Role of Heparan Sulfate Structure in FGF-Receptor Interactions and Signaling

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Abstract

Heparan sulfate (HS) belongs to the glycosaminoglycan family of polysaccharides and is found attached to protein cores on cell surfaces and in the extracellular matrix. The HS backbone consists of alternating hexuronic acid and glucosamine units and undergoes a number of modification reactions creating HS chains with alternating highly and low modified domains, where high degree of modification correlates with high negative charge. Fibroblast growth factors (FGFs) and their receptors (FRs) both bind to HS, which affect formation of the FGF–FR complexes on the cell surfaces. Activated FRs can trigger several intracellular signaling pathways leading thereby to diverse cellular responses.

Work presented in this thesis focuses on the effect of HS and its structures on FGF–FR complex formation and FGF-induced signaling. Studies with short, highly modified oligosaccharides and FGF1 and 2 combined with FR1c, 2c, 3c or 4 showed a correlation between the overall degree of modification and amount/stability of FGF–FR complexes. Our findings imply that several HS structures, differently modified but with the same negative charge density are equal in their ability to support complex formation. Co-application of oligosaccharides with FGF2 to HS-deficient cells and investigation of the thereby induced cell signaling confirmed our findings with a cell-free system. The oligosaccharide with the highest modification degree displayed the biggest impact on cell signaling, which was FGF2 concentration dependent. Studies with long HS polysaccharides with preserved high and low modified domains suggest that the proportion between these two types of domains and also the structure of the low modified domains are of importance for the FGF–HS–FR complex formation and cell activation capacity.

This work illuminates several aspects in how HS structure influences the interplay between FGFs and FRs and contributes to the understanding of what factors affect a cell’s response following FGF stimulation.

*Keywords:* heparan sulfate, oligosaccharide, fibroblast growth factor, fibroblast growth factor signaling

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Abbreviations

Akt  Protein kinase B
CS   Chondroitin sulfate
DAG  Diacylglycerol
DS   Dermatan sulfate
EXT  Exostosis
ERK1/2 Extracellular signal-regulated kinases 1 and 2
FGF  Fibroblast growth factor
FR   Fibroblast growth factor receptor
FRS2 Fibroblast growth factor receptor substrate 2
GAG  Glycosaminoglycan
Gal  Galactose
GlcA  Glucuronic acid
GlcNAc N-acetylglucosamine
GlcNS N-sulfated glucosamine
GPI  Glycosylphosphatidyl inositol
HS   Heparan sulfate
IdoA Iduronic acid
Ig   Immunoglobulin
IP3  Inositol-3-phosphate
MAPK Mitogen activated protein kinase
MEK1/2 Mitogen activated protein kinase/extracellular
       signal-regulated kinases 1 and 2
NA   N-acetylated
NS   N-sulfated
NDST N-deacetylase/N-sulfotransferase
PI3K Phosphatidylinositol-3-kinase
PIP2 Phosphatidylinositol-2-phosphate
PIP3 Phosphatidylinositol-3-phosphate
PLCγ Phospholipase Cγ
PG   Proteoglycan
PKC  Protein kinase C
SOS  Son of sevenless
UDP Uridine-5'-diphosphate
Xyl  Xylose
Heparan sulfates (HSs) are anionic polysaccharide chains linked to protein cores, found on cell surfaces and in the extracellular matrix. Through interactions with a large number of protein ligands, HS chains influence many biological events. Fibroblast growth factors (FGFs) are a family of polypeptides, which bind to and activate cell surface FGF receptors (FRs). Activated FRs trigger intracellular signaling, which can lead to proliferation, migration, apoptosis or differentiation. HS is an interaction partner of both FGFs and FRs and is required for efficient activation of FRs, affecting thereby FGFs’ actions.

The aim of the present work has been to investigate what HS structures support FGF–FR complex formation and what effect they have on FGF-induced signaling. Present findings suggest that formation of FGF–FR complexes and subsequent signaling are differently affected by diverse HS structures, where the charge density and the general chain structure is of importance.
Background

Proteoglycans

Proteoglycans (PGs) are proteins with covalently attached unbranched polysaccharide chain(s), which belong to the glycosaminoglycan (GAG) family (Kjellén and Lindahl, 1991). A GAG chain is composed of repeating disaccharide units consisting of a hexosamine and a hexuronic acid. Galactosaminoglycans, chondroitin sulfate (CS) and dermatan sulfate (DS), are composed of alternating galactosamine and glucuronic (GlcA) or iduronic acid (IdoA) units (Sugahara et al., 2003). Glucosaminoglycans, heparan sulfate (HS) and heparin, are composed of alternating glucosamine (GlcN) and GlcA or IdoA units (Lindahl et al., 1994). Keratan sulfate is also a glucosaminoglycan, but carries galactose (Gal) units instead of hexuronic acid (Funderburgh, 2000). Hyaluronan is a protein-free GAG composed of GlcN and GlcA (Evanko et al., 2007). All GAGs except hyaluronan are subjected to modifying sulfation reactions rendering the chains strongly anionic.

PGs are essential and abundant components of the extracellular matrix, but they are also found on cell surfaces and in intracellular vesicles. Their functions range from mechanical support to effects on cell proliferation, migration as well as tissue developmental events (Kjellén and Lindahl, 1991).

