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Endocytosis of therapeutic macromolecules in tumor cells - Mechanistic aspects of the proteoglycan receptor function

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- Mechanistic aspects of the proteoglycan receptor function

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Till Lina

"Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning"

Sir Winston Churchill

Abstract

Novel therapeutics to combat cancer are urgently needed. Most current pharmacological therapies have severe side effects and are seldom curative. Macromolecular drugs, and in particular nucleic acid based drugs, offer a potential remedy for this situation.

Currently, the absence of efficacious and safe methods to deliver nucleic acids to intracellular sites of action is the main impediment to the introduction of nucleic acid based therapies in the clinic. Viral delivery methods have been demonstrated to efficiently deliver nucleic acids, but also to be associated with severe, occasionally life threatening, immune reactions. Non-viral delivery methods are, so far, not sufficiently efficient for use in the clinic. Many viral and virtually all non-viral macromolecular delivery methods depend on cell surface heparan sulfate proteoglycans (HS PGs) for efficient uptake, however the details of this mechanism and the exact role of the PG has been unclear.

The aim of this thesis was to clarify the role of the cell surface PG in macromolecular uptake processes. It is demonstrated that mammalian cells can internalize extracellular DNA by a pathway strictly dependent on cell surface PGs and this pathway is characterized. Secreted, positively charged, proteins and peptides including the antimicrobial peptide LL-37, are shown to facilitate the uptake process. It is also demonstrated that specific HS epitopes, present on cell surface HS PGs, are pivotal for the uptake of diverse HS binding ligands including polyamines and macromolecular antibody complexes. Finally, using a newly developed method for the isolation of endocytic vesicles, it is demonstrated that both classes of cell surface HS PGs, syndecans and glypicans, are true internalizing receptors capable of intracellular macromolecular delivery.

This thesis advances our understanding of PGs as potential targets for macromolecular delivery vehicles. This understanding will be of aid for the development of future macromolecular drugs to the benefit of the patient.

Contents

List of publications

This thesis is based on the following papers referred to in the text by the roman numerals indicated below:

I.   Sandgren, S., **Wittrup, A.**, Cheng, F., Jönsson, M., Eklund, E., Busch, S. and Belting, M. (2004) The human antimicrobial peptide LL-37 transfers extracellular DNA plasmid to the nuclear compartment of mammalian cells via lipid rafts and proteoglycan-dependent endocytosis. *J Biol Chem*, **279**, 17951-17956.

II.   **Wittrup, A.**, Sandgren, S., Lilja, J., Bratt, C., Gustavsson, N., Mörgelin, M. and Belting, M. (2007) Identification of proteins released by mammalian cells that mediate DNA internalization through proteoglycan-dependent macropinocytosis. *J Biol Chem*, **282**, 27897-27904.

III. Welch, J.E., Bengtson, P., Svensson, K., **Wittrup, A.**, Jenniskens, G.J., Ten Dam, G.B., Van Kuppevelt, T.H. and Belting, M. (2008) Single chain fragment anti-heparan sulfate antibody targets the polyamine transport system and attenuates polyamine-dependent cell proliferation. *Int J Oncol*, **32**, 749-756.

IV.   **Wittrup, A.**, Zhang, S.H., ten Dam, G.B., van Kuppevelt, T.H., Bengtson, P., Johansson, M., Welch, J.E., Mörgelin, M., and Belting, M. (2009) ScFv antibody-induced translocation of cell-surface heparan sulfate proteoglycan to endocytic vesicles: Evidence for heparan sulfate epitope specificity and role of both syndecan and glypican. *J Biol Chem,* Sep 25, *(e-publication ahead of print)*.

Not included publications

The following papers are co-authored by the author of this thesis but are not included herein:

V.   Belting, M., Sandgren, S. and **Wittrup, A.** (2005) Nuclear delivery of macromolecules: barriers and carriers. *Adv Drug Deliv Rev*, **57**, 505-527.

VI. Magzoub, M., Sandgren, S., Lundberg, P., Oglecka, K., Lilja, J., **Wittrup, A.**, Göran Eriksson, L.E., Langel, Ü., Belting, M. and Gräslund, A. (2006) N-terminal peptides from unprocessed prion proteins enter cells by macropinocytosis. *Biochem Biophys Res Commun*, **348**, 379-385.

VII. Belting, M. and **Wittrup, A.** (2008) Nanotubes, exosomes, and nucleic acid-binding peptides provide novel mechanisms of intercellular communication in eukaryotic cells: implications in health and disease. *J Cell Biol*, **183**, 1187-1191.

VIII.Wittrup, A. and Belting, M. (2009) Characterizing peptide-mediated DNA internalization in human cancer cells. *Methods Mol Biol*, **480**, 101-112.

IX. Belting, M. and **Wittrup, A.** (2009) Developments in macromolecular drug delivery. *Methods Mol Biol*, **480**, 1-10.

X.   Belting, M. and **Wittrup, A.** (2009) Macromolecular drug delivery: basic principles and therapeutic applications *Mol Biotechnol* **43**, 89-94.

Abbreviations

AP2 – Adaptor Protein-2
CCP – Clathrin coated pit
CCV – Clathrin coated vesicle
CDC42 – Cell division control protein 42 homolog
CME – Clathrin mediated endocytosis
CPP – Cell penetrating peptide
CS – Chondroitin sulfate
CT – Cholera toxin
CTxB – Cholera toxin B subunit
DFMO – α-difluoromethylornithine
DRM – Detergent resistant membrane
dsRNA – Double stranded RNA
ECM – Extracellular matrix
EM – Electron microscopy
ER – Endoplasmic reticulum
FGF – Fibroblast growth factor
FGFR – Fibroblast growth factor receptor
GAG – Glycosaminoglycan
Gal – Galactose
GEEC – GPI-AP enriched early endosomal compartment
GlcA – Glucuronic acid
GlcNAc – N-acetylglucosamine
GlcNS – N-sulfoglucosamine
GlcN – Glucosamine
GPI – Glycosylphosphatidylinositol
GPI-AP – Glycosylphosphatidylinositol anchored protein
HS – Heparan sulfate
IdoA – Iduronic acid
LDL – Low density lipoprotein
LDLR – Low density lipoprotein receptor
l_d – Liquid-disordered
l_o – Liquid-ordered
MCD – Methyl-β-cyclodextrin
ODC – Ornithine decarboxylase
PEI – Polyethylenimine
PG – Proteoglycan
PNS – Post-nuclear supernatant
scFv – Single chain variable fragment
siRNA – Small inhibitory RNA
SV40 – Simian virus 40
TAT – HIV-1 trans activator of transcription
Tat – TAT protein transduction domain
Tf – Transferrin
TfR – Transferrin receptor
TNT– Tunneling nanotube
Xyl – Xylose

1. Introduction

There is a pressing need for the development of new anti-cancer therapeutics. Most current pharmacologic therapies have severe side effects and are seldom curative. Certain targeted therapies, introduced during the last few years, have had more beneficial efficacy versus side-effects profiles. These targeted therapies are directed towards tumor cell specific traits and defects such as oncogenic fusion proteins, amplified hormone and mitogen receptors or aberrant protein catabolism (1). However, the prospects of developing such targeted therapies for most cancers are limited, given the enormous heterogeneity of the cancer disease. Additionally, the ability of cancer cells to develop resistance against many therapies, and the difficulty and time required, to develop each new therapeutic molecule, limits the feasibility of highly targeted and personalized drugs. Macromolecular drugs and in particular nucleic acid based drugs offer a potential remedy for this situation.

The absence of efficacious and safe methods to deliver macromolecular drugs, is the main factor hindering introduction of, *e.g.*, nucleic acid based drugs in the clinic. Viral delivery methods are capable of efficient delivery of therapeutic nucleic acids, but also associated with severe, occasionally life threatening, immune reactions. Non-viral delivery methods have, so far, not been demonstrated to be sufficiently efficient for use in the clinic.

Many viruses and virtually all non-viral macromolecular delivery methods depend on cell surface heparan sulfate proteoglycans (HS PGs) for efficient uptake, however, the details of this mechanism and the exact role of the PG has been unclear. Importantly, both viruses and non-viral delivery vehicles capitalize on the process of endocytosis for cellular entry. An increased understanding of the basic mechanisms of endocytosis is thus highly warranted. The aim of this thesis was to clarify the role of cell surface PG in macromolecular uptake processes in malignant cells.

The outline of this book is as follows: In chapter 2, the endocytosis field is introduced. In chapter 3 the HS PG family of cell surface receptors and their functions are overviewed. In chapter 4, the field of macromolecular drug delivery with a focus on non-viral delivery vehicles, is briefly presented. Chapter 5 is a short summary of the uptake of endogenous macromolecules in mammalian cells and the implications for cell-cell communication. Finally in chapter 6, the experimental part of this thesis is presented and discussed.

2. Endocytosis

The plasma membrane constitutes an efficient barrier between the interior of the cell and the surroundings, for both charged and large molecules. Evolution has equipped cells with numerous strategies to overcome this barrier and transport molecules from the extracellular space to the interior: membrane channels for small charged molecules, *e.g.*, ions and water; endocytosis for macromolecules and certain solutes; and phagocytosis, a variant of endocytosis, for larger particles. This thesis is primarily concerned with mechanisms for the uptake of soluble macromolecules, and will for this purpose commence with a brief overview of the endocytosis field.

Through endocytosis cells take up material from its surroundings by enclosing it with a part of the plasma membrane and then pinching it off to form a membrane vesicle in the cytosol. The term endocytosis was coined in 1963 by de Duve to include both the ingestion of large particles (such as bacteria), *i.e.*, phagocytosis, and the uptake of fluids or macromolecules in small vesicles, *i.e.*, pinocytosis (2). The very first observations of an endocytotic process was made already at the end of the nineteenth century by Metchnikoff (3). He noted that cells ingested litmus particles whereby these changed color from blue to red indicating they had been transported to an acidic compartment within the cell. The observation earned him the Nobel Prize in physiology or medicine 1908 and accurately described the, today well studied, acidification of endosomes and phagosomes.

In mammalian cells several distinct mechanisms of endocytosis are known to exist, though the exact definition of these pathways are still under debate. In fig. 1, a recently proposed categorization of the different endocytic pathways is presented (4). Below is a summary of the characteristics of the main pathways and the evidence for their existence is critically reviewed.

2.1. Clathrin mediated endocytosis

In 1964 Roth and Porter first described, using electron microscopy (EM), electron dense "bristle-coated" pits and vesicles as the mediators of endocytic uptake of extracellular protein (5). Through pulse-chase experiments, they were able to deduce that a "coated" pit first formed at the cell surface and that this pit was then pinched off to form a coated vesicle. The electron dense coat was shown in 1975 by Pearse to consist of mainly one protein, that was given the name clathrin (6, 7) due to its cage-like structure (latin *clathra*: lattice).

In the 1970s Goldstein and Brown performed studies that subsequently would earn them the Nobel prize in physiology or medicine. Through binding and kinetic studies they demonstrated that cells have specific cell surface receptors for endocytosed extracellular macromolecules. They further showed that the receptor for low density lipoprotein (LDL)-particles must recycle back to the cell surface after ligand internalization and that the receptor thus is used repeatedly. The LDL-
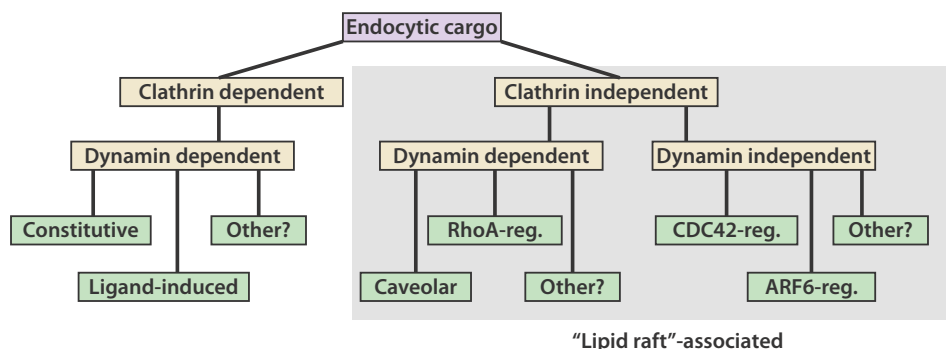
Figure 1. Categorization of endocytic pathways according to Mayor and Pagano (4). According to the scheme suggested, an endocytic cargo can be endocytosed by either clathrin dependent or clathrin independent mechanisms. Clathrin dependent mechanisms are all dynamin dependent but can vary in other mechanistic aspects. Clathrin independent mechanisms can be both dynamin dependent and dynamin independent. Clathrin independent mechanisms include caveolar endocytosis and pathways regulated by a variety of small GTPases (RhoA, CDC42, ARF6) as well as potentially other, as of yet, unidentified pathways. Common for most clathrin independent pathways is an association with so-called "lipid rafts" and a dependence on membrane cholesterol.

particles were concentrated above clathrin coated pits (CCPs) before being found inside intracellular clathrin coated vesicles (CCVs), thus implicating clathrin in this type of receptor mediated endocytosis. In a review article in 1979 they summarized their findings and outlined the main concepts, still valid today: transmembrane receptors recognize a ligand, the cytoplasmic tail then binds to and facilitate crosslinking of the clathrin coat, triggering endocytosis and internalization (8).

Over the last two decades numerous additional details of the mechanism of clathrin mediated endocytosis (CME) have emerged. Proteomic analysis of purified of CCVs have identified a large number of CCV associated proteins (9). These proteins fall into two broad categories of adaptor and accessory proteins (10). Adaptor proteins recognize and bind directly to endocytic motifs in the cytoplasmic tails of the endocytosed receptors, while accessory proteins perform various functions associated with the formation and stabilization of CCVs. Adaptor Protein-2 (AP2), the prototypic adaptor protein involved in CME, for example, recognizes canonical dileucine- and tyrosine-based endocytic motifs, *e.g.*, the YTRF motif of the transferrin receptor (TfR) (11), which destines this receptor for CME. Adaptor proteins recruit and promote the polymerization of individual clathrin homo-trimers (triskelia) into the clathrin lattice. Adaptors and other accessory proteins stabilize the clathrin lattice and also attaches it to the lipid membrane as clathrin does not bind directly to the membrane. Both AP2 and AP180 bind phosphoinositides that are enriched in CCVs (12). Other accessory proteins, *e.g.*, epsins are inserted in the membrane and give rise the to curvature necessary for further clathrin polymerization and budding (13). At the final stage of CME, the

large GTPase dynamin is recruited to the neck of the budding vesicle where it forms a polymeric ring that mediates the scission of the vesicle from the plasma membrane (14), possibly with the assistance of an actin driven pull (15). Once the vesicle has been released from the membrane the clathrin coat is removed by auxilin and hsc70 (16).

