REGULATED UPTAKE OF BIOPOLYMERS Role of cell surface proteoglycans Implications for drug and gene delivery

Sandgren, Staffan

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IMPLICATIONS FOR DRUG AND GENE DELIVERY

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Staffan Sandgren, medicine kandidat

Fakultetsopponent:
Professor Bernard Lebleu
Laboratoire des Défenses Antivirales et Antitumorales
Université Montpellier II
Montpellier, France

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ROLE OF CELL SURFACE PROTEOGLYCANS
IMPLICATIONS FOR DRUG AND GENE DELIVERY

STAFFAN SANDGREN

FROM:
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Lund University

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I. **Sandgren, S.**, and Belting, M. Suramin selectively inhibits carcinoma cell growth that is dependent on extracellular polyamines. *Anticancer Res* (2003) **23:** 1223-8.

II. Mani, K.*, **Sandgren, S.***, Weber, E., Cheng, F., Fransson, L-Å., and Belting, M. Common internalization mechanism for HIV-Tat protein transduction domain and polyamines in human carcinoma cells: Implications for tumor growth inhibition. Manuscript

III. **Sandgren, S.**, Cheng, F., and Belting, M. Nuclear targeting of macromolecular polyanions by an HIV-Tat derived peptide. Role for cell-surface proteoglycans. *J Biol Chem* (2002) **277:** 38877-83.

IV. **Sandgren, S.**, Wittrup, A., Cheng, F., Jönsson, M., Eklund, E., Busch, S., and Belting, M. 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* (2004) **279:** 17951-6.

V. **Sandgren, S.***, Wittrup, A.*, Lilja, J., Mörgelin, M., and Belting, M. Secreted components induce DNA internalization in mammalian cells. Manuscript

*) These authors contributed equally to this work

PEER-REVIEWED PUBLICATIONS NOT INCLUDED IN THIS THESIS

Belting, M., **Sandgren, S.*** and Wittrup, A.* Nuclear delivery of macromolecules: barriers and carriers. *Adv Drug Deliv Rev* (2005) **57:** 505-27 Review

Fransson, L-Å., Belting, M., Cheng, F., Jönsson, M., Mani, K. and **Sandgren, S**. Novel aspects of glypican glycobiology. *Cell Mol Life Sci* (2004) **61:** 1016-24 Review

Belting, M., Dorrell, M.I., **Sandgren, S.**, Aguilar, E., Ahamed, J., Dorfleutner, A., Carmeliet, P., Mueller, B.M., Friedlander, M. and Ruf, W. Regulation of angiogenesis by tissue factor cytoplasmic domain signaling. *Nat Med* (2004) **10:** 502-9

Mani, K., Cheng, F., **Sandgren, S.**, Van Den Born, J., Havsmark, B., Ding, K. and Fransson, L-Å. The heparan sulfate-specific epitope 10E4 is NO-sensitive and partly inaccessible in glypican-1. *Glycobiology* (2004) **14:** 599-607

Mani, K., Belting, M., Ellervik, U., Falk, N., Svensson, G., **Sandgren, S.**, Cheng, F. and Fransson, L-Å. Tumor attenuation by 2(6-hydroxynaphthyl)-beta-D-xylopyranoside requires priming of heparan sulfate and nuclear targeting of the products. *Glycobiology* (2004) **14:** 387-97

Belting, M., Mani, K., Jönsson, M., Cheng, F., **Sandgren, S.**, Jönsson, S., Ding, K., Delcros, J.G. and Fransson, L-Å. Glypican-1 is a vehicle for polyamine uptake in mammalian cells: a pivotal role for nitrosothiol-derived nitric oxide. *J Biol Chem* (2003) **278:** 47181-9

Ding, K., **Sandgren, S.**, Mani, K., Belting, M. and Fransson, L-Å. Modulations of glypican-1 heparan sulfate structure by inhibition of endogenous polyamine synthesis. Mapping of spermine-binding sites and heparanase, heparin lyase, and nitric oxide/nitrite cleavage sites. *J Biol Chem* (2001) **276:** 46779-91

Ding, K., Jönsson, M., Mani, K., **Sandgren, S.**, Belting, M. and Fransson L-Å. N-unsubstituted glucosamine in heparan sulfate of recycling glypican-1 from suramin-treated and nitrite-deprived endothelial cells. Mapping of nitric oxide/nitrite-susceptible glucosamine residues to clustered sites near the core protein. *J Biol Chem* (2001) **276:** 3885-94

*) These authors contributed equally to this work

ABBREVIATIONS

bFGF	basic fibroblast growth factor
CHO	Chinese hamster ovary
CS	chondroitin sulfate
CTxB	cholera toxin B
dcSAM	decarboxylated S-adenosylmethionine
DFMO	α-difluoromethylornithine
DS	dermatan sulfate
ECM	extracellular matrix
EGF	epidermal growth factor
eIF-5A	eukaryotic translation initiation factor 5A
FGF	fibroblast growth factor
GAG	glycosaminoglycan
GFP	green fluorescent protein
GPI	glycosylphosphatidylinositol
Hep	heparin
HIV	human immunodeficiency virus
HPO	horseradish peroxidase
HS	heparan sulfate
HTDP	HIV-TAT derived peptide
MGBG	methylglyoxal bis(guanylhydrazone)
MMP	matrix metalloproteinase
NDST	N-deacetylase-sulfotransferase
NO	nitric oxide
PDGF	platelet derived growth factor
PG	proteoglycan
PIP_2	phosphatidylinositol(4,5)bisphosphate
PLL	poly-L-lysine
SAM	S-adenosylmethionine
SAMDC	S-adenosylmethionine decarboxylase
Tat	active peptide derived from the HIV-1-TAT protein
TAT	trans-activator of transcription
VEGF	vascular endothelial growth factor

The nomenclature to differentiate between the HIV-1 TAT protein and the Tat peptide may appear somewhat inconsistent in the literature. Throughout the summary of this thesis "TAT" denotes the full length HIV-1 TAT protein, whereas "Tat" denotes the active peptide (e.g. a peptide such as GRKKRRQRRRPPQC, containing the essential stretch of basic amino acid residues). However, in paper III the Tat peptide is referred to as HTDP, short for HIV-TAT derived peptide. If used differently, "Tat" will be defined as peptide or protein.

INTRODUCTION

Cells continuously export, import, and recycle molecules over the plasma membrane. Internalization, i.e. cellular import of extracellular material, is a fundamental process, which provides cells with nutrients and enables the immune cells of higher organisms to remove debris, sample their surroundings for antigens and to fight microbes. Moreover, internalization regulates complex cellular signalling events involved in cellular division, motion, and communication with the surrounding extracellular matrix. However, the preserved routes of internalization are exploited by a large number of microbes and pathological factors such as bacterial toxins and viral proteins. The HIV-1 TAT protein was shown to enter cells and to target their nuclei, thus acting as a paracrine transcription factor, a finding that initiated the field of so called cell penetrating peptides (CPPs). Due to their ability to efficiently deliver macromolecular cargo over the plasma membrane, CPPs have proven to be useful tools in basic research. More importantly, the technology has been shown to enhance delivery of a number of macromolecular compounds *in vivo*, including anticancer drugs.

The proteoglycan family of molecules has previously been shown to participate in the interaction with and internalization of a number of ligands, including polyamines, growth factors, morphogens, and microbes. This thesis deals with the role of proteoglycans in cellular internalization of charged biopolymers, i.e. the polyamine family of growth factors, HIV-Tat peptide, antimicrobial peptides, and nucleic acids. The presented findings bring proteoglycans into focus as a general internalization pathway for charged macromolecules, with implications for drug and gene delivery.

BACKGROUND

PROTEOGLYCANS

Structure and function of proteoglycans

Proteoglycans (PGs) are a superfamily of molecules with a multitude of functions spanning from cartilage shock absorption to regulation of morphogenesis. PGs consist of a core protein substituted with one to more than a hundred glycosaminoglycans (GAGs). GAGs are linear polysaccharides that display high diversity due to the extensive modifications that occur during their synthesis and turnover. GAGs can be sub grouped depending on the type of saccharides that compose the basic polymeric structure [1-4].

The importance of PGs in physiology is reflected by the severe and often complex phenotypes of various PG/GAG null genotypes, ranging from mast cell abnormalities to early embryonic lethality (see Table 1). Herein, GAGs relevant to the present investigation will be reviewed.

GAGs and GAG attachment to core proteins

The GAGs chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS) and heparin (Hep) are polymers of repeating hexosamine-hexuronic acid units. In CS and DS, the hexosamine is N-acetyl galactosamine (GalNAc) whereas it is N-acetyl glucosamine (GlcNAc) in HS/Hep. These polysaccharides are therefore referred to as galactosaminoglycans and glucosaminoglycans, respectively. In both cases, the hexuronic acid moiety incorporated into the growing chain is D-glucuronic acid (GlcA). Some of the GlcA residues are converted to L-iduronic acids (IdoA) on the polymer level by the action of specific epimerases. DS is a galactosaminoglycan that contains IdoA whereas CS has only GlcA. The definitions of HS and Hep are not as straightforward and both species contain IdoA. However, Hep is generally more modified and contains over 70% IdoA whereas the IdoA content of HS normally is between 30% and 50% [2]. HS is made by most cells and has diverse functions, whereas Hep is only produced in mast cells.

All these polysaccharides are synthesized on protein cores and are attached in an O-linked fashion. The consensus sequence for CS/DS and HS/Hep substitution is serine-glycine (SG), usually with flanking acidic regions. Several PGs contain clustered SG sequences, an example being the GAG substituted region in human glypican-1: **D**V**D**FQ**D**AS**DD**G<u>S</u>G<u>S</u>G<u>S</u>G**D** (serines underlined and acidic residues in bold; [5]).

Table 1. Phenotypes of PG-related knockout animals

Species	Genotype	Phenotype	References
C. elegans	HS 2-OST -/-	Defective cell migration	[6]
C. elegans	Syndecan-1 -/-	Defective egg laying	[7]
C. elegans	Xyl-T -/-; Gal-TII-/-	Defective vulval morphogenesis	[8]
C. elegans	CS synthase -/-	Defective early embryogenesis and vulval development; defective embryonic cytokinesis and cell division	[9, 10]
Drosophila	HS 6-OST	Defective tracheal branching	[11]
Drosophila	Dally/Dally-like -/-	Impaired morphogen distribution	[12, 13]
Mouse	HS 2-OST	Renal agenesis, defective eye and skeleton development; defective cerebral cortex development	[14, 15]
Mouse	Syndecan-3 -/-	Resistance to diet-induced obesity; muscular dystrophy; enhanced level of long-term potentiation	[16-18]
Mouse	Syndecan-1 -/-	Resistance to mammary tumor development; defective wound healing	[19-21]
Mouse	HS polymerase -/-, conditional	Defect midline axon guidance	[22]
Mouse	Perlecan HS -/-	Defective lens capsule; defective acetylcholine esterase localization; impaired angiogenesis, wound healing and tumor growth	[23-25]
Mouse	Perlecan -/-	Defective cardiac morphogenesis; defective cartilage development, DDSH	[26, 27]
Mouse	Glypican-3 -/-	SGBS; Impaired hematopoietic differentiation	[28, 29]
Mouse	NDST-1 -/-	Neonatal mortality due to respiratory distress syndrome; Defective cerebral and craniofacial development	[14, 30]
Mouse	NDST-2 -/-	Abnormal mast cells	[14]
Mouse	NDST-1 -/-, conditional	Impaired neutrophil infiltration in inflammation	[31]
Mouse	HS polymerase (EXT1) -/-	Defective gastrulation and early embryonic development	[14, 32]
Mouse	HS epimerase -/-	Defective renal, lung, and skeletal development	[33]
Mouse	HS 3-OST-1 -/-	Normal hemostasis, intrauterine growth-retardation	[34]
Zebra fish	HS 6-OST -/-	Defective muscle development; defective angiogenesis	[35, 36]
Zebra fish	HS polymerase -/-	Defective axon sorting in optic tract	[37]
Zebra fish	UDP-Glc dehydrogenase -/-	Defective cardiac valve formation	[38]

DDSH, Dyssegmental dysplasia, Silverman-Handmaker type; SGBS, Simpson-Golabi-Behmel syndrome; 2,3 and 6-OST, respective O-sulfotransferase; Xyl-T, xylosyltransferase; Gal-TII, galactosyltransferase II; NDST, N-deacetylase-sulfotransferase; EXT, hereditary multiple exostoses; UDP, uridine-di-phosphate; Glc, glucose

CS/DS and HS/Hep are attached to the core protein via a common tetrasaccharide linkage region. First, xylose (Xyl) is attached to a serine residue, catalyzed by xylosyltransferase, using UDP-Xyl as donor. Subsequently, two galactose (Gal) residues and one GlcA are added by the action of specific enzymes, using the corresponding UDP-sugars as donors (see Figure 1). Core proteins with a defined number of SG sequences can be substituted with a varying number of GAG chains, possibly due to the fact that xylosylation is not a complete process. The Xyl residue can be transiently phosphorylated, which may act as a signal for quality control [39]. GAG initiation and polymerization occur during the passage of the core protein through the ER and Golgi compartments.

Addition of the first non-link region sugar determines whether the polysaccharide will be a CS/DS or HS/Hep type of GAG, where a GalNAc leads to CS/DS and GlcNAc initiates HS/Hep synthesis. It is still somewhat elusive what determines the type of GAG substitution, but the linear amino acid sequence in the attachment region and the three-dimensional structure of the core protein seem important [40-42]. It is clear that a nearby acidic cluster can be associated both with CS/DS and HS substitution. This feature however seems important for HS assembly, since mutations of acidic residues diminish HS synthesis, resulting in greater substitution with CS [42]. An adjacent tryptophan also stimulates HS substitution [42], and repetitive SG sequences appear to be a signal for HS substitution [41]. A recent study by Lander and co-workers reveals that efficient glycanation with HS is highly dependent on the presence of the glypican globular domain [40](defined below). The authors suggest that the function of the glypican globular domain may be to direct GAG substitution to the HS pathway. They show that the glypican-1 globular domain can shift glycanation to HS on other GAG attachment domains to which it is experimentally fused. Interestingly, expression of the globular domain stimulates HS substitution even on core proteins to which it is not fused.

Both CS/DS and HS can be heavily modified generating a very heterogeneous pool of GAGs and PG. To assemble a fully modified HS chain, at least a dozen genes (not taking into account the various enzyme isoforms) need to be expressed in addition to the gene coding for the core protein [4, 43]. General modifications occurring in the synthesis of an HS chain are: N-deacetylation/N-sulfation of GlcNAc residues; epimerization of GlcA to IdoA; sulfation at the 2-O position of IdoA; and sulfation at 6-O and 3-O positions of GlcNAc [1, 3, 4]. Occasional N-unsubstituted GlcN residues can also be found, but their mode of formation is still unknown [44]. An overview of HS assembly is presented in Figure 1.

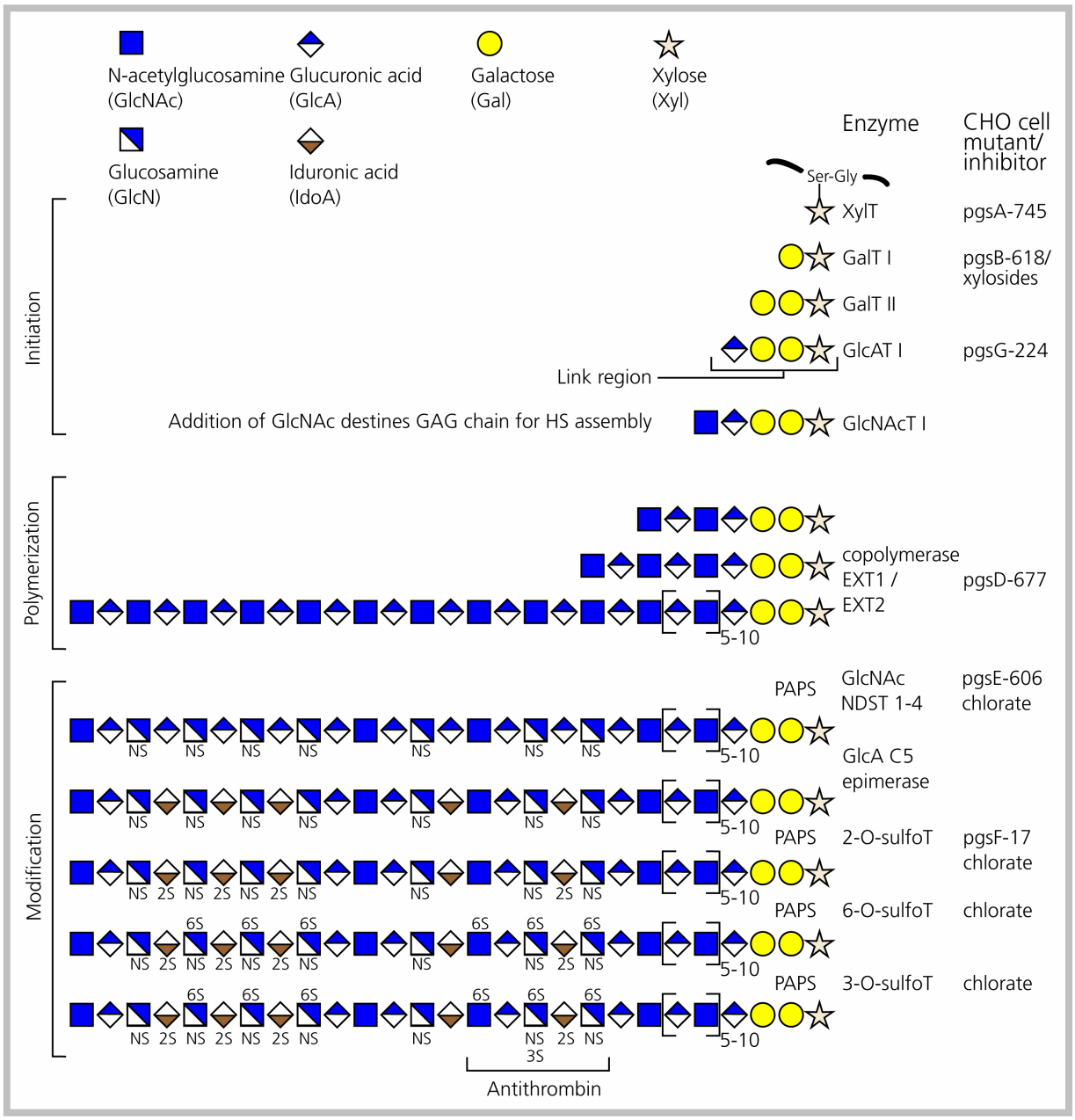


Figure 1. Biosynthesis of heparan sulfate. HS generally displays a patched appearance, with highly modified regions interspersed between regions of lower modification grade. The sequence responsible for antithrombin III binding is depicted. Esko and co-workers have developed a CHO cell system with mutants deficient in various steps of GAG synthesis. Mutants deficient in enzymes involving link region formation are deficient in all GAGs, i.e. the Xyl transferase mutant (pgsA-745 [45]), the Gal transferase I mutant (pgsB-618 [46]) and the GlcA transferase I mutant (pgsG-224 [47]). The EXT1 mutant (pgsD-677 [48]), is deficient in the co-polymerase responsible for HS chain polymerization and is thus HS deficient. Two mutants that produce under sulfated HS are available, i.e. the NDST-1 mutant (pgsE-606 [49, 50]) and the 2-O-sulfotransferase mutant (pgsF-17 [51]). The pgsC-mutant is deficient in sulfate transport but produces normal GAG-chains due to endogenous cellular production of sulfate from cystein residues [52]. Monosaccharide symbols in accordance with Nomenclature Committee, Consortium for Functional Glycomics. T, transferase; sulfoT, sulfo-transferase; NS, N-sulfate; (2, 3 or 6)S, (2, 3 or 6)-O-sulfates; PAPS, 3'-phosphoadenosine 5'-phosphosulfate (sulfate donor)

Core proteins and functions of cell surface proteoglycans

As mentioned above, PGs are important extracellular matrix (ECM) components. Aggrecan, versican, decorin and biglycan are all secreted CS/DS PGs present in connective tissue like cartilage and fibrous membranes, where they participate in various processes such as ECM remodeling; growth factor sequestering; structure maintenance; and shock absorption [53]. Neurocan and brevican are secreted CD/DS PGs present in the brain. Aggrecan can, in addition to CS/DS, also carry keratan sulfate (KS, a GAG with repeating Gal-GlcNAc units). Other KS substituted PGs are lumican, keratocan and fibromodulin, all of which have wide tissue distributions. Perlecan and agrin are HS carrying PGs present in basement membranes. Serglycin, present in myeloid cells, can carry both CS/DS and HS/Hep, where the GAG functions as a reservoir for granule-associated cationic proteins [54]. Serglycin is the only known heparin substituted PG [2].

