cell cycle regulation and is one of the most widely studied eukaryotes. The last non-mammalian cell line in paper I was insect cells derived from the pupal ovarian tissue of the Fall armyworm, *Spodoptera frugiperda* (Sf9), a moth larva to compare with bacteria and yeast.

Mammalian cells (Paper II-III)
Chinese hamster ovary (CHO-K1) cells are an epithelial cell line taken from a biopsy of an adult Chinese hamster. The CHO cells are extensively used in biological and medical research and were therefore used in paper II and III for ease of comparison.

Henrietta Lacks (HeLa) is involuntary a well-known name in the research community. Cervical adenocarcinoma cells were taken from her without her knowledge after her death in 1951 and have since then been used all over the world in cancer research. HeLa cells are a rapidly growing immortalized epithelial cell line known to be easily transfected and were previously utilized to internalize ghost217. The transfection efficacy of ghosts containing the GFP plasmid239 was analyzed in paper IV.

Minimum inhibitory concentration (Paper I and IV)
The minimum inhibitory concentration (MIC) is used to investigate if a peptide is antimicrobial or not. By performing the broth microdilution method it
is possible to measure at what concentration the peptide is able to kill bacteria or yeast. The principle is to grow cultures overnight, transfer aliquots to fresh culture medium the next day and incubate for an additional 3–5 h to reach mid-logarithmic phase organisms. The cultures are then diluted in medium together with peptides in a 96-well plate or spread onto agar plate and incubated for 16 h. The antimicrobial activity of the peptide is then analyzed at 595 nm in a microplate reader. The aim was to optimize the MIC values and time points of incubation. Therefore bacteria were incubated in medium or 10 mM NaH2PO4 pH 7.4 for one or two hours, since incubation with buffer gave the lowest MIC value.

Optimization of ghost production

A variant of the MIC assay was used to determine peptide concentrations lethal to E. coli. The same procedure was followed except that after reaching mid-logarithmic phase the bacteria was centrifuged for 5 min at 1000 g and resuspended in broth or buffer. Bacteria of different optical densities (ODs) were incubated with peptides in broth or buffer for 1 or 2 h and then a small aliquot of the peptide-bacteria incubation solution was transferred to fresh broth in a 96-well plate and onto agar plates followed by 16 h incubation to determine the lethal dose of peptide at a certain time point. Survival was measured as absorbance at 595 nm on a Digiscan absorbance reader where zero absorbance was interpreted as a lethal dose.

Agarose gel analysis of DNA (Paper IV)

Bacteria with different ODs were treated with 10 or 100 µM MAP in 10 mM Na2HPO4 buffer (pH 7.4) or LB for 1 h to perforate the plasma membrane and release the cytoplasmic contents of the bacteria. To confirm that the plasmid still was inside the bacterial ghosts plasmid DNA was extracted and prepared as described previously and analyzed on an agarose gel.

Uptake of cell-penetrating peptides

High performance liquid chromatography (Paper I-III)

Quantitative data of CPP uptake was obtained by using an HPLC coupled to a fluorescence detector. The advantage of this method is that the degradation pattern of each CPP can be examined and thus the half-lives can be calculated. In all studies relating to peptide uptake it is critically important to
separate membrane attached peptide from internalized peptide. To achieve this, cells were treated, prior to cell lysis, with diazotized 2-nitroaniline, which is a chemical agent that reacts with primary amino groups. Since the diazotized 2-nitroaniline is a small molecule compared to the more commonly used trypsin, it should lead to less over-estimation of internalized peptide. Primary amino groups of lysine residues will attack the diazo compound and form a highly unstable triazene which decomposes and the carbon stretch of lysine transforms to either a ring structure or an alcohol. Thus the modified peptide will become more hydrophobic and have a longer retention time in the HPLC. Internalized and extracellular peptides are separated since the reagent cannot cross the plasma membrane and modify internalized peptides. The disadvantage with this method is that it requires lysine residues in the peptide since these are the only amino acids with primary amino groups. Intact and degraded peptides were identified in the HPLC by analyzing 10 pmol of the parent peptide and comparing the area under the curve.