2.1.1. Cargo selectivity in clathrin mediated endocytosis

Relatively recently, through live cell microscopy, it has become evident that CME can proceed through two distinct modes of clathrin recruitment. CCVs can either be formed *de novo* by the recruitment of clathrin as described above, or from stable CCPs at the cell surface from which several CCVs can bud iteratively while the CCP remains intact (17). This observation suggests a possible heterogeneity in CME. This concept, *i.e.*, that not all CCVs are created alike, has further been strengthened by the discovery of cargo selective adaptor proteins (18). For instance, cells depleted of AP2 are still able to internalize the epidermal growth factor receptor (EGFR) and LDLR while TfR endocytosis is completely abrogated (19).

   Numerous studies have since demonstrated cargo selectivity for several different adaptor proteins, including, epsins for ubiquitinated receptors, arrestins for GPCRs and Dab2 for LDLR (20-22). Recently it was also elegantly demonstrated, through live-cell microscopy, that endocytosed ligands that utilize these cargo selective adaptors are internalized by a specific subset of cell-surface CCPs, and that the internalized vesicles are specifically sorted to highly motile rapidly maturing vesicles (23). Tf was in contrast indiscriminately internalized by all CCPs and enriched in a separate pool of static, slowly maturing, early endosomes. Thus, CME is probably not a single endocytosis pathway but rather a collection of several different pathways sharing certain characteristics, most notably the clathrin coat.

2.2. Caveolar endocytosis

Already when Roth and Porter described coated endocytic vesicles in 1964 it was apparent that not all endocytosis proceeds through coated compartments (5). However, possibly due to successful purification of coated vesicles, or simply due to the sheer success of the concept of coated pits and vesicles, most endocytosis studies during the last few decades have focused on CME. Lately, the interest in non-coated pathways has been growing with numerous studies describing several novel potentially separate endocytotic pathways (4). The non-clathrin mediated endocytotic pathway generally considered to be the most well studied and characterized, is the caveolin mediated pathway. Caveolin-1 is a 22 kDa, lipid bilayer inserted, protein originally identified as the main structural component of caveolae (24). Two additional homologues of caveolin-1 (2 and 3), present in the mammalian genome, are also involved in caveolae formation (25). Caveolae are morphologically highly characteristic "flask"-shaped 50-80 nm invaginations of the

plasma membrane present on many cell types. Caveolae are very prominent on certain cells, *e.g.*, endothelial cells where they constitute a large fraction of the cell surface area, while being absent from others, *e.g.*, many immune cells (25). The morphological similarity of caveolae to that of CCPs just prior to scission tentatively suggested, early on, that caveolae were involved in endocytosis and membrane trafficking. In 1986 Simionescu and coworkers proposed caveolae were responsible for albumin transcytosis over endothelial cells (26). However, the existence and importance of a caveolin mediated endocytotic pathway was highly controversial from the beginning and astonishingly, is just as controversial today, more than 20 years later (27).

While caveolin-1 involvement in albumin endocytosis and transcytosis is relatively well established (10) it is extremely difficult to visualize actual trafficking of caveolin-1 positive structures across the 100-200 nm thin endothelial cell barrier. Indeed, given the very thinness of the endothelial cell it is difficult to visualize, even with EM, structures that are convincingly non-continuous with the plasma membrane (27). Through careful and well controlled sectioning of samples for EM analysis, Sandvig and coworkers have demonstrated that caveolar structures, even seemingly distant from the plasma membrane, most often can be shown to be continuous with the plasma membrane when the cell is sectioned perpendicular to the cell surface (28). To complicate matters further, caveolae can acquire a long (several 100 nm) tubular morphology with the caveolin-1 coat remaining only at the tip rendering both thin-sectioned EM studies and fluorescence microscopy studies difficult to interpret.

The caveolar tubulation process seems to be regulated by a newly identified caveolae-enriched protein called SDPR/cavin-2 (29). This protein is member of a growing class of homologous caveolae associated proteins, the cavins. PTRF/cavin(-1) is the founding member of this class and was one of the very first non-caveolin proteins identified to have a function in caveolae biogenesis (30). SRBC/cavin-3 has been suggested to act as an adaptor for the caveolar endosomal pathway analogous to the adaptors of CME, though this conclusion is probably somewhat premature given the data presented so far (31).

Fluorescence bleaching (FRAP) studies have shown that cell surface caveolae at steady-state are highly immobile structures (on μm distances) with very low turnover (32). Pelkmans and coworkers have in more detailed live-cell microscopy studies, identified three distinct pools of caveolar structures, based on their dynamics: 1, static and stable membrane associated structures; 2, structures cycling rapidly between the cell surface and a nearby membrane adjacent domain; and 3, highly motile caveolin positive vesicles transported on microtubuli (33). Whether the rapidly cycling pool of caveolae are truly severed from the plasma membrane or whether they remain connected by an elongated neck, as described (29), has not yet been addressed.

Constituitive endocytosis through caveolae seems to be limited, but certain ligands, *e.g.*, simian virus 40 (SV40) viral particles and certain sphingolipids can

induce the internalization of caveolar domains (34). However, SV40 is still internalized in cells devoid of caveolae (caveolin-1 -/-), through a non-caveolin, non-clathrin mediated pathway (35). Indeed, this pathway, which is also present in cells containing caveolae, exhibits substantially more rapid uptake kinetics relative to the caveolar uptake pathway ($t_{1/2} \approx 20$ min vs. $t_{1/2} \approx 100$ min). In accordance with these findings, it has been suggested that caveolin-1 is not actually a mediator of endocytosis, but rather a negative regulator of endocytosis that stabilizes lipid membrane domains at the cell surface (36, 37).

One of the most commonly used "markers" for caveolar endocytosis has been intact cholera toxin (CT) or the B subunit of the toxin (CTxB). While initial reports clearly suggested caveolar uptake of CT/CTxB, this was mainly inferred from cholesterol depletion, which is not a specific perturbation of caveolin mediated endocytosis (38). Recent reports have rather suggested that the quantitatively most important pathways for CT/CTxB uptake are CME as well as a non-clathrin, non-caveolar pathway (27, 37). Detailed ultrastructural studies have confirmed that only a small fraction of internalized CTxB is found in caveolin-1 positive structures (37). Other groups have seen more significant fractions of internalized CTxB in caveolin-1 positive structures including the Pelkmans laboratory (39). Our experience is that internalized CTxB and caveolin-1 define distinct compartments in both CHO and HeLa cells (40, and Wittrup, A., unpublished observation).

2.3. Non-clathrin, non-caveolin mediated endocytosis

While the existence of one or more non-clathrin, non-caveolin mediated endosomal pathways has been apparent for some time, the significance and the molecular basis for this or these pathways have until recently, been more or less unknown. A unifying theme for most observations of non-clathrin mediated endocytosis has been the requirement for membrane cholesterol and hence sensitivity to cholesterol depleting agents (34, 38, 41, 42). Due to this cholesterol dependence it has been speculated that so-called "lipid rafts" are important platforms for clathrin-independent endocytosis.

2.3.1. Lipid rafts

Lipid rafts are lipid microdomains suggested to be present in the plasma membrane of most cells and to have important roles in cell signaling and membrane trafficking (43). Rafts are considered to constitute liquid-ordered (l_o) membrane domains (stabilized by saturated lipids and cholesterol) phase separated from, and floating like "rafts" in, the surrounding liquid-disordered (l_d) membrane (containing unsaturated lipids) (44). Theoretical and model-membrane studies suggested the liquid-ordered (l_o) membrane domains to be enriched in glycolipids and cholesterol, that would contribute to the ordered state. Membrane constituents from cells extracted with cold non-ionic detergent (Triton X-100) partition into

two fractions: a soluble and a non-soluble fraction. Cholesterol, sphingolipids and certain membrane proteins are found in the non-soluble, detergent resistant membrane (DRM) fraction while other proteins and lipids are found in the soluble fraction. The non-soluble proteins include numerous signaling molecules, *e.g.*, Src family tyrosine kinases, growth factor receptors, IL-2R, glycosylphosphatidyl-inositol-anchored proteins (GPI-APs) as well as flotillins and caveolins. These proteins were assumed to be present in DRM domains of the plasma membrane, enriched in specific lipids and lipophilic molecules (43). The lipid raft associated proteins were further hypothesized to have characteristic diffusive properties (45). It was also noted that certain proteins, *e.g.*, GPI-APs acquire increasing detergent insolubility during transport to the plasma membrane through the endoplasmic reticulum (ER) and Golgi pointing to a specific sorting mechanism into DRMs (46).

However, the lipid raft concept has been contested as it has been very difficult to detect or visualize any l_o membrane microdomains in living cells (47). Indeed, detailed photo-bleaching (FRAP) studies in live cells have demonstrated that different putative raft associated proteins have very different diffusion constants (D) varying over an order of magnitude (48). These observations run contrary to the concept that DRM proteins reside and diffuse together in a common membrane microdomain. In fact, GPI-APs have been shown to be very diffusive while likewise DRM-associated caveolin-1 has been shown to be virtually static and non-diffusive (32, 48). GPI-APs are, furthermore, usually homogeneously distributed on the cell surface (on scale of the resolution limit for standard light microscopy, *i.e.*, ~250 nm) while caveolin-1 and flotillin-1 can be seen to be present in distinct and separate microdomains (42). Recent highly sensitive FRET studies indicate, that GPI-APs most probably form concentrated "nano-clusters" where 3-4 individual GPI-APs are held together tightly within 5-10 nm in a cholesterol dependent manner (49).

Despite certain limits of the lipid raft hypothesis, several observations suggest the concept has some merit. Indeed, the differential sensitivity to cholesterol depletion for different proteins and processes seem to correlate with whether these proteins fraction into DRMs or not. This is also true for endocytosis; CME of TfR or other non-DRM fractionating proteins are substantially less sensitive to cholesterol depletion than either endocytosis of caveolar ligands, GPI-APs or glycolipids, all of which are found in DRM fractions (34, 38, 41, 42).

2.3.2. Endocytosis of DRM enriched proteins

Since DRM enriched GPI-APs are known to be endocytosed but have no cytoplasmic tail to interact with specific adaptors and coat proteins, a radically different mechanism of endocytosis must be involved. Other DRM enriched proteins have been shown to be endocytosed in cells without caveolae or intact CME, reinforcing the notion of a non-clathrin, non-caveolin mediated endocytosis pathway. Currently there is an emerging consensus on the existence of at least two

different such pathways, possibly more. The two pathways can be categorized based on their dependence on dynamin and reliance on different mechanistic and regulatory components, especially small GTPases involved in actin dynamics (4 and fig. 1).

The most well characterized, so far, of these alternative pathways is the dynamin independent uptake of GPI-APs (50). The uptake seems to proceed through a very distinct class of tubular endocytic compartments that also internalize bulk fluid (dextran) and CTxB (37, 50), and are referred to as GPI-AP enriched early endosomal compartment (GEEC). The internalization is regulated by the small GTPase CDC42, and by several Golgi-enriched proteins including ARF1 and CtBP1 (50, 51). Recently it was shown that the CDC42 regulatory protein, GRAF1, localizes to GEECs and is a specific marker for this pathway, as well as being necessary for its proper function (52). The role of dynamin in the GEEC uptake pathway is currently not absolutely clear. The data presented, so far, seem to suggest that the initial uptake is dynamin independent, but that further sorting, possibly to Golgi, require dynamin (37, 52).

How proteins are selected for GEEC endocytosis is not clear. Recently, it was suggested that endocytosis through GEEC is not dependent on a positive sorting signal. Instead, GEEC endocytosis was proposed to be a default pathway for lipid-anchored proteins not exhibiting CME sorting signals (53).

Another, increasingly well defined, non-clathrin, non-caveolin mediated uptake pathway was initially identified as responsible for the dynamin dependent uptake of interleukin 2 receptor β (IL-2Rβ). In cells with abrogated CME through over-expression of dominant negative Eps15, IL-2Rβ uptake proceeds unperturbed. The uptake through this pathway seems to be dependent on a specific small GTPase involved in actin remodeling, RhoA (54), which has opposite effects on this pathway and CME. Recently, several additional regulatory components of RhoA regulated endocytosis were identified (55). Other non-clathrin, non-caveolar endocytosis pathways possibly exist, among which the support for an ARF6 regulated pathway, currently is the strongest (4).

2.3.3. Macropinocytosis

Macropinocytosis is the receptor independent uptake of bulk fluid, often detected by inert macromolecules such as dextran (56). Initial characterization of macropinocytosis was mainly done in macrophages activated with macrophage-colony stimulating factor (M-CSF) where increased bulk fluid uptake was envisaged to mediate the uptake of possible immunogens (57). Later, other non-immune cells where also seen to be able to internalize large volumes of fluid upon growth factor stimulation, *e.g.*, epidermal growth factor (EGF) stimulation of human epidermoid carcinoma cells (58). The process of macropinocytosis begins with membrane ruffling and protrusion mediated by actin remodeling. The protruding membrane encloses a large volume of extracellular fluid and forms a macropinosome, *i.e.*, a large often irregularly shaped non-coated vesicle. The

macropinosome possibly merges with the other endocytotic pathways at the level of the early endosome as it has been noted that internalized material eventually end up in EEA1 positive compartments (59). Little is known of the regulation of macropinocytosis but ARF6, Rab5 and Rab34 have all been suggested to be involved (57). Macropinocytosis has, furthermore, been suggested to be both dynamin independent and dynamin dependent though is most often considered to not require intact dynamin for proper function (57).