The two major families of cell surface associated HSPGs are the syndecans and the glypicans (Figure 2). The syndecan family comprises four members (1-4) in mammals with varying tissue distribution. Syndecan-1 is expressed predominantly in epithelial and plasma cells; syndecan-2 in epithelial, fibroblast and neuronal cells; syndecan-3 almost exclusively in neuronal and musculoskeletal tissue; and syndecan-4 in virtually every cell type [55]. Syndecans are structurally similar with a cytoplasmic, an extracellular and a transmembrane domain (depicted in Figure 2). The transmembrane region is highly conserved, whereas the extracellular and the cytoplasmic domains are varied, yielding core proteins with molecular weights ranging from 22-43 kDa [56]. Syndecans can be substituted with both CS and HS chains. The core proteins all have N-terminal GAG attachment domains that are generally substituted with HS. Other GAG attachment sites, generally substituted with CS, can be located near the transmembrane region [55].

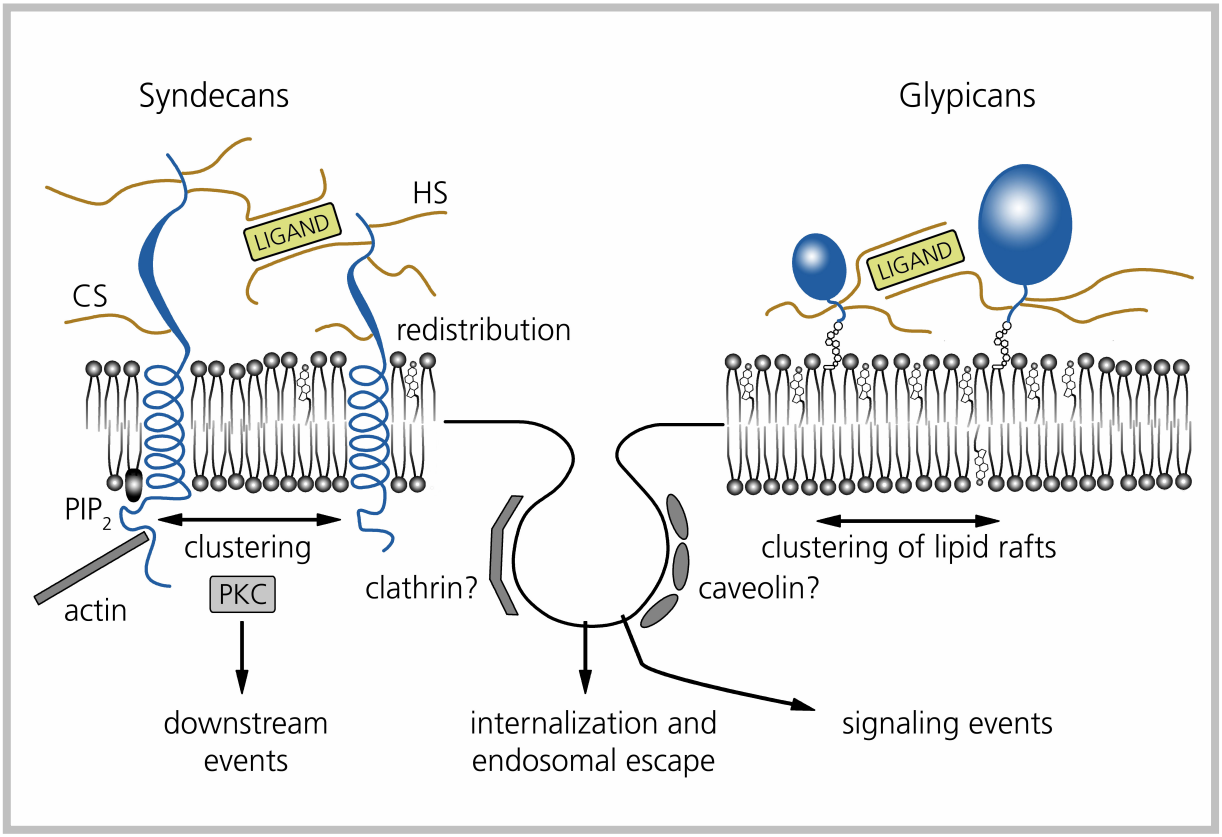


Figure 2. General characteristics of syndecan and glypican proteoglycans. General characteristics using syndecan-4 and glypican-1 as models. Clustering of syndecan molecules induces intracellular signaling events; redistribution into the lipid raft partition of the plasma membrane; and internalization via endocytosis. Ligand binding to HS on glypican molecules induces clustering of lipid rafts, which may trigger endocytosis. Once internalized, further destiny may include signaling events or endosomal escape.
PIP_2, phophoinositol (4,5) bisphosphate; PKC, protein kinase C

The short cytoplasmic domain contains three important regions; conserved regions 1 and 2 (C1 and C2) and a variable region (V). These regions all interact with specific intracellular proteins which e.g. anchor syndecan-4 to the actin cytoskeleton [57]. The V domain displays a high degree of heterogeneity and has been studied particularly well in syndecan-4. In syndecan-4 the V domain contains a phosphatidylinositol-4,5-bisphosphate (PIP_2) binding site. PIP_2 binding to the V domain allows syndecan-4 to interact with, and activate, protein kinase Cα, initiating intracellular signaling events of yet unknown importance [56] that are possibly involved downstream of basic fibroblast growth factor (bFGF) binding [58]. Due to V domain homologies and functional similarities it has been suggested that syndecan-1 and 3 should be regarded as one group and syndecan-2 and 4 as another. Syndecan-1 and syndecan-3 expression is often associated with inhibition of cell growth, whereas syndecan-2 and syndecan-4 expression leads to cell proliferation [56]. It is still unknown how syndecans engage in signal transduction and affect cellular

proliferation. An often used model suggests that cell surface HSPG in general serve as co-receptors for Hep binding growth factors, e.g. bFGF, epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF). Cell surface HSPG sequesters Hep binding growth factors and presents them to their respective high affinity tyrosine kinase receptor. It is clear that GAG interactions are important for syndecan-associated signal transduction. However, protein-protein interactions are also of vital importance, as exemplified by TGFβ's interaction with syndecan-2 [59]. Furthermore, presentation to high affinity receptors is not the only means by which syndecans mediate signaling. As mentioned above, the syndecan cytoplasmic tail interacts with a large number of proteins and is itself capable to initiate intracellular signaling. In addition to interacting with soluble factors, syndecans are important regulators of cell-matrix interactions and cell-cell interactions, especially so during development [60]. Although integrins are essential components of focal adhesions, assembly of focal adhesions seems to be regulated by syndecan-4 [61, 62]. Further expanding the role of syndecans in signaling and cell-cell interaction, it has been demonstrated that protein kinase Cγ mediates phosphorylation of the cytoplasmic domain of syndecan-2 in right side, but not left side, ectodermal cells, as an instructive function in *Xenopus* left-right development [63]. The same report demonstrates that syndecans can also be involved in inside-out signaling to adjacent cells.

The glypican family has six members (1-6) in mammals. Mature glypican core proteins have a molecular weight of 60–70 kDa. The bulk of the protein is thought to form a tight globular domain due to the presence of 14 conserved cystein residues, presumably engaged in disulfide bond formation [44].

The function of the globular domain is a matter of speculation. It has been suggested to regulate HS assembly onto glypican core proteins, as mentioned above. Glypicans have three GAG attachment sites in direct succession located between the globular domain and the C-terminal glycosylphosphatidylinositol (GPI) anchor attachment sequence. The GPI anchor attaches glypicans to membranes and directs glypicans to regions of the plasma membrane with specific lipid characteristics, i.e. lipid rafts and caveolae (see further below), which is thought to be a general behavior of GPI-anchored proteins [64]. Interestingly, the tissue distribution of glypicans varies during the life cycle of an organism. The expression pattern is dramatically different in adult mammals as compared to embryos. In adults, only glypican-1 is ubiquitously distributed. Several glypican members are primarily found in the CNS and neurons. The species represented are however different in embryos than in adults [43]. Glypican-1 has been ascribed a role in FGF-2 signaling; glypican-2 is believed to be important for axonal guidance; and glypican-3 is suggested to be important for insulin growth factor signaling. The functions of glypicans 4-6 are

not known [44]. Glypican homologues in *Drosophila*, Division abnormally delayed (*Dally*) and *Dally-like*, are involved in morphogenesis and are associated with signaling of *wingless*, *hedgehog* and *decapentaplegic* [44, 65, 66]. *Dally* and *Dally-like* are thought to exert their effects by regulating the distribution of *wingless*, *hedgehog* and *decapentaplegic*, establishing morphogen gradients [66, 67]. Glypicans and syndecans are both involved in the formation of morphogen gradients and developmental patterning, having partly overlapping and partly distinct effects on developmental processes [68].

A growing number of proteins and peptides are reportedly dependent on interaction with HS for proper function (see Table 2). Due to their polyanionic nature, natural binding ligands for HS-chains are positively charged molecules. In the case of proteins, consensus sequences such as xBBBxxBx, xBBxBx, BBxBB or TxxBxxTBxxxTBB (where B is a basic amino acid, x is a hydropathic amino acid and T is a turn) have been proposed [69, 70]. Non-peptide ligands such as the polyamine spermine (with positive charges derived from arginine), have also been reported to bind avidly to highly sulfated HS, as well as DS [71, 72] and will be discussed further below.

Table 2. Examples of proteins/peptides whose function depend on interaction with HS

Protein/peptide	Function in:
VEGFs, angiostatin, endostatin	Angiogenesis
laminin, fibronectin, thrombospondin collagen types I, II, and V, fibrillin, tenascin, vitronectin	Cell-matrix interactions
antithrombin III, heparin cofactor II, tissue factor pathway inhibitor, thrombin, protein C inhibitor, tissue plasminogen activator, plasminogen activator inhibitor-1	Coagulation/fibrinolysis
FGFs and FGF receptors; Wingless factors; Hepatocyte growth factor (HGF, scatter factor); Transforming growth factors (TGFs)β 1 and 2; Bone morphogenic proteins (BMPs) 2, 3, 4, and 7; Hedgehog factors; Decapentaplegic factor;	Cell proliferation/ differentiation
Chemokines (e.g., MIP-1b); cytokines (e.g., IL-2, -3, -4, -5, -7, -8, and -12); L- and P-Selectins; Extracellular superoxide dismutase; Antimicrobial peptides	Inflammation
Lipoprotein lipase, hepatic lipase, apoliprotein E	Lipid metabolism

Adapted from [4]

POLYAMINES

Definition and physiological function of polyamines

The polyamines (putrescine, spermidine and spermine) are low molecular weight amines that are protonated at physiological conditions. Putrescine is in fact a diamine, but is generally referred to as a polyamine [73, 74].

Due to their positive charge, polyamines interact avidly with nucleic acids. They are ubiquitous in all mammalian cells, fungi, protozoa, and bacteria [75]. Although hard to define, their role in cellular processes such as replication, transcription, and translation is essential [76]. High polyamine levels are associated with rapid proliferation, whereas low polyamine levels are associated with quiescent cells. A simplistic description of polyamines may be that they serve as the grease in the cellular machinery. A very specific role of spermidine has however been identified, i.e. the posttranslational formation of the amino acid hypusine, unique to the eukaryotic translation initiation factor 5A (eIF-5A) [77]. The first enzymatic step in hypusine formation is the transfer of the 4-aminobutyl moiety of the polyamine spermidine to the epsilon-amino group of a single specific lysine residue in the eIF-5A precursor protein to form deoxyhypusine which is hydroxylated to form hypusine [78]. Although the actual function of eIF-5A is only partially known, it promotes the formation of the first peptide bond during the initial stage of protein synthesis. This effect is dependent on the hypusine residue [79] which highlights the importance of spermidine in basic cellular mechanisms like protein synthesis. Modulation of protein, receptor and ion-channel activity has been discussed as a role for polyamines [73, 80]. Direct interaction with lipid bilayers causing destabilizing or stabilizing effects depending on the length of the polyamine has been observed [81]. Recent data obtained from *E. Coli* suggest that polyamines may function to protect cells from oxidative stress [82]. However, polyamine catabolism produces H_2O_2, a mediator of oxidative stress and DNA damage [83], when the polyamine oxidase degrades acetylated species of spermine and spermidine [84]. Contradictory results have been reported regarding the role of polyamines in apoptosis. An appealing explanation is that the effect on apoptosis is dependent on polyamine concentration combined with the presence of other pro- or anti-apoptotic signals [85].

Polyamine metabolism

Intracellular levels of polyamines are tightly regulated via anabolic and catabolic pathways (see Figure 3) as well as transport into and out of cells.

The biosynthesis of polyamines is intricately associated with basic metabolic pathways, as the precursors are amino acids (primarily arginine and methionine but also lysine and glutamate) and products of amino acid degradation. Degradation of arginine to ornithine via the action of arginase is a key event in the urea cycle, and provides ornithine for polyamine biosynthesis [86]. Ornithine can however be formed from alternative pathways, e.g. from conversion of glutamate [86].

The rate limiting step in polyamine biosynthesis in mammals is the decarboxylation of ornithine to putrescine, performed by the enzyme ornithine decarboxylase (ODC) [86]. Another enzyme with decarboxylation activity, S-adenosylmethionine decarboxylase (SAMDC), catalyses the formation of decarboxylated S-adenosylmethionine (dcSAM) from S-adenosylmethionine (SAM). dcSAM is the aminopropyl donor in the synthesis of spermidine and spermine. Both ODC and SAMDC activity display unusually rapid changes in response to different stimuli. Especially ODC has been extensively studied regarding its regulation in physiological and pathological situations, and a strong correlation between ODC induction and events of cellular proliferation has been observed. ODC activity is modulated by polyamine mediated feedback inhibition and regulation at transcriptional as well as translational and other posttranscriptional levels [86]. ODC regulation includes a specific inhibitor, the protein antizyme [87]. Interestingly, expression of antizyme involves a specific +1 translational frameshift, induced by high polyamine levels [88]. Antizyme not only binds ODC to inhibit its activity, but also mediates its degradation by the 26S proteasome in a ubiquitin independent manner [89]. Antizyme is itself degraded by the proteasome after ubiquitination, a process inhibited by spermine as shown in yeast [90]. In addition, antizyme has been found to downregulate the polyamine transport system [91, 92]. Antizyme in turn has its own protein inhibitor, namely antizyme inhibitor, mediating the release of ODC from ODC-antizyme complexes, resulting in increased ODC activity and higher polyamine levels. A recent investigation suggests that increased ODC activity in cancer cells partly results from the sequestration of antizyme and subsequent rescue of ODC from degradation, via enhanced antizyme inhibitor expression [93]. Altogether, having one of the shortest half lives described for mammalian proteins [94], ODC activity is regulated in a very strict manner, and can be swiftly increased in times of need as well as reduced when cell proliferation and growth is no longer needed.

After putrescine has been formed by the action of ODC, spermidine synthase transfers the aminopropyl group from dcSAM to putrescine creating spermidine. Spermine is in turn formed by a similar reaction catalyzed by spermine synthase [83].

Spermine and spermidine can be recycled back to spermidine and putrescine by the action of spermidine/spermine acetyltransferase and polyamine oxidase via an acetylated intermediate [83]. Recently, an enzyme was cloned that has the ability to oxidase spermine directly into spermidine without the necessity of prior acetylation. It has been named spermine oxidase [95].

Abnormal polyamine levels have been observed in a number of pathological conditions [96]. The elevated polyamine levels associated with cancer have received particular interest. Several inhibitors of polyamine biosynthesis have been synthesized, primarily to be used as anti-cancer drugs. Although *in vitro* experiments showed promising results, early compounds displayed much less promising data *in vivo* [96]. All the components of the polyamine biosynthetic machinery have been targeted for inhibitor development. Due to their key roles in polyamine biosynthesis, SAMDC and ODC have been of particular interest. One of the best known inhibitors of ODC is the suicide inhibitor α-difluoromethylornithine (DFMO). In theory, inhibition of ODC would deplete the entire polyamine pool resulting in abolished cell proliferation. Early *in vitro* studies using rat hepatoma cells showed that the levels of putrescine and spermidine are depleted within one day of DFMO treatment. However, this initial decrease does not affect cell number. The spermine concentration takes somewhat longer to reduce but its decline is paralleled by a reduced proliferation rate [97]. Cell cycle arrest induced by DFMO *in vitro* has been shown to take place in different phases of the cell cycle depending on cell line [98]. In analogy with other compounds, DFMO has less dramatic effects *in vivo*. The reason has been attributed to increased polyamine uptake [99, 100] as well as homeostatic changes in metabolism such as upregulation of enzymes and increased interconversion of polyamines keeping intracellular levels within the normal range [74]. Amplification of the ODC gene in the presence of DFMO has also been reported [101]. Although DFMO as the sole treatment in cancer is generally going to be of little value, it has been proven beneficial in some niches, e.g. has DFMO alone been suggested as an effective palliative therapy for recurrent anaplastic gliomas [102]. DFMO has few side effects, with occasional reversible hearing loss reported in a study on oral DFMO treatment over 3-12 months [103]. This makes it a potentially valuable component in cancer combination therapy, an approach that is currently under investigation. Aside from its use as an anti-cancer agent, DFMO is used in the treatment of human African trypanosomiasis, or sleeping sickness. In an WHO coordinated 5-year effort, the Aventis and Bayer companies assure free production of five essential antitrypanosomal drugs, amongst them DFMO, for distribution in Africa [104].

Methylglyoxal bis-guanylhydrazone (MGBG) is a potent competitive inhibitor of SAMDC. It depletes spermidine and spermine content but causes

significant accumulation of putrescine [105]. The compound bears structural resemblance to spermidine and uses the polyamine transport system to enter cells. In accordance, DFMO treatment will result in increased internalization of MGBG [99]. MGBG has little use in the treatment of cancer due to severe toxicity, but it has been a convenient tool in the isolation of mutant cell lines deficient in polyamine uptake.

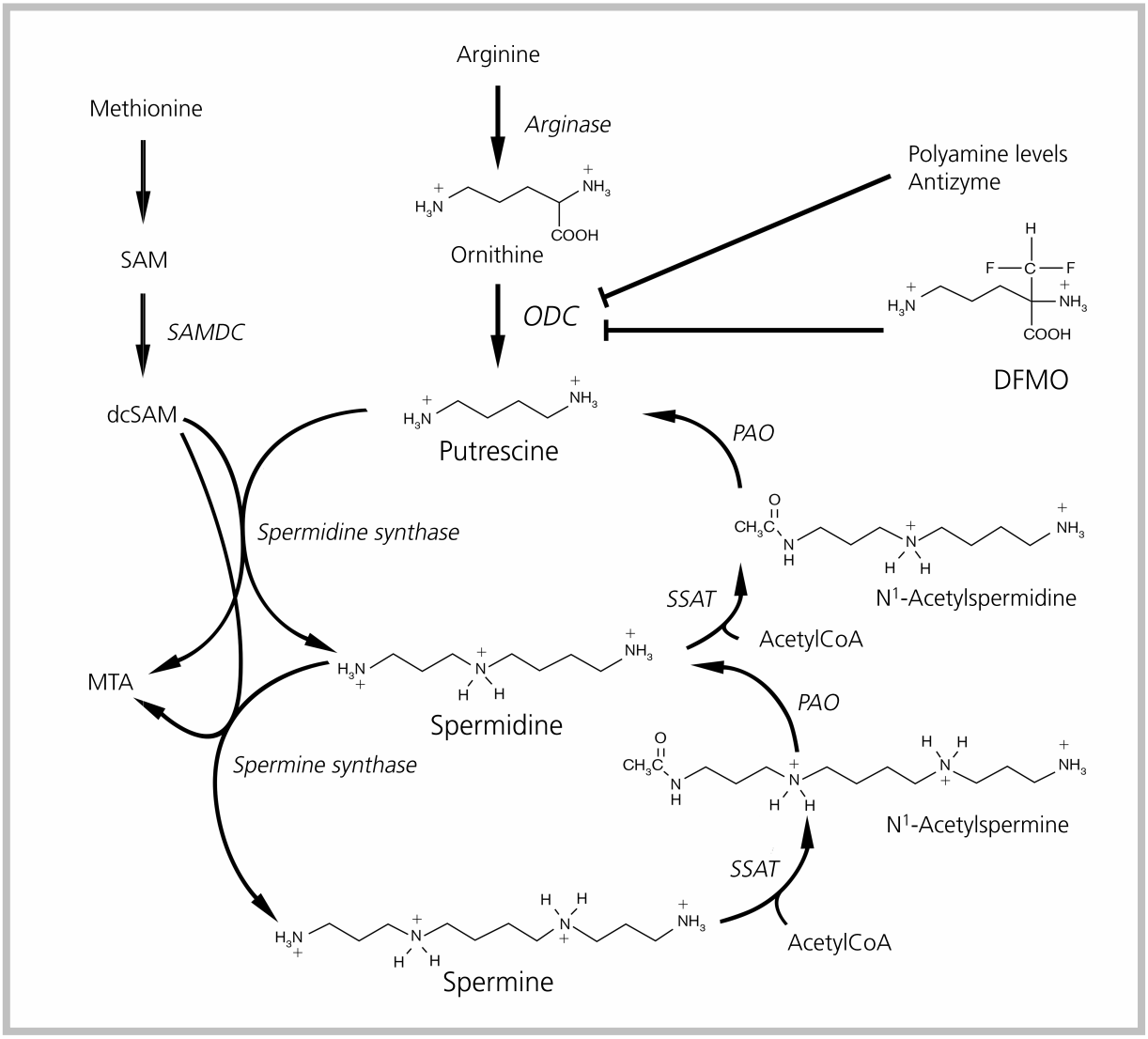


Figure 3. Key features in polyamine metabolism. Adapted from [73, 74, 86]. ODC, ornithine decarboxylase; PAO, polyamine oxidase; SAMDC, S-adenosylmethionine decarboxylase; SAM, S-adenosylmethionine; dcSAM, decarboxylated S-adenosylmethionine; SSAT, spermidine/spermine acetyltransferase; MTA, methylthioadenosine; DFMO, α-difluoromethylornithine

Polyamine transport

Proper polyamine levels are ensured not only by biosynthesis and degradation, but also by transport into and out of the cell. As discussed above, inhibition of endogenous biosynthesis results in upregulated uptake maintaining intracellular polyamine levels in the presence of extracellular polyamines [99]. Exogenous polyamines are derived both from dietary intake [106] and from the intestinal flora [100]. Transport systems for polyamines have been cloned in *E. Coli* [107]. Uptake in *E. Coli* is mainly catalyzed by two energy dependent systems. They are both ABC (ATP binding cassette) transporters [108] consisting of a substrate-binding protein in the periplasm, two channel-forming proteins and a membrane-associated ATPase. One of the systems is specific for putrescine and the other is a spermidine-preferential system. *E. Coli* has a third transporter (PotE), specific for putrescine, that is capable of both uptake and excretion. PotE is a 46 kDa protein containing 12 transmembrane segments. Uptake by this system is dependent on membrane potential, whereas excretion involves a putrescine/ornithine antiport mechanism [107].