**Fluorescence activated cell sorter (Paper III-IV)**

Fluorescence activated cell sorter (FACS) analysis is a sensitive tool to study uptake of e.g. different macromolecules. Concurrently toxicity can be investigated either by adding dyes such as propidium iodide (PI), that fluoresces when it binds DNA in cells with damaged plasma membrane, due to the several fluorescence filters situated in the FACS, or by investigating if the cell population is homogenous. Thus it is possible to investigate if the internalization of molecules is due to toxicity. In paper III FACS was used to corroborate the uptake established by HPLC and fluorescence microscopy. However, it is not possible to use the diazo method in the FACS studies; therefore cells were treated with trypsin. In addition, trypan blue was added to quench any membrane bound fluorescing peptide. In paper IV, FACS was used to analyze the morphology of peptide-produced bacterial ghosts and compare the morphology to live untreated bacteria. Moreover, FACS was used to investigate the transduction of the ghosts into HeLa cells and to show that GFP plasmids in the internalized bacterial ghosts were expressed.

**Fluorescence microscopy (Paper III-IV)**

Quantitative analysis of CPPs is important in order to estimate half-lives of peptides and to investigate degradation and uptake kinetics. However, it does not say anything about the localization inside the cell. In paper III fluorescence microscopy was used to investigate how the new peptides distributed inside the cell. Both diffuse cytoplasmic staining of peptides was found together with endosomal vesicles. Confocal laser scanning microscopy (CLSM) was used to photograph sections in the vertical plane of cells to prove that the peptides did not reside in the plasma membrane but were actu-
ally inside the cells. Moreover, it was used to visualize mobilization of lysosomes to the plasma membrane which would occur during the MRR. This was done by detecting the lysosomal luminal membrane protein LAMP-2 on the plasma membrane with an antibody indicating that the lysosomes had translocated to the plasma membrane to donate their membrane, thus exposing the luminal lysosomal membrane protein LAMP-2 on the surface of the plasma membrane. Both CLSM and PI were used to confirm that the antibodies were not inside the cell but on the plasma membrane and that the membrane was intact. In paper IV uptake of GFP plasmid carrying ghosts was visualized with fluorescence microscopy.

**Electron microscopy (Paper III)**

The high resolution of transmission electron microscopy (TEM) makes it possible to deduce more specifically where inside the subcellular compartments peptides are localized and how they are affecting the membrane. In TEM a beam of electrons is transmitted through an ultrathin section of the cell and recognizes different densities in the material. As described above the peptides were labeled with 1.4 nm nanogold coupled via a maleimide bond. Undecagold with a size of 0.8 nm was also investigated but without success. Cells incubated with gold labeled peptides were fixed and prepared by silver enhancement to be able to visualize the particles in TEM. After the enhancement processes the cells were mounted into an epoxy resin followed by thin sectioning with a diamond knife into approximately 40 nm thick slices. The main drawback with TEM is the extensive sample preparation it requires to produce a specimen thin enough to be electron transparent and still able to withstand the high vacuum present inside the instrument.

**Cytotoxicity assays**

**Lactate dehydrogenase leakage (Paper II-III)**

Cytotoxicity can be determined by measuring the leakage of the enzyme lactate dehydrogenase (LDH) which catalyzes the interconversion of lactate to pyruvate at the same time as NAD$^+$ is reduced to NADH. If the cell membrane is compromised, leakage of LDH can be measured by the increase in NADH which catalyzes the reaction of resazurin to resorufin which can be measured in a fluorometer. Although LDH is a large molecule, the test has been shown to correlate with leakage of smaller molecules such deoxyglucose$^{162,242}$. 
Calcium measurements (Paper III)

The MRR is activated by calcium influx and therefore calcium measurements were performed in paper IV. The cell permeable compound FURA-2 acetoxyethyl (AM), which can enter cells in the form of an AM ester, was used. Well inside the cell the ester is hydrolyzed, thus FURA-2 cannot escape the cell. A ratio is measured between unbound (380 nm) and calcium bound (340 nm) FURA-2 with an emission of 520 nm. Even though there was no leakage of LDH a clear concentration dependent increase of calcium was seen with all CPPs. To test that not any peptide caused a calcium influx two non-penetrating peptides were used as negative controls. Ionomycin was used as positive control, which is a pore-forming calcium ionophore.