2.4. Regulation of endocytosis

The observation that non-CME fluid phase uptake was substantially up-regulated upon inhibition of CME was an early indication that the activity of different endosomal pathways might be actively and compensatory regulated (60). CME is generally considered to be constitutive and internalize cell-surface receptors irrespective if a ligand is bound or not. An exception to this rule is the ligand induced internalization of GPCRs (61). Caveolar endocytosis of SV40 is also believed to be induced by ligand binding and to involve kinases, as the tyrosine kinase inhibitor genistein strongly inhibits SV40 uptake (35). Conversely, the phosphatase inhibitor okadaic acid has been demonstrated to induce caveolar/raft budding (62). In fact, a genome-wide human kinase RNA interference screen identified a large number of kinases involved in SV40 uptake, many of which showed both analogous and opposite effects on the CME pathway (63). The findings suggest there are distinct kinase requirements for the clathrin and caveolar/raft mediated uptake pathways. The large number of regulators with both opposite and similar effects on different endosomal pathways point towards a complex network-based regulatory system that possibly will require systems biology approaches to be deciphered (64).

2.5. Endosomal sorting

In eukaryotic cells there is continuously an extensive traffic of membrane proteins and other components between different cellular compartments. This traffic includes the transport of newly synthesized proteins to their final destinations, transport of old proteins and organelles to degradative compartments and exocytic as well as endocytic processes. The Rab family of small GTPases are the main supervisors of this kind of regulated vesicle trafficking. There are more than 70 mammalian Rab and Rab-like GTPases among which almost 40 have been associated with specific functions (65).

Rab GTPases regulate the directed transport of vesicles through initiation and control of vesicle budding, cytoskeletal transport and targeted docking and fusion of vesicles. The control of such diverse events is mediated by spatially and temporarily controlled recruitment of specific effector proteins that carry out the specific functions.

Sorting of endocytic cargo has been shown to proceed through a specific set of intermediary vesicular and tubular compartments defined by different Rabs and effectors. Uncoated CME derived vesicles have been shown to initially be sorted to the so called early or sorting endosome in a Rab5 dependent manner. The early endosome is a slightly acidic (pH 5.9-6-0) tubulovesicular compartment where the lower pH facilitates ligand and receptor dissociation. Specific Rabs can subcompartmentalize the early endosome and mediate pinching off of specific domains and constituents (66). Often, *e.g.*, in the case of the LDLR, the receptor (and the lipid membrane) is recycled back to the cell surface through Rab4 dependent recycling endosomes, for another round of internalization (67). The internalized ligand is instead further sorted, to multivesicular late endosomes (pH 5.5-6.0) (68) and lysosomes (pH 5.0-5.5) where it is degraded (69). Early
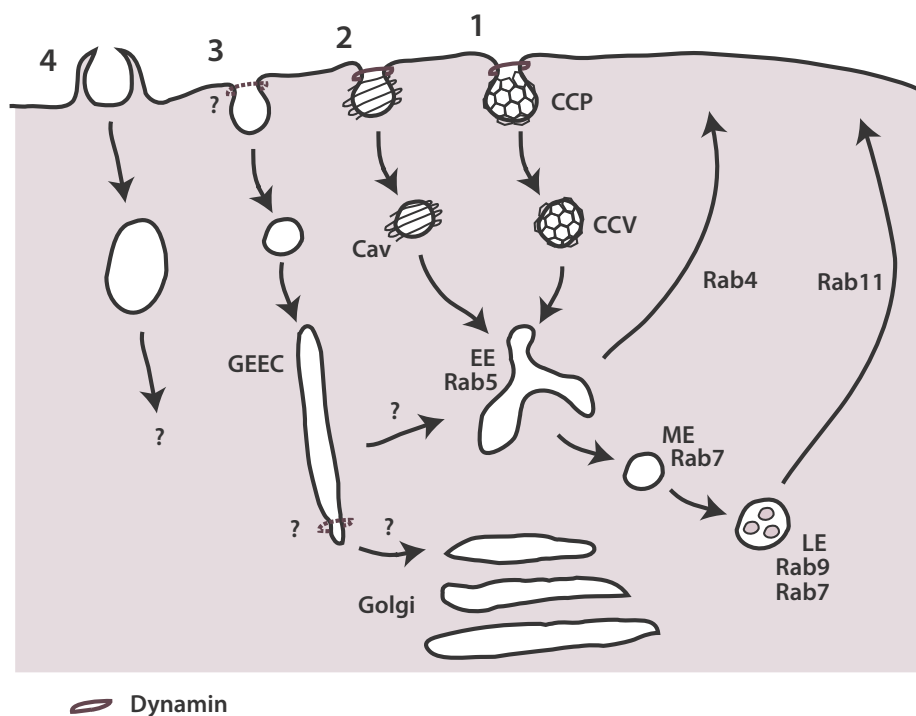
Figure 2. Intracellular sorting of cargoes from different endocytic pathways. Cargoes from different endocytic pathways are sorted through defined vesicular compartments. Both CME (1) and caveolar endocytosis (2) merge at the level of Rab5 decorated early endosomes (EE). Whether ligands of non-clathrin, non-caveolin mediated pathways such as the GEEC pathway (3) and macropinocytosis (4) are sorted through the same compartment, is currently less clear. Early endosomes are further sorted to multivesicular late endosomes (LE) via maturing endosomes (ME) through a Rab7 dependent pathway. Recycling back to the plasma membrane can proceed through two distinct pathways dependent on Rab4 and Rab11, respectively. Cav, caveosome.

endosomes gradually mature into late endosomes or fuse with already existing late endosomes. Further sorting to lysosomes is believed to require fusion with pre-existing lysosomes (70).

The function of Rab GTPases in endosomal sorting has mostly been studied in connection to CME. By consequence, most of what is known about Rabs during endocytosis applies specifically to CME. However, for example Rab5 and its effector EEA1 seem to be involved in many and perhaps most endocytic pathways. This suggests the early endosome might be a integrator and a common sorting station for most internalized material (4, and fig. 2).

3. Proteoglycans

The PGs are a family of proteins with the common characteristic of one or several glycosaminoglycans (GAGs) attached through a hydroxyl bond to serine residues in the protein. The GAG substituents are heterogenous disaccharide polymers consisting of 20-150 disaccharide units. The PG family of proteins is very diverse and while some PGs contain only a single GAG (*e.g.*, decorin) others contain more than 100 (*e.g.*, aggrecan). The PGs can be divided into several distinct groups depending on localization and structural properties. The major PGs found in the extracellular matrix (ECM) belong to the aggrecan family and the small leucine rich PGs (SLRPs) and contain GAG substituents of chondroitin sulfate (CS), dermatan sulfate (DS) or keratan sulfate (KS) type. The so-called basement membrane PGs are all heparan sulfate (HS) substituted and include perlecan, agrin, and collagen type XVIII. The secretory granule PG, serglycine, can be either CS or heparin (highly sulfated HS) substituted (71, 72). This thesis addresses the function and trafficking of cell-surface PGs, among which the syndecan and glypican family of HS substituted PGs dominate. The following presentation will focus primarily on these PGs.

3.1. Heparan sulfate synthesis
3.1.1. Backbone synthesis

GAGs are synthesized by (A) formation of a region linking the GAG chain to the protein, (B) polymerization of the polysaccharide chain, and (C) enzymatic modification of the chain to yield the specific saccharide sequences and structural organization that are responsible for protein binding. The synthesis is initiated by *O*-glycosylation of a serine residue in the core protein by transfer of xylose (Xyl) from UDP-Xyl by xylosyltransferase. The consensus sequence recognized by xylosyltransferase is a serine-glycine (SG) sequence usually surrounded by acidic residues and sometimes repeated, *i.e.*, SGSGSG (73). The synthesis then proceed by sequential addition of galactose (Gal), followed by Gal and glucuronic acid (GlcA) to complete the -GlcA-Gal-Gal-Xyl linkage region. This region is identical for HS and CS/DS chains (71). Addition of the next sugar residue determines whether the chain is to become an HS or CS/DS chain. Addition of *N*-acetylglucosamine (GlcNAc) is the first step of HS synthesis while addition of *N*-acetylgalactosamine (GalNAc) initiates CS/DS synthesis. The chain elongation then proceeds through the addition of GlcA-GlcNAc disaccharides in the case of HS synthesis, and GlcA-GalNAc in the case of CS/DS synthesis. Exactly what determines whether HS or CS/DS is synthesized is not absolutely clear. Properties in the core protein amino acid sequence or structure seem to be decisive. Repetitive SG sequences are preferentially substituted with HS (73) and the glypican globular domain is a positive signal for attachment of HS chains. The default pathway in the absence of the globular domain is to attach a CS/DS chain (74). When the glypican globular domain is fused to other normally CS/DS substituted proteins,

these instead become HS decorated. Possibly, there are also signals that do not emanate from the specific core protein, as overexpression of the glypican globular domain shifts the substitution towards HS also for proteins not directly fused to the globular domain (74). It is, furthermore, clear that the very same core protein can be decorated with CS/DS chains in some cells while bearing HS/(heparin) in other cells (72).

3.1.2. Generation of heparan sulfate structural diversity

After (or during) backbone synthesis the HS chain is extensively and variably modified. The first step of these modifications is the *N*-deacetylation and sulfation by *N*-deacetylase/*N*-sulfotransferase that replaces the *N*-acetyl group of GlcNAc with a sulfate group (75). This is followed by C5-epimerization of adjacent GlcA into iduronic acid (IdoA), 2-*O*-sulfation of IdoA and GlcA, and subsequent 6-*O* and more seldom 3-*O*-sulfation of the GlcNS. The modifications are clustered around the initial sites of *N*-deacetylation resulting in a domain structure of the final HS with highly sulfated, GlcNS rich domains (NS domains, 6-10 disaccharides) (76) interspaced with larger relatively unmodified domains rich in GlcNAc (NA domains) (see schematic over HS composition in fig. 3). The modification and sulfation of HS is of fundamental developmental and physiological importance. Mutants for *N*-deacetylase/*N*-sulfotransferase 1 (NDST-1), C5-epimerase and 2-*O*-sulfotransferase (HS2ST) are all neonatal lethal in the mouse (77-79).

The modifying enzymatic reactions do not go to completion and the synthesis is not fully deterministic. In theory there are 48 possible disaccharides, however only 23 have been identified in HS, heparin or synthetic intermediates (80). The result is substantial structural heterogeneity where an octasaccharide in theory can be modified in a quarter of million different ways and it has been suggested that virtually every HS chain is unique (81). In contrast to protein and nucleic acid synthesis, GAG synthesis is not template directed. Still, the generation of HS structure diversity is a regulated process and the disaccharide composition of HS has been shown to be differential during development and in different tissues (82). The HS chains on a specific core protein can vary substantially between different cell types with regard to chain length, domain structure and sulfation level (83). However, the general HS composition is believed to be similar on different core proteins in the same cell (84).

Relatively recently, van Kuppevelt and coworkers have isolated multiple single chain variable fragment (scFv) anti-HS antibodies using phage display technology. These antibodies recognize specific HS epitopes composed of 3-4 disaccharide units with specific sulfation patterns, some of which have been relatively well characterized (table 3). Using these antibodies, specific distribution of HS modifications and epitopes have been observed in several different tissues including kidney, lung and spleen (85-87). The mechanisms behind this is currently not clear. However, all HS-modifying enzymes, except C5-epimerase and

Figure 3. Composition of HS chains. HS is composed of a linkage region consisting of Xyl-Gal-Gal-GlcA linked to a serine residue in the core protein. This is followed by 20-150 glucosamine/uronic acid disacharides. The HS chain is initially synthesized with GlcNAc/GlcA disacharides. These sugar residues are then modified by *N*-deacetylation and sulfation of GlcNAc (R_3), epimerization at the C5 position of GlcA (into IdoA) and by specific sulfotransferases (R_1, R_2) to yield heavily modified NS domains interspersed in relatively unmodified NA domains. Monosaccharide symbols are in accordance with Nomenclature Committee, Consortium for Functional Glycomics. 2S, 2-*O*-sulfation; 6S, 6-*O*-sulfation; NS, *N*-sulfation.

2-*O*-sulfotransferase, exist in multiple isoforms with different substrate specificities (88). The expression of these isoforms is developmentally regulated and varies between different tissues (77). It is thus possible that differences in the specific isoform repertoire available in a cell, can explain the differences in HS epitopes between and within tissues.

3.1.3. Heparan sulfate structure-specific protein interactions

Proteins interact with PGs both through interaction with the core protein and the GAG-chains. Usually, highly polybasic domains, containing several clustered basic amino acids, are the binding motifs for the negatively charged GAGs. A large number of growth factors, cytokines and ECM components contain such HS-binding domains. Certain HS-binding ligands, *e.g.*, FGF1, FGF2, transforming growth factor (TGF)-β, platelet factor 4 and antithrombin III have all been shown to recognize specific HS sequences (71). Two different ways of obtaining HS structure selectivity have been identified. Either a specific relatively uncommon modification is recognized as exemplified by antithrombin III that bind to a specific pentasaccharide, containing a rare internal 3-*O*-sulfation or the herpes simplex glycoprotein D that also require an uncommon 3-*O*-sulfation for efficient

viral internalization and infectivity (89). Alternatively, specific spatial organization of more common structures are necessary for optimal HS and ligand interaction. In case of FGF and FGFR, both bind a relatively common highly sulfated HS domain. For maximal FGFR signaling, however, two such domains must be spaced appropriately relative to each other in the interacting HS chain (71).

Although major efforts have been made in HS structure analysis, there is still relatively limited knowledge in the context of specific protein and HS interactions. Recent developments with mass-spectrometry based methods, and especially the introduction of HS-epitope specific antibodies, have opened up for substantial progress in the field (85, 90). Indeed, in this thesis evidence is presented that the uptake of growth promoting polyamines is specifically dependent on relatively unmodified and sulfation scarce HS domains (**III**). It is also demonstrated that an anti-HS antibody recognizing a specific 2-*O*-sulfated domain, but not antibodies recognizing other prevalent epitopes, is efficiently internalized by several cell lines (**IV**).