Recently the first polyamine transporters in eukaryotes were identified. Four genes encoding polyamine transport proteins (TPO 1-4) on the vacuolar membrane in yeast have been identified [109]. These proteins all contain 12 putative transmembrane segments, and three glutamate or aspartate residues suitable for polyamine interactions in positions similar to those of the critical acidic amino acid residues in the bacterial PotE protein. Interestingly, two transporters located to the plasma membrane in yeast have also been recently identified, namely Gap1p [110] and Agp2p [111]. Gap1p accepts putrescine and to a lesser degree spermidine, whereas Agp2p preferentially recognizes spermidine. Agp2 is more potent, and seems to have a central role in polyamine uptake in yeast. Neither Gap1p nor Agp2p accepts spermine as a substrate.

Intriguingly, no gene encoding a plasma membrane polyamine transport protein has of yet been identified in mammalian cells. The presence of an elaborate transport system is however obvious, and a large number of reports have dealt with defining the properties of this system [80]. Mammalian cells take up polyamines in an energy dependent and carrier-mediated manner and in many cell lines it seems as if the polyamines share a single carrier system. This is exemplified by the CHO-MGBG cell line. The CHO-MGBG is a Chinese hamster ovary (CHO) cell mutant isolated after chemical mutagenesis in the presence of the SAMDC inhibitor MGBG, a regime producing mutants that do not internalize MGBG. The CHO-MGBG cell line is not only deficient in spermidine uptake. It is completely incapable of internalizing polyamines [112], suggesting a non-selective transport system. This approach (chemical mutagenesis

in the presence of MGBG) has been used on several cell types rendering similar results [113-115].

Transport deficient mutants have been used for rescue approaches trying to identify polyamine transporter genes. After transfection with total cellular DNA, cells that were able to grow in the presence of DFMO and exogenously supplied polyamines were selected. A number of clones with polyamine uptake capacity and sensitivity for MGBG were identified. No distinct gene could however be identified and the authors suggested that the results imply a multiplicity of polyamine-transport systems [116].

Recent data from our group indicate an important role for HSPG in polyamine uptake [117]. Interaction between polyamine and HS is particularly strong in the case of spermine (which also binds DS [71]), but binding to HS is evident also for spermidine [72]. Spermine uptake is competitively inhibited by exogenous GAGs. Various approaches that deprive cells of functional PGs all result in reduced spermine uptake, i.e. enzymatic cleavage of GAG chains; use of competitive inhibitors of GAG chain synthesis or use of sulfation inhibitors. Mutant CHO cells deficient in different aspects of PG synthesis (pgsA, pgsB, pgsD and pgsG; see Figure 1) established by Esko and co-workers also display decreased polyamine uptake. These mutants exhibit inability to thrive in polyamine substituted medium in the presence of DFMO, indicating dependence on endogenous polyamine biosynthesis. The mutant with the most severe phenotype, pgsA, virtually forms no tumor foci *in vivo* in the presence of DFMO, as shown in a mouse model of haematogenous lung metastasis [118]. Focusing on glypican-1, our data suggest that this species in particular is important for polyamine uptake [119].

An obstacle when studying polyamine transport is the fact that the polyamines are small molecules and that the covalent attachment of a reporter such as a fluorophor severely affects the overall appearance of the compound and hence possibly its interactions with a putative transport system. A recent report on polyamine uptake uses spermidine-C2-BODIPY, a compound that according to the authors faithfully mimics polyamine transport in CHO cells, although the behavior of the compound in yeast cells bears no resemblance to that of natural polyamines [120]. An objection to this compound is that it is functionally a diamine, like putrescine, since the BOPIDY reporter occupies the second amino group. The study reveals vesicular localization of internalized spermidine-C2-BODIPY [121]. Vesicles are acidic and partly co-localize with the Golgi compartment. Using the END1 CHO mutant (displaying slowed internalization of transferrin [122]) the authors argue that uptake is independent of receptor mediated endocytosis. Using bafilomycin A_1 and monensin, with the intent to disrupt vesicular membrane proton gradient, the authors show that overall

cellular accumulation and vesicular retention of spermidine-C2-BODIPY is dependent on a proton gradient. Two possible models for internalization are discussed: A) an internalization pathway involving a plasma membrane transporter that delivers polyamines into the cytosol followed by sequestration into pre-existing vesicles via a vesicular membrane transporter, or B) a receptor dependent endocytotic pathway where the polyamine-receptor complex is internalized to form vesicles. The authors favor model A, based primarily on the END1 mutant results and the effects of disruption of vesicular proton gradient. Since the END1 mutant hardly can be considered deficient in endocytosis (70% decrease in transferrin uptake [123]; other endocytic pathways than clathrin dependent endocytosis less well studied) and indeed displays defects in endosomal and lysosomal acidification, the data presented do not convincingly exclude uptake according to model B. The fact that considerable efforts to identify a plasma membrane carrier for polyamines in mammalian cells have been unsuccessful [109] also disfavors model A. The report however presents an attractive model for visualization of putrescine/diamine uptake and clearly reveals that internalization involves a vesicular compartment. The importance of proper pH conditions is also evident.

Accumulated data thus indicate that polyamine internalization is a highly regulated process. Transport in and out of cells is an integral part of polyamine homeostasis. Although several genes encoding polyamine transporters have been identified in bacteria and in yeast, no plasma membrane transporter has been identified in mammalian cells. Uptake of polyamines is dependent on proper pH configuration of cellular compartments and seems to involve a vesicular compartment [121]. The tetra amine spermine is internalized via a mechanism dependent on cell surface HSPG [117].

CELLULAR MECHANISMS OF INTERNALIZATION

Evolutionary biology describes the universal cell ancestor as a conglomeration of non-individual cells, i.e. progenotes, which lacked the organization and structural integrity of present time cells. A prerequisite for rapid development at this stage in evolution was the absence of efficient barriers defining different cells, or even distinguish extracellular from intracellular. However, as ever more advanced cellular processes evolved, the need for compartmentalization and protection from nonself genetic material arose [124, 125]. Modern cells rely heavily on their capacity to compartmentalize various processes such as energy conversion, handling of genetic material and protein production. This is done using a system of barriers and carriers that selectively allow passage of molecules to satisfy various cellular needs, e.g. nutrition, waste disposal and signal transduction.

The most apparent barrier to cellular internalization is the phospholipid bilayer of the plasma membrane. With time, molecules diffuse down their concentration gradient through a lipid bilayer. The addition of size, and even more so charge, rapidly decreases the permeability coefficient. Thus, membrane passage of small charged molecules is generally controlled by the use of specific channels and pumps. Uptake of larger molecules, such as the charged biopolymers of this treatise, is thought to be achieved by endocytosis, or according to some reports direct interaction with and penetration of the plasma membrane. Traditionally, endocytosis is divided into phagocytosis (cell eating) and pinocytosis (cell drinking). Phagocytosis is typically performed by phagocytes, i.e. macrophages, neutrophils and dendritic cells. Pinocytosis is a more widespread phenomena utilized by essentially all mammalian cell types. It occurs by a number of different more or less well defined pathways, including clathrin-mediated (classical) endocytosis; caveolin-mediated endocytosis; clathrin and caveolin independent endocytosis; and macropinocytosis [126].

Clathrin-mediated endocytosis

Endocytosis dependent on clathrin is thought to involve a number of distinct steps: 1) formation of nucleation site for assembly; 2) invagination and maturation of a clathrin coated pit including recruitment of transmembrane proteins; 3) membrane fission and formation of a clathrin coated vesicle; 4) translocation of the vesicle into the cytosol and 5) uncoating of the vesicle and redistribution of coat components to the plasma membrane [127-130]. The detailed succession of the process is still under debate [128].

Clathrin mediated endocytosis was first discovered due to the distinct appearance of the clathrin coat in electron micrographs. The bulk constituents of

the polygonal coat are two oligomeric proteins: the clathrin triskelion and the adaptor protein complexes.

Clathrin triskelions consist of three identical heavy chains and three regulatory light chains of two different types (a and b) that are distributed randomly giving rise to a heterogeneous triskelion pool. The three-legged shape of the triskelion oligomer is the structural basis of the polyhedral appearance of clathrin coats. Under the right conditions triskelions can be made to self-assemble into cages *in vitro* [127].

The adaptor protein complexes have multiple roles and come in four heterotetrameric forms, AP1-4 [131]. APs trigger clathrin assembly and link the clathrin coat to membranes by interacting with the cytoplasmic portion of transmembrane proteins and with PIP_2 [130]. AP1 and AP2 localize clathrin coat formation to the trans golgi network (TGN) and the plasma membrane respectively, making AP2 responsible for clathrin dependent endocytosis. APs are also thought to concentrate cargo into forming clathrin coated pits by interacting with and recruiting transmembrane receptors. Finally, APs are supposed to have regulatory functions and interact with a large population of proteins [127, 129, 132].

Another key player in clathrin mediated endocytosis, as well as in other types of vesicle formation, is the guanosine triphosphatase dynamin. Its role in endocytosis was identified in the *Drosophila* mutant *shibire,* expressing a temperature sensitive dynamin homologue. The fly exhibited a rapid and reversible paralysis upon exposure to non-permissive temperature due to blocked endocytosis affecting synaptic signaling [132, 133]. Dynamin is capable of generating force and polymerizes into rings when purified [134]. It is thought to polymerize around the neck of invaginating buds and to pinch off coated vesicles, with the exact mechanism still being inexplicable [133]. There are three isoforms of dynamin in mammals (1-3), with several splice variants supposedly involved in membrane fission at distinct cellular locations. Dynamin-1 is expressed exclusively in neurons; dynamin-2 is expressed in all tissues; and dynamin-3 is restricted to the testis, the brain, the lung, and the heart (for refs see [135]). Several mutant dominant negative dynamin constructs have been used by researchers to investigate the role of dynamin in different aspects of endocytosis. The action of dynamin is regulated by protein-protein interactions, primarily via its proline rich regions and SH3 binding sites [133].

A role for actin filament rearrangement in clathrin mediated endocytosis has been implied by several findings. A number of proteins involved in the endocytotic machinery regulate actin turnover, e.g. dynamin. Microscopy techniques have revealed a spatial and temporal co-incidence of actin dynamics and clathrin mediated endocytosis [136, 137]. Actin has accordingly been

suggested to play a role in clathrin nucleation, vesicle budding and transport of newly formed vesicles [127, 137]. However, studies on the action of actin inhibitors in clathrin mediated endocytosis in mammalian cells show variable and sometimes contradictory results [127]. Two recent studies indicate an essential role for actin dynamics in clathrin mediated endocytosis. Zhu *et al.* report that cortactin (an F-actin binding protein) and dynamin drive the fission of clathrin-coated pits in an actin polymerization dependent manner [138], whereas Yarar *et al.* state that actin dynamics is essential for the processes of coated pit formation as well as maturation into coated vesicles and translocation into the cytosol [137].

A plethora of accessory proteins are involved in clathrin mediated endocytosis and their respective roles remain to be fully elucidated. Eps15, epsin and CALM are thought to be involved in AP2 recruitment to the plasma membrane. Amphiphysin and endophilin are believed to be involved in the invagination process whereas intersectin, Hip1 and Hip1R appear to play a role in regulating actin polymerization [128]. Many components of the endocytic machinery, including AP2, AP180, amphiphysin, epsin, and endophilin have domains that interact selectively with inositol phospholipids, most importantly PIP_2 [126, 139]. Turnover of inositol phospholipids seems important for the un-coating process, since knockout of synaptojanin-1, a polyphosphoinositide phosphatase, results in accumulation of clathrin-coated vesicles and pits paralleled by an accumulation of PIP_2 [139].

The ability to accumulate transmembrane proteins in clathrin coated pits is attributed to the presence of a number of alternative mechanism involving adaptor proteins. Transmembrane protein receptors, channels, transporters and other integral membrane proteins can all be internalized via clathrin-mediated endocytosis, and are thus regulated in their cell-surface expression and function [129].

The contribution and importance of clathrin mediated endocytosis have been investigated in a multitude of contexts. Long used methods to inhibit endocytosis included low pH shock treatment, potassium depletion and brefeldin A treatment [127]. Since these methods all lack in specificity, alternative methods have been developed. The drug chlorpromazine has been used as a tool to inhibit clathrin mediated endocytosis. It causes loss of coated pits from the cell surface and appearance of clathrin coated pits on endosomes, possibly by affecting AP2 affinity for different binding sites [140]. Acute cholesterol depletion seems to affect clathrin mediated endocytosis by limiting membrane curvature capacity [141]. However, cholesterol depletion more readily affects other modes of endocytosis.

Overexpression of fragments or mutants of proteins associated with the formation of clathrin coated vesicles, e.g. mutants of Eps15 [142], the μ2 subunit

of AP2 [143], and dynamin [144], inhibit endocytosis dependent on clathrin. A recent approach is RNA interference directed against the same subset of proteins [129], e.g. AP2 [145] or the clathrin heavy chain [146]. These methods also suffer from drawbacks, primarily the probable existence of a residual pool of unaffected and functional proteins that keep clathrin coated vesicle formation from reaching zero. In the case of RNA interference, effects have been very modest. This has been explained by the long half life of most proteins involved in clathrin mediated endocytosis and by functional redundancy [129].

A recent study using a range of fluorescent latex beads of defined sizes (50–1000 nm) on non-phagocytic B16 melanoma cells defines the upper size limit for clathrin mediated endocytosis to approx. 200 nm [147]. In the same study, increasing bead size over 200 nm redirected internalization to other, non-clathrin dependent pathways such as caveolae mediated endocytosis.

Caveolae/raft-mediated endocytosis

The term caveola intracellularis, intracellular cave, was coined by Yamada in 1955 after studying mouse gall bladder epithelium by electron microscopy [148]. Caveolae appear as 50-100 nm bulb-like invaginations of the plasma membrane. Associated with caveolae is a striated protein coat that decorates the cytosolic side. The protein coat consists of a 22 kDa protein, named caveolin [149]. The caveolin family comprises three members in mammals (1-3) that differ in their tissue distribution. Caveolin-1 and -2, having two and three isoforms respectively, are co-expressed in most cell types, and are very abundant in endothelial cells and fibroblasts. The expression of caveolin-3 is restricted to muscle cells, astrocytes and chondrocytes [150, 151]. Caveolin-1 is known to homo-oligomerize or hetero-oligomerize with caveolin-2 to form complexes of 14-16 units [152, 153]. These complexes are thought to be the assembly units for caveolar coat formation. Introduction of the caveolin-1 gene in lymphocytes devoid of caveolin induced formation of caveolae [154]. In the absence of caveolin-1, caveolae formation is abolished and caveolin-2 seems to be completely retained at the level of the Golgi complex. Upon reintroduction of caveolin-1, caveolin-2 transport is restored resulting in the formation of hetero-oligomers and caveolae [155], indicating that caveolin-2 is functionally dependent on caveolin-1.

Studies on caveolin knockout mice have gained many insights into the biology of caveolins. Surprisingly, caveolin knockout mice are viable and show only mild phenotypes [156]. Aberrations in caveolin-1 null mice are related to lung function and vasculature while caveolin-3 null mice display mild-to-moderately dystrophic muscle tissue [152].

Investigations on the endocytosis and transcytosis of albumin, which are thought to be caveolae-dependent events, have produced contradictory results. Embryonic fibroblasts from caveolin-1-null mice failed to show surface labeling or internalization of fluorophor-labeled albumin, while transfection of caveolin-1 into the null cells restored uptake [156]. Likewise, injected albumin conjugated with 5 nm gold is readily internalized by pulmonary endothelium in wild type mice, whereas no uptake can be seen in caveolin-1 null mice as shown by electron microscopy [157]. However, both albumin concentration in cerebrospinal fluid, which is presumed to depend on caveolar transcytosis, and albumin dependent extravascular osmotic pressure are normal in caveolin-1 null mice [158].

The original view of caveolae as simply an alternative pathway for endocytosis has been questioned lately [159, 160]. Endocytic pathways in general have been shown to be highly involved in signaling functions. Over 200 kinases are associated with internalisation routes like caveolae/raft mediated endocytosis and clathrin mediated endocytosis [161]. Not only do signalling pathways govern these routes of entry. Lipid rafts, caveolae and endosomes, clathrin associated or not, also seem to function as hubs for a large number of signalling pathways controlling cell adhesion, growth and proliferation [159, 161-164].

It also appears that the role of the caveolin coat in caveolar endocytosis may not be in analogy to that of the clathrin coat in clathrin mediated endocytosis. Rather it seems that the role of caveolin is to stabilize the caveolae onto the plasma membrane [160, 165]. Other components of caveolae and the closely related membrane structures called lipid rafts have thus come into focus. It has recently been suggested that caveolae are a subpopulation of lipid rafts. Accordingly, rafts are detergent-insoluble, low-density membrane fractions rich in cholesterol and sphingolipids, whereas caveolae are cholesterol- and sphingolipid-rich invaginations of the plasma membrane that partition into raft fractions and whose expression is associated with caveolin-1 [166]. The fact that caveolae share many characteristics with membrane lipid rafts suggests that caveolae and rafts share a common endocytic pathway, which is defined by clathrin independence, dynamin dependence, sensitivity to cholesterol depletion, and similar membrane lipid composition [166]. This definition, although debated, unites caveolar endocytosis with non-caveolar raft endocytosis and is referred to as caveolae/raft dependent endocytosis. Still, the exact roles of caveolin in caveolar/raft formation and transport remain unknown. Data have been presented to indicate that plasma membrane caveolae are rather immobile structures [160], and that the presence of caveolin stabilizes the caveolae structures at the cell surface thus inhibiting internalization [167]. Caveolin is, however, also associated with internalized vesicles, called caveosomes [168]. Nabi and Le suggested a model [166], where caveolin-association with lipid raft membrane domains may be one way to

regulate the dynamics of a general clathrin-independent endocytosis pathway, and that this regulation can be influenced by a number of different signaling events.

As in clathrin-mediated endocytosis, dynamin is essential for the scission of the forming vesicle from the plasma membrane [135]. In the case of caveolae mediated endocytosis it has recently been shown that dynamin-2 interacts directly with caveolin-1 via the dynamin proline rich domain [169]. Expression of a dominant negative dynamin-2 construct drastically reduced caveolae dependent SV40 internalization and infectivity [168].