B-hexosaminidase leakage (Paper III)

If the plasma membrane is damaged lysosomes are mobilized by the MRR to the site of disruption to patch the broken membrane. When the lysosomes donate their membranes, lysosomal enzymes are released to the extracellular milieu. One of those enzymes is β-hexosaminidase which activity can be measured colorimetrically by the conversion of 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside to 4-methylumbelliferone.
Results and discussion

The aim of the thesis was to understand how and why CPPs internalize into cells. Throughout the thesis two structurally different peptides, MAP and penetratin, have been analyzed in order to be able to compare results. In paper I, translocation of CPPs into non-mammalian cells were investigated to understand the significance of the composition of different biological membranes and the antimicrobial activity of the analyzed CPPs. The importance of peptide degradation both inside and outside cells was assessed in both non-mammalian and mammalian cells in paper I and II, respectively. The first two papers indicated that there might be a direct penetration mechanism involved in CPP uptake. Paper III investigates this possibility and offers a possible explanation to why there is no leakage of endogenous molecules during this direct penetration. Finally, in paper IV, the antimicrobial effects of CPPs were used to produce delivery vectors, known as bacterial ghosts, to deliver plasmids.

Evaluation of CPP uptake and antimicrobial effects in non-mammalian cells (Paper I)

There are many similarities between CPPs and antimicrobial peptides as aforementioned. Several antimicrobial peptides have been shown to translocate across cell membranes. Therefore it was necessary to investigate if CPPs showed any antimicrobial characteristics and if they could translocate across non-mammalian plasma membranes. Four different non-mammalian cell lines were assessed, namely B. megaterium (gram-positive bacteria), E. coli (gram-negative bacteria), S. cerevisiae (yeast) and Sf9 cells (insect) together with four CPPs of different origins. MAP, a model amphipathic, non-protein-derived peptide, shares many characteristics with antimicrobial peptides. Penetratin is a non-mammalian protein-derived peptide from Drosophila, composed of both hydrophilic and hydrophobic patches, however not as regularly spread as MAP. The third CPP, pVEC is derived from a vascular endothelial mammalian protein cadherin (VE-cadherin), found in blood vessels of murine and consist of a hydrophobic N terminus, shown to be important in uptake, and a hydrophilic C terminus. In order to evaluate
the effect of degradation, a D-analog of pVEC was analyzed. To ascertain that not any peptide could translocate across the cells, a sequence from the VE-cadherin protein, was used as a negative control.

The antimicrobial effects of the CPPs were assessed by the broth microdilution method. All peptides were antibacterial against gram-positive and gram-negative bacteria but at different MICs. B. megaterium showed the highest sensitivity with a MIC of 1 µM for each peptide. In E. coli, the MIC was 25 µM for each peptide. No antimicrobial activity was found in S. cerevisiae at the highest concentration tested (25 µM), indicating that the yeast membrane is more resistant to peptide activity, followed by gram-negative bacteria and gram-positive bacteria. Nevertheless the four CPPs translocated all four cell lines, although with different efficacy and degradation patterns. Interestingly, degradation of pVEC was only detected in E. coli and Sf9 cells. In B. megaterium and S. cerevisiae all CPPs were highly degraded except the D-analog of pVEC. Moreover, in cells that could degrade pVEC to a high degree, pVEC was also the most efficient CPP. In cells not degrading pVEC, MAP was most efficient. This indicated that the degradation of the CPP is important in uptake. To investigate this further we compared the uptake of pVEC with its D-analog. Both the ratio of intact pVEC versus the D-analog and total uptake of pVEC versus the D-analog was compared. The comparison showed that at low peptide concentrations, higher uptake of pVEC was found and at high peptide concentrations, the D-analog was taken up more efficiently, suggesting that two uptake mechanisms are involved. The first mechanism, is dominating at low concentrations, where pVEC is most efficient, thus dependent on chirality but with low capacity. The second mechanism, is independent of chirality but has high capacity, dominating at high concentrations, where the D-analog is most efficient.

In conclusion, the CPPs were antibacterial and the uptake and degradation varied widely with pVEC illustrating the greatest difference in degradation between the cell lines. The uptake mechanism dependent on chirality, suggests the involvement of a transporter protein with high specificity and low capacity. The other mechanism with high capacity, low specificity and independent of chirality, may be a direct penetration through the membrane.
Determining the importance of peptide degradation in CPP uptake (Paper II)