3.2. Cell surface heparan sulfate proteoglycans

HS has been known to be present on the cell surface since the early 1970s (91, 92). In the 1980s the first prototypic membrane intercalated HS PG, now referred to as syndecan-1, was identified and later cloned (93, 94). This was followed by identification of three homologous proteins (syndecan-2-4) and the identification of a GPI anchored and membrane associated HS PG. This PG was given the name glypican in reference to its GPI anchor (71, 95). Subsequently in total 6 mammalian glypicans have been identified (96). Syndecans and glypicans are always HS decorated (and sometimes also CS decorated). In contrast, so-called part-time PGs including CD44 and betaglycan bear HS chains only in some cells or under certain circumstances (71). It has been proposed that one of the main functions of cell surface HS PGs are to trap HS-binding signaling molecules in the three-dimensional space surrounding the cell, and then present the ligands to specific receptors on the cell membrane (97). In the case of HS and FGF2 interaction, however, the HS chain seems to take active part in the signaling process by being a required component of a ternary complex of FGF2, FGFR1 and HS that elicits maximal tyrosine receptor activation (80, 98).

3.2.1. Syndecans

All four members of the syndecan family (1-4) are type I transmembrane proteins possessing an extended conformation (fig. 4) due to a large number of interspersed charged amino acids in the primary sequences (71). The syndecans are relatively small proteins (25-40 kDa) that bear their HS substituents distal from the cell surface (94). From sequence homology they can be divided into two groups with syndecan-1 and -3 in one group and syndecan-2 and -4 in the other. Syndecan-1

and -3 can also have short CS/DS substituents closer to the plasma membrane (71, 99).

Syndecans are developmentally regulated and present in specific tissues at specific time-points throughout development and in the adult organism. In the adult organism syndecan-1 is found mainly on epithelial and plasma cells. Syndecan-2 is present on mesenchymal cells while syndecan-3 is almost exclusively found on neuronal cells (71). Syndecan-4 is widely expressed on most cell types throughout the organism (100). However, syndecans are often deregulated and both overexpressed and suppressed on tumor cells, thus exhibiting non-typical expression patterns (101).

Syndecans interact with the ECM and control of cell attachment, spreading and migration, often in cooperation with integrins. Syndecan-1 interacts with integrin $\alpha_v\beta_3$ (102) and antibody mediated clustering of syndecan-1 redistributes it sites of cytoskeletal interaction and promotes the reorganization of the actin cytoskeleton (103). Syndecan-2 has also been shown to interact with the actin cytoskeleton and influence the formation of focal-adhesions and filipodia (104-106). Syndecan-4, as well, is intimately involved in focal-adhesion formation; overexpression induce their formation while a truncated variant has dominant-negative effects and prevents focal-adhesion formation (107).

3.2.2. Glypicans

Glypicans are a family of six homologous, virtually purely HS substituted, GPI-linked membrane proteins of approximately 60 kDa (96, 108). 1-3 HS chains are attached to an extended domain of the protein close to the plasma membrane (71, 95) (fig. 5). Distal to this region, there is globular domain containing 14 conserved cysteines. All glypicans contain an N-terminal signal peptide and a hydrophobic domain in their C-terminal region, required for attachment of the GPI anchor. The six mammalian glypicans can be categorized into two groups based on sequence homology, consisting of glypican-1, -2, -4, and -6 as one group and glypican-3 and -5 as the other. The *Drosophila melanogaster* glypicans Dally-like and Dally bear similarity to each of the two groups respectively (96, 109).

The GPI anchor of glypicans suggests sorting to "lipid rafts" or DRMs (110). However, glycosylation seem to be a negative regulator of DRM sorting as a significant fraction of glypican is excluded from DRMs while non-glycosylated glypican is primarily found in DRMs (111).

Glypicans are important regulators of growth factor and morphogen signaling (96). For example, glypican-3 has been shown to be a positive regulator of Wnt signaling in hepatocellular carcinoma cells (112). At the same time, glypican-3 has also been shown to be a negative regulator of hedgehog signaling. Loss-of-function mutations in glypican-3 results in the Simpson-Golabi-Behmel overgrowth syndrome (SGBS) through over-activation of the hedgehog signaling pathway (113). Glypican-1 is frequently overexpressed in human gliomas and potentiate FGF signaling (114). Glypican-1 also mediate the mitogenic response to

vascular endothelial growth factor (VEGF) in endothelial cells (115) and positively modulate the mitogenic, angiogenic and metastatic potential of pancreatic tumor cells *in vivo* (116). Another well-documented role for glypicans is in the formation of morphogen gradients which has been extensively studied in *D. melanogaster* where the two glypicans Dally and Dally-like have been shown to have distinct roles (117).

3.2.3. Proteoglycan turnover

It has long been known that cell surface HS PGs are endocytosed (118). It was noted early on that the catabolism of internalized PGs followed two different pathways (118). Later, it was shown that GPI-linked glypicans were rapidly degraded upon internalization without any discernible degradative intermediates of the HS chains. Syndecans were instead found to be degraded substantially slower over 2-3 h with clearly detectable HS degradation intermediates (119). A large number of HS binding ligands have since been shown to be endocytosed and to require cell surface HS PG expression for this endocytosis (120). Studies on virus internalization suggested virus particles first bound to cell-surface HSPGs which then presented the virus to secondary internalization or fusion receptors (121). Uptake of lipoprotein particles have been shown to be mediated by both syndecan and perlecan, both independently and in cooperation with other lipoprotein binding receptors, *e.g.*, lipoprotein receptor related protein (LRP) (122, 123). Simons and coworkers have demonstrated that FGF or antibody mediated cross-linking of syndecans trigger their internalization. Cross-linking seem to shift syndecan into DRM fractions from where they are internalized (124). The internalization of FGF-triggered cross-linked syndecan-4 was shown to proceed through dynamin-independent non-clathrin and non-caveolin mediated endocytosis, regulated by CDC42 (125).

Few specific components or mediators of syndecan endocytosis have been identified. However, the syndecan interacting protein syntenin is present at the cell membrane and in syndecan containing intracellular vesicles (126). Syntenin was also identified as a protein specifically localized to apically derived endocytic vesicles in MDCK cells (127). Syntenin has since been demonstrated to specifically regulate, in concert with ARF6, syndecan recycling back to the cell surface (128).

Glypicans are also known to be internalized and to recycle to the plasma membrane. In the case of glypican-1, this has been suggested to primarily proceed through caveolar endocytosis, and that the recycling back to the plasma membrane is controlled by both enzymatic and non-enzymatic degradation of the HS substituents (129). Glypican-3 has also been shown to be endocytosed, whereby it can downregulate hedgehog signaling through endocytosis of ligand sonic hedgehog bound to the core protein (113).

Both syndecans and glypicans are shed into the surrounding environment (fig. 4) and this shedding can control the availability of growth factors and other HS binding ligands for receptors on the cell surface (130). In the case of glypicans
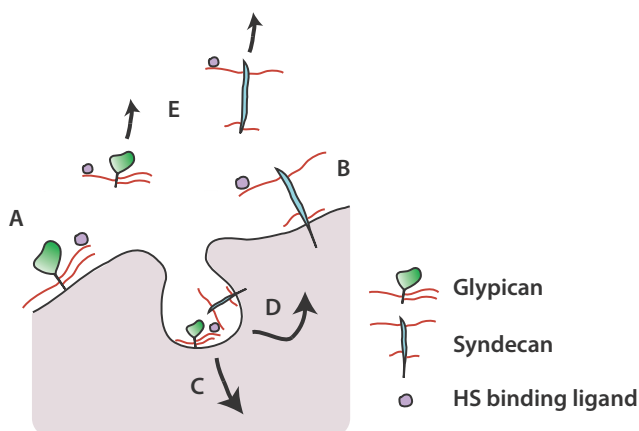
Figure 4. Turnover of cell surface HS PG. Both glypicans (A) and syndecans (B) are known to sequester a wide variety of HS binding ligands. Both are also endocytosed either together with a ligand as depicted here (C) or constitutively. (not shown). In paper (**IV**) it is demonstrated that syndecans and glypicans can be endocytosed in concert together with a ligand as seen above. Both glypicans and syndecans can also be endocytosed on their own, possibly through separate pathways. Both syndecans and glypicans can furthermore recycle back to the cell surface once they have been endocytosed (D). Finally, both syndecans and glypicans can be shed through the activity of metalloproteases or the lipase Notum, respectively (E).

this shedding has been demonstrated to be mediated by the phospholipase Notum, thus becoming a negative regulator of Wnt signaling (131). Syndecans are released to the environment by protease activity, a mechanism that can be triggered by FGF2, rendering cells dependent on glypican-1 for FGF2 signaling (132). The shedding of syndecan-1 has been shown to be regulated by Rab5, linking the endosomal pathway with HS PG shedding (133). This link is possibly of functional significance as both shedding and internalization constitutes two mechanisms whereby the cell can downregulate HS PG dependent cell signaling. This exemplifies the dual roles of cell surface HS PGs in being able to both promote and inhibit growth factor signaling, described as their "on" and "off" mechanisms (120).

3.3. Proteoglycan redundancy

An open question is to what degree different members of the HS PG family can substitute for each other. The phenotypes of knock-out animals and loss-of-function mutations of individual HS PGs are generally relatively mild, while abrogated or impaired HS synthesis has severe developmental consequences, suggesting substantial redundancy between different core proteins (79). However, conserved regions of the different syndecans suggest rather specific functions for the different members (71). Concerning any potential redundancy between syndecans and glypicans, the results are so far somewhat ambiguous. For example,

in the case of HS PG-mediated growth factor tyrosine kinase receptor activation, syndecans and glypicans have been shown to have both redundant (134) and core protein specific functions (132, 135). In this thesis both syndecans and glypicans are demonstrated to mediate internalization of an HS binding ligand (**IV**).

4. Macromolecular drug delivery

4.1. Macromolecular drugs

The "Holy Grail" of the drug delivery field is a versatile and non-toxic delivery vehicle, by which it is possible to deliver macromolecules, *i.e.*, DNA, RNA or proteins, intracellularly, in a tissue or cell of choice. In order to accomplish this goal a large number of both physical and scientific barriers need to be overcome: the delivery vehicle must be stable in serum, the tissue or cell of interest must be targeted, the vehicle must be internalized and possibly escape from an endosome and, in the end, navigate to the final destination and site of action in the interior of the cell. Despite these challenges, considerable efforts have been made in the field, as the potential gains are huge. The discovery of RNA interference, *i.e.*, the possibility to selectively knock-down any given gene through the use of small inhibitory RNAs (siRNAs) offer a previously unthinkable freedom in the selection of potential drug targets . SiRNAs are 21-23 nucleotides long, double stranded RNA (dsRNA) with one strand complementary to the gene that is intended to be knocked-down (136). Since RNA can be synthesized to any specified sequence and the human genome is known, siRNAs against any given gene can be produced relatively easily. Traditional small molecule drugs or antibody drugs must be synthesized to fit the specific three-dimensional protein conformation of each individual drug target. Consequently, siRNA drugs offer the possibility to vastly facilitate the development of new drugs. A hypothetical future siRNA drug could be a cocktail containing hundreds of individual siRNAs directed towards tens of different drug targets. Indeed, in the case of anti-cancer therapies, this kind of massively parallel drug cocktails are probably an absolute requisite; by just changing a single nucleotide in the sequence recognized by the siRNA, resistance could be aquired towards a drug containing only one unique siRNA sequence. It would thus be very interesting to see a proof-of-principle demonstration *in vitro*, whether an siRNA cocktail targeted towards driving oncogenes in a cultured tumor cell line is inherently more difficult, for the tumor cells, to develop resistance towards, than against individual siRNAs. To the author's knowledge such a demonstration has not been made.

There are additional potential benefits with sequence based macromolecular drugs, as they would interface extremely well with modern and future diagnostic methods in the cancer field. These methods include microarray and deep-sequencing techniques (137), and sequence based drugs are virtually the only conceivable class of drugs that could match the demand for personalized drugs that these methods give raise to. The possibility of obtaining detailed diagnostic knowledge of oncogenic mutations and fusion proteins in an individual tumor, is already a reality in the research setting, as demonstrated by the recent characterization of "driving" and "passenger" mutations in a variety of cancers through the use of deep-sequencing (138, 139).

4.2. Artificial non-viral delivery methods
4.2.1. Naked DNA

In 1990 Wolff and coworkers demonstrated that "naked" plasmid DNA injected intramuscularly in mice resulted in uptake of DNA in myocytes and subsequent reporter gene expression (140). Intradermal injection of DNA has also been shown to result in reporter gene expression but generally the expression level after "naked" DNA injections has has been too low for most purposes (141). However, in 1999 Wolff and Liu independently of each other demonstrated that intravenous injection of "naked" DNA plasmid in the tail vein of mice with a large volume of buffer (hydrodynamic method) resulted in significant expression levels in hepatocytes (142, 143). Typically, 1.6 to 2 ml of DNA solution in saline, for a 20 g mouse (~10% of the body weight) was injected over a period of ~5 seconds. The hydrodynamic tail vein procedure has been shown to be efficient and practical. Long-term transduction after a single injection has been demonstrated for both transgene expression (up to 1/10 of initial expression after 1 year) and short hairpin RNA mediated gene knock-down (efficient knock-down for up to 20 weeks) (144, 145).

Initially "naked" DNA uptake was believed to occur only *in vivo* and not in cell culture (146). However, naked DNA plasmids have been demonstrated to be internalized and result in reporter gene in cultured primary hepatocytes, keratinocytes and macrophages (141, 147, 148). In this thesis, it is demonstrated that also certain tumor cell lines can internalize "naked" DNA and a detailed characterization of the internalization mechanism is presented (**III**).

4.2.2. Cationic lipids and polymers

Due to the general inefficiency of "naked" plasmid as gene delivery vehicle (except for the hydrodynamic method) other approaches have been tried. The discovery and introduction of cationic lipids was important and its use has become widespread (149). Cationic lipids bind and condense negatively charged DNA to form so-called lipoplexes that are efficiently internalized by cultured cells. Neutral lipids in the reagent formulation then usually interact with the endosomal membrane and lead to the intracellular release of DNA. However, most cationic lipids are inhibited by serum proteins and have shown limited efficiency *in vivo*, mainly transfecting endothelial cells in the pulmonary vasculature after systemic administration (150). Lipoplexes are also highly toxic and acute reactions have been reported in animals after airway instillation or intravenous injection (150).