Actin filaments are important for caveolae function. Caveolae have been shown to anchor to the actin cytoskeleton. The interaction is mediated by the actin binding protein filamin, which associates with the N-terminus of caveolin-1 [170]. As a consequence, disruption of actin filaments results in increased lateral movement of caveolae [160]. Studying the SV40 virus, that induces its own internalization via caveolae, it has also been shown that actin disruption gives rise to a highly laterally mobile caveolae pool [168]. The same study indicates a role for actin dynamics in the internalization of SV40 containing caveolae. After viral entry into caveolae, filamentous actin was reduced and actin redistributed to patches and tails associated with caveolae, mimicking the actin tails that propel pathogens such as *Listeria* through the cytosol [171]. SV40 internalization and transport were inhibited by actin disruption. However, a recent study indicates that actin depolymerisation can induce caveolae mediated endocytosis, making the story more complex [172]. Thus, the exact role of actin in caveolae mediated endocytosis remains to be elucidated. For features of caveolae/lipid raft-mediated endocytosis as well as other endocytic pathways, see Table 3.

Table 3. Features of endocytic pathways

	Clathrin mediated endocytosis	Caveolar/lipid raft endocytosis	Macropinocytosis	Phagocytosis
Important plasma membrane constituents	Cholesterol [141] Phosphoinositides [139]	Cholesterol Glycosphingolipids GPI-anchors [166]	Cholesterol [173]	
Receptor mediated	Yes [129]	Yes [174]	No [175]	Yes [176]
Actin dependent	Yes [137]	Yes [160]	Yes [175]	Yes [177]
Dynamin dependent	Yes [132]	Yes [135]	#	Possibly [178]
Extracellular ligands/ markers	Transferrin [179, 180] LDL [180, 181]	Albumin [156] Cholera Toxin B [165]	Dextran [182]	Antibody or complement opsonized particles [178]
Inhibitors [183]	Low pH shock Chlorpromazine Potassium depletion DN Eps15 mutant DN AP2 μ2 mutant	Cholesterol depletion β-MCD Fillipin Nystatin DN caveolin mutant	Amiloride [184] Dimethylamiloride [184] Cholesterol depletion β-MCD [173] etc	

An important conclusion from this table is that any attempt to inhibit a specific pathway is likely to have other, unwanted effects. Especially so in approaches involving drugs and ligands. Approaches directed at actin will not surprisingly affect all pathways of endocytosis. Regarding dynamin, this may also be the case. Cholera toxin B binds to ganglioside M1 that is associated with lipid rafts and caveolae, but the toxin has been shown to enter cells both by means of clathrin-independent pathways and via clathrin mediated endocytosis [185]. Approaches that affect intracellular pH will have effect on several of the routes discussed. Furthermore, the cholesterol extracting drug methyl-β-cyclodextrin (β-MCD) not only inhibits clathrin-independent endocytosis but also clathrin-dependent endocytosis [141]. #)Macropinocytosis has been reported to be dependent on dynamin in stimulated cells [186], whereas a recent report indicates a role for BARS (defined below) in macropinocytotic membrane fission [187].
DN, dominant negative; β-MCD, methyl-β-cyclodextrin; LDL, low density lipoprotein; GPI, glycosylphosphatidylinositol

Macropinocytosis

Macropinocytosis was first described in the 1930s by studying macrophages using time lapse microscopy techniques [188]. The process was named pinocytosis but later renamed to macropinocytosis to distinguish it from micropinocytosis (clathrin dependent, caveolar and uncoated endocytosis by means of small vesicles). For long considered a somewhat artefactual phenomenon of stimulated cells, macropinocytosis remains less well studied than other modes of endocytosis [175]. It has become apparent however, that macropinocytosis is an active and highly controlled event [189]. A role for macropinocytosis has been suggested in processes such as satisfying nutritional requirements, sampling of soluble antigens

in dendritic cells, and membrane turnover in motile cells [175]. Macropinocytosis is mechanistically in many ways similar to phagocytosis. The process starts with formation of ruffles, i.e. actin driven plasma membrane protrusions. The ruffles organize to form large folds and cups that are then either relaxed and flattened again, or progressively more invaginated to form a flask like appearance similar to those seen in micropinocytosis [175]. Actin dynamics have a profound role during all phases of macropinocytosis. Ruffle formation has been shown to require cholesterol [173].

Macropinocytosis differs from other types of endocytosis in that it supposedly is receptor independent. Phagocytosis as well as clathrin- and caveolae mediated endocytosis all involve receptors and transmembrane proteins that offer selectivity in cargo, whereas macropinocytosis is thought to mediate unspecific uptake of surrounding solutes [175]. The membrane of the forming macropinosome most certainly contains a multitude of receptors, but these are thought not to be specifically concentrated, in contrast to clathrin and caveolae dependent internalization. Since the volume to surface area ratio is high, macropinocytosis may be an efficient way to internalize nutrients.

A role for dynamin has been suggested also in macropinocytosis. Platelet derived growth factor (PDGF)-induced macropinocytosis was abolished by expression of dominant negative dynamin-2, as determined by measuring fluid phase uptake of fluorescent dextran and horseradish peroxidase (HPO) [186]. The authors point out that dominant negative dynamin constructs had little effect on the basal rate of fluid phase uptake, i.e. in non-stimulated cells. However, going from a flask like appearance to a vesicle requires membrane fission and a function like that of dynamin is likely involved also in unstimulated cells. Interestingly, a very recent report indicates the existence of an alternative membrane fission machinery with the protein BARS (brefeldin A-ribosylated substrate) at a key position [187]. BARS is reportedly active in macropinocytosis but not in clathrin mediated endocytosis.

The intracellular destination of macropinosomes appears to depend on the cell type. In macrophages, macropinosomes migrate to the centre of the cell, decrease in size and form an acidic endosome-like organelle that merges with lysosomes [190]. In A431 human carcinoma cells macropinosomes do not acidify, hardly interact with endosomal compartments and form a distinct vesicle population exhibiting a high rate of recycling to the plasma membrane [191].

Tools for the study of macropinocytosis include growth factor stimulation and phorbol ester treatment, since they transiently induce membrane ruffling and consequently macropinocytosis [173, 192]. Dextran sulfate is considered a marker for fluid phase uptake and is thus used to investigate the signaling pathways involved in macropinocytosis [192]. Amiloride and

dimethylamiloride, both inhibitors of the plasma membrane Na^+/K^+ pump, have been used to prevent macropinocytosis, probably due to acidification of the cytosol [184]. Cytosolic acidification however also affects clathrin mediated uptake [193], but mild acidification seems to affect primarily macropinocytosis [194, 195]. Cholesterol depletion by use of β-MCD has also been used to inhibit macropinocytosis [173].

Phagocytosis

Phagocytosis is the mechanism by which primarily the professional phagocytes of the innate immune system, neutrophils and macrophages, engulf and eliminate pathogens and cell debris [196]. In the case of pathogen internalization, the process is also the initiation of the adaptive immune response via phagocytic antigen presentation on class II major histocompatibility complex molecules [197]. Although much less efficient than in specialized cells, most cell types display various degree of phagocytic activity, perhaps reflecting the fact that lower eukaryotes use phagocytosis as a means to acquire nutrients [196, 198].

The process of phagocytosis is heavily dependent on actin and membrane rearrangement [198]. The traditional model of phagocytosis suggested that the phagosome was formed by actin driven protrusions and invaginations of the plasma membrane. This implied that the phagocytic process would decrease phagocyte surface area due to internalization of plasma membrane components. The importance of actin rearrangement remains unquestioned [177], whereas the view on membrane dynamics has changed. Loss in net surface area has not been observed and recent work suggests the contrary: surface area is in fact increased [199]. The suggested explanation is a process named focal exocytosis [200], referring to the contribution of membrane components from several cellular compartments, e.g. the endoplasmic reticulum [201, 202], directly to the area of the phagocyte involved in phagocytosis.

A central theme of phagocytosis is its receptor dependency. Phagocytes are equipped with receptors that recognize a limited number of conserved motifs on pathogens, e.g. lipopolysaccharides, but also receptors that recognize targets coated with opsonizing molecules. Fc receptors and complement receptors recognize the Fc part of antibodies and complement system components such as the C3bi fragment respectively [176, 196]. Receptor binding triggers a signaling cascade that results in actin dynamics and membrane recruitment. In the case of Fcγ receptor induced phagocytosis, an initial event is Src-family kinase phosphorylation of tyrosine residues in the cytoplasmic regions of clustered Fcγ receptors [198].

A role for dynamin in macrophage phagocytosis has been suggested by investigations using a dominant negative dynamin-2 mutant [178]. Phagocytosis

of red blood cells opsonized with IgG or complement was reduced with 65-85% upon expression of the dominant negative dynamin-2 construct. As the point of blockade was prior to the completion of the phagocytic cup, it was later suggested that dynamin might modulate focal exocytosis [203]. An interesting question is whether the newly discovered BARS-system that seems to mediate membrane fission in macropinocytosis also has a role in phagocytic membrane fission.

Internalization via phagocytosis is generally thought to be restricted to large molecules, and a size limit of >0.5 μm is commonly mentioned [196]. Interestingly, it has long been known that smaller particles can be aggregated on the surface of the phagosome until a critical mass is reached and phagocytosis is induced [204].

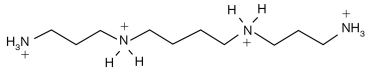
Phagocytosis is a complex process with high redundancy, and a common conclusion is that the process may differ slightly between separate cell types [177] or even within a single cell depending on the circumstances [196].

Role of cell surface HSPG in cellular internalization

Not only do PGs interact with a great number of factors. PGs are also emerging as multifunctional carriers for the internalization of extracellular ligands [205, 206]. PGs as an entry pathway is utilized not only by physiological factors, but indeed by a large number of pathogens, as exemplified in Table 4. Several viruses and intracellular bacteria are thus dependent on PGs for efficient internalization and infectivity.

Internalization as a means of activity regulation is a well established concept for many receptor-ligand complexes. In the case of the fibroblast growth factor (FGF) family of growth factors, evidence has accumulated pointing in the direction that internalization is a requirement for full activity [218]. Internalization of basic-FGF (bFGF) has been shown to depend on HSPG *in vivo* [208]. Recently, it was established that syndecan-4-bFGF complexes are co-internalized after bFGF induced clustering of syndecan-4 molecules [219]. These results were obtained using an Fc-receptor-syndecan-4 chimera previously shown to form heterodimers with co-expressed native syndecan-4, a system that according to the authors reports the behavior of native syndecan-4 [220]. The authors conclude that bFGF binding induces oligomerization of syndecan-4, leading to endocytosis of the complexes (see Figure 2). Macropinocytosis is suggested as the involved endocytotic pathway since internalization appears lipid raft-dependent, clathrin and dynamin-independent and amiloride sensitive [219]. Indeed, a shift of plasma membrane localization has been observed for syndecan-4 when clustered by ligands. Unclustered syndecan-4 resides predominantly in the non-raft membrane partition, whereas clustering induces redistribution of syndecan-4 into the non-caveolae raft partition [220].

Table 4. Examples of ligands and pathogens that are internalized via HSPG

Ligand	Function in	GAG-binding motif
Apolipoprotein E [207]	Lipid metabolism	SHL**RK**L**RKR**LL**R**DADD
Basic fibroblast growth factor [208]	Cell-growth and wound healing	GHF**K**DP**KR**LYC**K**NGGF
TFPI-Xa complex [209, 210]	Regulation of coagulation	GGLI**K**T**KRKRKK**Q**R**V**K**IAY (in TFPI)
Spermine [117]	Cell growth and differentiation	

Pathogen	Associated disease
Neisseria gonorrhoea [211]	Acute gonorrhoea; pelvic inflammatory disease and infertility in women
Enterococcus faecalis [212]	Nosocomial bacteremia and endocarditis
Herpes Simplex virus [213]	Cold sores; genital herpes. Encephalitis. During pregnancy, herpes may cause miscarriage or stillbirth
Adeno Associated Virus-2 [214]	Possible role in miscarriage and trophoblastic disease [215]
HIV-1 [216, 217]	Acquired immunodeficiency syndrome

TFPI, tissue factor pathway inhibitor; Xa, activated coagulation factor X

As mentioned above, several GPI-anchored proteins have been reported to recycle between the plasma membrane and intracellular compartments [221, 222]. Such a behavior has also been suggested for HSPG [223, 224], and may be a means by which PGs function to shuttle cargo from the exterior of the cell to the correct intracellular compartment for processing or signal induction [205, 206]. During glypican recycling, the GAG chains are thought to be largely degraded by both enzymatic and non-enzymatic processes, possibly as a way to release cargo. The non-enzymatic process is a deaminative cleavage of HS catalyzed by nitric oxide (NO). NO is produced by NO-synthases and may be sequestered by the glypican core protein as SNO groups, a possible role for the 14 preserved cystein residues of the glypican globular domain. Upon arrival to the Golgi compartment, GAG chains are re-synthesized so that complete and functional glypicans arrive at the plasma membrane to initiate another cycle [44]. An interesting link between glypican turnover and polyamine transport has been observed. Under circumstances where cells are dependent on extracellular polyamines, i.e. inhibition of endogenous polyamine synthesis by DFMO treatment, PGs in general and glypicans in particular exhibit increased affinity for spermine [117, 119]. Possibly, the GAG synthesis machinery can be modulated in times of need to produce GAG species with increased affinity for specific growth factors or extracellular ligands, e.g. polyamines. As discussed above, no

mammalian plasma membrane located polyamine transporter has been identified. The role of PGs in polyamine internalization may still just be to present the ligand to an as yet elusive high affinity receptor. Alternatively, loaded PGs are internalized via endocytosis and processed to release their polyamine cargo in a suitable compartment.

CELL PENETRATING PEPTIDES

Definition

In 1988, Frankel and Pabo reported that a protein could be internalized by cells and subsequently transported into the nucleus [225]. The protein in question was HIV-1-trans activator of transcription (TAT). TAT is produced from HIV-1 infected cells, transported in a paracrine fashion and internalized by surrounding cells. Once inside the nucleus of a HIV-1 infected cell, TAT binds the viral trans activation responsive (TAR) region, a hairpin loop structure located near the 5' end of the HIV-1 transcript. TAT binding is stabilized by interaction with Cyclin T and together they recruit other factors, e.g. Cdk9. The complex functions as an anti termination factor allowing RNA polymerase II to progress through arrest sites and transcribe the entire HIV-1 DNA, resulting in viral replication [226].

A few years later the field was expanded as Prochiantz *et al.* reported that the *Drosophila* Antennapedia homeodomain peptide was internalized by nerve cells and transported to the nucleus, inducing morphological differentiation [227]. The ability to enter cells was in both cases found to arise from a short stretch of amino acids [228, 229]. In the case of HIV-TAT the sequence is GRKKRRQRRRPPQC (from now on referred to as Tat, whereas the full protein will be referred to as TAT), and in the case of Antennapedia RQIKIWFQNRRMKWKK (named penetratin). A common name, protein transduction domain or PTD, was adopted based on the fact that the active fragment is part of a native protein that is internalized as a whole [230]. As the family of peptides with carrier properties expanded to include artificially designed and chimerical peptides not found in native proteins, the wider term cell penetrating peptide (CPP) was introduced [231]. The term CPP and the concept of CPPs as they were originally proposed [231] contain implications on the mechanism of internalization and may thus be inappropriate, as will be discussed below. As no consensus on terminology or classification has of yet been accepted, the term CPP will in this thesis refer to a peptide, with or without cargo, with the ability to efficiently enter either the cytoplasmic and/or the nuclear compartment of cells.

Examples of cell penetrating peptides

The list of CPPs is rapidly expanding. Excitement regarding the possible implications of peptides as delivery vehicles has generated a pool of peptides that may have little or nothing in common except for the reported ability to enter cells. A general theme amongst the most commonly used CPPs is a positive net charge due to a high frequency of the basic amino acids arginine (R) and lysine

(K). Not all peptides with the capability to enter cells however exhibit positive net charges [231]. Herein, emphasis will be on basic CPPs. For examples, see Table 5.

Table 5. Examples of basic CPPs

Name	Sequence	Source	Net charge
Tat [229]	G**RKKRR**Q**RRR**PPQCa	Protein derived, aa 48-60 of the HIV-1 TAT protein	+8
Penetratin [228]	**R**QI**K**IWFQN**RR**M**K**W**KK**	Protein derived, aa 43-58 of the third helix of the Antennapedia protein	+7
LL-37 [232]	LLGDFF**RK**S**K**E**K**IG**K**EF**KR**IV Q**RIK**DFL**R**NLVP**R**TESCa	Intact human protein, expressed in hemaotpoietic and epithelial cells	+6
Prion peptide [233]	MV**K**S**K**IGSWILVLFVAMWSDV GLC**KKR**P**K**P	Protein derived, aa 1–28 of the bovine prion protein	+5
LMW protamine [234]	VS**RRRRRR**GG**RRRR**	Thermolysine degraded salmon protamine	+10
Protamine [235]	P**RRRR**SSS**R**PV**RRRRR**P**R**VS **RRRRRR**GG**RRRR**	Intact peptide, expressed in spermatides (In this case protamine A1 from salmon)	+21
MAP [236]	**K**LAL**K**LAL**K**AL**K**AAL**K**LA	Model amphipathic peptide	+5
Homopolymers of basic aa	$\mathbf{R_n}$* [237] and $\mathbf{K_n}$ [238]	Synthetic	+n
Transportan [239]	GWTLNSAGYLLG**K**IN**K**ALAAL A**K**ISILa	Chimera of active part of galanin (aa 1-12) and mastoparan	+3

* In the case of Poly-Arg, number of residues seems important for efficient internalization. Optimal size is reportedly ~8 Arg residues [237]. This result was however obtained after fixation of cells. LMW, low molecular weight; a, amide; aa, amino acids

Cargo

CPPs have been used to deliver a wide range of cargos, i.e. iron beads; synthetic macromolecules; fluorophors; peptides; proteins; oligonucleotides; and DNA plasmids to cells both *in vitro* and *in vivo* [231, 240]. The addition of CPPs such as penetratin and Tat to liposomes has proven to increase uptake of liposomes [241]. The drugs doxorubicin and methotrexate, used in cancer chemotherapy, have also been delivered conjugated to Tat [242] and poly-L-lysine (PLL) [243] respectively.

In theory, intracellular delivery of proteins conjugated to CPPs such as Tat would mimic the physiological function of the peptide and should be a feasible approach that would provide a valuable tool in the study of biological processes. Indeed, a large number of Tat-protein and Tat-peptide conjugates have

been shown to be efficiently delivered to the inside of cells with maintained biological activity of the cargo protein [244]. Dowdy and co-workers have presented numerous reports where the biological functions of proteins associated with the cell cycle, apoptosis etc. have been investigated, especially in the context of anti cancer therapy. Examples include Tat-p16, Tat-p27, Tat-Cdk2 dominant-negative, Tat-caspase-3, Tat-p73 dominant-negative, Tat-E2F-1 dominant-negative, and Tat-pRb (for refs see [244]).

One of the first reports on peptide mediated delivery of proteins into cells is however that of Shen and Ryser from 1978 [245]. They provide evidence that PLL conjugated to albumin or HPO greatly enhances protein uptake into cultured fibroblasts. The authors measured HPO activity in cell extracts after trypsin treatment of intact cells and visualized uptake by electron microscopy and light microscopy after staining for HPO.

CPP-conjugates have also been used to deliver biologically active proteins in vivo. Intraperitoneal administration of a Tat-β-galactosidase conjugate, results in delivery to most tissues in mice [230]. Fusion proteins of Tat and glial line-derived neurotrophic factor [246] or the anti apoptotic proto-oncogene Bcl-xL [247, 248] are protective after focal cerebral ischemia when administered intravenously or intraperitoneally. Intraperitoneal administration of a fusion protein of Tat and caspase-3 with specificity for hypoxic tissue due to the addition of the (oxygen-dependent) degradation domain of hypoxia-inducible factor-1, had anti tumor effects [249].