Heparan sulfate proteoglycans

PGs carrying HS or heparin chains are called HSPGs. The major families of HSPGs are the transmembrane type syndecans, the glycosylphosphatidyl inositol (GPI) linked glypican (Bernfield et al., 1999) and the extracellular perlecan, collagen XVIII and agrin (Iozzo, 2005). The only known heparin PG is serglycin, which is synthesized in mast cells and is stored in intracellular granules (Kolset and Tveit, 2007).

The syndecans are a family of four members, syndecan-1 to -4. A syndecan consists of a short conserved cytoplasmic domain, a transmembrane domain and an extracellular domain with three to five attachments sites for HS or CS (Fig. 1A). Due to the cytoplasmic domain, syndecans have interac-
tion partners not only outside the cells but also inside, which affect the cytoskeletal organization, cell adhesion properties and signal transduction events (Tkachenko et al., 2005).

The glypican family consists of six members, glypican-1 to -6. They are linked to the cell membrane through a GPI-anchor; carry three to four HS attachment sites close to the membrane and a globular domain further away from the membrane (Fig. 1B). Glypicans affect growth factor signaling and play a role in cellular uptake of small compounds with net positive charge (Fransson et al., 2004). Although the cell surface HSPGs have common features, they display protein core dependent differences. The differences apply to degree of shedding of the extracellular domains from the cell surfaces, kinetics of turnover and the following fate of the co-internalized ligands and differences in protein core binding ligands (Bernfield et al., 1999).

![Figure 1. Schematic depiction of HS/heparin proteoglycans.](image)

The extracellular HSPGs are found at the periphery of cells in the basement membranes throughout the body. Perlecan has a protein core of size ~470 kDa, which makes it to one of the largest single-chain polypeptides found in vertebrate and invertebrate animals (Fig. 1C). It contains three HS attachment sites in the N-terminus and one in the C-terminus (Iozzo, 2005). Collagen XVIII combines properties of a collagen and a PG; it has several collagenous triple-helical domains interrupted by non-collagenous parts carrying in total three HS chains. Proteolytic cleavage of the C-terminal non-collagenous domain releases endostatin, an HS-binding anti-angiogenic factor (Marneros and Olsen, 2005). Agrin also has a very large protein core
(~225kDa) and carries three HS chains. This PG has several isoforms in different tissues (Bezakova and Ruegg, 2005). Each of these PGs has a number of important biological functions, ranging from maintenance of basement membrane homeostasis to effects on angiogenesis (Iozzo, 2005) and neuromuscular junctions (Bezakova and Ruegg, 2005).

The only intracellular PG, serglycin, is found in connective tissue type mast cells in storage granules. It has a small protein core, which is heavily substituted with heparin chains (Fig. 1D). Heparin chains bind histamine and proteases helping thereby in retaining these inflammatory mediators in the granules (Kolset and Tveit, 2007)

Heparan sulfate biosynthesis

Heparin and HS chains are synthesized by a number of enzymes located in the Golgi apparatus (Bernfield et al., 1999; Esko and Lindahl, 2001; Esko and Selleck, 2002). The synthesis is initiated by transfer of xylose (Xyl) from uridine-5’-diphosphate (UDP)-Xyl to a serine residue in a core protein. Stepwise attachment of two Gal molecules and GlcA forms the tetrasaccharide ‘linkage region’, GlcA-Gal-Gal-Xyl (Fig. 2A). Up to this point heparin and HS share the biosynthetic machinery with CS and DS. The transfer of the next sugar residue, either N-acetylglucosamine (GlcNAc) or N-acetylgalactosamine, determines whether the GAG will become a glucosaminoglycan or a galactosaminoglycan. Alternating addition of GlcA and GlcNAc monosaccharide units from their respective UDP-sugar nucleotides forms the HS chain (Fig. 2A–B). The chain elongation reactions are performed by an HS polymerase consistent of two gene products, the exostosis related enzymes EXT1 and EXT2 (Zak et al., 2002). The length of the HS chain is generally 50–150 disaccharides.

While the (GlcA-GlcNAc)n polymer is being synthesized it is subjected to a series of sequential enzymatic modification reactions in which the product of one reaction is the substrate for the next (Fig. 2A). The initial modification is replacement of the N-acetyl group of GlcNAc residues with a sulfate group forming an N-sulfated GlcN (GlcNS) performed by N-deacetylasen sulfotransferases (NDSTs) (Kjellén, 2003). Consequently, some of the GlcA residues next to GlcNS residues are epimerised at C5 to IdoA by C5-epimerase (Li et al., 1997) and a part of them are O-sulfated at C2 by 2-O-sulfotransferase to IdoA2S (Bai and Esko, 1996) (Fig. 2B). Finally, a number of GlcNS and a few of GlcNAc residues are O-sulfated at C6 by 6-O-sulfotransferases to GlcNS6S and GlcNAc6S, respectively (Habuchi et al., 2000). The most rare modification reaction is O-sulfation at C3 of GlcNAc, GlcNS or GlcNH+ by 3-O-sulfotransferases (Shworak et al., 1999). All modification enzymes except C5-epimerase and 2-O-sulfotransferase have several isoforms.
Figure 2. Scheme of heparin/HS biosynthesis and domain organization. (A) Biosynthesis. Components of the ‘linkage region’ GlcA-Gal-Gal-Xyl are added to a serine residue of the core protein, followed by alternating addition of GlcNAc and GlcA. The chain is then modified by a number of enzymes creating polysaccharides with distinct modification patterns, which depend on the cell type. (B) Structure of the unmodified disaccharide: GlcA\(^{\beta1-4}\)GlcNAc (white circle and square). Example of a strongly modified disaccharide: IdoA\(^{2S}\)GlcNS-6S,3S (grey circle and square with black dots). (C) Domain organization. The upper structure represents heparin and the lower HS. Consecutive disaccharides with \(N\)-sulfated GlcN constitute the NS-domains. Unmodified stretches are NA-domains, whereas alternating \(N\)-acetylated and \(N\)-sulfated disaccharides make up the NA/NS-domains.