In 1995 Behr and coworkers presented a new gene-delivery vehicle, the cationic polymer polyethylenimine (PEI) (151). PEI is also efficient *in vitro* and condense DNA through charge interactions. The exit mechanism from the endosome is thought to depend on endosomal acidification in a so-called "proton-sponge" mechanism. The PEI polymer is believed to buffer the endosomal acidification by becoming increasingly protonated, resulting in an accumulation of

Cl$^-$ in the vesicle and finally osmotic lysis and intracellular release of the cargo (151). Despite the high effciency *in vitro*, PEI has generally been plagued by the same limitations *in vivo* as cationic lipids (152).

4.2.3. Cationic peptides

An early indication that certain proteins, other than bacterial toxins, could enter cells came from a viral protein. In 1987 Frankel and Pabo demonstrated that the HIV-1 protein, transactivator of transcription (TAT) was able to shuttle between and enter the cytosol of cells. Through this pathway TAT could activate dormant virus in cells adjacent to where it had initially been synthesized (153). TAT binds the trans activation responsive (TAR) region of HIV-1 transcript and promote the replication of the virus (154). A similar process was later reported by Prochiantz and Joliot for the homeodomain of the *D. melanogaster* homeobox transcription factor Antennapedia. The Antennapedia homeodomain was shown to be able to enter cells, functionally intact, be transported to the nucleus and elicit a morphogenetic response (155). In both Antennapedia and TAT a short stretch of approximately 15 amino acids (referred to as penetratin and Tat, respectively) was shown to mediate the uptake. These sequences are rich in the basic amino acids arginine and lysine and can be fused to other proteins and thereby mediate their uptake (156, 157).

A large and growing number of sequences of diverse origins, that can mediate the uptake of functionally intact proteins, have since been discovered and are referred to as protein transduction domains (PTDs) or cell-penetrating peptides (CPPs) (158). In this thesis the human antimicrobial peptide LL-37, as well as proteins released during cell cultivation, are demonstrated to possess CPP-like properties (**I**, **II**). The term cell-penetrating peptide hint towards a mechanism of direct penetration of the plasma membrane, as was initially generally believed to be the mechanism of internalization. However, this term is probably somewhat of a misnomer given what has been learnt about the mechanism of uptake of CPPs (see section 4.2.4 and **IV**) but the term will anyway be used in this thesis to refer to peptide sequences with an ability to enter mammalian cells.

Many CPPs can be used to deliver macromolecules both when fused to a cargo protein or nucleic acid and when used to bind and condense DNA through electrostatic interactions similarly to cationic lipids and polymers. CPPs have been used to deliver a multitude of different cargoes both *in vitro* and *in vivo*. Poly-L-lysine could be considered to be the first identified CPP, as it was shown as early as in 1978 to efficiently promote the uptake of albumin and horseradish peroxidase into cultured cells (159). In other early studies, Lebleu and coworkers used poly-L-lysine to deliver antisense oligonucleotides to inhibit the viral protein synthesis of the vesicular stomatitis virus (160) and also to protect against the cytopathic effect of acute HIV-1 infection (161). Among other notable cargoes that have been delivered using CPPs, is the Cre-recombinase for the genetic reprogramming of cells both *in vitro* and *in vivo* (162, 163). The penetratin sequence has been used to

deliver peptides that interfere with the translation initiation factor eIF4E, leading to apoptosis of the transduced cells (164). CPPs have also been used to deliver siRNA both *in vitro* and *in vivo* (165, 166).

4.2.4. Mechanism of internalization of cationic drug delivery vehicles

A common feature of the above described gene and protein delivery vehicles is that they are almost all cationic. The positive charge is necessary for complex formation with nucleic acids but also directly influence the mechanism of entry of these vehicles; most have been shown to be dependent on negatively charged cell surface HS PG for efficient entry and delivery (167). Mislick and Baldeschwieler demonstrated in 1996 in a seminal paper that the uptake of poly-L-lysine/DNA complexes was strongly dependent on HS PG as digestion of cell surface HS PG decreased complex uptake by approximately 80% and the protein expression level was 53-fold lower in transfected PG deficient cells compared to wild-type CHO cells (168). In later studies the uptake and expression of both lipoplexes and polyplexes were shown to be strictly dependent on cell surface HS PGs (169-171). In all cases the uptake of complexes was believed to involve endocytosis as no uptake or expression was seen after incubation at 4°C. Initial reports on the uptake of penetratin and Tat, however, suggested a non-endocytotic mechanism of entry, as uptake and nuclear targeting proceeded at 4°C, was non-saturable and did not depend on chiral receptors (157, 172). Uptake of free Tat did furthermore not seem dependent on cell surface HS PGs, while Tat fused to a cargo protein was dependent on HS PG for endocytic uptake (173). A model was proposed for the uptake of the aliphatic CPP penetratin in which it formed micelles and translocated directly through the plasma membrane by aid of tryptophan residues within its sequence (172, 174). However, other reports have suggested that many of these studies have been plagued by technical artifacts arising from insufficient cell surface rinsing, to remove non-internalized cell surface ligands, before microscopic and flow cytometric evaluation (175, 176). Also, artifactual translocation of especially small free CPPs to the nuclei of cells during fixation has proven to be a significant issue, resulting in a consensus that cells ideally should be evaluated live, at least for the localization of free basic CPPs (177, 178). However, there is still some controversy whether non-endocytic internalization pathways exist, especially for small free CPPs (179). Current data indicate that high concentrations (>10 µM) of CPPs can induce the formation of "nucleation zones", where significant direct membrane penetration of peptides can be seen to occur (180). The therapeutic and physiological relevance of this mechanism is probably limited but should be kept in mind when evaluating data pointing towards non-endocytic mechanisms of entry.

While an endocytic mechanism of internalization has been agreed on for many cationic peptides, polymers and lipids, at reasonable physiological

concentrations, no consensus has been reached on the exact endocytic pathway by which these diverse compounds enter cells. Tat fused to cargo proteins, for instance, has been suggested to be internalized by either CME, caveolar endocytosis or macropinocytosis in different studies, and both CME and macropinocytosis have been proposed for free Tat peptide uptake. In table 1 the mechanism of entry (as determined in the different studies) for a number of cationic delivery vehicles is presented. Among the reasons for the observed discrepancies, are probably the use of unspecific tools (most notably unspecific drugs), varying experimental conditions (ligands, cells, *etc.*) and also the fact that cationic ligands recognize a

Table 1. Suggested entry mechanism for selected cationic delivery vehicles

Mechanism	PTD/cargo	Cells analysed	Evidence provided	Ref.
Clathrin-dependent endocytosis	GST–TAT–EGFP	Jurkat T-cells	Inhib: Eps15-DN, Dyn-DN	(185)
	Tat–FITC	CHO, HepG2	Inhib: CPZ, K^+-depletion	(230)
	Lipoplex/DNA	HeLa	Inhib: CPZ, K^+-depletion no inhib: MCD, genistein	(231)
Caveolar endocytosis	GST–TAT–EGFP	HeLa, Jurkat T-cells, COS-1	Coloc: cav1, CTxB, Dx10, no coloc: Tf, inhib: MCD, cytoD	(184)
	PEI/DNA	HeLa	No inhib: CPZ, K^+-depletion inhib: MCD, genistein	(231)
Macropinocytosis	Tat-Cre-HA	CHO	Coloc: Dx70, no inhib: Eps15-DN, Dyn-DN, inhib: EIPA, MCD, nyst., no inhib: CPZ	(183)
	Tat-Alexa Fluor	CHO	Coloc: actin, activ: Rac1, inhib: EIPA	(181)
	Cationic proteins/DNA	CHO	Coloc: Dx10, no coloc: cav1, no inhib: Eps15-DN, Dyn-DN, inhib: Amil., MCD, no inhib: CPZ	**(II)**
Non-clathrin, non-caveolar endocytosis	PEI/DNA	BS-C-1	Coloc: flot1 Inhib: siDyn, no inhib: siCav1, siClath	(182)
Multiple endocytosis pathways	Tat–FITC, Antp-FITC, R9-FITC	HeLa	Inhib: CPZ, MCD, EIPA	(180)
Non-endocytic uptake	Tat–FITC, R9-FITC >10µM	HeLa	EM, light microscopy live cells	(180)

Inhib, inhibition; coloc, colocalization; activ, activation; CPZ, chlorpromazine; cytoD, cytochalasin D; EIPA, ethylisopropylamiloride; nyst., nystatin; Dx10, 10 kDa dextran; Dx70, 70 kDa dextran; cav1, caveolin-1; flot1, flotillin-1; Dyn-DN, Dynamin dominant negative; Eps15-DN, Eps15 dominant negative, siDyn, dynamin-2 siRNA; siClath, clathrin heavy chain siRNA; siCav1, caveolin-1 siRNA; Antp, penetratin; R9, nona-arginine.

multitude of cell surface receptors, including (but not limited to) cell surface HS PGs (see also section 6.4).

The use of different experimental procedures complicate comparisons between studies and make it hard to draw firm general conclusions from the available data. However, certain themes are starting to emerge from the latest studies, especially those in live cells. Most studies point towards a dependence on membrane cholesterol, limited effect of clathrin inhibitors and, possibly, an involvement of the actin cytoskeleton in the uptake of PG dependent delivery vehicles (181-184). Dynamin dependence has varied between the studies. The suggested pathways of internalization have mostly been macropinocytosis or non-clathrin, non-caveolar endocytosis, and in an early live-cell study also caveolar endocytosis (184). A notable exception to this theme is an impressive study by Beaumelle and coworkers on the uptake of intact TAT in T-cells devoid of caveolae. In these cells TAT is sorted (slowly over several hours) via CME to first early endosomes, then to late endosomes from where TAT exits into the cytosol (185). In T-cells, however, TAT is known to be able to utilize numerous cell surface receptors including CXCR4 and low-density lipoprotein receptor–related protein (LRP) in addition to HS PG (186, 187). In the study by Zhuang and coworkers, PEI polyplexes and lipoplexes are instead sorted through a flotillin dependent non-clathrin, non-caveolar pathway that partially bypasses the early endosome, for direct delivery to late endosomes (182). Clearly, new approaches with more focused studies on the individual receptor proteins are needed.

5. Endogenous macromolecular uptake

The plasma membrane constitutes an efficient barrier not only for man made synthetic therapeutics, but is also a limit for the spread of endogenous macromolecules. A notable exception to this general rule is sterol based lipophilic and membrane permeant hormones, *e.g.*, estrogen. Information dense macromolecules such as nucleic acids and proteins have, on the other hand, been thought to be restricted to the outside of receiving cells during cell-cell communication. Cell membrane receptors, activated by specific signaling molecules, overcome the membrane barrier by transmitting signals to the interior of the receiving cell through a cytoplasmic domain of the protein.

Lately, accumulating evidence is pointing towards a role for internalized macromolecules in mammalian physiology during cell-cell communication. Below is a brief summary of this emerging concept.

5.1. Polyamines

One extensively studied class of membrane impermeable molecules known to be taken up by cells, is the polyamines (188). Polyamines (putrescine, spermidine, spermine) are low molecular weight amines synthesized with arginine and methionine as primary precursors. Under physiological pH, polyamines are protonated and thus positively charged. Polyamines are important for a wide variety of cellular processes and are generally believed to function as unspecific trancription and translation factors by interaction with negatively charged DNA and RNA (189). Polyamines are necessary for cell growth and are known to be more abundant in highly proliferative conditions, *e.g.*, cancer, and has thus been considered a possible target for anti-cancer therapies (190). The intracellular levels of polyamines are tightly regulated through both synthetic and import/export pathways . The synthetic pathway is well characterized while much less is known about the import/export pathways. Several inhibitors of polyamine synthesis have been developed. One of these, α-difluoromethylornithine (DFMO), a specific suicide inhibitor of the rate-limiting polyamine synthesis enzyme, ornithine decarboxylase (ODC), is *in vitro* an efficient antiproliferative agent (190). The results from the use of DFMO in clinical settings against a variety of tumors have however, generally been disappointing. Uptake of exogenous polyamines has been shown to compensate for inhibited synthesis (190). Interference with the uptake pathway offer a possible rational addition to DFMO treatment (see paper **III** and section 6.3.3)

While several yeast polyamine importers have been identified and cloned, no mammalian polyamine importer has so far been cloned. Cell surface HS PG has, however, been shown to be involved in the uptake of polyamines in mammalian cells (191). Polyamines, especially spermine, have high affinity for HS (192) and the PG glypican-1 seems to have a central role in the uptake of polyamines (193). In paper **III** evidence is provided for a dependence on specific HS epitopes for the

uptake of polyamines, In this context, it is worth noting, that several cationic lipids (*e.g.*, lipofectamine and transfectam) have polyamine derivatives as their polar head moieties. These cationic lipids have been demonstrated to rely on cell-surface PG for efficient transfection (170). Possibly these cationic lipids utilize the same internalization mechanism as free polyamines.

5.2. Cationic peptides and proteins

The discovery of the internalization of the homeobox transcription factor Antennapedia suggested that homeoproteins possibly could act as paracrine molecules as well as cell-autonomous transcription factors (194). This suggestion has been reinforced by subsequent discoveries by especially the Prochiantz lab and collaborators. For instance, it has been demonstrated that the homeoprotein Engrailed-2 can be secreted through an unconventional secretion pathway and taken up by neuronal cells. It can thereby influence axonal guidance by directing the movement of the axonal growth cone. The effect requires uptake of the homeoprotein and seems to be mediated by an influence on protein translation rather than on transcriptional activity, unlike the typical action of homeoproteins (195, 196).

Recently, it was also demonstrated that the homeoprotein Otx2, involved in visual cortex plasticity, exerts its function in a non-cell-autonomous way that requires the transfer of intact protein between physically separated neurons. Among other things it was demonstrated that biotinylated Otx2 could be injected in the mouse eye and subsequently found in the visual cortex, necessitating transfer of intact protein over at least two synapses in a selective and directed manner (197). How the apparent directionality and targeting of the transport is obtained is currently unknown.