Specific and efficient delivery of bio active nucleic acids is an attractive tool for biomedical researchers as well as clinical practitioners. A large number of methods have been developed for *in vitro* delivery of nucleic acids, including viral delivery but also non-viral delivery methods, e.g. lipofection, electroporation, microinjection and calcium phosphate precipitation [250]. A general drawback with these methods is their lack in specificity and/or high level of toxicity. The use of most of these methods is in addition completely restricted to cell culture models. The many drawbacks of using viruses as vectors include immunogenicity and cytotoxicity, but also insertional mutagenesis in which ectopic chromosomal integration of viral DNA either disrupts the expression of a tumor-suppressor gene or activates an oncogene leading to malignant transformation of cells [251]. The discovery of CPPs and their nuclear homing abilities suggested a new approach for efficient and non-toxic nucleic acid delivery with *in vivo* potential. Delivery of nucleic acids using CPPs has been accomplished using two seemingly disparate approaches: conjugation and complex formation. A year before the discovery that HIV-TAT can enter cells, Lebleu and co-workers performed a study using PLL conjugated to an oligonucleotide complementary to the initiation region of vesicular stomatitis virus N-protein mRNA. Addition of this

construct to infected cell cultures resulted in specific inhibition of viral protein synthesis and strong antiviral activity at concentrations as low as 100 nM, i.e. at much lower doses than required when only oligonucleotide was administered [252]. The same group later showed that PLL-conjugated antisense oligonucleotides can be used to protect cells from the cytopathic effect of HIV-1 in acute infection assays, with an EC50 two orders of magnitude lower than the EC50 for the unconjugated oligonucleotide [253]. After the discovery that the Antennapedia homeodomain and penetratin were able to enter cells, Prochiantz and coworkers developed a system for delivery of peptides and oligonucleotides using these "Trojan peptides" as conjugated carriers (reviewed in [254]). Peptide-oligonucleotide conjugates using Tat as carrier have also been constructed [255]. It seems as if though efficient nucleic acid delivery with conjugated CPPs as carriers is restricted by a nucleic acid size limit, making the conjugated approach suitable primarily for oligonucleotide delivery [254]. The suggested explanation is that the translocation ability of the peptide is lost as it binds double stranded DNA, i.e. when the oligonucleotide is long enough to interact with and quench its own carrier. CPP-cargo conjugates are intended to interact with the cell and be internalized as monomers. As mentioned above, the most frequently used CPPs all have a positive net charge. If the cargo is a molecule with profound negative net charge, e.g. a nucleic acid, multimeric complexes of many constructs are likely formed in addition to the self quenched monomers. Indeed, CPP-oligonucleotide conjugates display low solubility [254, 256].

However, delivery of nucleic acids (or other polyanions for that matter) using CPPs is not dependent on covalent conjugation between the two. By mixing the polyanionic DNA with a polycationic peptide, interpolyelectrolyte complexes (polyplexes) spontaneously assemble due to formation of a cooperative system of interchain electrostatic bonds. The physicochemical characteristics of these polyplexes, i.e. solubility, dimensions, and surface charge, can be varied by altering the composition of the complex and the chemical structure of the constituents [257]. Efficient uptake of polyplexes has been shown to be especially dependent on the ratio of added positive and negative charges, with a slight positive net charge greatly enhancing internalization [257-259]. Formation of polyplexes has been shown to condensate DNA [260] and to protect it from nuclease activity [234, 257]. Protection and cellular uptake of RNA by use of polyplex formation with protamine and histones was reported early [235]. The protective features of polycations and the fact that the size of the nucleic acid to be delivered is less restricted than in conjugation approaches, make polyplexes a suitable method for delivery of DNA plasmids. An inherent problem with the polyplex approach is difficulty to predict the characteristics of the formed complexes. Moreover, these characteristics must be regarded as dynamic, since

interactions with factors present in medium, serum and at the cell surface are likely to affect the composition and appearance of the polyplexes. *In vivo*, the situation becomes even more complex. Polyplexes are quickly taken up by the liver and accumulate or deposit in tissues with very fine capillaries, i.e. the lung, skin and intestine, rendering short plasma half lives [261-263].

It is clear that the cargo greatly influences carrier mediated internalization. Especially so if the cargo itself has extreme physicochemical characteristics, as in the case of nucleic acids.

Mechanism of internalization

Since the discovery that certain peptides had the ability to enter cells there has been an intense debate regarding the mechanism of entry. Alongside with reports on energy independent uptake of CPPs, other investigators have firmly stated the opposite, in many cases about the same peptide. Initial studies on the uptake mechanism of penetratin indicated that uptake was present at 4°C, non-saturable and seemingly independent of a chiral receptor [228, 264]. Similar results were obtained with other CPPs, e.g. Tat [229]. The inverted micelle model was proposed [264, 265] suggesting that the peptide interacts directly with the plasma membrane. The interaction is in this model mediated via electrostatic interactions between the positive charges of basic amino acids in the peptide and negative charges present on the membrane, e.g. on phospholipids and gangliosides. Accumulation of peptides in the water membrane interface would then destabilize the membrane to form inverted micelles, with a hydrophilic pocket encapsulating the peptide and allowing passage through the lipid phase of the membrane. Once the inner face of the membrane is reached, the inverted micelle supposedly performs the opposite maneuver, releasing the peptide into the cytoplasm [254]. The tryptophan residues with their aromatic side chains have been suggested essential for the internalization of penetratin by some authors, whereas others have found it dispensable [266]. The inverted micelle model has stimulated studies of peptide-membrane interactions. The bulk of these investigations have been performed in model membrane systems with defined composition in terms of lipids and other components. Whether results obtained with these models can be extrapolated to intact cells and organisms is questionable. It is however clear that penetratin interacts with model membranes and that this interaction involves basic as well as hydrophobic peptide residues [267]. With evidence in favor for the inverted micelle model being scarce, other models for CPP internalization have been suggested. Almedia and co-workers observed graded fluorescein efflux from phosphatidylcholine vesicles in the presence of the δ-Lysin peptide. This observation led the authors to propose the

sinking raft model, where transient pore formation is induced alongside with peptide internalization [268]. The δ-Lysin peptide is however a hemolytic peptide with neutral net charge at physiological pH. These characteristics make it very different from the peptides in the CPP family, and probably disqualify it as an appropriate tool for the study of CPP internalization. Several other models for direct membrane penetration have been discussed (see [268]).

As will be discussed below, the CPP field has been forced to re-evaluate several of its findings since it has become apparent that fixation may induce artefacts [269, 270] and that flow cytometry fails to discriminate between cell attachment and true internalization [270, 271]. Moreover, models like the inverted micelle have never been able to explain internalization of peptide-cargo constructs, as the mere size of the conjugates rules out intra-plasma membrane localization. Neither do models involving transient pore formation sound applicable on the most frequently used CPPs (i.e. not including haemolytic peptides etc.), since the cytotoxicity such models would imply has not been observed. It is however clear that cargo internalization by CPPs is not an artefact, since delivery of CPP-protein and CPP-oligonucleotide conjugates clearly have biological effects that require intracellular localization, and delivery of CPP-DNA plasmid polyplexes results in plasmid expression. Thus, other mechanisms of internalization must be taken into consideration, although it cannot be completely ruled out that certain peptides may have the capacity to directly traverse the plasma membrane. The existence of other routes of internalization is especially evident in the context of macromolecular cargo delivery.

Initial reports on PLL-protein [245] as well as PLL-oligonucleotide [272] constructs suggested uptake via endocytosis. Early observations made it evident that polyplexes also enter cells via endocytosis [257, 273], as suggested also by later studies [274]. A number of recent studies clearly demonstrate that uptake of Tat occurs in an energy dependent manner by means of endocytosis, as shown both for fluorophor conjugated Tat, 5 nm gold Tat conjugates and Tat-protein constructs [271, 275-279]. Endocytosis has also recently been reported to be the mechanism of entry for fluorophor conjugated penetratin [266] as well as a penetratin-Ser-Gly-Biotin construct using fluorophor conjugated streptavidin as a reporter [280].

An elaborate method to prove that CPP-protein conjugates do indeed reach the nuclei of target cells is the Cre-loxP system [281]. Cre (cyclization recombination) is a 38-kDa site-specific DNA recombinase that recognizes a 34-bp site called loxP (locus of X-over of P1). Cre catalyzes DNA recombination between pairs of loxP sites, excising the DNA sequence located between them. A DNA sequence containing two loxP sites surrounding a STOP region can be inserted in a reporter gene construct. The construct can then be stably introduced

into cells. The reporter gene will only be expressed by such cells if Cre is present to excise the STOP region. This approach has been used to provide evidence that Tat-Cre [276, 282] and penetratin-Cre [283] in fact reach cell nuclei when administered to cells in culture. In the case of Tat-Cre uptake, it has been suggested that uptake occurs via macropinocytosis since: 1) uptake occurs via endocytosis as evidenced by co-localization with an unspecific marker for endocytosis, FM4-64; 2) cholesterol depletion using β-MCD and nystatin inhibited uptake and activity, speaking in favor of lipid raft/caveolae mediated uptake or possibly macropinocytosis; 3) fluorescent Tat-Cre did not co-localize with caveolin-1-red fluorescent protein, excluding caveolae mediated uptake; 4) expression of a dominant negative dynamin construct did not hamper Tat-Cre activity whilst uptake of fluorescent transferrin was inhibited, speaking against clathrin mediated uptake [276].

Other investigators have recently reported that clathrin mediated pathways are crucial for the uptake of fluorophor conjugated Tat [278]. In this study, nystatin had no effect, whereas chlorpromazine and potassium-depletion markedly reduced Tat internalization. The notion of clathrin mediated endocytosis as the internalization route for Tat conjugated to low molecular weight cargo has been argued in an elaborate report by Vendeville and co-workers [275]. Not only do these authors present electron micrographs of Tat-5 nm gold conjugates localized to coated pits. Using dominant negative constructs of Eps15 and intersectin they also demonstrate the importance of these clathrin associated proteins. Dominant negative dynamin constructs also affected internalization in the same study.

Caveolae/lipid raft mediated uptake has likewise been reported as the mode for internalization [284, 285]. In these reports, Tat-GFP and TAT-GFP administered to cells displayed co-localization with caveolin-1 positive vesicular structures and CTxB, whereas no co-localization with transferrin was observed. β-MCD severely reduced uptake in these studies. To conclude, the route of entry for TAT protein and Tat peptide seems to elude concise definition. Whether this reflects the actual mechanism of internalization, i.e. a pan-endocytic route of entry, or not remains to be determined.

Also in the case of polyplex uptake virtually all endocytic routes have been suggested as the one responsible for internalization. Polyplexes of reporter gene plasmids and histidinylated PLL reportedly enter cells both by means of macropinocytosis and clathrin mediated endocytosis [286]. The same study suggests that uptake via macropinocytosis is non-productive whereas clathrin mediated uptake results in efficient reporter gene expression. In contrast, another study on the uptake of polyplexes, in this case of polyethyleneimine and DNA, concludes that uptake via clathrin-mediated endocytosis results in delivery to the

lysosomal compartment and degradation, whereas polyplexes internalized via caveolae manage to escape degradation permitting efficient transfection [287]. It has also been discussed that internalization of polyplexes can in part be due to phagocytosis or endocytosis via lipid rafts [288]. Ignatovich *et al.* report that the mode of internalization of Tat-DNA polyplexes varies depending on the cell line used [274].

Studies of the uptake of ligand-devoid, fluorescent latex beads in non-phagocytic B16 melanoma cells showed that the internalization pathway varies depending on the size of the latex beads [147]. Particles with a diameter <200 nm entered predominantly via clathrin-coated pits and were delivered to lysosomes. Increasing bead size was associated with increasing occurrence of caveolin-1 co-localization. Given that the generally accepted size of caveolae is 50-100 nm these results are somewhat surprising. Moreover, 100 nm particles never co-localized with 500 nm particles, suggesting completely disparate pathways. Large particles were delivered to lysosomal compartments much slower than were small particles. The authors suggest that the latter observation may explain why larger lipoplexes give rise to higher transfection efficiency.

It can not be excluded that the administration of positive charge itself stimulates endocytosis. Early studies on protein uptake in cultured cells revealed that addition of non conjugated polycationic proteins and peptides such as histones, PLL and poly-L-ornithine enhanced albumin uptake up to 50-fold [289]. Poly-L-ornithine was the most effective polycation in terms of stimulating albumin uptake, without forming complexes with albumin. Dowdy *et al.* recently reported that Tat stimulates macropinocytosis [277]. They incubated cells with a 70-kDa neutral dextran-texas red fluid phase marker that supposedly does not interact with polybasic peptides. Addition of polybasic peptides, e.g. Tat and variants thereof, resulted in increased dextran internalization. The ability to stimulate macropinocytosis correlated with peptide cell surface binding capacity, which directly depended on net charge of the peptide, i.e. replacement of two basic amino acids with alanine resulted in a reduction to near background levels of macropinocytosis.

That interaction between positive charges of basic CPP residues and negative charges associated with the cell membrane is a prerequisite for internalization has been argued since the dawn of the CPPs. Initially, negative charged species of phospholipids and gangliosides were pointed out as the most probable binding partners in CPP-interaction with the plasma membrane. However, accumulating data recognize another group of negatively charged plasma membrane-associated molecules as essential for CPP internalization, namely cell surface HSPG. Using PG deficient mutant cells; inhibitors of GAG synthesis; competitive inhibition by GAG mimetics; and GAG degrading

enzymes, the role of cell surface HSPG in internalization of positively charged compounds has recently been studied intensely. Since the early findings that efficient transfection using PLL-DNA polyplexes and cationic lipid-DNA lipoplexes requires cell surface PG [259, 290], the role of cell surface HSPG as a plasma membrane carrier has been firmly cemented [205]. It has been shown that the polyamine spermine interacts with GAGs and is dependent on cell surface HSPG for efficient internalization [71, 72, 117]. The fact that HSPG mediated internalization of spermine stimulated investigations on the internalization of spermine-based cationic lipids, e.g. Lipofectamine [291, 292]. Regarding CPP internalization, it has convincingly been reported that cell surface HSPGs are essential for the uptake of fluorophor conjugated Tat [278], Tat-protein constructs [276, 280] and Tat-DNA polyplexes [293]. Indeed, cell surface HSPG has proven essential for entry of a penetratin-protein conjugate [280]. Cell surface HS has been suggested as an essential part of a ubiquitous internalization pathway for arginine rich peptides [294], i.e. positively charged CPPs.

The concept of endocytic mechanisms as the pathway of CPP entry delays a central delivery problem. Relocation from the outside of the cell into a vesicle brings the cargo no closer to the cytosol - there is still a lipid bilayer to cross. As focus has turned from direct plasma membrane penetration to vesicular uptake the mechanism of endosomal escape has gained much interest. Knowledge about pathogen entry into host cells has proven valuable in attempts to optimize this process. The influenza virus hemagglutinin protein is a pH sensitive fusogenic protein that destabilizes membranes at low pH. The influenza virus uses this protein to escape the endolysosomal compartment after its endocytotic entry. Dowdy *et al.* show that co-incubation of Tat-Cre with Tat-HA2 (HA2 being the active part of the hemagglutinin protein, a 20 amino acid peptide), markedly increased Cre recombination, arguably as a result of enhanced endosomal escape [276]. However, mechanisms inducing efficient release from endosomal compartments are also utilized by our own cells. Froelich *et al.* have investigated the process of cytotoxic granule-mediated apoptosis and delivery of the cytotoxic effectors (e.g. granzyme B) to target cells. The cytotoxic cells secrete complexes of the PG serglycin, perforin (a putative membrane perturbing protein) and granzymes. Serglycin with its GAGs serves as a scaffold, maintaining the integrity of the complex. Once the target cell is reached, either of two mechanisms occurs. The complex may be internalized as a whole and once inside the endosomal compartment, perforin forms a pore in the endosomal membrane resulting in cytosolic delivery of the active granzymes [54]. More recently the same group revised this model [295]. They now suggest that upon reaching the target cell, the basic granzyme B is translocated from serglycin to cell surface HSPG, after which endocytosis mediates internalization of the effector.

Hypothetically, perforin may accompany granzyme B and induce endosomal escape also in this model. Either way, this physiological polyplex may be viewed as nature's design of a drug-delivery system.

Seeking to understand the mechanism of CPP entry has thus proven a complicated task. The complexity is greatly enhanced by the fact that individual results cannot easily be compared or scrutinized. Groups use different assays; cell lines; instruments; concentrations; amino acid sequences; and indeed – different definitions and different scientific perspectives. Moreover, the accumulated data indicate that unconjugated CPPs, CPP-conjugates and CPP containing polyplexes may well be internalized by different routes, depending on size, charge and other factors. Recent advances have also added a great deal of complexity to the field of endocytosis, as discussed above. It has become apparent that formerly unknown pathways of entry may be of vital importance for the uptake of numerous ligands, and that crosstalk between different internalization routes modifies the notion of separate and unrelated endocytic pathways.

THE PRESENT INVESTIGATION

OBJECTIVES

The objective of this thesis has been to investigate mechanisms by which mammalian cells interact with and internalize charged biopolymers. In this context, charged biopolymers may be as diverse as polycationic growth factors and nucleic acids. Especially in focus for this investigation has been the role of cell surface PGs, a group of molecules that has gained much interest as of late due to the vast number of potent ligands with which they interact [4].

METHODS

The quantitative aspects of biopolymer internalization have been characterized using either fluorophor conjugated or radio labeled compounds. In the case of fluorophor conjugated compounds, internalization has been quantified using flow cytometry (Figure 4). Cells in a mono-cellular suspension are passed in a single-file through a laser beam by continuous flow of a fine stream of the suspension. Each cell scatters some of the laser light, and also emits light from the fluorophors excited by the laser. Photomultipliers detect the different aspects of light which are converted to values describing cell size, granularity and fluorescence intensity. By using different optical filter settings, multicolor analyses on the same subset of cells can be performed. Flow cytometry is a convenient method since it determines the amount of fluorescence associated with individual cells. Hence, the readout will include not only a mean or median value, but also the distribution and potential existence of subpopulations amongst cells. The method will in parallel deliver a fairly good picture of the wellbeing of a cell culture. This is very useful, since aberrances from the normal situation will affect internalization rate and indeed result in false positives in cell cultures that are particularly bad off, due to loss of membrane integrity.

In the present work, flow cytometry has been used to quantify uptake of nucleic acids, GAGs and Tat peptide. The readouts have been related to negative and positive controls. We have not attempted to convert the arbitrary values delivered by the flow cytometer to absolute values, a process that in the presence of the appropriate controls in theory could deliver fairly accurate estimations of net cellular uptake. A problem with using fluorescent conjugates and flow cytometry is the inability to differ between cell surface associated and internalized material. This will be discussed in the section below.

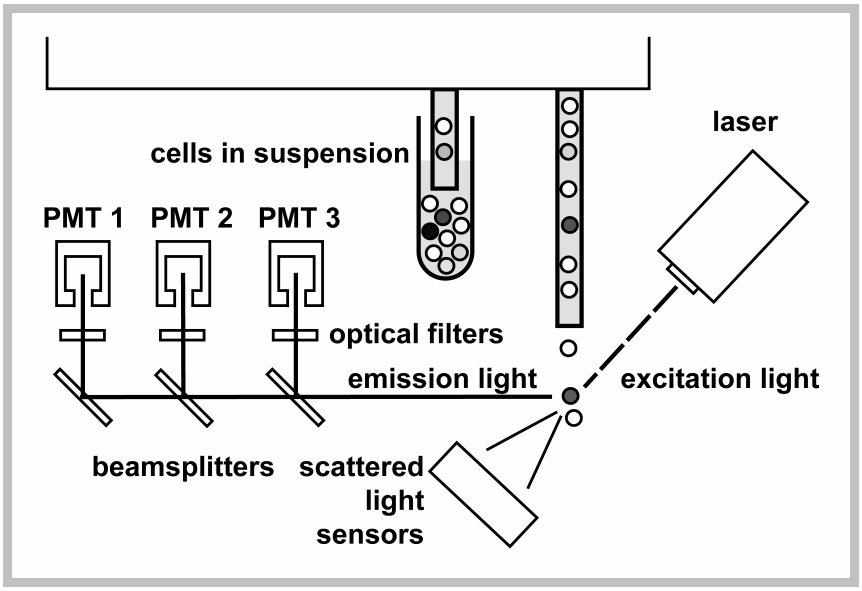


Figure 4. Principles of flow cytometry. Cells in monocellular suspension are passed in a single-file through a laser beam. Each cell scatters some of the laser light, and also emits light from the fluorophors excited by the laser. Photomultipliers (PMTs) detect the different aspects of light which are converted to values describing cell size, granularity and fluorescence intensity. By combination of optical filter settings and the appropriate dichroic mirrors (beamsplitters) multicolor analyses on the same subset of cells can be performed.

An often used complement to flow cytometry is laser scanning confocal fluorescence microscopy (confocal microscopy). The method delivers a qualitative conception of the localization of the fluorescent signal in three dimensions (Figure 5). In a confocal microscope all structures out of focus are suppressed at image formation. This is obtained by an arrangement of diaphragms which, at optically conjugated points of the path of rays, act as a point light source and as a point detector respectively. Rays from out-of-focus are suppressed by a detection pinhole. To obtain a full image, the image point is moved across the specimen by mirror scanners. The emitted light passing through the detector pinhole is transformed into electrical signals by a photomultiplier and displayed on a computer monitor screen. By using lasers with different wavelengths and appropriate optical filters, multiple emission wavelengths can be analyzed in parallel. Thus, collecting data regarding the location of several different fluorescent compounds is possible, and presence of co-localization can be recorded. We employed confocal microscopy to determine intracellular localization; to study co-localization; and to exclude cell surface adherence. The method faithfully reports the appearance of presented samples and it is therefore of great importance to choose representative and artefact free specimens, as will be discussed below.