Heparin is extensively and rather homogenously \(N\)- and \(O\)-sulfated and is characterized by a high IdoA/GlcA ratio (Fig. 2C). HS on the contrary is subjected to substantially fewer modifications, which are clustered into
highly sulfated domains (NS-domains) interrupted by unmodified stretches (NA-domains) and alternating domains (NA/NS-domains) (Lindahl et al., 1994) (Fig. 2C). The biosynthesis scheme leads to an enormous structural heterogeneity that seems to be cell and tissue specific (Maccarana et al., 1996; van Kuppevelt et al., 1998; Ledin et al., 2004). In most studies dealing with functions of HS, heparin is used as a substitute because it is much more easily available.

Role of heparan sulfate proteoglycans *in vivo*

*In vivo* functions of HSPGs have been studied using genetically modified invertebrates, *Danio rerio* (zebrafish), *Drosophila melanogaster* (fruit fly), *Caenorhabditis elegans* (nematode) and *Xenopus Laevis* (frog) (Bułow and Hobert, 2006). Functions of HSPGs in mammalian physiology have been approached by creating a number of transgenic and knockout mice (Bułow and Hobert, 2006; Bishop et al., 2007), which will be focused on below.

Loss of the HS polymerization enzymes, Ext1 (Lin et al., 2000) or Ext2 (Stickens et al., 2005) in mice, leads to lack of HS and cause embryonic lethality showing that HS is essential for gastrulation and early embryonic development. Disturbed morphogen gradients and thereby abnormal morphogen signaling is a possible explanation for such severe effects. Knockout mice lacking Ndst1 (mammals have four NDST isoforms), the enzyme performing the first HS modification step (Fig. 2A), die perinatally due to respiratory failure (Ringvall et al., 2000) and exhibit severe developmental defects of the forebrain and forebrain-derived structures (Grobe et al., 2005). Loss of Ndst2 leads to abnormal mast cells caused by defective heparin in the HSPG serglycin (Forsberg et al., 1999).

Inactivation of the single isoform enzymes C5-epimerase (Li et al., 2003) and 2-O-sulfotransferase (Bullock et al., 1998) results in perinatal lethality, lack of kidneys and skeletal malformations. The 6-O-sulfotransferases have three and the 3-O-sulfotranferases six isoforms in mice, respectively. Knockout mice lacking 6-O-sulfotranferase-1 (Izvolsky et al., 2008) and 3-O-sulfotranferase-1 (Shworak et al., 2002) are viable and have substantially milder phenotypes, such as growth retardation, suggesting overlapping functions or compensatory mechanisms of the various isoforms.

An alternative approach for illuminating HSPGs functions has been to remove the various HS protein cores. Mice lacking syndecan-1 (Alexander et al., 2000), -3 (Reizes et al., 2001) or -4 (Echtermeyer et al., 2001) are all viable and exhibit milder defects, such as abnormal feeding behaviour (syndecan-3) and delayed wound healing (syndecan-4). The glypican-3 deficient mice display developmental overgrowth, perinatal death, kidneys and lungs affected (Cano-Gauci et al., 1999). Inactivation of the extracellular PGs perlecan (Arikawa-Hirasawa et al., 1999) and agrin (Gautam et al., 1996) lead
to perinatal lethality due to general severe developmental defects (perlecan) and disruption of neuromuscular junctions (agrin). Mice lacking collagen XVIII are viable and have specific eye developmental defects (Fukai et al., 2002). All these studies illustrate how many general and specific functions various HSPGs have and how important the presence of correctly modified HS is for normal development.

In humans, mutations in HS biosynthetic enzymes and protein cores have been associated with several disorders. Heterozygous null mutations in EXT1 (Ahn et al., 1995) and EXT2 (Stickens, et al., 1996) cause exostosis, formation of bony outgrowths on long bones. The Simpson-Golabi-Behmel syndrome is caused by mutations in the glypican-3 gene and is characterized by pre- and postnatal overgrowth with visceral and skeletal abnormalities (Pilia et al., 1996). Mutations in perlecan have been linked to the Schwartz-Jampel syndrome with skeletal and eye defects (Nicole et al., 2000), whereas mutations in collagen XVIII are the cause of Knobloch syndrome, where patients have ocular abnormalities leading to blindness (Sertié et al., 2000).