Homeoproteins are also known to shuttle between plant cells, primarily through the plasmodesmata, a network of fine channels connecting cells in plants. Plant homeodomains can also be shuttled between mammalian cells, suggesting a common or convergent evolutionary origin of this type of cell-cell communication (198). One homeoprotein known to be transported between plant cells is knotted1 that can be shuttled together with its mRNA (199). Whether this mechanism of communication exist in mammalian cells is currently not known, but in papers **I** and **II** a role for endogenous released cationic proteins in the uptake of nucleic acids, through a PG mediated pathway, is presented.

5.3. Other avenues for cellular macromolecular exchange

During the last few years several new potential avenues for the exchange of macromolecules between mammalian cells have started to emerge (outlined in fig. 5). One of these pathways is the so-called tunneling nanotubes (TNTs). TNTs are exceedingly thin plasma membrane protrusions up to several tens of µm long, that can connect distant cells (200). Another mechanism for cellular exchange of
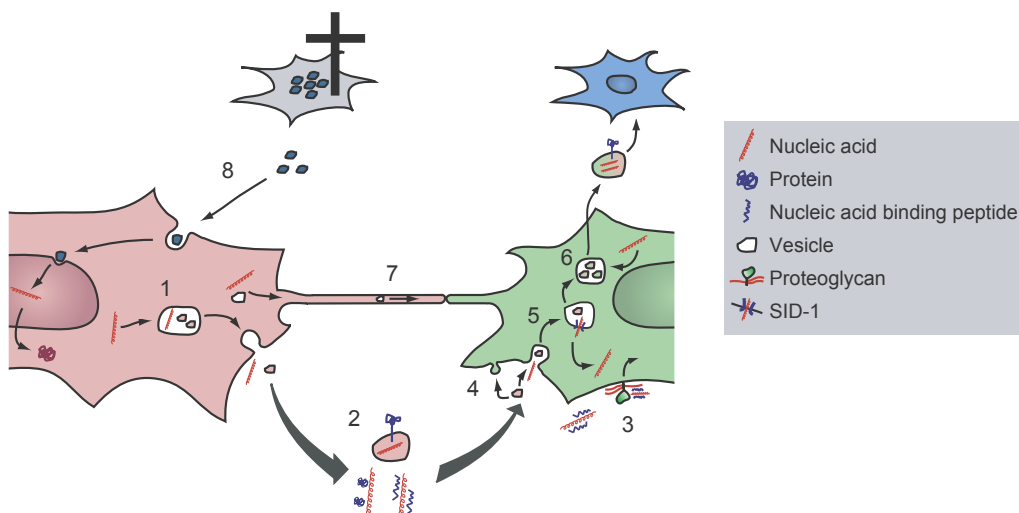
Figure 5. Possible routes for exchange of macromolecules between cells. The classical pathway for cells to communicate to each other through macromolecules is by secretion of signaling molecules. Secreted molecules are usually relayed through a secretory vesicular compartment (1) that subsequently fuses with the plasma membrane. Exosomes are released in a similar fashion, while microvesicles bud off directly from the plasma membrane. Such vesicles have been shown to contain specific proteins and nucleic acids (2) that hereby can shuttle between cells. The internalization mechanisms for peptide or protein bound nucleic acids are addressed in this thesis and involve the endocytosis of cell surface PGs (3). The uptake mechanism in the recipient cell of exosomes and microvesicles is, however, largely unknown but reasonably involve either direct fusion or endocytosis (4). How endocytosed macromolecules escape the endosome and gain access to the cytoplasm is possibly even less known than the initial uptake. Recent findings suggest a role for SID-1 in the import of nucleic acids into the cytosol from the endosomal compartment (5). Internalized vesicles might also intersect with the exosomal biogenesis machinery in the late endosome/multivesicular body (6) thus giving rise to compound vesicles that can deliver an integrated message to yet other cells (shown in blue). An alternative pathway for macromolecules shuttling between cells is through TNTs (7). Vesicles of endosomal origin have been prominent among the cargoes demonstrated to be transported this way. However, a multitude of other cargoes probably remain to be identified, including nucleic acids and proteins. Finally it has been demonstrated that apoptotic bodies released from apoptotic tumor cells (8) can be taken up by adjacent cells and deliver oncogenic DNA to the recipient cell and thus transform non-malignant surrounding cells. Adapted from (206, Belting, M. and Wittrup, A.).

macromolecules in mammalian cells that is just recently starting to emerge, is the exchange of secreted vesicles, so-called exosomes and microvesicles. In both exosomes and microvesicles, specific subsets of proteins are present in the vesicles. Specific RNAs, especially miRNAs, are also enriched in exosomes (201). How exosomes and microvesicles are taken up by recipient cells are currently virtually unknown, but they have been demonstrated to exert biological function on recipient cells. For instance, glioma cells with an oncogenic constitutively active

EGFR have been shown to transfer this mutated receptor via microvesicles. The oncogenic receptor is, thereby, transferred to clones within the tumor, not bearing the EGFR mutation (202). This is similar to how apoptotic bodies from tumor cells have been shown to be taken up by adjacent stromal cells by phagocytosis and result in expression of tumor genes, possibly conferring tumor desired traits to the stroma (203, 204).

Another intriguing pathway of nucleic acid transfer between mammalian cells are through the dsRNA plasma membrane pore SID-1. SID-1 was identified in *Caenorhabditis elegans* as an 11-pass transmembrane protein capable of dsRNA uptake from the extracellular space (205). In *C. elegans* SID-1 mediates the spread of the systemic silencing signal, possibly in response to foreign RNA. Interestingly, mammalian genomes contain two SID-1 homologues. The physiological function and relevance of this uptake is still unknown (206).

6. Present investigation

6.1. Aim

The main aim of this thesis was to clarify the role of cell-surface HS PG in the uptake of potentially therapeutic macromolecules in mammalian cells and, more specifically, in tumor cells. The rational behind this aim is, that an increased understanding of mechanisms for macromolecular uptake, may eventually lead to construction of efficient and non-toxic macromolecular drug delivery vehicles, for cancer and other diseases. The four papers contained herein address different aspects of the cell-surface HS PG receptor function in macromolecular uptake. The specific aims of the four papers (**I-IV**) are:

I.   To determine the role of PG mediated endocytosis in the uptake of DNA in complex with the antimicrobial peptide LL-37.

II. To elucidate the mechanism of the uptake of "naked" or free DNA.

III. To evaluate the possibility of interfering with HS PG receptor function in polyamine uptake using epitope specific anti-HS antibodies.

IV. To evaluate whether macromolecular uptake through HS PG is dependent on specific HS epitopes or certain core proteins and whether the PG acts as a true internalizing receptor in this process.

6.2. Methods

6.2.1. The study of endocytosis

In all of the studies in this thesis, fluorescent compounds have been utilized. Fluorescent substances, so called fluorophores, are excited by light at certain wavelengths and then emit light with a different (longer) wavelength. Fluorescent compounds can be used in several different contexts in the study of endocytosis. Using fluorescence microscopy, internalized labeled compounds can be visualized and localized. Traditional epi-fluorescence microscopy suffers from a low signal-to-noise ratio due to significant contribution of out-of-focus light, emanating from focal planes above or below the plane in focus. Using laser scanning confocal microscopy, the out-of-focus light can be removed (at the cost of speed and some sensitivity) resulting in high-contrast visualization of only the focal plane. With confocal microscopy, the sample can be optically sectioned (down to 500 nm thin sections) and several such images taken at different "depth" (z-stack) can be merged to construct a three-dimensional representation of the sample. Alternatively, the individual images in the z-stack can be superimposed on each other to create a new two-dimensional image containing all the information from the different focal-planes, in effect resulting in an image of a flattened sample.

Confocal microscopy has been used extensively in this thesis. In some instances the possibility to label different cellular structures with fluorphores that

emit light with different wavelengths has been used and qualitative colocalization studies have been performed. In a colocalization analysis one seeks to determine whether two (or more) labelled ligands or cellular components (markers) are present in the same locality of the cell (see table 2 for examples).

For most quantitative analysis of endocytosis, flow cytometry (FACS) has been performed instead of microscopy. A flow cytometer can measure the total fluorescence of large numbers of individual particles, *e.g.*, cells, very rapidly. This means that statistically confident quantitative measures of the average cellular ligand uptake, for instance, can be obtained easily.

When the uptake of fluorescent ligands are quantified using both flow cytometry and quantitative microscopy techniques, negative samples must be used as controls, to compensate for inherent autofluorescence in most cells and tissues. Both methods also yield only relative measures, not absolute amounts, of ligand uptake. In this thesis several drugs and genetic constructs that interfere with endocytosis have been used and these are presented in table 2.

An additional method for the study of endocytosis of fluorescent compounds has been developed during the course of this thesis. In paper **IV** (fig. 3C and suppl. fig. 2) cells were incubated with, and endocytosed, a fluorescent ligand. The cells were then mechanically disrupted to yield a "soup" of membrane fragments and vesicles that finally was analyzed with flow cytometry. Using a flow cytometer specifically adjusted to detect small particles, it was thereby possible to measure the abundance, size and also the relative ligand amount in each vesicle (fig. 6). By using fluorescent markers for specific cellular compartments, it is also possible to do colocalization studies on a "per particle" basis using this technique, as exemplified in fig. 6. The advantage of a flow cytometry based colocalization

Table 2. Methods used to discriminate between different endocytic pathways

Pathway	Ligand	Endogenous marker	Genetic inhibitor (dominant negative or siRNA)	Pharmacologic perturbation
Clathrin-dependent endocytosis (245)	*Tf*, LDL	Clathrin	*Dynamin*, *Eps15*, AP2, AP180	*Chlorpromazine*, K+-depletion
Caveolar endocytosis (30, 34)	SV40, *CTxB*	*Caveolin-1*, cavins	*Dynamin*, *caveolin-1*	*Cholesterol depletion*, genistein, *CytoD*
GEEC (50, 52)	*CTxB*, *dextran*	GPI-AP, GRAF1	GRAF1	*Cholesterol depletion*
Macropinocytosis (57)	*Dextran*	Actin remodeling	-	*Cholesterol depletion*, *amiloride*, EIPA, *CytoD*

Cholesterol depletion include: MCD, nystatin, filipin; CytoD, cytochalasin D; EIPA, ethyl-isopropylamiloride; Methods used in this thesis are italicized
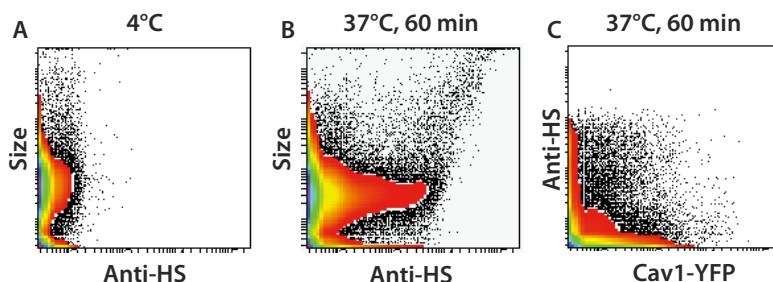
Figure 6. Flow cytometry analysis of endocytic vesicles. Cells were incubated with fluorescent anti-HS antibody complexes. After internalization for 60 min at 37°C (B) or at 4°C (A), remaining surface associated ligand was removed by trypsinization and extensive washing. Cells were then mechanically disrupted and nuclei and cell fragments were removed to yield a post-nuclear supernatant (PNS) consisting of both ligand-containing vesicles and other cellular components. The PNS was analyzed with flow cytometry to determine the size and fluorescence intensity of the particles/vesicles. C, cells expressing caveolin-1-YFP were incubated with anti-HS complexes, processed as described above and analyzed for YFP and anti-HS fluorescence in two different channels, clearly indicating absence of significant colocalization.

analysis compared to the classical confocal microscopy based, is the fact that the two parameters are measured in single particle and not within a certain volume (usually ~250x250x500 nm with confocal microscopy). Such a volume of the cell can potentially house multiple vesicles or other structures, possibly confounding the analysis. There are, however, several drawbacks with a flow cytometer based approach, among which a limited sensitivity and a risk for a selection bias towards larger particles are among the more difficult to address.

6.2.2. The study of proteoglycans

PGs have several unique physicochemical properties, enabling the study of them with a wide variety of specific techniques. The extensive sulfation of the GAG chains of PGs enable relatively selective radiolabeling through incubation of cells with [^{35}S]-sulfate in sulfate depleted medium. The high anionic charge of GAGs also render the entire PG highly anionic, making purification by anion exchange chromatography possible. Often, including in this thesis, PGs are purified using DEAE-sepharose, which with standard protocols result in partially purified PGs with the [^{35}S]-sulfate almost exclusively incorporated into PGs. [^{35}S]-radioactivity can then be used as highly sensitive quantitative tool, as well as for detection using autoradiography after, *e.g.*, size separation by electrophoresis (SDS-PAGE). Most intact PGs are also unusually large proteins thanks to their GAG substituents. Intact PGs, however, do not have a well defined molecular weight but rather

Table 3. Selected epitope specific scFv anti-HS antibodies and their reactivity

Clone	CDR3	GAG	Required modifications	Inhibiting modifications	Ref.
RB4EA12	RRYALDY	HS	NAc, NS, 6-OS		(86)
AO4B08	SLRMNGWRAHQ	HS	NS, IdoA, 2-OS, 6-OS		(241)
HS4C3	GRRLKD	HS	NS, 2-OS, 3-OS, 6-OS		(86)
HS4E4	HAPLRNTRTNT	HS	NAc, NS, IdoA	2-OS, 6-OS	(241)
EV3C3	GYRPRF	HS	NS, IdoA, 2-OS	6-OS	(87)
HS3A8	GMRPRL	HS	NS, IdoA, 2-OS, 6-OS		(85)
LKIV69	GSRSSR	HS	NS, IdoA, 2-OS	6-OS	(237)
EW3D10	GRTVGRN	Hep	-OS		(238)
NS4F5	SGRKGRMR	HS	NS, IdoA, 2-OS, 6-OS		(239)
MW3G3	QKKRPRF	AS	GlcNS, 2-OS		(240)

CDR3, amino acid sequence of the complementarity determining region 3 (a major determinant in antigen recognition); -OS, *O*-sulfation; NS, *N*-sulfation; NAc, *N*-acetylation; GAG: class of glycosaminoglycan against which the antibody has been raised; AS: acharan sulfate; Hep: heparin; Clones used in this thesis are italicized.

migrate as a broad smear when separated using, *e.g.*, electrophoresis or size-exclusion chromatography as can be seen in paper **IV** (fig. 5A and F).