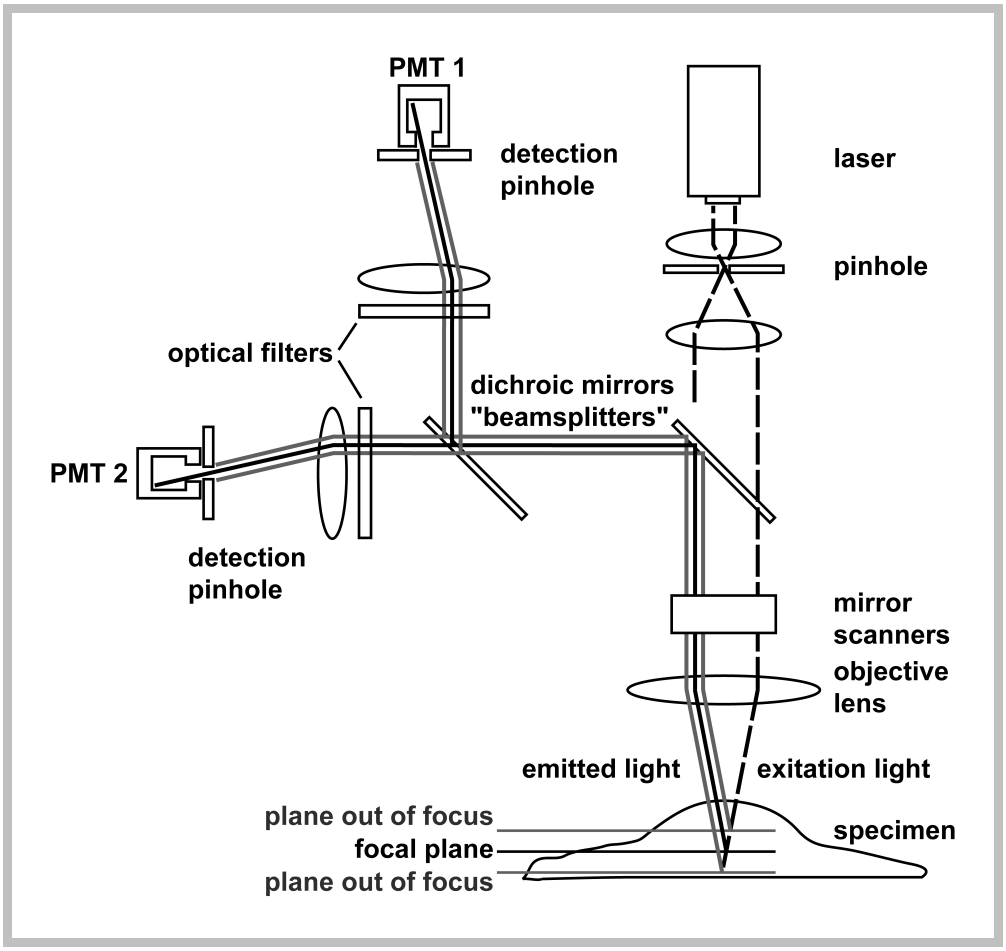


Figure 5. Principle of laser scanning confocal fluorescence microscopy. Light rays are only partly depicted for simplicity. Excitation light from the laser is passed through a pinhole to produce a point light source. As the light hits the sample, fluorophors are excited and emit light with longer wavelengths. Light is emitted both from the plane of interest, the focal plane, and from planes out of focus. Emitted light is passed through a system of dichroic mirrors and optical filters that separate different wavelengths. Specific windows of wavelengths are then led to photomultiplier tubes and converted to electronic signals. Detection pinholes make sure that only emitted light from the focal plane is detected. Mirror scanners move the image point across the specimen. PMT, photomultiplier tube

In the case of polyamine uptake, we have relied on radio labeled species to quantify uptake. The method used cannot determine uptake in individual cells. Instead, the total amount of radioactivity internalized by a culture is measured. Given a known number of cells and a known specific activity, a mean value of internalized molecules per cell can be calculated.

To measure productive internalization of nucleic acids, i.e. nucleic acids reaching the nucleus for expression, we used a luciferase reporter gene assay. A pGL3-luciferase plasmid containing the firefly luciferase gene behind a

cytomegalovirus promoter was administered to cell cultures alone or in CPP polyplexes. This approach will result in very low luciferase expression (measured as emitted light after administration of its substrate, luciferin, to lysed cultures) in cells to which naked plasmid has been administered. In cells incubated with polyplexes, productive internalization mediated by CPPs can be measured as emitted light exceeding that emitted after incubation with naked DNA plasmid. The output signal using this approach will have an arbitrary value.

Internalization of polyamines such as spermine is essential for cells whose endogenous polyamine production is inhibited. Cell surface HSPGs have been identified as essential components of the internalization machinery. By inhibiting endogenous polyamine synthesis using DFMO we have studied the effect on cell proliferation and tumor growth of compounds that assert their effect on PGs. *In vitro*, this has been done using the crystal violet cell proliferation assay [296] and the thymidine incorporation assay. The crystal violet proliferation assay determines the amount of DNA present in a culture by spectroscopic methods. After a growth period of 3-4 days, the amount of DNA present in a drug treated culture can be compared to a control culture and to a culture harvested at day 0. The thymidine incorporation assay determines effect on cellular growth over a shorter time period. The assay utilizes the fact that administered radio active thymidine will be incorporated into cells relative to the degree of DNA synthesis. To evaluate the effect of combined inhibition of endogenous polyamine biosynthesis and administration of drugs affecting internalization of polyamines *in vivo*, we employed a tumor growth model in nude, immune deficient, mice. Tumor cells were injected subcutaneously and tumor mass in control animals and drug treated animals was determined at a defined time point.

Methods for studying glycosaminoglycans and proteoglycans

A number of biochemical methods have traditionally been used to study the structure of GAGs. GAGs and PGs can be isolated from cell lysates due to their polyanionic properties. An often used protocol to isolate PGs and GAGs from cell cultures starts with ion exchange chromatography on DEAE cellulose with a thorough rinsing protocol to remove unbound material. Size exclusion chromatography and gradient ion exchange chromatography using high pressure liquid chromatography is often used. GAGs can be metabolically labeled using radioactive precursors, e.g. [^{35}S]sulfate, [^{3}H]GlcN or [^{3}H]Gal. After isolation of PGs, GAGs can be detached from the core protein by treating the sample with alkaline borohydride. The composition of GAGs can then be analyzed by using degradation methods specific for certain motifs. Nitrous acid at pH 1.5 cleaves HS at all GlcNS residues (N-sulfated GlcN) degrading HS to disaccharides,

whereas nitrous acid at pH 3.9 cleaves at GlcN with unsubstituted amino group, a less frequent moiety. GAG-lyases that cleave CS/DS and HS/Hep at specific sites were originally isolated from bacteria, and are commercially available.

Other methods are used to identify PG dependent processes. Several animal knockout models have been generated (see Table 1). Listed in Table 6 are tools used for investigation of PG involvement in cellular processes.

Table 6 . Tools for studying the function of cell surface proteoglycans

Tool	Function/Phenotype
GAG lyases [297]	
Hep lyase I (heparinase)	Cleaves at GlcNS6S-IdoA2S, a heparin-like domain
Hep lyase II	Cleaves at GlcNAc/S +/- 6S-IdoA +/- 2S
Hep lyase III (heparitinase)	Cleaves at GlcNAc/S-GlcA
Chondroitin lyase AC I	Cleaves at GalNAc4S/6S-GlcA
Chondroitin lyase AC II	Cleaves at GalNAc4S/6S-GlcA and GalNAc4S/6S-GlcA-Gal-
Chondroitin lyase B	Cleaves at GalNAc4S-IdoA
Chondroitin lyase C	Cleaves at GalNAc6S-GlcA
Chondroitin lyase ABC	Cleaves at GalNAc4S/6S-GlcA/IdoA
Chemical degradation of GAGs [298]	
HNO_2 at pH 1.5	Cleaves HS at GlcNS
HNO_2 at pH 3.9	Cleaves HS at GlcN moieties with unsubstituted amino groups
Mutant CHO cell lines	
pgsA-745, XylT def. [45]	GAG-deficient, less than 5% PG as compared to WT cells
pgsB-618, GalT I def. [46]	GAG-deficient, less than 10% PG as compared to WT cells
pgsG-224, GlcAT I def. [47]	GAG-deficient, about 10% PG as compared to WT cells
pgsD-677, EXT I def. [48]	HS-deficient, overproduces CS/DS
pgsE-606, NDST I def. [49, 50]	70% loss in N-sulfation and N-deacetylation, decreased O-sulfation
pgsF-17, 2-OST def. [51]	No 2-O sulfate, affected N-sulfation, increased 6-O sulfation
Mutant mice	
loxP EXT1 [22]	Conditional HS knockout
Drugs affecting PG turn-over	
suramin	Inhibition of heparanase [299] Inhibition of GAG interaction with growth factors [300]
chlorate [301]	Unspecific reduction of sulfation due to depletion of major sulfate donor, PAPS. In HS, O-sulfation is most severely affected
xylosides [302]	False substrate for GAG synthesis. Competes with core proteins by occupying the GAG biosynthetic machinery
Competition by addition of exogenous polyanions	

On the subject of artefacts in methods used to study CPP internalization

Initial findings regarding the ability of CPPs to enter cells with very efficient nuclear localization evoke a massive interest in these peptides as new tools for drug and gene delivery. When studying the nuclear homing kinetics of the herpes simplex virus VP22 protein, Lundberg and Johansson noted that even a ten second incubation at 4°C was sufficient to visualize nuclear localization of a VP22-GFP fusion protein. They found the very rapid nuclear import unreasonable and sought an alternative explanation for the observed phenomena. After comparing cellular localization of VP22-conjugates before and after fixation with methanol, they were able to conclude that the nuclear localization was an artefact induced by fixation. In unfixed living cells, the fluorescent signal was entirely associated with the plasma membrane after short incubations at both 37°C and 4°C. The authors suggested that cell surface binding of VP22 will allow the protein to remain attached to cells during washing prior to fixation. The cell surface bound material would then constitute a reservoir that is released upon methanol fixation and concentrated in the nucleus due to the high affinity of peptide basic residues for nuclear DNA [269]. In consecutive reports, Lundberg and Johansson extended their artefact concept to include other CPPs. In a recent report they show that GFP fusion proteins of VP22, Tat, Arg_8 as well as Lys_8 all adhere strongly to the plasma membrane and that this localization is the only one that can be observed in live cells after short incubations, independent of temperature. After longer incubations (1-24 hours) at 37°C, GFP-VP22 could be seen in vesicles, consistent with endocytic internalization. No cytoplasmic or nuclear localization was seen at any time point. The Tat, Arg_8 and Lys_8 GFP fusion proteins all displayed the same behavior [270].

The fact that basic CPPs adhere so strongly to the plasma membrane also implies that flow cytometry analyses may give false positive results. Unless very stringent rinsing procedures are applied, flow cytometry may severely overestimate the amount of CPP internalized, as exemplified by VP22 internalization at 4°C [270]. Recent protocols to remove cell surface associated material include trypsination [271]; competition with cell surface binding using excess polyanions (e.g. heparin; [270]); or combinations thereof [277].

In the light of the nuclear localization artefact and the potential pitfalls with flow cytometry the CPP field was prompted to re-evaluate its data. Several studies have been published during the past few years that agree with those of Lundberg and Johansson: CPP internalization is an energy dependent endocytic process and nuclear localization of fluorescent conjugates is not seen in live cells with unperturbed integrity [271, 275-279]. However, it is also clear that fusion proteins containing these CPPs do enter cells and reach the nuclear

compartment, as evidenced by results obtained with Tat-Cre and penetratin-Cre conjugates [276, 283]. The attention has therefore swiftly turned from plasma membrane penetration to the concept of endosomal escape and its modulation as a means to enhance intracellular activity of CPP-constructs.

As a consequence of what is discussed above, some of the data presented in this thesis also need to be re-evaluated. The fixation artefact as it has been presented in the literature concerns the localization of fluorescently labeled peptide. The data presented in this thesis however predominantly relate to the localization of polyplex delivered polyanions, i.e. GAGs and DNA and not to the localization of the CPP *per se*. Although polyanions are likely to have high affinity for the nuclear compartment, the fixation artefact has less prominent effects on the localization of polyplex delivered polyanions. Firstly, delivered polyanions are markedly larger than CPPs and secondly, the affinity between the components in the polyplexes is likely going to inhibit rapid diffusion from an extracellular or intracellular depot. Indeed, we have observed the latter phenomena regarding labeled CPPs. In Paper III, Figure 2, panels B and G can be compared to panels D and I. From this figure it is evident that co-administration of Tat-Texas Red with HS largely prevents nuclear re-localization of the peptide upon fixation. However, both paper III and IV studies the intracellular localization of polyplex delivered polyanions using confocal fluorescence microscopy. The cells in these experiments have all been fixated using 4% paraformaldehyde in PBS after incubation and thorough rinsing including a 1 M NaCl in PBS step. Four percent paraformaldehyde is generally considered milder than methanol fixation, but evidently permeabilizes cells enough to allow counterstaining with nuclear stains and fluorescent phalloidin (Sandgren, unpublished data; discussed in [271]). Therefore, we tested whether intracellular localization in unfixed cells after incubation with polyplexes of Tat and Rhodamine labeled HS-6 (highly sulfated HS) differed from the intracellular localization observed in paper III. As can be seen in Figure 6, vesicular staining is unaltered upon fixation. In both live and fixated cells, plasma membrane staining is hardly present. Nuclear localization of delivered polyanion on the other hand, is common in fixated cells (D, E) but hardly detectable in live cells (A-C). However, procedures such as fixation using paraformaldehyde and permeabilization using 0.1 % Triton X-100 in PBS does not at all equal nuclear redistribution, as can be seen in panel F where phalloidin counterstain evidently has gained access to the cell without the nucleus being stained with the polyanion associated fluorophor. Based on these observations, the quantitative aspects of nuclear localization as visualized by confocal pictures in papers III and IV should be considered with caution. The conclusion that Tat and LL-37 are able to mediate nuclear delivery of intact DNA plasmid however remains valid, since reporter gene assays reliably show

high levels of expression in the presence of these CPPs. Conceivably, the bulk of the plasmid is held back in an endolysosomal compartment, whereas a minor portion is able to escape and end up in the nucleus for expression.

To exclude that the observed distribution of internalized material is not a cell specific phenomenon, we have studied intracellular localization of delivered polyanions in several cell lines, including CHO-K1 hamster epithelial cells, COS-7 monkey fibroblasts, HFL-1 human lung fibroblasts and human T24 urinary bladder carcinoma cells. The results obtained from these cell lines are very similar. Figure 6, panel E, depicts the intracellular localization of delivered polyanion in COS-7 cells, which can be compared to that observed in CHO-K1 cells (panel D).

As for flow cytometry as a tool for quantification of uptake, our results have all been obtained after a rinsing protocol consisting of: 3 washes with cold PBS; trypsination for 2 minutes; blocking with 10% fetal calf serum; 2 consecutive wash/centrifugation cycles using 1% bovine serum albumin (BSA) in PBS followed by pelleting of cells and suspension of cells in 1% BSA in PBS before flow cytometry measurements. This procedure leaves negligible amounts of dextran sulfate releasable material remaining at the cell surface when the CPP in use is Tat, LL-37 or endogenously produced heparin binding factors (Sandgren, Wittrup, Lilja and Belting, unpublished observations).

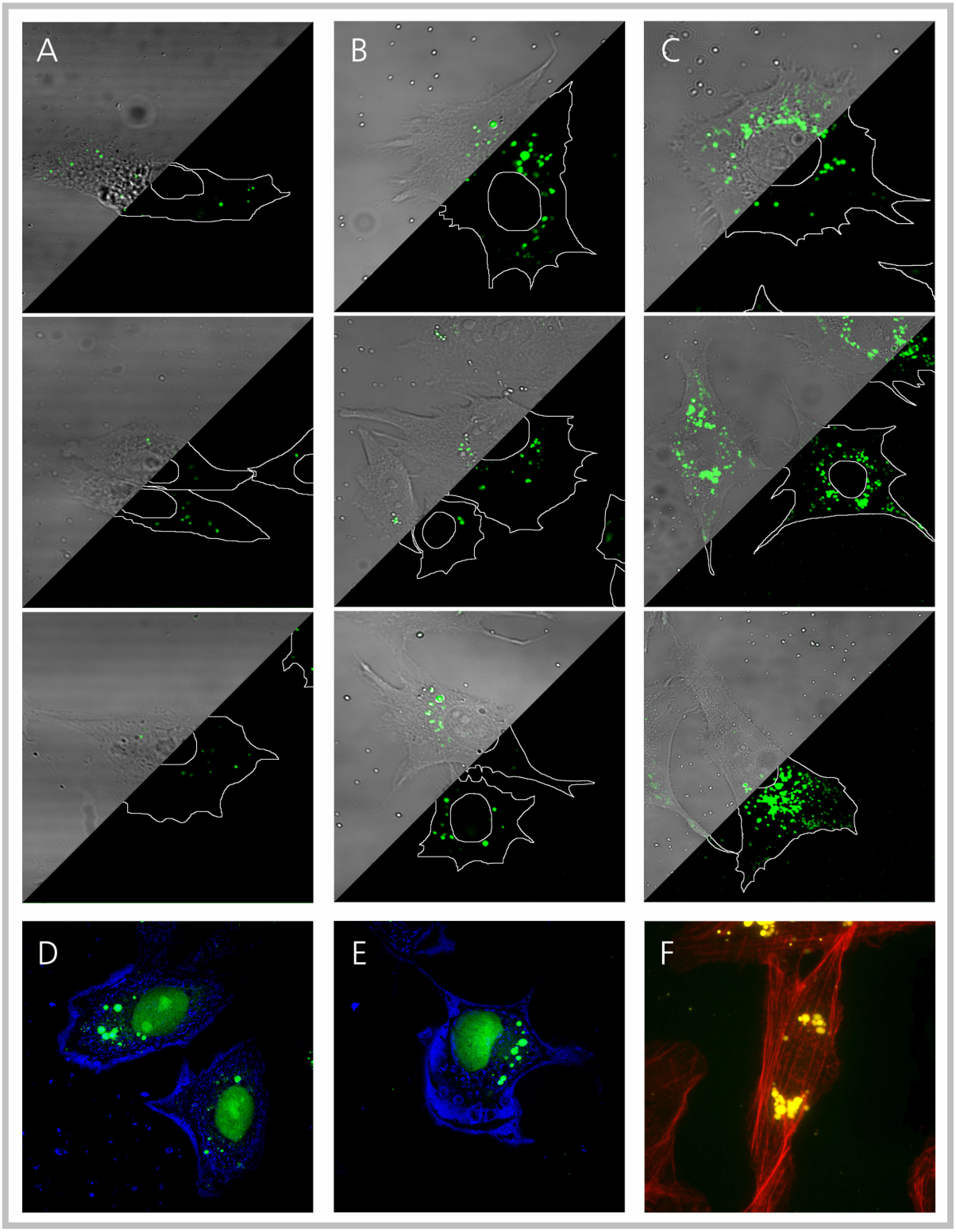


Figure 6. Effect of fixation on localization of internalized material. CHO-K1 cells (except for E) were incubated with polyplexes of HS-6 RhodamineGreen (green in the pictures) and a 4-5 times excess of Tat (w/w). Panels A, B and C show cells that were incubated for 30 minutes, 3 hours and 24 hours respectively, thoroughly rinsed and subjected to confocal microscopy (triplets shown). Panels D, E and F all show cells that were incubated for 24 hours, thoroughly rinsed and then fixated using 4% paraformaldehyde in PBS for 30 minutes. Panel E displays COS-7 cells. Panel F shows a cell that was counterstained using phalloidin-AlexaFluor-568 (red) subsequent to permeabilization using 0.1% Triton X-100 in PBS.

Results and Comments

Paper I

Suramin selectively inhibits carcinoma cell growth that is dependent on extracellular polyamines

The effect of polyamine biosynthesis inhibition by DFMO in cancer treatment has been disappointing [303]. When endogenous production is inhibited intracellular polyamine levels are maintained by efficient cellular uptake of polyamines [99], a process dependent on cell surface PGs [117]. Previous investigations show that combined inhibition of polyamine biosynthesis and polyamine uptake has drastic effects on cellular proliferation [118]. To inhibit uptake of polyamines, approaches directed at PGs have been attempted, e.g. xylosides that act as false substrates for GAG biosynthesis [117]. A drug that in theory would be suitable for co-administration with DFMO is the well established anti-parasite drug suramin. Suramin is a polysulfonated naphtylurea whose polyanionic nature has rendered it the epithet heparin mimetic. As such, suramin may interfere with polyamine uptake mediated by cell surface HSPG by two mechanisms: either direct interaction with polyamines or disturbance of HSPG turnover.