Heparan sulfate–protein interactions

The multitude of biological processes affected by HSPGs is thought to be mainly due to HS–protein interactions. The list of proteins, which can interact with HS, is long and includes among others growth factors (e.g. fibroblast growth factors, vascular endothelial growth factors, hepatocyte growth factor), cytokines (e.g. interleukins, tumor necrosis factor α, platelet factor 4), enzymes (e.g. lipoprotein lipase), protease inhibitors (e.g. antithrombin III) and extracellular matrix proteins (e.g. fibronectin, laminin, thrombospondin) (Capila and Linhardt, 2002; Whitelock and Iozzo, 2005). HS binding sites in proteins often contain clusters of basic residues (lysines and arginines), which create a positive electrostatic potential and attract negatively charged HS chains (Hileman et al., 1998). Binding of growth factors to HS promotes growth factor–receptor complex formation and affects thereby the subsequent signaling events (Powers et al., 2000; Stringer, 2006). HS may localize bound cytokines to various extracellular compartments and affect the activity of these molecules (Mulloy and Rider, 2006). HS binding to lipoprotein lipase affects activity of the enzyme and thereby clearance of lipoproteins from the circulation (van Barlingen et al., 1997). Antithrombin III on the other hand becomes a much more powerful inhibitor of thrombin when bound to heparin (Bourin and Lindahl, 1993). Besides being an important mediator in many physiological events, HSPGs also act as coreceptors for many pathogens, e.g. Leishmania spp., Plasmodium falciparum, human immunodeficiency virus, herpes simplex virus and Helicobacter pylori (Rostand and Esko, 1997).
The polysaccharide structures providing interaction sites for proteins and HS binding surfaces on proteins have been intensively studied using a number of different approaches (Capila and Linhardt, 2002; Powell et al., 2004; Imberty et al., 2007). The HS structures required for the different proteins have been found to vary in length and sulfation pattern. In a few cases a specific, well-defined HS structure is needed, where minor changes lead to loss of binding, e.g. interactions with antithrombin III (Bourin and Lindahl, 1993) and herpes simplex virus type-1 gD protein (Shukla et al., 1999). For many interactions studied a number of similar HS structures fulfil binding requirements (Kreuger et al., 2006; Gorsi and Stringer, 2007).

Fibroblast growth factors

Fibroblast growth factors (FGFs) are polypeptide growth factors found in multicellular organisms ranging from the nematode Caenorhabditis elegans to the human. In humans FGFs make up a large family of 22 members, divided into 7 subfamilies based on phylogenetic analysis (Itoh, 2007). These growth factors are 17–34 kDa in size and share 13–71 % amino acid identity. Members of the FGF family are expressed throughout life and are needed for successful mesenchymal–epithelial communication and organogenesis during development. FGFs are for example involved in the development of the nervous system (Mason, 2007), kidneys (Bates, 2007), lenses (Robinson, 2006) and skeleton (Su et al., 2008). In the adult organism they regulate tissue homeostasis and affect processes such as wound healing and tissue repair (Ornitz and Itoh, 2001; Alzheimer and Werner, 2002; Werner and Grose, 2003).

Mice knockout studies with a number of FGF family members have shown that lack of some FGFs, such as Fgf4 (Feldman et al., 1995) or Fgf8 (Meyers et al., 1998) result in embryonic lethality. Lack of several other members give no or only mild phenotypes, illustrated by Fgf1/− (Miller et al., 2000) and Fgf2/− mice (Ortega et al., 1998), respectively. Analysis of mice with mild phenotypes show, that some FGFs have very specialized biological functions (Fgf5/− mice have long hair (Hebert et al., 1994)), whereas other FGFs affect a multitude of properties (Fgf2/− mice have neuronal and cardiovascular phenotypes (Ortega et al., 1998; Zhou et al., 1998)).

Mutations in several members of the FGF family have been linked to human hereditary diseases, where in all cases except FGF23 these are due to loss-of-functions. Michel aplasia, where congenital deafness is one of the characteristics, is caused by mutations in FGF3 (Tekin et al., 2007). Aplasia of lacrimal and salivary glands and lacrimo-auriculo-dento-digital syndrome are both caused by FGF10 mutations (Entesarian et al., 2005; Milunsky et al., 2006). One type of spinocerebellar ataxia is linked to mutations in
FGF14 (van Swieten et al., 2003). Mutations in FGF23, a FGF controlling phosphate and vitamin D homeostasis, result in phosphate wasting disorders (Yu and White, 2005).

Fibroblast growth factor receptors

FGFs exert their biological actions by binding, dimerizing and activating cell-surface FGF receptors (FRs) (Powers et al., 2000). FRs are tyrosine kinase receptors containing an ectodomain consisting of three extracellular immunoglobulin (Ig)-like domains (I-III) with an ‘acidic box’ between IgI and II, a transmembrane helix and two cytoplasmic kinase domains (I-II) (Fig. 3A). The FR gene family is made of four members (1, 2, 3 and 4) of which FRs 1, 2 and 3 are subjected to alternative splicing. This results in receptors having two (II-III) or three (I-III) Ig-like domains and two variants of the Ig-like III-domain, IIIb and IIIc (Johnson and Williams, 1993).