In two of the papers in this thesis (**III** and **IV**) epitope specific phage display derived scFv anti-HS antibodies have been used. The most well-characterized anti-HS antibodies are presented in table 3. The main techniques to study HS PGs, used in this thesis as well as by others, are summarized in table 4.

6.2.3. Organelle purification

In paper **IV,** a method for the isolation and purification of ligand containing endocytic vesicles, is presented. Traditionally, purification of specific subcellular structures and organelles has been performed using differential and density gradient centrifugations. These methods rely on the assumption that the sought for organelle exhibit a specific density or sedimentation propensity relative to other components of the cell. Such techniques have proven to be extremely successful for the isolation of as diverse organelles as mitochondria, lysosomes and CCVs (207). Optimized centrifugation protocols can yield virtually homogeneously pure CCVs. From such pure samples, characterization of the "molecular anatomy" of the vesicles can be made, both at the level of protein and lipid constituents, including stoichiometric relationships (208).

However, not all organelles are possible to purify using such procedures. The purification of lipid rafts, caveolae and caveosomes has instead relied on detergent solubilization in, *e.g.*, cold Triton X-100, and subsequent extraction of the insoluble, detergent resistant membrane fraction (DRM) (209). There are obvious drawbacks to such approaches, most notably the significant risk of both artificially

Table 4. Methods used to study HS PGs

Method	Aspect studied	Ref.
Radiolabeling and purification		(235)
[35S]-sulfate	Sulfation specific GAG-labeling and detection	
[3H]-glucosamine	General GAG labeling	
Anion exchange chromatography	PG/GAG purification and structure analysis (charge)	
size-exclusion chromatography	PG/GAG purification and structure analysis (size)	
Gel electrophoresis	Primarily size separation	
Interference with HS synthesis		
Chlorate	General sulfation inhibition	(244)
CHO-cell mutants	pgsA; pan-GAG def. (XT-1: xylosyltransferase) pgsB; pan-GAG def. (GalT-I: galactosyltransferase I) pgsG; pan-GAG def. (GlcAT-I: glucuronyltransferase I) pgsD; HS def. (EXT-1: GlcA and GlcNAc transferase) pgsE; undersulf. HS (NDST1: GlcNAc *N*-deacetylase/ *N*-sulfotransferase) pgsF; 2-OS def. (HS2ST: HS 2-*O*-sulfotransferase) pgsH; 6-OS def. (6OST-1: HS 6-*O*-sulfotransferase)	(232)
Conditional knockouts	EXT-1, sulfotransferases	(242, 243)
Interference with HS PG function		
Degradative lyases	Hep lyase I, II and III	(233)
competition/blocking	Dependent on selectivity of competitor, *e.g.*, heparin	**(II)**
HS PG antibodies for, *e.g.*, immunoblot analysis		
Core protein specific antibodies	specific HS PG core proteins	**(IV)**
ΔHS ("anti-stub") antibody (3G10)	All HS PG core proteins (recognizes a neoepitope generated by Hep lyase III digestion)	(234)
Epitope specific scFv anti-HS antibodies	HS epitopes, see table 3	
Other anti-HS antibodies	10E4 (GlcNAc/GlcNS mixed seq.), JM13 (2-OS, GlcNS or GlcN), HepSS1 (GlcNS), JM403 (GlcN)	(236)
Modulation with HS PG expression		
siRNA core protein knockdown	specific HS PG core proteins	**(IV)**
Core protein over-expression	specific HS PG core proteins	**(IV)**

-OS, *O*-sulfation; Methods used in this thesis are italicized

creating structures and disrupting native structures by the detergent solubilization. One strategy to improve the specificity of these isolation procedures has been to do parallel isolations of two samples differing in some fundamental component of the studied compartment; *i.e.*, DRMs have been extracted from cells with and without

caveolin-1 or from both cholesterol depleted and untreated cells (30, 210). By quantitatively comparing the protein composition of the two samples, it is possible to determine which proteins that are localized to a certain compartment, specifically because of the assayed parameter, *i.e.*, caveolin-1 or cholesterol, in these two examples respectively.

Another alternative is to perform immunoisolation with antibodies against specific components on the outside of a given organelle. Such techniques have been applied successfully for both caveolae and vesicles decorated with different Rabs (211, 212). However, the approach requires a known component to be exposed on the surface, as well as availability of specific antibodies.

In paper **IV** a method is presented where ligand containing endocytic vesicles are isolated and purified despite the absence of a known specific marker exposed on the surface of the vesicle, nor a specific physical property of the vesicles such as size or density. The method relies on magnetic purification, after endocytosis of a ligand, in this case anti-HS antibody complexes, coupled to a superparamagnetic 100 nm sized nanoparticle. The method is schematically presented i fig. 7.
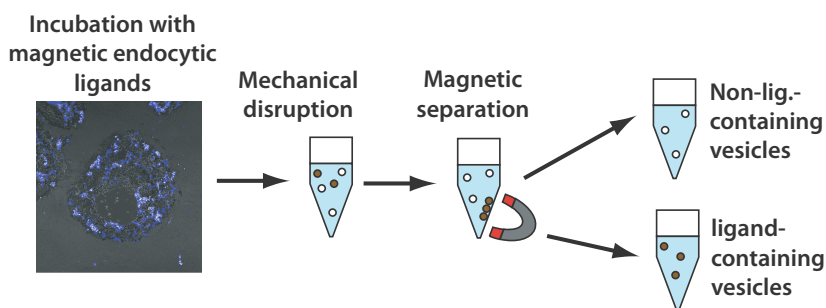

Figure 7. Magnetic isolation of ligand containing endocytic vesicles. Cells are incubated with anti-HS antibody complexes conjugated with magnetic nanoparticles (visualized in blue). After internalization for a given period of time, remaining surface associated ligand is removed by trypsinization and extensive washing. Cells are then mechanically disrupted and nuclei and cell fragments are removed to yield a post-nuclear supernatant (PNS) consisting of both ligand-containing vesicles and other cellular components. The PNS is separated into a magnetic fraction with ligand-containing vesicles and a non-magnetic fraction with the remaining cellular components.

6.3. Results

6.3.1. Paper I - Antimicrobial peptide transports DNA to cell interior

Introduction – The antimicrobial peptide LL-37 has high positive net charge (+6) and thus share an important characteristic with many CPPs. LL-37 is actively secreted and present in high concentrations at sites of tissue injury, inflammation and in certain body fluids (213). The potential of LL-37 mediated transport and internalization DNA is of considerable interest given the ability of the peptide to lyse bacteria and release bacterial DNA to the extracellular space.

Results – LL-37 was shown to bind and condensate plasmid DNA and protect it from serum nucleases. LL-37 also mediated PG dependent endocytosis and subsequent nuclear translocation of intact plasmid DNA resulting in reporter gene expression. Both DNA uptake and reporter gene expression were dependent on LL-37 concentration and increased with higher concentrations. LL-37 mediated DNA endocytosis and reporter gene expression were shown to be sensitive to cholesterol depletion using MCD and nystatin, suggesting involvement of non-clathrin mediated endocytotic mechanisms. Confocal microscopy colocalization experiments demonstrated that internalized DNA did not localize to caveolin positive structures but colocalized with the lipid raft marker CTxB. It was also demonstrated that LL-37 was highly cytotoxic towards bacteria but virtually non-toxic to mammalian cells.

Given the selective bacterial cytotoxicity at physiological peptide concentrations and the ability of the peptide to transport intact plasmid DNA to the interior of mammalian cells, a hypothetical model for lateral gene transfer at sites of LL-37 mediated bacterial lysis is presented.

6.3.2. Paper II - Naked DNA is internalized via proteoglycans

Introduction – Despite previous efforts to identify cell surface receptors for "naked" DNA, no convincing candidate receptor or mechanism of uptake have been presented. In order to improve our understanding of "naked" DNA uptake in mammalian cells, DNA uptake and reporter gene expression were analyzed in cultured cells. The kinetic properties and substrate specificities of the uptake mechanism were evaluated using quantitative methods. A better understanding could potentially improve on a safe and non-toxic route of gene-delivery, *i.e.*, the "naked" plasmid.

Results – Uptake of "naked" DNA in cultured cells (CHO cells) was time, concentration and temperature dependent. Cultured cells were also shown to exhibit significant levels of reporter gene expression after addition of naked reporter

gene plasmid to the culture medium. Reporter gene expression was concentration dependent and demonstrated similar saturable kinetics to the cellular DNA uptake. Uptake of fluorophore labeled DNA could be competitively inhibited with excess unlabeled DNA as well as HS but not with CS or hyaluronic acid, all indicating a specific and common uptake mechanism for DNA and HS.

Unexpectedly, a role for cell surface PG was demonstrated in that cultured cells were shown to release heparin binding proteins that could condensate extracellular DNA and mediate PG dependent DNA internalization. Several of these heparin binding proteins were identified and included nucleolin, HMGB1, moesin and histones, all of which had previously been shown to be involved in the uptake of macromolecules, by unknown mechanisms.

DNA uptake was through endocytosis but was not inhibited by dominant negative dynamin or Eps15. Internalized DNA, furthermore, did not colocalize with caveolin-1 and uptake was unperturbed in caveolin-1 deficient (caveolin-1 -/-) cells. Instead, internalized DNA was shown to colocalize with the fluid phase marker dextran and be sensitive to cholesterol depletion and actin cytoskeleton disruption. Taken together the data pointed towards a macropinocytotic mechanism of uptake of "naked" DNA through interaction with secreted polybasic proteins and cell-surface PGs.

6.3.3. Paper III - Anti-HS antibodies interfere with PG-dependent polyamine uptake

Introduction – Tumor cells are dependent on polyamines for proliferation. The highly specific polyamine synthesis inhibitor α-difluoromethylornithine (DFMO) is *in vitro* an efficient antiproliferative agent. However, in clinical trials DFMO has generally not improved significantly on tumor progression or survival (190). Uptake of exogenous polyamines has been shown to be a salvage mechanism for polyamine depleted cells (190). Uptake of polyamines is dependent on cell surface PGs and this mechanism is upregulated upon polyamine depletion (191). Indeed, combined inhibition of polyamine and PG synthesis, strongly inhibited tumor growth in a mouse tumor metastasis model (214). DFMO treatment combined with administration of the polybasic peptide Tat acting as a polyamine uptake competitor was also shown to inhibit tumor growth *in vivo* (215).

In order to test whether the uptake of polyamines is dependent on specific HS epitopes present on tumor cells several epitope specific scFv anti-HS antibodies were tested for their potential to block polyamine uptake and polyamine dependent cell proliferation.

Results - Both polyamine binding and uptake was shown to be specifically inhibited by the anti-HS scFv antibody RB4EA12 but not by the antibodies AO4B08 or HS4E4. RB4EA12 recognize relatively low sulfated domains (see also table 3) indicating such domains are important for polyamine uptake. RB4EA12 anti-HS

antibody also efficiently inhibited proliferation of tumor cells made dependent on exogenous polyamines through pharmacological (DFMO) or genetic means (polyamine synthesis deficient ODC -/- cells). Incubation of tumor cells with RB4EA12 resulted in compensatory induction of ODC. Combined treatment of tumor cells with DFMO and RB4EA12 was furthermore shown to result in cytostatic rather than cytotoxic inhibition of cell proliferation.

Taken together, the study demonstrated the potential of using epitope specific anti-HS antibodies to modulate the accessibility to growth promoting polyamines for tumor cells.

6.3.4. Paper IV - Internalization of epitope specific anti-HS antibodies is mediated by both syndecan and glypican

Introduction – Significant controversy has surrounded both the mechanism of uptake as well as the exact role of cell surface HS PG in the uptake of especially CPPs and their cargoes (167). The PG has been proposed to act either as a true internalizing receptor or merely as an initial attachment receptor (182, 216). In the latter case, ligand binding and concentration by the HS PG has been suggested to result in subsequent ligand presentation to alternative, internalizing receptors, or in ligand uptake through receptor-independent pinocytosis. Direct membrane penetration through phospholipid interactions has also been suggested as a means for CPP uptake (172, 180). Furthermore, it has been unclear to what extent different members of the family of cell surface HS PGs can mediate internalization of macromolecular cargoes. Neither has it been known whether the actual HS epitope recognized by HS binding ligands influences their fate and potential for internalization.

In order to address these questions, epitope specific scFv anti-HS antibodies were applied to the analysis of HS PG mediated macromolecular internalization. Additionally, a method for the selective purification of ligand containing endocytic vesicles, was developed, in order to analyze the HS PG composition specifically in endocytic vesicles.

Results – Among several epitope specific anti-HS antibodies screened for uptake in tumor cells, only one (AO4B08) was shown to be efficiently internalized. Several of the other screened antibodies recognized epitopes present on the evaluated cells but were not internalized. The uptake of AO4B08 was shown to be endocytic and specifically dependent on the presence of 2-*O*-sulfations. Importantly, incubation of cells with both internalizing AO4B08 antibodies as well as with the CPP Tat, resulted in consumption of cell surface HS PG, indicative of ligand induced internalization. Using the magnetic purification method developed in the paper it was shown that HS PGs co-internalized with AO4B08 and remained intact for several hours.

Finally it was demonstrated both syndecan and glypican could mediate the uptake of anti-HS antibody complexes. A fraction of the internalized anti-HS antibodies was internalized by vesicles containing both syndecan and glypican suggesting a common uptake pathway for the two classes of cell surface HS PG.

6.4. Conclusions and discussion

In this thesis, the function of cell surface PG, in its role as a receptor for several different endocytic ligands, has been examined and clarified. Below is a brief summary of the main conclusions and their implications.