We hypothesized that a combination regimen of DFMO and suramin would strongly inhibit carcinoma cell proliferation *in vitro* and *in vivo,* due to disturbed intracellular polyamine levels.

Results

T24 human urinary bladder carcinoma cells were assayed to determine the kinetics of an active polyamine transport system. Cells were then made dependent on extracellular polyamines. At 5 mM of DFMO, cell proliferation could be restored in a dose dependent manner by the addition of extracellular polyamines. One µM of spermine completely restored cell growth. The rescue effect could in turn be reduced in a dose dependent manner by the addition of suramin. Growth dependent on extracellular polyamines was almost completely abolished at 0.5 mM suramin. Suramin alone had little effect on proliferation at all concentrations. Hence, suramin specifically interferes with proliferation that is dependent on extracellular polyamines.

To gain further insight into the mechanism by which suramin exerts its growth inhibitory effects, suramin affinity to polyamines was investigated. Using a spermine substituted HiTrap affinity column and a gradient salt elution step it was concluded that suramin interacts avidly with polyamines. Suramin eluted as a single peak at ~1.5 M guanidine, and thus bound the column markedly stronger

than heparin, which eluted as a broad peak ranging from 0.5-1.5 M guanidine. Not surprisingly, suramin readily eluted heparin from the polyamine column.

Subsequently it was established that suramin inhibits uptake of both spermidine and spermine in a dose dependent manner.

To evaluate the effects of combined DFMO and suramin treatment *in vivo*, immunodeficient mice were subcutaneously inoculated with T24 tumor cells and then received either no treatment, suramin only, DFMO only or combined DFMO and suramin treatment for 3 weeks. Suramin alone had little or no effect on tumor growth, whereas DFMO alone under these conditions inhibited tumor growth by two thirds. Combination of DFMO and suramin resulted in an additional halving of tumor load as compared to DFMO alone.

To conclude, the combination of DFMO and suramin affects polyamine turnover by combined inhibition of synthesis and HSPG mediated uptake. The combination has distinct effects on cell proliferation *in vitro*, and markedly reduces tumor load *in vivo* at concentrations were no adverse effects or toxicity can be observed.

Paper II

Common internalization mechanism for HIV-Tat protein transduction domain and polyamines in human carcinoma cells: Implications for tumor growth inhibition

With combined targeting of polyamine biosynthesis and polyamine uptake being an efficient method to inhibit proliferation of cancer cells both *in vitro* and *in vivo* (Paper I), we sought more potent methods to inhibit polyamine uptake. In a wide sense, the polybasic Tat peptide is much like a doubled spermine. Tat contains eight positive charges derived from its arginine residues, whereas spermine contains four positive charges partly derived from arginine rests. Indeed, arginine containing peptides bind stronger to heparin than other polybasic peptides [70], suggesting that arginine rich peptides like Tat would be potent competitive inhibitors of polyamine-GAG interactions.

We hypothesized that the Tat peptide would function as a polyamine analog displaying competitive inhibition of polyamine-GAG interactions, resulting in efficient inhibition of cellular proliferation *in vitro* and *in vivo* upon simultaneous administration with DFMO.

Results

In accordance with the literature, Tat affinity for heparin markedly exceeds spermine affinity for heparin, as determined by gradient affinity chromatography on a Heparin HiTrap affinity column.

As concluded above, DFMO treatment of T24 human urinary bladder carcinoma cells upregulates cellular internalization of both spermidine and spermine. Interestingly, DFMO treatment also induces a slight increase in the uptake of fluorophor labeled Tat, suggesting a common internalization pathway for Tat and polyamines. The notion of a common uptake mechanism is further supported by the observation that Tat inhibits cellular uptake of both spermidine and spermine in an energy dependent manner.

The compound methylglyoxal bis(guanylhydrazone) (MGBG) is a highly toxic polyamine analogue that enters cells via the polyamine transport pathway. It has been used to establish polyamine uptake deficient CHO cell clones. Interestingly, CHO-MGBG cells that are incapable of internalizing polyamines display no decrease in Tat internalization when compared to wild type CHO cells, as determined by flow cytometry after a rigorous rinsing protocol. Moreover, the gross appearance of the HSPG pool in CHO-MGBG cells does not differ from the HSPG pool in wild-type CHO cells, suggesting that the polyamine uptake defect in CHO-MGBG cells lies downstream of HSPG interaction.

Thymidine incorporation and crystal violet proliferation assays reveal that the effect of Tat on polyamine uptake has functional consequences, as Tat administration inhibits polyamine dependent DNA synthesis and cell growth *in vitro*.

To evaluate the effects of a combined DFMO and Tat regimen *in vivo*, immunodeficient mice were subcutaneously inoculated with T24 tumor cells and then received either no treatment, DFMO only, Tat only or combined DFMO and Tat treatment for 18 days. Tat alone had no effect on tumor growth. DFMO alone had no significant effect under these conditions, although a tendency towards tumor mass reduction was recorded. The combination of DFMO and Tat however resulted in a 91.5% reduction of tumor mass, with tumors appearing as pale avascular nodes.

To conclude, as in the case of the DFMO/suramin combination regimen, combination of DFMO and Tat affects polyamine turnover by combined inhibition of synthesis and HSPG mediated uptake. The combination inhibits *in vitro* cell proliferation, and reduces tumor load with 91.5% *in vivo* at concentrations were no adverse effects or toxicity could be observed.

Paper III

Nuclear targeting of macromolecular polyanions by an HIV-Tat derived peptide. Role for cell-surface proteoglycans

Cell surface HSPGs are emerging as important carriers for cellular internalization of various ligands, e.g. PLL-DNA polyplexes and cationic lipid-DNA lipoplexes

[259]; polyamines [117]; and parasites (Table 4 this thesis). A common feature of these ligands is binding to GAGs via polybasic sequences. We have shown that the polybasic peptide Tat can compete with polyamines for binding to cell surface PGs, inhibiting polyamine uptake (Paper II). The internalization mechanism for Tat itself is however still a matter of debate. Due to the abundance of polybasic compounds that utilize cell surface HSPGs as receptors for internalization it seems plausible that Tat also enters cells via cell surface HSPG.

We hypothesized that polyplexes of the Tat peptide (in the paper denoted HTDP, HIV TAT derived peptide) and various polyanions would be internalized in a PG dependent manner. The mechanism of internalization in general and the importance of cell surface PGs in particular, are studied,

Results

Polyplexes of Tat and GAGs or DNA are internalized in an energy dependent manner and by means of endocytosis, as shown by flow cytometry and confocal microscopy. Polyplexes consisting of Tat and DNA or Tat and GAGs are likely taken up via the same mechanism, as suggested by their reciprocal inhibition.

Upon internalization, delivered polyanion is directed to an acidic compartment, as polyplex containing vesicles stain with the Lysotracker-Red reporter.

Internalization is dependent on cell surface PGs, as shown by the use of the PG-deficient CHO cell mutant pgsA-745; chlorate treatment of WT cells; and degradation of GAG chains using GAG lyases. Notably, both HS and CS degrading enzymes affect internalization.

The notion of negatively charged cell surface PG as the carrier is further supported by the finding that efficient internalization requires that polyplexes possess an excess of positive charges, i.e. the ratio of positive charges over negative charges being higher than 1. Optimal PG dependence is seen at a ratio of just below 3. At negative net charges, uptake in PG-deficient cells exceeds that in wild type cells. At very high positive net charges, i.e. at a ratio of 7, uptake in PG-deficient cells seems restored.

Moreover, cells exposed to exogenous Tat as well as cells that over-express TAT-GFP exhibit effects on PG/GAG turnover.

In conclusion, this paper demonstrates that Tat-polyanion polyplexes enter cells via an endocytotic pathway strongly dependent on PGs. Nuclear delivery is evident since administration of Tat-DNA plasmid results in reporter gene expression.

Paper IV

THE HUMAN ANTIMICROBIAL PEPTIDE LL-37 TRANSFERS EXTRACELLULAR DNA PLASMID TO THE NUCLEAR COMPARTMENT OF MAMMALIAN CELLS VIA LIPID RAFTS AND PROTEOGLYCAN-DEPENDENT ENDOCYTOSIS

Previous results from our group support the notion of a common internalization mechanism for arginine rich peptides (Paper III). The internalization pathway is dependent on cell surface PG and mediates uptake of other polybasic compounds as well, e.g. polyamines (Papers I and II; [117]). The pathway allows for Tat-DNA polyplex delivery into cells, resulting in efficient reporter gene expression (Paper III). Curious as to whether the route was accessible for more physiological vectors, we sought a human peptide with potential CPP features. The antimicrobial peptide LL-37 was chosen due to its distinct positive net charge (+6) and reported high local concentrations. Moreover, its high expression in infected wounds is intriguing since it suggests potential interaction with DNA released from lysed bacteria. LL-37 along with other antimicrobial peptides constitute an important part of the innate immune system. LL-37 is primarily expressed in myeloid cells but indeed also in other cells and tissues, e.g. epididymis, spermatides and epithelial cells. Upon infectious stimuli LL-37 expression is increased and can reach extremely high concentrations. Its specific antimicrobial activity has been ascribed to interaction with negatively charged phospholipids that are especially frequent in the outer leaflet of bacterial plasma membranes.

We hypothesized that the polybasic antimicrobial peptide LL-37 would function as a CPP and have the capacity to deliver nucleic acids into mammalian cells at physiological concentrations.

Results

LL-37 has drastic effects on bacterial growth at concentrations (1-10 μM) where mammalian cell proliferation is unaffected, as shown by colony forming unit determination and crystal violet proliferation assay respectively.

LL-37 is capable of protecting DNA plasmid from nuclease activity present in serum. After 16 hours of incubation at 37°C in serum, agarose gel electrophoresis reveals that DNA plasmid is still largely intact in the presence of LL-37.

Polyplexes consisting of LL-37 and DNA (or HS) enter mammalian cells in an energy dependent manner. Both DNA internalization, measured by flow cytometry, and luciferase reporter gene expression increase with higher LL-37 concentrations at constant DNA concentrations. Confocal microscopy makes evident that polyplexes enter cells by means of endocytosis.

Treating cells with cholesterol depleting drugs, i.e. nystatin and β-MCD, inhibited both uptake of polyplexes and expression of delivered reporter gene. In combination with confocal microscopy studies revealing no co-localization with caveolin-1 but co-localization with CTxB, these results suggest a role for lipid raft mediated endocytosis excluding caveolae. Interestingly, expression of non-complexed reporter gene plasmid (i.e. the control) is increased in the presence of both cholesterol depleting drugs.

Both uptake of DNA and reporter gene expression are severely reduced in PG-deficient mutant cells.

To conclude, the cationic human antimicrobial peptide LL-37 has CPP features comparable to those of Tat at physiological and bactericidal concentrations. We speculate that peptides such as LL-37 may be involved in lateral gene transfer from bacteria to vertebrates.

Paper V

Secreted components induce DNA internalization in mammalian cells

A central role for PGs as carriers in the internalization of positively charged compounds has been indicated by numerous reports (reviewed in [205]; Papers I-IV). In parallel, cellular exchange of polybasic molecules has been suggested to be an important way to mediate intercellular communication. Regarding polyanions, highly sulfated HS species administered to cells show antiproliferative activity upon internalization and nuclear localization [304]. In plants, systemic translocation of RNA exerts control over plant development and defense [305, 306]. Moreover, administration of naked DNA plasmid results in high levels of transgene expression *in vivo* [307]. Separate discoveries thus suggest the existence of a cellular system for uptake of polyanions, and that this uptake has important physiological functions. Although several attempts have been made to characterize a cellular receptor for DNA, no convincing candidates have been presented [308]. The intriguing and counter-intuitive discovery that efficient uptake of naked polyanions is dependent on cell surface PGs led us to investigate the mechanism by which mammalian cells internalize naked polyanions.

We hypothesized that PG dependent uptake of administered polyanions is mediated by endogenously produced polybasic cellular factors.

Results

Naked, i.e. non-complexed, polyanions such as DNA and GAGs are internalized into CHO-cells. Internalization of the luciferase reporter gene results in low, but clearly detectable gene expression. Both internalization and expression are temperature dependent, saturable processes. The polyanion uptake mechanism

appears non-selective since DNA, low sulfated HS as well as highly sulfated HS are all taken up whilst displaying reciprocal inhibition. Degree of sulfation or net charge of the polyanion is important. Highly charged polyanion species (heparin like HS-6) readily inhibit uptake of less densely charged species (e.g. DNA), whereas the opposite situation affects uptake to a lesser extent, i.e. DNA does not easily inhibit HS-6 uptake. Competitive inhibition of uptake is also dependent on the co-operative effect of the many charges of an intact GAG chain. Degraded HS displays decreased inhibitory effect on DNA uptake as compared to intact HS chains, and disaccharides do not affect DNA uptake at all.

Although very much less prominent than when complexed to Tat or LL-37, internalized polyanions localize to vesicles, as shown by confocal microscopy.

Thus far, all characteristics of naked polyanion uptake mimic the characteristics of CPP-DNA polyplex uptake. Moreover, internalization is enhanced non-linearly after longer incubations and conditioned medium (CM) enhances uptake up to ten-fold over fresh medium. The stimulatory effect of CM on polyanion uptake is completely dependent on PGs, as shown by experiments with wild type CHO cells and PG-deficient CHO mutants (pgsA-745 and pgsB-618). Altogether, these results suggest the existence of endogenously produced factors with CPP features. The factors can be concentrated from CM using heparin substituted affinity columns, and are hence heparin binding compounds in analogy with polybasic CPPs. Sensitivity to boiling supports the notion of the factors being proteins or polypeptides. In analogy with Tat, CM components are capable of forming complexes with polyanions, as shown by fluorophor quenching experiments.

Exogenously added spermine did not induce polyanion internalization, excluding polyamines as the active components in CM.

CM-induced uptake was sensitive to actin disruption by latrunculin A, and cholesterol depletion by β-MCD. Amiloride treatment also inhibited uptake.

To conclude, we show that mammalian cells produce factors that bind to and induce PG dependent uptake of polyanions such as DNA and GAGs. Uptake occurs by means of endocytosis and results in reporter gene expression. More detailed studies are needed to determine the exact route of entry.

GENERAL DISCUSSION

The data presented in this thesis indicate the existence of a common internalization pathway for charged biopolymers, with cell surface PGs holding a key regulatory position. In agreement with previous studies on uptake of cationic lipid-DNA lipoplexes and PLL-DNA polyplexes (reviewed in [205] and [206]) we demonstrate the importance of cell surface PGs for efficient internalization and transfection of positively charged vectors. We however extend the emerging role of PGs as plasma membrane carriers beyond the realms of synthetic vector internalization. We show that PGs hold a key position in the internalization of polyamines, and that combined inhibition of polyamine biosynthesis and PG dependent internalization efficiently reduces proliferation of cancer cells *in vitro* and *in vivo* (Papers I and II). These findings have implications for the treatment of human cancer that may be closer to clinical use than first imagined. DFMO and suramin have been in clinical use for quite some time and their behavior and side effects are well known. In contrast to the rather moderate effects of DFMO in models where cancer is already established, DFMO has been shown to be a potent inhibitor of carcinogenesis. Chemoprevention trials with DFMO are therefore currently performed [303], a fact that illustrates that the drug is rather nontoxic and harmless. Any approach that awakes the anti-tumor potential of DFMO would therefore contribute a valuable tool with few side effects to the chemotherapy arsenal. Our results indicate that considerable reduction of tumor burden can be achieved by a combination regimen based on DFMO and an inhibitor of polyamine uptake, e.g. suramin or a polyamine analog such as Tat. The fact that administration of suramin (a heparin mimetic), HS or xylosides (that compete with HSPG synthesis) all reduce polyamine uptake and inhibit cellular proliferation dependent on exogenous polyamines [117], suggests that heparin may be an efficient anticancer drug when administered in combination with inhibitors of endogenous polyamine biosynthesis, e.g. DFMO. Indeed, heparin and low molecular weight heparin have been shown to have beneficial effects unrelated to their anticoagulant effect when administered to cancer patients. Clinical studies show prolonged survival and regression of primary tumors in patients with lung cancer. The effects have been related both to the development of primary tumors (e.g. effects on growth factors, angiogenesis and the immune system) and to the metastatic process (e.g. effect on cell adhesion and penetration of the ECM) [309]. With heparin entering the stage as an attractive anti-cancer drug, it may be time for DFMO to join in and reclaim its place as an anti-tumor agent.

In addition to their role in polyamine uptake, cell surface PGs regulate internalization of polyplexes. We show that the antimicrobial peptide LL-37 has

CPP features comparable to those of Tat (paper IV). Moreover, we demonstrate that mammalian cells have the ability to internalize naked (i.e. not pre-complexed) polyanions, which in the case of DNA results in reporter gene expression. Cellular ability to internalize naked polyanions is related to endogenous production of heparin binding factors that induce PG dependent polyanion internalization. Uptake of naked DNA *in vivo* has been demonstrated to be surprisingly efficient, whereas experiments in cell cultures have resulted in much lower yields [307]. In the light of the results presented in this thesis it may be speculated that part of this divergence arises from the differing levels of heparin binding factors present in the two situations. *In vivo,* DNA is delivered into an environment where endogenously produced factors are at equilibrium, whereas in the cell culture experiments pre-rinsing or medium change are likely to eliminate the bulk of such factors. Indeed, during short incubations in fresh medium, cell culture uptake of naked DNA is very low and is not facilitated by PGs (Paper V).

A voiced argument against polyplex delivery is the often applied non-physiological conditions that may induce events of questionable existence in biology. Studying uptake going from a situation with high concentrations of non physiological polyplexes (Tat and polyanion; Paper III), via polyplexes where the viral protein fragment is exchanged for an intact mammalian peptide (LL-37-polyanion; Paper IV) to a situation where a so far undefined endogenously produced cellular factor replaces the role of the previously administered CPP (Paper V) is not only an attempt to explain delivery of charged biopolymers but also a quest for physiological function of PG-dependent transport. The fact that internalization in all cases is highly PG dependent and mediated by means of endocytosis supports the view that polyplexes are hitch-hiking on a ubiquitous and preserved route for cellular uptake, a route that a plethora of viruses already utilize [213, 214, 216]. Especially the results presented in paper V support this notion, i.e. mammalian cells produce factors with CPP features capable of inducing reporter gene expression up to ten-fold. As mentioned before, endocytosis is central to a multitude of processes. It may be that PG-dependent uptake is just a reflection of basic endocytic aspects like feeding and scavenging. This view is supported by the general observation that the major part of internalized CPP material gets stuck in acidic vesicles (lysosomes?). However, PG dependent endocytosis seems to be more specific and functionally important than so. As discussed above, internalization of bFGF is dependent on PGs and important for optimal activity. Intercellular transport of proteins is essential to several cellular processes, e.g. embryogenesis and organogenesis [310]. Distribution of several morphogens, e.g. *wingless*, *hedgehog* and *decapentaplegic*, is dependent on cell surface HSPG [311-314]. Studies using the dynamin loss of

function *Drosophila* mutant *shibire* revealed that endocytosis is required for efficient *decapentaplegic* gradient formation [315]. The authors suggest that planar transcytosis is responsible for distribution of *decapentaplegic.* Moreover, they discuss the potential involvement of endocytosis in regulation of *wingless* and *hedgehog* gradient formation.

Not only proteins are transported between cells. MicroRNAs (miRNAs) are approximately 22 nucleotide long non-coding RNA molecules, believed to play important roles in gene regulation by functioning as negative regulators of specific target mRNAs, a type of RNA interference. In mammals, 250 putative miRNA genes have been characterized [316]. In plants, exciting results regarding systemic transport of miRNA and other small RNAs have been obtained. miRNAs expressed by a subpopulation of cells have been shown to traffic the entire plant, regulating protein expression in the organism as a whole [305]. To achieve this, miRNAs not only need to be exported from the donor cell but indeed internalized by the receiving cell. RNA interference was first discovered in *C.Elegans.* Systemic RNA interference can readily be induced in these nematodes, e.g. by feeding them bacteria expressing dsRNA [317]. Although not yet described, similar RNA transport systems may be present also in other eukaryotic families. Other models for intercellular delivery of molecules include the argosome theory. In this model, membrane fragments form exovesicles that are transported from cell to cell by means of endocytosis. The argosomes are thought to function in morphogen distribution and colocalize with *wingless*. Interestingly, argosome mediated distribution of *wingless* is inhibited by enzymatic degradation of HS [318].