![Figure 3. Schematic picture of a FR (A) and a model of a ternary complex involving FGFs, HSPG and FR molecules (B).](image)

FGFs bind with variable selectivity to the different variants of FRs. FGF1 is the only FGF capable to activate all receptor types, whereas FGF10 is a good activator of only FR2IIIb (Zhang et al., 2006). Ig-like domains II and III and the linker between them are responsible for ligand binding and specificity, i.e. what FGFs can bind to a certain receptor (Yeh et al., 2003; Mohammadi et al., 2005). Ig-like domain I and the linker between domains I and II are dispensable for FGF binding and are thought to act as autoinhibitory element in FRs (Olsen et al., 2004). Expression of the IgIIIb and IgIIIc isoforms is tissue specific and is found in epithelial and mesenchymal cell lineages, respectively (Orr-Urtreger et al., 1993).

Functions of the different receptors have been studied by targeted gene disruption in mice. Lack of several receptors caused lethality due to defective cell migration events when Fr1−/− (Deng et al., 1994; Yamaguchi et al., 1994) or Fr1c−/− (Partanen et al., 1998) were nonfunctional. Fr2−/− mice die due to defects in placenta (Xu et al., 1998) and Fr2b−/− due to impaired func-
tion of the lung (De Moerlooze et al., 2000). Mice with disrupted Fr2c show impaired skull and bone development (Eswarakumar et al., 2002), Fr3−/− have bone overgrowth and defects in inner ear (Colvin et al., 1996), but both are viable. Fr1b−/− (Partanen et al., 1998) and Fr4−/− (Weinstein et al., 1998) have no obvious phenotype.

As in the case with FGFs, there are also several human hereditary disorders connected to mutated FRI, 2 and 3 (Wilkie, 2005). Mutations in FRs may lead to increased protein stability, changed ligand–receptor specificity, enhanced FGF–FR affinity and increased kinase activity in a ligand independent manner (Eswarakumar et al., 2005). FR mutations cause impaired cranial, skeletal and digital development leading to craniosynostosis (premature fusion of cranial sutures) syndromes, such as Crouzon, Pfeiffer and Apert syndromes. The other group of disorders is dwarffing chondrodysplasia syndromes, such as hypochondroplasia, achondroplasia and thanatophoric dysplasia (Wilkie, 2005). In addition to skeletal disorders mutations in FRs have also been associated with cancer development. Bladder and cervical carcinoma, gastric and colorectal cancers, acute myelogenous leukemia can all be a result of an abnormal FR (Chaffer et al., 2007).

**Heparan sulfate and fibroblast growth factor–receptor complex formation**

HS has been shown to function as a secondary receptor for FGFs and is required for efficient activation of FRs (Yayon et al., 1991; Rapraeger et al., 1991). HS, which in most studies is substituted by heparin or heparin-derived oligosaccharides, is interacting with both the growth factors and the receptor molecules, thus assisting in formation of a ternary complex and stabilizing it (Fig. 3B). The precise stoichiometry and the interactions of the complex components are still under debate (Harmer et al., 2004; Ibrahimi et al., 2005), with two main models based on crystallographic analyses.

In one of the models, the so-called ‘symmetric’, the dimeric FGF–HS–FR complex has two components of each kind (2:2:2) and heparin is functioning as a stabilizer of ligand–receptor and receptor–receptor contacts (Schlessinger et al., 2000). In the second ‘asymmetric’ model, there is only one heparin molecule present (2:2:1), functioning as a bridge between ligand and receptor molecules, which have no protein–protein contacts on their own (Pelligrini et al., 2000). A recent study suggests that both interaction modes are possibly dependent on the length and structure of heparin oligosaccharide used (Goodger et al., 2008).

Determination of HS structural requirements for successful interaction with different FGFs, FGF–FR pairs and for induction of FGF signaling have been investigated by several groups (Guimond et al., 1993; Pye et al., 1998;
Despite all the work done, it is still a matter of debate what HS structures are required and how changes in modification type/degree affect the binding. Some studies suggest dependence on the different HS modification types (Guimond et al., 1993; Lundin et al., 2000; Pye et al., 1998) and considerable impact from subtle structural changes (Pye et al., 1998; Guimond and Turnbull, 1999). Results from other studies on the hand suggest that lack of one type of modification can be tolerated or compensated by another (Kariya et al., 2000; Merry et al., 2001; Wu et al., 2003; Kamimura et al., 2006).

Fibroblast growth factor induced downstream signaling

Binding of FGFs together with HS to the extracellular part of FR leads to dimerization of receptor molecules followed by trans autophosphorylation of tyrosine residues in the cytoplasmic kinase domains. A number of different signal transduction pathways can be activated in response to FGFs (Eswarankumar et al., 2005).

One of the most studied pathways is the Ras–mitogen activated protein kinase (MAPK) pathway (Fig. 4). The pathway is started by the docking of FR substrate 2 (FRS2) to the activated FR, phosphorylation of FRS2, subsequent recruitment of Grb2/son of sevenless (SOS) complexes and phosphorylation of Raf. Raf phosphorylates MAPK/extracellular signal-regulated kinases 1 and 2 (MEK1/2) that in turn phosphorylate extracellular signal-regulated kinases 1 and 2 (ERK1/2). Activated ERK1/2 phosphorylate a variety of cytoplasmic substrates; ERK1/2 also translocate to the nucleus and phosphorylate transcription factors (Schlessinger, 2000; Katz et al., 2007). This pathway is activated in all cell types in response to FGFs and is crucial for the mitogenic response, but is also involved in cell migration (Dailey et al., 2005).