Due to the rapid degradation of CPPs in non-mammalian cells, it was natural to continue to investigate the degradation of CPPs in mammalian cells. Most \textit{in vitro} and \textit{in vivo} studies, have not taken into account, how quickly peptides are degraded both inside and outside cells. If CPPs shall be used to transport drugs or oligonucleotides \textit{in vivo}, it is critically important to know the extracellular half-life of the peptide. Therefore the degradation of the structurally different CPPs, MAP and penetratin, was analyzed in CHO-K1 cells, extracellularly and intracellularly. An important difference, in addition to amphipathicity and origin, is that MAP does not have any arginine residues, only lysines, whereas penetratin contains both. This is an important feature because arginine residues are often given as a characteristic of CPPs. Moreover, in this study detailed Fl-HPLC examination was used to obtain quantitative data on uptake and degradation of CPPs. In all studies relating to peptide uptake, it is critically important to separate membrane attached peptide from internalized peptide. To achieve this, cells were treated prior to cell lysis, with diazotized 2-nitroaniline, a chemical agent that primary amino groups react with. After the modification, the peptide will be more hydrophobic and have a longer retention time in the HPLC. Internalized and membrane bound peptides are separated since the reagent cannot cross the plasma membrane and modify internalized peptides. However, it might be possible that the detergents, used for cell lysis or components in the cell lysates, form strong complexes that cannot be separated even in the HPLC. These complexes may be eluted with differing retention times, compared to the parent peptide, which would decrease the amount of the intact peptide deceptively. Therefore intact diazotized labeled peptides were mixed with cell lysate, to confirm that this was not the case.

The extracellular degradation of MAP and penetratin was rapid (Figure 8). Shorter degradation fragments of the CPPs could be found inside the cell within minutes. Surprisingly, intact MAP and penetratin could still be found inside the cell after one hour, even though the extracellular peptides should have been degraded by that time, indicating that the extracellular degradation might be determining the CPP uptake. This was corroborated by comparing the curve corresponding to uptake, with the curve corresponding to the increase in extracellular degradation products. Moreover, the fact that there was degraded peptide within minutes inside the cell but intact peptide still remaining after one hour, indicates that there might be at least two uptake processes. One pathway resulting in intact peptide, possibly corresponding to endocytosis, shielding the peptide from proteases at least until the peptide reaches the lysosomes and another pathway that results in degraded peptides, possibly corresponding to direct translocation.
Table 3. Protease and endocytosis inhibitors used in the study.

<table>
<thead>
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<th>Name</th>
<th>Target/Activity</th>
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<tbody>
<tr>
<td>Phenylmethylsulfonyl fluoride (PMSF)</td>
<td>Serine proteases</td>
</tr>
<tr>
<td>Phenanthroline</td>
<td>Matrix metalloproteinases (MMP)</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>Serine and Cysteine proteases</td>
</tr>
<tr>
<td>Bacitracin</td>
<td>Most proteases</td>
</tr>
<tr>
<td>Bovine serum albumin (BSA)</td>
<td>Saturation of proteases</td>
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<tr>
<td>Cytochalasin B</td>
<td>Inhibiting the assembly of actin filaments</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>Inhibits PI-3K, fusions of early endosomes</td>
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<tr>
<td>Nocodazole</td>
<td>Depolymerizing microtubules</td>
</tr>
<tr>
<td>NaN3/Deoxy-glucose</td>
<td>Energy depletion</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>Endosomolytic reagent, prevents formation and maturation of endosomes</td>
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</table>

Next an attempt was made to decrease the extracellular degradation with different protease inhibitors and substrates, to investigate if it influenced the uptake (Table 3). Phenanthroline, which is a MMP inhibitor, decreased the intra- and extracellular degradation of MAP and penetratin. A corresponding increase in uptake was found for penetratin, indicating that TOP could be involved in the intracellular degradation. On the contrary, the uptake of MAP was decreased, which was later correlated to toxicity. MAP and phenanthroline alone did not induce any LDH leakage but together they had a synergistic effect that could lead to peptides leaking out of the cell, resulting in a decreased uptake. Moreover, BSA was added to the peptide solution in an attempt to saturate extracellular proteases and thereby increase the extracellular half-lives of the peptides. BSA, together with MAP, resulted in more intact peptide on the outside but surprisingly, there was no increase in uptake. To investigate if this was a result of electrostatic interactions between cationic MAP and anionic BSA, shielding the peptide from proteases but preventing it from internalizing cells, MAP was preincubated with BSA or bacitracin for thirty minutes before incubation with cells. Penetratin was unaffected at the utilized concentrations but the uptake of MAP was reduced by 50%, indicating that electrostatic interactions were involved. A similar study with polyarginine peptides, found that the peptides bound to BSA, which reduced the uptake. Since the data suggested both endocytosis and direct translocation, the effect of several endocytosis inhibitors was eva-
 evaluated (Table 3). Chloroquine, which in addition to buffering endosomes, prevents maturation and formation of endosomal vesicles, wortmannin, which inhibits PI3K thus, preventing the formation of early endosomes to late endosomes, cytochalasin B, which inhibits the assembly of actin filaments and thereby block intracellular transport and, nocodazole, which depolymerizes microtubules, were analyzed. Chloroquine and cytochalasin B decreased the uptake significantly, indicating that endocytosis is involved and that it requires actin filaments. Nocodazole and wortmannin decreased the uptake of penetratin, although not significantly at these concentrations.