These examples indicate that cellular systems for exchange of molecules are much more intricate and regulated than general feeding and scavenging mechanisms would suggest. Accumulating evidence indicate that HSPG dependent endocytosis is functionally important. More detailed knowledge on the mechanisms that control uptake in these physiological examples of delivery, alongside with insight into the entry of pathogens, are likely the means by which the scientific community will optimize applications like gene and drug delivery.

Specificity

A central aspect of CPPs in drug and gene delivery is specificity. Is it possible to target CPPs to specific subsets of cells in an organism? Which endocytic route is the most efficient one and how to specifically target one of the pathways? Concentrating on HSPGs as receptors for polybasic CPPs, the matter is still complex. HSPGs are present on the surface of virtually all mammalian cells. Protein binding to GAG chains can be very specific, as exemplified by the heparin-antithrombin interaction with a dissociation constant of 50 nM [319].

The sequence responsible for antithrombin binding is a penta-saccharide, as depicted in Figure 1 [319]. Loss of the 3-O-sulfate group in the central glucosamine unit increases the dissociation constant to well above 2 mM [319], illustrating that very specific modifications may be essential for HS-protein interactions. Specificity in protein binding is mediated by GAG modifications such as deacetylation, epimerization and sulfation, generating a high diversity of GAG sequences. Twenty-three different disaccharides have been identified in heparin, heparan sulfate, or as intermediates in biosynthesis. In theory, the potential number of disaccharides is 48 [4]. The GAG synthesis machinery is expressed in a cell type specific manner, suggesting that some specificity in terms of target cells may be reached [4]. The expression of GAG modifying enzymes is however regulated in response to external stimuli. We have previously reported that inhibition of endogenous polyamine synthesis induces synthesis of HS GAG chains with increased polyamine affinity [117]. The population of presented GAG sequences must therefore by considered somewhat dynamic.

Tat, on the other hand, seems to bind HS/Hep rather unspecifically, with several sites on each GAG chain [320, 321]. Ziegler and Seelig conclude that the binding capacity, i.e. the number Tat units per molecular weight of HS/HEP, roughly follows the extent of sulfation. Accordingly, they report that a 1 kDa HS fragment binds 0.46 Tat, whereas a 1 kDa Hep fragment binds 0.69 Tat [321]. Although promiscuous, Tat interaction with HS/Hep is rather strong. The affinity is however also dependent on degree of sulfation, with Kd values of ~1 µM and 30 nM reported for low sulfated HS and Hep respectively [320, 321]. From this it follows that specificity in terms of target cells can hardly be reached by the use of CPPs like Tat.

A large number of proteins containing grouped positive charges have affinity for GAGs [70]. This fact is utilized when using heparin substituted sepharose to isolate DNA binding proteins. The presented consensus sequences [70] imply that almost any arginine or lysine rich peptide would interact with PGs. In our hands, polybasic peptides (Tat, LL-37, protamine, a polybasic prion peptide and the M1 peptide from S. pyogenes [322]) with the ability to form complexes with polyanions also induce PG dependent uptake of the polyanion (Papers III and IV; unpublished observations). Efficient internalization is in all cases dependent on a surplus of positive charges over negative charges. Indeed, the same charge ratio dependence for efficient polyplex uptake has been observed previously [257-259], supporting the view that proper interactions between polyplexes and cell surface HSPG are needed to induce internalization. The common internalization mechanism for arginine rich peptides proposed by Suzuki *et al.* [294] does not only apply to the peptides per se, but also to polyplexes containing these peptides.

Is it perhaps the abundance of cell surface HSPG that makes the PG dependent internalization mechanism seem efficient? For example, the surface of endothelial cells has been estimated to carry as many as one million syndecan-1 molecules [323]. As a comparison, primary endothelial cells in culture have been reported to have about 100.000 high affinity binding sites for transferrin [324]. Transferrin has been applied as a targeting ligand to deliver for example anticancer agents, proteins, and genes into cells that express transferrin receptors [325]. If a typical syndecan-1 contains three HS chains and each HS chain is capable of binding about six Tat peptides [321], the number of sites for Tat to bind cell surface HSPG would be at least two orders of magnitude higher than the number of sites for high affinity transferrin binding. The dissociation constant for transferrin binding to the transferrin receptor is on the other hand two orders of magnitude lower than the dissociation constant for Tat and HS interaction [321, 326]. The dissociation constant for both interactions is however at or lower than 1 μM. When studying CPP internalization, concentrations in the interval 0.1 μM – 10 μM are often used [278], meaning that Tat binding to the cell surface would be up to two orders of magnitude more frequent than transferrin binding to the cell surface.

Polybasic CPPs such as Tat do evidently posses the capacity to deliver biologically active molecules to the inside of cells both *in vitro* and *in vivo*. However, target cell specificity may be hard or impossible to achieve, as discussed above. Can CPP mediated delivery nevertheless be used in treatment of specific cells, e.g. the cells of a tumor? One approach may be to deliver proteins that restore loss of function in the tumor cells, e.g. tumor suppressors like pRb and p53 [244]. Another approach may be to utilize various gain of functions that arise in tumor cells, e.g. tumor cell overexpress matrix metalloproteinases (MMPs) and heparanase as a means to breach the ECM. Tsien and coworkers have developed a model where a CPP is blocked by a polyanionic stretch linked to the CPP by a cleavable linker, e.g. containing a MMP cleavage site. In the presence of MMPs, as in a tumor, the linker will be cleaved and the CPP released. If a relevant payload is also coupled to the CPP, it will in theory be specifically delivered to tumor cells [327]. Yet another method is to make use of the fact that the intratumoral milieu may be different from the rest of the organism. The inside of tumors is generally thought to be hypoxic. A Tat-caspase-3 construct supplemented with the (oxygen-dependent) degradation domain of hypoxia-inducible factor-1 has shown increased stability in hypoxic cell cultures. Moreover, degradation of the construct *in vivo* was markedly increased in normal tissue, whereas accumulation of the construct was observed in hypoxic tumor regions [249]. To conclude, various degrees of specificity may be obtained by careful design of CPP constructs.

Specificity in terms of endocytic route

Does CPP interaction with cell surface components induce internalization? Clustering of syndecan-4 by binding of the ligand bFGF has been shown to induce internalization of both syndecan and bFGF [219]. Clustering of lipid rafts into larger structures, e.g. induced by ligand binding to GPI-anchored proteins, has been suggested as a way to induce internalization [166]. Using latex beads coated with antibodies directed at HS, it was found that HSPG clustering induces endocytosis [328]. It may be speculated that binding of polybasic compounds induces cell surface HSPG clustering by simultaneous interaction with side chains from different HSPG monomers. In the case of syndecan-4 this would result in intracellular kinase activity and downstream events leading to internalization, whereas in the case of glypican clustering would induce lipid raft mediated endocytosis (see Figure 2). In the case of polyplexes exhibiting positive net charge, clustering of cell surface HSPG is even more plausible due to the vast number of possible GAG binding sites per polyplex unit. This model would explain the common internalization mechanism for arginine rich or polybasic peptides. It also gives a plausible explanation to the observed induction of endocytosis and uptake of non-conjugated and non-complexed proteins in the presence of polybasic proteins and peptides [277, 289].

Can the fact that CPPs and CPP containing formulations enter cells dependent on cell surface HSPG tell us something about the specificity of the endocytic route involved? As mentioned, GAG synthesis is cell type specific rather than core protein specific, i.e. HS on glypicans are the same as HS on syndecans. However, the topographical localization of GAG chains on these two PG species differ. Glypican HS chains are attached to the core protein in close vicinity to the plasma membrane and syndecan HS chains are attached at more peripheral sites. HS binding ligands may therefore be exposed to syndecan HS prior to glypican HS. Unless this or other unknown facts are of vital importance, administered CPPs should be distributed to the GAG chains of all cell surface PG species. If so, what does that imply in terms of internalization route? Due to their GPI-anchor attachment glypicans are supposed to reside in membrane lipid rafts and thought to be internalized via lipid raft and caveolae mediated endocytosis. Syndecans normally reside in the non-lipid raft membrane fraction but (at least in the case of syndecan-4) seem to re-distribute to the lipid raft fraction upon ligand induced clustering. Both species thus potentially utilize the raft/caveolae pathway. Macropinocytosis has also been suggested as a way of entrance for bFGF-syndecan-4 complexes [219]. Moreover, a recent study suggests that internalization of Tat involves both PGs and clathrin dependent endocytosis [278]. It may thus be speculated that internalization via cell surface HSPG will be distributed across the entire range of endocytic routes depending on 1) the subset

of PG core proteins present; and 2) the preference of these core proteins for specific endocytic pathways. This may explain the differing results obtained as of late regarding which internalization route Tat uses when entering cells via HSPG. Indeed, clathrin mediated endocytosis; caveolar/lipid raft mediated endocytosis; and macropinocytosis have all been suggested as the PG dependent route of entry for Tat [277, 278, 284, 285]. In this perspective, it may well turn out that the exact internalization pathway is of little importance and that the key to optimizing delivery is endosomal escape.

The term CPP implies that the action of internalization is an inherent feature of the peptide and not the receiving cell, excluding mechanisms such as endocytosis. Since one of the most commonly used CPPs, Tat, has been shown to enter cells by means of endocytosis the term CPP can only be accepted if the word penetration is used in its most lax meaning.

FUTURE PERSPECTIVES

The CPP field has developed at a remarkable pace and has surely had its share of revolving discoveries as of late. Due to methodological pitfalls in early studies, many groups have had to re-evaluate part of their work. But for all that, or perhaps because of it, the number of exciting questions has rather multiplied than declined. The implications for drug and gene delivery are still to be fully investigated and will likely result in the production of new treatment strategies for the benefit of the patient. Regarding gene delivery, approaches that aim at constructing synthetic viruses with defined characteristics and controlled interaction with both serum components and target cells, are under development [329]. Moreover, research on CPP mode of internalization has brought focus onto endocytic pathways and the physiological aspects of macromolecular delivery. Especially intriguing is the possible existence of intercellular communication via regulated transfer of nucleic acids.

A central question in the CPP field is as discussed the mode of entry. Although the field has shifted so as to generally accept the notion that polybasic CPPs enter cells by PG dependent endocytosis [205, 206], the exact mechanism and the specific molecules involved remain to be determined. As mentioned, virtually all known endocytic pathways have been suggested as the one responsible for CPP internalization. An approach that might finally answer several of the remaining questions may be to isolate endocytic vesicles and to characterize their molecular components. Do isolated vesicles belong to a certain subspecies of endosomes? Do they contain a certain subspecies of PGs? Is the lipid composition different to that of the regular plasma membrane? Or is it perhaps so that the vesicles are a heterogeneous population containing representatives of all cell surface PGs and lipid compositions? Our next step will thus be to isolate vesicles for proteomic analysis of their contents. We plan to do so using magnetic nanoparticles coupled to the cargo or the peptide itself. So far, we have tested a polyplex approach using magnet-conjugated DNA complexed with Tat as depicted in Figure 7. After a defined incubation time, cells are harvested. Internalization of magnetic polyplexes is evident (A and B) and extensive enough to easily allow for magnetic isolation of cells that have taken up complexes, with close to zero yield of cells that have not internalized magnetic polyplexes (C). Importantly, internalization of magnet-containing polyplexes is strictly PG dependent (C). Electron micrographs reveal vesicular localization of internalized magnetic polyplexes (D). Magnetically isolated cells are then subjected to nitrogen cavitation, which disrupts cell integrity leaving vesicular compartments largely unaffected. Upon disintegration of cells, magnetic vesicles are obtained by magnetic isolation (E). To asses the integrity of isolated vesicles

and the purity of the samples, electron microscopy will be employed. Pure isolates will then be subjected to extensive molecular characterization (F).

Regarding our findings on the role of PGs in cancer therapy, we extend our investigations to include the role of HSPGs in angiogenesis. Angiogenesis is central to tumor growth. Moreover, PGs are intimately connected with the process of angiogenesis, e.g. via the actions of HS binding growth factors like VEGFs and FGFs [330, 331]. In more long term, we shall capitalize on our discovery that PGs mediate polyplex internalisation to deliver bioactive macromolecules to tumor vasculature with the ultimate goal to achieve anti-tumor effects in cancer patients.

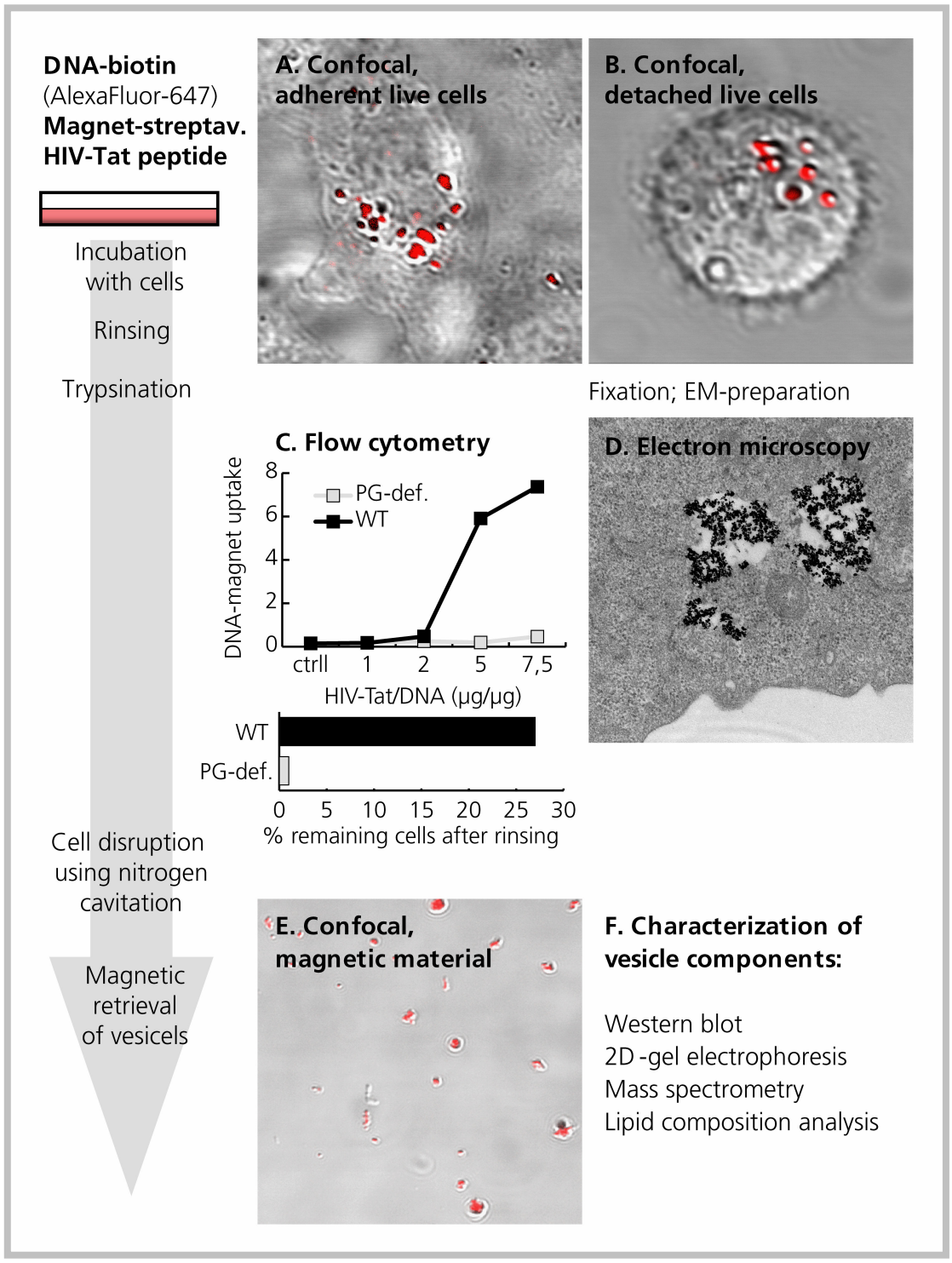


Figure 7: General approach for isolation and characterization of cargo-containing vesicles. Biotinylated DNA-AlexaFluor-647 is rendered magnetic by the addition of streptavidin conjugated magnetic particles. Polyplexes are formed by the addition of Tat. Magnetic and fluorescent polyplexes are incubated with cells and then subjected to: confocal microscopy before (A) and after (B) trypsination; flow cytometry (C); and electron microscopy (D) to assess the characteristics of polyplex uptake. Magnetically purified cells are then disrupted by nitrogen cavitation. After yet another magnetic separation step, the purity of the sample is determined by confocal microscopy (E) and electron microscopy. Vesicle isolates will then be subjected to extensive molecular characterization (F).

SAMMANFATTNING PÅ SVENSKA

Människans kropp är uppbyggd av ett stort antal celler. Celler är komplexa och känsliga maskinerier vars olika processer måste skyddas dels från varandra, dels och kanske framförallt från en omvärld vars inverkan hade varit omedelbart förödande för en ”oskyddad” cell. Våra celler har därför ett stort antal barriärer och portar som försäkrar att rätt saker befinner sig på rätt plats och förhindrar att objudna gäster tar sig in. Den allra mest framträdande barriären hos en cell är dess yttre skal som kallas plasmamembranet. Detta skal är i sitt grundutförande i stort sett ogenomträngligt för de flesta vattenlösliga ämnen. Emellertid behöver celler kunna ta upp en stor mängd olika sorters ämnen från sin omgivning, bland annat för att tillgodose sitt näringsbehov, för att bekämpa bakterier och för att kommunicera med varandra. Därför finns ett antal mekanismer, eller portar, för transport genom plasmamembranet och in i cellen. För små ämnen räcker det med pumpar och kanaler, men för större ämnen behövs mer avancerade processer som kallas cellätande (phagocytos) och celldrickande (pinocytos). Alla dessa kontrollerade vägar in i cellen behövs för att en cell skall må bra och fungera optimalt.

Barriärerna och portarna har dock betydelse för mer än cellernas vanliga liv. Det visar sig nämligen att bakterier, virus och en mängd gifter har lärt sig att ta sig in i våra celler genom deras olika portar. Genom årmiljonerna har det pågått en ständig kamp mellan olika parasiters försök att ta sig in och cellernas försök att förhindra intrång, och båda sidor har utvecklat en mängd metoder i sina försök att vinna striden. Exemplen på parasiter som tar sig in i celler genom att utnyttja cellernas egna portsystem är otaliga och inkluderar bland annat vanligt influensavirus, HIV-virus, koleratoxin (det gift som produceras av kolerabakterien) och klamydia-bakterien.

Också för läkemedelsbehandling är cellernas barriärer och portsystem av stor betydelse. Läkemedel som är verksamma inuti celler är föga effektiva om de inte lyckas ta sig förbi plasmamembranet. För läkemedelsföretag är därför kunskap om de olika portsystemen och möjligheterna att utnyttja dessa av stor betydelse för konstruktion av effektiva läkemedel. Omfattande resurser investeras på ett område som på svenska skulle kallas läkemedelsleverans (drug delivery). Inte minst gäller detta det område som kallas genterapi där läkemedlet som skall levereras utgörs av genetiskt material.

Denna avhandling studerar hur stora, positivt laddade molekyler tas upp av celler via ett portsystem som innehåller en typ av molekyler som kallas proteoglykaner. Genom att studera celler som saknar proteoglykaner och jämföra med vanliga celler, kan vi visa att proteoglykaner är helt avgörande för att detta portsystem skall fungera. Upptaget sker via en mekanism som involverar bildning

av små fickor i plasmamembranet som sedan knips av och blir små blåsor (endosomer). Dessa blåsor transporteras sedan inåt i cellen och en del av det material som tagits upp anländer slutligen till cellens kärna.

Bland de positivt laddade ämnen som normalt tas upp via det här portsystemet finns en grupp ämnen, polyaminer, som har stor betydelse för cancer-tumörers tillväxt. Genom att manipulera proteoglykanerna med hjälp av olika läkemedel kan vi minska funktionen i portsystemet, vilket får till följd att upptaget av polyaminer sänks och tillväxthastigheten av tumörer minskar.

Vi har också studerat huruvida detta proteoglykan-beroende portsystem kan användas för genterapi. Genom att utnyttja kunskaper om hur HIV tar sig in i och aktiveras i celler har vi utvecklat en metod där genetiskt material (DNA) levereras in i celler. DNA paketeras med hjälp av ett HIV-protein och paketet erhåller då egenskaper som gör det lämpligt för transport via det proteoglykan-beroende portsystemet.

Denna princip har vi sedan utnyttjat för att visa att också kroppens egna celler kan producera ämnen som har förmågan att leverera DNA in i celler - återigen via samma proteoglykanberoende portsystem. Om detta är något som normalt sker i celler kan det vara ett betydelsefullt och hittills outforskat sätt för kroppens olika celler att kommunicera med varandra. Det kan också vara en förklaring till varför den mänskliga arvsmassan innehåller runt 200 gener av bakteriellt ursprung.

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