Novel pathway for the cellular uptake of DNA
In paper **I** and **II** a novel mechanism for the uptake of extracellular DNA is presented. It is shown that endogenously produced peptides and proteins with a positive charge can interact with negatively charged extracellular DNA, analogously to synthetic CPPs, and mediate uptake of the condensed DNA through, likewise negatively charged, cell surface HS PGs (schematically illustrated in fig. 8). The physiological and pathophysiological importance of this mechanism is still unclear. However, emerging evidence suggest a fundamental role for peptide and protein mediated DNA uptake in the pathogenesis of autoimmunity towards self-DNA. Self-DNA induced immune responses, *e.g.*, interferon production, is commonly seen in autoimmune diseases, including *psoriasis* and *systemic lupus erythematosus* (SLE). Normally, the organism is protected against immune recognition of self-DNA, abundantly released during necrotic and apoptotic cell death, through the activity of extracellular DNases and the endosomal localization in immune cells of DNA sensing Toll-like receptors (217). Toll-like receptors are part of the innate
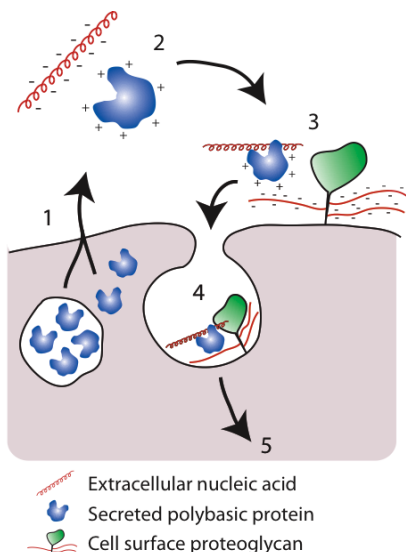

Figure 8. Novel pathway for the cellular uptake of DNA (1) Cells secrete polybasic proteins through both classical (vesicular) and non-classical, possibly non-vesicular pathways. (2) The positively charged proteins bind electrostatically to extracellular DNA. (3) positively charged protein/DNA complexes bind to negatively charged cell surface HS PG. (4) Protein/DNA complexes are internalized by cell surface HS PG and are further sorted to other intracellular locations including the nucleus for expression (5).

~~~~~~~ Extracellular nucleic acid
🔵 Secreted polybasic protein
🟢 Cell surface proteoglycan

immune system and recognize structurally conserved molecules derived from microbes, *e.g.*, DNA, RNA and LPS (218). LL-37 mediated nuclease protection and transport of self-DNA to endosomes, in plasmacytoid dendritic cells, was recently identified as the driving force of self-DNA autoimmunity in *psoriasis* (219). LL-37 complexed self-DNA was shown to trigger an interferon response through activation of Toll-like receptor 9 after DNA/LL-37 complex uptake into endosomes. LL-37 was also recently demonstrated to transport self-RNA to endosomes where an immune response was triggered through activation of Toll-like receptors 7 and 8 (220). It is also noteworthy that HMGB1, one of the several heparin binding proteins identified to mediate DNA uptake in paper **II**, was recently shown to trigger an immune response by complexing and mediating uptake of self-DNA in plasmacytoid dendritic cells and B-cells (221).

The emerging pathophysiological consequences of DNA uptake through the described PG dependent pathway, suggest this pathway is not commonly utilized under normal homeostasis. Indeed, purification of heparin binding serum proteins from healthy donors failed to produce any DNA transporting proteins (Wittrup A. and Pallon J., unpublished observation). Whether serum from autoimmune disease subjects would contain such proteins is an open question. Likewise, it is of interest whether the described mechanism is involved in clearance of circulating DNA during the massive release of DNA from necrotic and apoptotic cells, commonly seen in cancer (222).

Cell surface HS PGs are true internalizing receptors for macromolecular cargoes
In paper **IV** several different techniques are used to demonstrate that cell surface HS PGs are internalized, subsequent to binding of HS binding ligands. Incubation of cells with Tat peptide, Tat/DNA complexes and an internalizing anti-HS antibody all resulted in the cell surface consumption of HS PG, indicative of endocytosis. The ability of a well characterized, and highly HS-specific, antibody to induce the internalization is of great importance. This demonstrates that it is HS binding/crosslinking *per se* that is triggering endocytosis and not an interaction with other internalizing receptors. Such interactions cannot be ruled out with other less HS-specific endocytic cargoes such as FGF or potentially promiscuous peptides such as Tat and penetratin.

For the first time an internalized PG, together with its bound ligand, is biochemically isolated (**IV**). This enabled the identification of the core proteins directly responsible for the studied macromolecular uptake. The identification of both syndecan-2 and glypican-1 was done with no *a priori* assumption of which HS PGs that were responsible for the uptake. Previously, using a variety of more focused techniques, the uptake of several different HS binding ligands have been shown to be dependent on specific core proteins: lipoprotein uptake on syndecan and perlecan; HIV-1 uptake, in the absence of CD4, on syndecan-1 but not on glypican-1; and heparanase uptake on syndecan-1 but not on glypican-1 (122, 123, 223, 224). Uptake of PEI/DNA complexes has been suggested to require

syndecan-1 but to be inhibited by syndecan-2. However, no quantitative data was provided in this study, clearly demonstrating the inhibitory effect of syndecan-2, on complex uptake (225). There are several processes known to be mediated by both syndecans and glypicans, most notably FGF signaling (134), but to our knowledge, paper **IV** constitutes the first report of an HS PG dependent uptake process, mediated by both glypicans and syndecans.

Non-clathrin, non-caveolin mediated, PG dependent endocytosis
In paper **II** it is shown that PG mediated endocytosis of DNA/protein complexes proceed through non-classical, non-clathrin, non-caveolin mediated endocytosis. The endocytosis pathway is not dependent on dynamin but sensitive to disruption of the actin cytoskeleton and depletion of cholesterol. Colocalization of internalized complexes with the fluid-phase marker dextran together with the fact that the drug amiloride inhibited uptake, made us conclude that the main uptake pathway was macropinocytosis. This conclusion is in agreement with other reports, with several different HS PG binding ligands being internalized via macropinocytosis, including free Tat peptide and proteins fused with Tat, as well as the HS binding growth factor FGF (125, 181, 183). There is however not universal agreement on macropinocytosis being the main mechanism of internalization of PG binding ligands in general and CPPs in particular.

A possible explanation for the conflicting reports on the endocytic pathway, is the use of different cell lines with radically different HS PG core protein repertoires. As demonstrated in paper **IV**, both glypican and syndecan can internalize at least certain HS binding ligands. Glypican and syndecan possibly follow different endocytic pathways (see section 6.5). Thus, the core protein repertoire, in the specific cell type, could determine which pathway is chosen for a given HS binding ligand.

Specific HS epitopes are involved in the uptake of polyamines and anti-HS antibodies
In paper **III** it is demonstrated that RB4EA12, an epitope specific scFv anti-HS antibody, inhibits the uptake of growth promoting polyamines in tumor cells. This study is one of the very first studies to demonstrate interference of HS PG function with an epitope specific anti-HS antibody. The study suggests it might be possible to harness the structural diversity of HS for therapeutic purposes.

In paper **IV** it is demonstrated that an epitope specific scFv antibody can be utilized to deliver macromolecular and nanoparticular cargoes to the interior of tumor cells. Surprisingly, only one (AO4B08) of the several screened antibodies were shown to promote endocytosis and to be internalized. The reason for this selectivity is not absolutely clear at the moment. However, steric reasons are the most probable explanation. The epitope recognized by the internalized AO4B08 antibody might be relatively more accessible and amenable to cross-linking than the epitopes recognized by the other antibodies. In this context, it is of interest to

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note that the 2-*O*-sulfate modifications recognized by the AO4B08 antibody have been shown to be preferentially localized to the distal ends of GAG chains (226).

The study in paper **IV** also demonstrates the possibility of utilizing epitope specific anti-HS antibodies for the delivery of therapeutic macromolecules and nanoparticles in, *e.g.*, cancer therapy. Given the tissue specific distribution of HS epitopes, epitope targeted delivery could enable delivery to specific locations within a tissue. However, the observation that not all HS binding antibodies are efficiently internalized, underline the difficulties in achieving targeted intracellular delivery.

Organelle purification of ligand containing vesicles
In paper **IV** a novel method for isolating ligand containing endocytic vesicles is presented. Up until now, several endocytic ligand have been shown to be internalized by non-classical internalization pathways (see section 2.3). The molecular details of these non-classical pathways are however, so far, very scarce. The presented method offer the possibility to substantially improve on this status by making it possible to isolate virtually any endocytic compartment in which antibodies can be used to trigger the internalization.

There are a few reports previously in the literature of similar approaches, most notably for the isolation of larger phagosomes (227). In studies of endocytic processes, non-targeted magnetic particles, following a non-specific fluid-phase uptake pathway, have mostly been utilized (42, 228). In one study, a more specific approach, with signaling molecule decorated magnetic nanoparticles, was undertaken (229). The method presented in paper **IV** with magnetically tagged antibodies is however more versatile and can hopefully aid in improving our understanding of non-classical endocytosis pathways (see also discussion in section 6.5).

6.5. Future perspectives
In this thesis it is conclusively demonstrated that HS binding ligands, including CPPs, indeed trigger the internalization of the cell surface HS PG and that the PG, thus, is a true internalizing receptor for HS-binding ligands. However, many questions remain to be addressed in the field. Especially the finding that both syndecan and glypican can mediate the internalization of an HS binding ligand is of considerable interest. Are the ligands sorted through two distinct pathways or do syndecan and glypican utilize a common pathway? Previous studies of the constitutive endocytosis of glypican and syndecan have suggested that they follow distinct pathways (119). However, in a significant fraction of ligand containing vesicles both glypican and syndecan were detectable within 10 min. of HS ligand internalization (**IV**). What pathway do these hybrid vesicles follow? Syndecan-4 has been shown to be internalized through CDC42 regulated macropinocytosis upon clustering by FGF2 (125). It is unclear whether this sorting is dependent on FGF binding to FGFR or whether a "pure" HS binding ligand would be routed the same pathway. The described pathway has striking similarity to the recently

described GEEC endocytic pathway, including dynamin independence, CDC42 regulation and bulk fluid phase uptake. Glypican-1, on the other hand, has been suggested to be endocytosed via caveolae (129). Emerging evidence, however, is pointing towards the GEEC pathway being the main route of entry for GPI-APs, though cross-linked GPI-APs possibly follow a caveolin associated pathway (110). Thus, more detailed characterization of endocytic sorting of both syndecan and glypican as well as HS binding (*i.e.*, both syndecan and glypican binding) ligands would be of great interest.

The magnetic isolation procedure described in paper **IV** is also a powerful tool for a more detailed characterization of the endocytic pathway taken by both syndecan, glypican and HS binding ligands. Indeed, preliminary results point towards non-clathrin, non-caveolin, cholesterol dependent and CDC42 regulated uptake of anti-HS antibody complexes. Additionally, at least one novel, functionally important, protein component in this pathway has been identified through proteomic analyses. Hopefully, further analysis will reveal additional components and improve our understanding of this potentially very useful macromolecular delivery route.

7. Populärvetenskaplig sammanfattning

Det finns ett stort behov av nya läkemedel mot cancer med bättre effekt och mindre biverkningar. En ny klass av läkemedel baserade på stora biologiska molekyler, som utövar sin effekt inne i cellen, d.v.s. intracellulärt, har potential att erbjuda detta. För närvarande finns dock inte några effektiva och säkra leveransmetoder, som kan användas kliniskt, för dessa stora molekyler,. Försök har gjorts, och görs, med virus som modifierats för att kunna leverera stora molekyler med läkemedelsverkan, t.ex. DNA och RNA. Sådana virusbaserade metoder har dock visat sig vara förenade med svåra, ibland dödliga immunreaktioner. Det finns flera icke-virusbaserade metoder för att leverera molekyler intracellulärt. Dessa metoder är mycket effektiva och välanvända för leverans till celler odlade i cellkultur. Dock har metoderna visat sig svåra att tillämpa kliniskt på patienter.

Ett gemensamt drag hos såväl icke-virusbaserade som flera virusbaserade leveransmetoder, är att de använder sig av en grupp proteiner vid namn proteoglykaner, som finns på ytan på de flesta celler. Proteoglykaner är proteiner med en särskild typ av kolhydratstrukturer fästa på sig. Deras exakta roll vid cellens upptag och internalisering av de olika leveransverktygen har inte varit känd. Det har bland annat varit oklart om proteoglykanen följer med leveransverktyget in i cellen eller om den förblir på ytan och alltså bara utgör ett fäste för verktyget på ytan av cellen. Leveransverktyget skulle i så fall behöva förlita sig på andra molekyler och proteiner på ytan av cellen för att ta sig vidare in i cellen.

I den här avhandlingen belyses proteoglykanens roll vid upptaget av ett flertal molekyler och potentiella leveransverktyg. Bl.a. visas det att proteoglykanen följer med in i cellen vid upptaget av leveransverktygen. Det visas också att medlemmar ur två av undergrupperna till proteoglykanerna, nämligen syndekaner och glypikaner, båda har förmågan att transportera leveransverktyg in i cellen.

I den här avhandlingen visas också att mänskliga celler har förmåga att ta upp DNA från sin omgivning. Upptaget sker genom att DNA:t kan binda till starkt positivt laddade proteiner som utsöndras av celler, vilket i sin tur gör att DNA:t kan bindas upp av den negativt laddade proteoglykanen på cellytan, som sedan transporterar in DNA:t in i cellen. Bland de starkt positivt laddade proteiner som visade sig besitta ovan beskrivna förmåga, var bl.a. ett kroppseget proteinbaserat antibiotikum, LL-37. Även ett flertal andra proteiner kända att frisättas vid celldöd, ett tillstånd som också är associerat med riklig förekomst av DNA i cellernas omgivning, visade sig kunna transportera in DNA i celler. Dessa fynd har, något överaskande, bidragit till vår förståelse för hur utveckling av autoimmunitet mot ens eget DNA går till. Detta är ett vanligt tillstånd vid autoimmuna sjukdomar och verkar vara intimt förknippat med upptag av DNA via proteoglykaner. Framförallt har dock fynden i avhandlingen betydelse för vår förmåga att utveckla nya leveransverktyg för stora molekyler. Dessa verktyg har potential att kunna revolutionera behandlingen av ett flertal sjukdomstillstånd där vi i dag saknar fungerande behandlingar, bl.a. cancer.

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9. References

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