The phosphatidylinositol-3-kinase–protein kinase B (PI3K–Akt) pathway becomes activated via Grb2 and Gab1 complex formation or through direct interaction of PI3K with phosphorylated FR (Fig. 4). PI3K generates formation of phosphatidylinositol-3-phosphate (PIP3) at the plasma membrane, which leads to translocation of Akt to the membrane and its subsequent phosphorylation. Activated Akt can then phosphorylate its cytoplasmic and nuclear substrate proteins (Fayard et al., 2005). This pathway is cell type specific and is mainly associated with an anti-apoptotic response (Schlessinger, 2000).
The third main FGF induced pathway is the phospholipase Cγ (PLCγ) pathway (Kaibuchi et al., 1986) (Fig. 4). PLCγ becomes phosphorylated after binding to phosphorylated receptor and hydrolyzes phosphatidylinositol-2-phosphate (PIP2) to diacylglycerol (DAG) and inositol-3-phosphate (IP3). IP3 stimulates release of Ca^{2+} from intracellular stores. DAG together with free cytosolic Ca^{2+} activates protein kinase C (PKC), which then phosphorylates its substrates proteins. The PLCγ pathway is also cell type specific and activates calcium dependent protein kinases, thus affecting the cytoskeletal organization (Schlessinger, 2000).

Besides the pathways mentioned above, FGFs is also able to activate additional signaling pathways. Activation of the other MAPK cascades following FGF stimulation have been detected, p38 (Tan et al., 1996), ERK5 (Kesavan et al., 2003) and c-Jun amino-terminal kinase (JNK) pathway (Liu et al., 1999). Transcription factors called signal transducer and activator of transcription (STAT) have also been shown to undergo phosphorylation following FR activation (Sahni et al., 1999).

In order to induce an appropriate response, the amplitude and duration of the signaling pathways are tightly regulated by limiting and terminating mechanisms. One of the major approaches is the activity of phosphatases, which dephosphorylate FRs and many other signal transducing proteins. Other important points are the control of subcellular localization of the sig-
naling molecules, interaction with scaffold proteins, endocytosis of the activated receptors and cross-talks between the various signaling pathways (Schlessinger, 2000; Katz et al., 2007; Shaul and Seger, 2007).

Cellular response to fibroblast growth factors

Cell activation by FGFs is usually associated with proliferation and/or migration (Boilly et al., 2000), although a number of other responses have been observed. Osteoblasts and Ewing’s sarcoma cells have been shown to undergo apoptosis after FGF stimulation (Mansukhani et al., 2000; Williamson et al., 2004). FGF stimulation can lead to cell cycle arrest in PC12 cells (Hondermarck et al., 1994) and chondrocytes (Sahni et al., 1999). During neural development, FGFs induce several important differentiation processes (Mason, 2007). For myoblasts FGFs function on the contrary as inhibitors of differentiation (Brunetti and Goldfine, 1990).

Diverse effects are observed in different cell types, but even the same cell type can respond differently to a certain FGF dependent on its developmental stage (Mansukhani et al., 2000). It is still unclear what determines cellular response to FGF signaling. Obviously, the combination of FGF and FR forming the complex and the cell type are important determinants, but they alone cannot explain the diversity of cellular responses observed. Presence of specific signal transducing molecules, transcriptional modulators and interactions with other signaling networks are some other potential factors suggested to influence the biological response (Dailey et al., 2005). HS, which stabilizes the FGF–FR complexes, is most probably also affecting the cellular signaling and response, but it’s involvement has so far received little attention.

If FGF signaling becomes for some reason perturbed, the effects may be deleterious. Pathological conditions resulting from mutations in FGFs or FRs, which disturb FGF signaling are described above. In addition, in a number of studies with cancer patients, overexpression of FGFs and down-regulation of negative regulators of the FGF signaling has been detected (Chaffer et al., 2007). For example, overexpression of FGF8 has been reported in breast and prostate cancers (Tanaka et al., 1998), whereas overexpression of FGF7 has been reported in gastric carcinoma (Shaoul et al., 2006). Lower expression levels of Sprouty-1, a negative regulator of the Ras–MAPK pathway, have been observed in prostate cancer (Kwabi-Addo et al., 2004).
Present investigations

Aims of the study

The participation of HS in the FGF–FR complex formation on the extracellular part of the cell is well established. The effects of HS on the intracellular events following FGF-induced cell stimulation are on the other hand very poorly understood. Since appropriate cell activation by FGFs is of major importance for many physiological events and disturbed FGF stimulation may result in pathological conditions, a better understanding of the factors involved is needed.