In summary, the data indicated two uptake mechanisms, resulting in rapid or delayed degradation of the peptide, which could be interpreted as direct penetration versus endocytosis. Alternatively, the rapid degradation may correspond to fast endocytosis, with an even faster endosomal escape. The rapid extracellular degradation is an important parameter that should be taken into account and determined the uptake at least in this system.

Figure 8. Uptake pathways and corresponding half-lives derived from Gepasi simulation program. The extracellular degradation half-lives of penetratin (Pen) and MAP were 5 and 10 min, respectively. Intact Pen and MAP were found inside the cell after about 2 and 6 min, respectively, whereas degraded peptides were found within about 4 and 3 min approximately. There was a delayed degradation of the peptides corresponding to 20 and 86 min for Pen and MAP, respectively.
The MRR masks membrane damages caused by CPPs (Paper III)

Our previous studies indicated that CPPs could translocate across the membrane via a direct penetration mechanism. In paper II, the kinetics indicated that one mechanism lead to almost instant degradation of the peptides, whereas the other mechanism resulted in a delayed degradation. Therefore the focus was on investigating if such a direct penetration mechanism existed, how was it possible, and why was there no leakage of endogenous molecules out of the cell? Moreover, if CPPs coupled to cargo are supposedly trapped in endosomes, how can they induce a biological effect and if some cargo-coupled CPPs leak out from the endosomes, why are not all CPPs able to disrupt the endosomes? Since paper II suggested that there was a direct penetration concurrently with endocytosis, perhaps the CPPs are trapped in the endosomes and the biological effect comes from the fraction of peptides that end up in the cytoplasm, after the direct penetration. With that in mind, we read about the protein perforin, involved in target cell apoptosis, which is exocytosed in granules together with granzymes and granulysin during target cell apoptosis\textsuperscript{94}. Perforin, known to be a pore forming protein\textsuperscript{91}, caused necrosis in cells at high concentrations, whereas at physiologically relevant concentrations, it promoted granzymes to induce apoptosis. During the release of granzymes, it was found that perforin activated the MRR, which was triggered by the calcium influx caused by the membrane disturbing effects of perforin, resulting in translocation of intracellular vesicles to patch up the membrane at the site of entry. Altogether, the characteristics and mechanism of perforin, granzymes and granulysin resembled the actions of CPPs. Thus, the MRR could be involved in CPP uptake.

Potential CPPs from the three native proteins were synthesized together with two well-known CPPs, MAP and penetratin, to investigate if the peptides alone were able to induce the MRR, as the native protein perforin. To confirm that not any peptide could induce the MRR, two control peptides from perforin were synthesized and utilized as negative controls. The novel peptides translocated across cells although not as efficiently as MAP and penetratin. Nevertheless, all peptides were found to induce calcium influx, supporting the involvement of the MRR. Furthermore, the peptides caused translocation of a lysosomal protein LAMP-2, as the protein perforin, from inside the cell to the plasma membrane, corroborating the activity of the MRR. The discovery in EM, that all CPPs were found in the cytoplasm with and without vesicular structure surrounding them was an important finding (Figure 9), as well as the insertion into the membrane without vesicle formation, corroborating that the direct penetration mechanism could exist.
Figure 9. EM photos of CPP distribution in HeLa cells. The black dots correspond to CPPs, coupled to 1.4 nm nanogold, magnified to approximately 8-12 nm with silver-enhancement. Penetratin (A, B), pGrB (C) and MAP (D) were found in both vesicular and non-vesicular structures. In some vesicles the conjugate was inserted into the endosomal membrane. Interestingly, penetratin seemed to affect the structure of the nuclear membrane (B).
Production of bacterial ghosts for plasmid delivery by CPPs (Paper IV)