Work presented in this thesis aims at gaining more insight into the impact of HS and its structure in FGF-induced events. In order to approach the various structural features of HS, GAGs from different sources were used. Heparin-derived oligosaccharides, which are highly modified/sulfated, were used for studying NS-domains and effect of their composition. Full-length HS chains were used in order to illuminate the effect of NS-, NA/NS- and NA-domain composition of the chain. More precisely the aims of the present studies have been:

- To elucidate what effect structurally different heparin-derived oligosaccharides have on the ability of FGF1 and FGF2 to form complexes with FR1c, 2c, 3c and 4 (Paper I)

- To investigate the effect of these oligosaccharides on FGF2-induced Ras-MAPK and Akt signaling in HS-deficient cells (Paper II)

- To analyze the effect of different types of endogenous HS chains on ligand binding, FGF–HS–FR complex formation and signal induction capacity (Paper III)
Results

Heparan sulfate-related oligosaccharides in ternary complex formation with fibroblast growth factors 1 and 2 and their receptors (Paper I)

In order to elucidate which HS structures support complex formation between FGF1 and 2 and FR1c, 2c, 3c and 4, HS-related heparin-derived oligosaccharides of varying length and structure were tested. Their ability to support FGF–FR complex formation was studied using affinity chromatography with immobilized FR ectodomains, where the salt-elution profiles were used to interpret stability of the complexes formed.

Heparin octamers were found to be the shortest oligosaccharides supporting FGF–FR complex formation with both FGF1 and FGF2. Heparin decamers increased both the number of complexes formed and their affinity, the longer oligosaccharides only displayed a slight further increase in affinity. Therefore biosynthetic libraries were generated from octa- and decamer oligosaccharides. These libraries were made by chemical and enzymatic treatment of heparin and resulted in N-sulfated radiolabeled oligosaccharides with systematically varied 2-O- and 6-O-sulfation. Binding experiments were conducted under conditions preventing interactions between oligosaccharides and FRs (FR1c–3c) in the absence of FGFs.

We found that FGF1 needed octa- or decamers with at least five O-sulfate groups for binding to FR1c–3c (elution >0.2–0.4 M NaCl), and decamers with seven O-sulfate groups for strong binding (elution >0.4–2.0 M NaCl). The corresponding requirements for FGF2 were octa- or decamers with at least four O-sulfate groups for binding to FR1c–3c and at least six for strong binding. FR4 differed from the other receptors, as it could retain oligosaccharides at the chosen conditions in the absence of FGFs and displayed a reversed preference for FGFs. FGF1 required an octa- or decamer with only three O-sulfate groups to form complexes with FR4, whereas FGF2 needed six O-sulfate groups. In all combinations studied increased O-sulfation degree led to increased amounts and stability of the FGF–FR complexes, where 2-O- and 6-O-sulfate groups could compensate for each other. Our findings suggest that the overall sulfation level of oligosaccharides rather than the precise distribution of sulfate groups correlate with complex formation.
Heparan sulfate-related oligosaccharides modulate cell signaling induced by fibroblast growth factor 2 (Paper II)

Keeping in mind our previous findings (Paper I) with oligosaccharide libraries using a cell-free system, we wanted to investigate what kind of effect these oligosaccharide libraries have on FGF2-induced cell signaling. Chinese hamster ovary 745 (CHO745) cells lacking endogenous HS and CS due to mutated xylosyltransferase gene and expressing a low level of FRs (mostly FR1) were used for that purpose. Two out of three main FGF-induced signaling pathways were studied, the Ras–MAPK pathway and the PI3K–Akt pathway. Cells were stimulated with FGF2 alone and in combination with decamer long oligosaccharides, followed by cell lysis at various time points and determination of the amounts of phosphorylated ERK1/2 and Akt by immunoblotting.

Stimulation with FGF2 alone (10 ng/ml) led to transient phosphorylation of ERK1/2 and Akt, which displayed maximum phosphorylation before 10 min and a continuous decline thereafter. Addition of decamers carrying three to five O-sulfate groups had a slightly prolonging effect on the phosphorylation period of both ERK1/2 and Akt signals, displaying no preference for either 2-O- or 6-O-sulfation. Co-application of FGF2 with the highest sulfated decamers carrying six O-sulfate groups had a much stronger effect. These decamers both increased the amplitude and prolonged the phosphorylation period of ERK1/2 and Akt signals, which showed no decline during the studied time (80 min).

Changing the FGF2 concentration while keeping the oligosaccharide concentration constant affected the activation pattern of both, ERK1/2 and Akt, with a differential impact of oligosaccharides on the two pathways. ERK1/2 phosphorylation was no longer affected by addition of oligosaccharides at higher or lower concentrations of FGF. Akt phosphorylation responded to the highest sulfated decasaccharide with an increased signal at low and a decreased signal at high FGF2 concentration, respectively. Our findings imply that moderately sulfated oligosaccharides, independent of the sulfation pattern, prolong the ERK1/2 and Akt phosphorylation possibly by protecting FGF2 from degradation and/or stabilizing the FGF2–FR complexes, whereas the highest sulfated oligosaccharide also affected the signal amplitude by as yet unknown mechanism.

Binding of FGF2 to size-fractionated heparan sulfate from porcine tissues (Paper III)

HS chains present on cells are long and have a tissue dependent domain organization with NS-domains, which were in focus in the previous studies (Papers I and II), interspersed by NA- and NA/NS-domains. In order to understand how chain organization with domains of highly and low sulfated
stretches influence HS binding properties, we studied the ability of endogenous HS chains to form FGF2–HS and FGF2–HS–FR1c complexes and to support FGF2 induced signaling. Full-length HSs of varying sizes, pools I–III, were isolated from porcine liver and intestine, radiolabeled and studied with regard to type and degree of sulfation as well as domain organization.