The information that some CPPs had an antimicrobial activity, found in paper I, was used in this study. Instead of acting as a drug delivery vector, the CPP MAP was used to produce a drug delivery vector by using a new strategy. The novelty of the method was to use the antimicrobial activity of MAP, to produce bacterial ghosts by disrupting the membrane of *E. coli*, causing an empty bacterial envelope that could be used to deliver plasmids into cells. Usually bacteria are loaded with a plasmid encoding lysis gene E, expressing a 91 amino acid membrane protein that disrupts the membrane of the bacterium, resulting in an empty shell that can be loaded with DNA and used for delivery to other cells\textsuperscript{223,247}. In the new strategy, MAP was utilized to perfor-rate the membrane and produce a ghost, containing the desired plasmid. To further investigate the potential of the ghosts to act as delivery vehicles, HeLa cells were incubated with the ghosts in an attempt to deliver the plasmid by simply letting the HeLa cells phagocytose or endocytose the ghosts. Recently melanoma cells were shown to internalize ghosts containing DNA via phagocytosis\textsuperscript{217}, with much higher efficacy than a non-liposomal lipid transfection reagent, and it was suggested that the LPS of the ghosts, activated the melanoma cells through TLR-4 that is constitutively expressed in these cells, resulting in an increased production of IL-8 and cell adhesion\textsuperscript{229,248}.

Remarkably our strategy worked as the CPP MAP, was able to kill the bacteria without distorting the structural features of the bacteria, which still retained the plasmid after treatment. After optimization, a rapid method was produced comprising incubation of bacteria with peptide for one hour in buffer, significantly reducing the experimental steps and excluding lysis gene E. The ghosts were then incubated with HeLa cells, followed by 45 hour incubation in complete medium and assessed by FACS. It is difficult to explain how the DNA escapes the phagophagolysosome in which the bacterial ghosts should be entrapped after internalization. However, it has been suggested that the phosphatidylethanolamine, present in the membrane of the ghosts, mediate the DNA escape as previously reported for dioleoylphosphatidylyethanolamine delivery of DNA\textsuperscript{218,249}.
Conclusions

Bacterial ghosts and CPPs are two relatively new and exciting delivery vehicles. However, peptides are believed to be rapidly degraded and only a few studies have analyzed the degradation pattern of CPPs. Although CPPs have been under investigation for about 15 years, the proposed mechanism has gone from direct penetration to endocytosis and back again. Therefore one of the purposes with the thesis was to study CPP entry in different organisms, i.e. mammalian and non-mammalian cells to investigate how the difference in membrane composition affected uptake. Furthermore, degradation of CPPs in various cell lines was investigated to understand how it affected internalization. The other aim was to design and evaluate a new peptide-based strategy to produce bacterial ghosts, to be utilized as delivery vehicles in gene delivery and DNA vaccination.

The conclusion of paper I and II were that CPPs indeed were rapidly degraded both in non-mammalian and mammalian cells and that it had a high impact on the CPP uptake. While analyzing the difference in membrane composition, the analyzed CPPs appeared to be antibacterial further corroborating the similarities between CPPs and AMPs. In addition, data suggested that CPPs utilized at least two uptake pathways, which could correspond to direct penetration and endocytosis in eukaryotic cells, and direct penetration and transporter-mediated uptake in prokaryotic cells. With these results in mind the MRR mechanism suddenly appeared and, as suggested in paper III, it was the perfect explanation to the controversial direct penetration mechanism. Furthermore, the conclusion that some CPPs were lytic against bacteria, led to the development of the new CPP-based strategy to produce bacterial ghost and as illustrated in paper IV, the ghosts proved to be successful delivery vehicles of plasmids.


När bakterier kommer in i kroppen, detekteras de av kroppens immunförsvar, som har till uppgift att hitta och oskadliggöra bakterierna. Immunförsvars cirkulerande celler, känner igen bakterierna på grund av att de har speciella sockermolekyler på plasmamembranen, så kallade lipopolysaccharider. När immunförsvars celler åker runt i kroppen, känner de igen lipopolysacchariderna och kan då binda till dessa, för att sedan äta upp bakterierna och på så sätt oskadliggöra dem. Jämfört med mammala celler, är det lätt att införa olika gensekvenser i bakterier, vilka sen kan uttryckas i bakterierna till olika proteiner. Proteiner kan sedan, likt läkemedel, ha olika funktioner i cellen som motverkar sjukdomsförloppet. Alternativt kan gensekvensor användas för vaccinering.

Bakteriella spöken är döda bakterier som fortfarande har kvar sin ur sprungliga struktur, som till exempel lipopolysacchariderna på plasmamembranen.


Tack till alla på Södertörn, speciellt Pontus och Peter, för att ni är såna sköna kil-lar och för allt kul både på Södertörn och på Neurokemi. Ina, Lotta, Marianna och Ullis för alla fester och roliga stunder. Tack Birgitta för att du gjorde min återkomst till Södertörn så lätt och rolig.

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