Formation of FGF2–HS complexes was found to increase with chain length, showing very similar values for liver and intestine HS, suggesting similar FGF2–HS binding capability with HS from these tissues. Formation of FGF2–HS–FR1c complexes displayed a reverse correlation between HS chain length and number of complexes formed, liver HS being more efficient in supporting complex formation than intestine HS. HS structure analysis showed that the overall N- and O-sulfation degree increases with decreasing chain length and that liver NS-domains are more densely sulfated than intestine NS-domains, explaining thereby preference for liver HS and the shorter chains of both liver and intestine for FGF2–HS–FR1c complex formation.

Co-application of FGF2 with intestine HS chains and the longer liver chains (pools I and II) to cells led to prolonged phosphorylation time of ERK1/2 and Akt compared to stimulation with FGF2 alone. The shortest liver chains (pool III) also increased the signal amplitude, leading thereby to the strongest ERK1/2 and Akt response. Together these findings suggest that for strong cell activation, HS chains must contain appropriately sulfated NS-domains and a limited number of NA- and NA/NS-domains. In our case this requirement was only fulfilled by liver pool III. The longer liver chains contained a larger proportion of low sulfated stretches, but were efficient FGF2 binders, thereby possibly limiting access of FGF2 to the receptors.

Discussion

The major difference between proteins and GAGs, besides the different building blocks, is that the structure of a protein is reflecting a DNA sequence present in the genome. GAGs and other glycans are secondary gene products. That means that there is no template for them in the genome and therefore the availability of enzymes and their substrate specificity define the structure. Biosynthesis of HS involves more than 20 enzymes, if one considers the different enzyme isoforms. Why do we need so many enzymes? The obvious answer is for creation of a great variety of HS structures. HS structure are cell and tissue type dependent (Maccarana et al., 1996; van Kuppervelt et al., 1998; Ledin et al., 2004) and change during development (Brickman et al., 1998) and aging (Feyzi et al., 1998). But what is all this enormous structural variety needed for? Why is HS biosynthesis so strictly regulated? What interaction partners can discriminate between slight variations in length, domain organization and modification type/degree of an HS chain? These questions represent a great challenge in the HS field today.
Heparan sulfate structure—the question of specificity

The FGF family belongs to some of the most studied HS-binding proteins. The only other protein having received more attention is probably anti-thrombin. After the discovery of the specific pentasaccharide sequence needed for a successful interaction with antithrombin (Lindahl et al., 1979; Petitou et al., 2003), one was expecting to find similar stringent requirements for other HS-binding proteins. FGFs, in particular FGF1 and 2, and their receptors have been major targets in that respect. So far no specific saccharide sequences have been found, instead one finds preferences or selectivity for certain types of structures. The structures found to be required for FGF–FR complex formation by different groups show substantial discrepancy in modification type/degree and tolerance to structural changes (Guimond et al., 1993; Pye et al., 1998; Guimond and Turnbull, 1999; Kariya et al., 2000; Lundin et al., 2000; Kreuger et al., 2001; Merry et al., 2001; Ostrovsky et al., 2002; Allen and Rapraeger, 2003; Wu et al., 2003; Kreuger et al., 2005; Kamimura et al., 2006).

In most studies, the variation in polysaccharide structures lacking different modification types (N-, 2-O- and 6-O-sulfation) were compared to the fully modified GAG and in some cases could show dependence on the modification (Guimond et al., 1993; Lundin et al., 2000), but not in others (Kariya et al., 2000; Merry et al., 2001; Wu et al., 2003; Kamimura et al., 2006). This kind of approach makes it however difficult to compare the importance of the modification types, because the N-, 2-O- and 6-O-desulfated saccharides have usually different overall degree of modification/negative charge and are therefore not readily comparable to each other. Variations in degree of modifications are HS source dependent and might partly explain the contradicting findings by different groups. In one study the degree of 6-O-sulfation was varied and found to have a great impact on FGF2–FR1c complex formation, but that was done with oligosaccharides having the same degree of 2-O-sulfation (Pye et al., 1998).

In our work we have concentrated on variation in both 2-O- and 6-O-sulfation in constant N-sulfated background, thereby attempting to illuminate the impact of these two modification types under similar conditions. With that approach we could not detect any preference for either 2-O- or 6-O-sulfation and instead found a dependence on overall charge density in both cell-free and cell based assays (Paper I and II) (Fig. 5). Therefore our findings support the idea that 2-O- and 6-O-sulfate groups can compensate for each other and that a specific well-defined HS structure is not a requirement, at least not for FGF1 and 2, to form complexes with FRs.
FGFs constitute a big family, where FGF1 and 2 belong to the same subfamily and consequently share more properties with each other than with other family members (Itoh, 2007). Therefore it is fully possible that other FGFs have other kind of requirements on HS structure and would tolerate fewer structural variations in the binding HS sequence than FGF1 and 2. Findings from in vivo studies with mice deficient in enzymes required for HS biosynthesis (C5-epimerase and 2-O-sulfotransferase) leading to deranged HS structure show that some of the major organs requiring appropriate FGF signaling appear normal, whereas a number of others are affected (Li et al., 2003; Wilson et al., 2003). These data suggest that more strictly defined HS structures may be necessary for normal FGF-induced signaling in some cases, but not always. Considering the large number of proteins that have been shown to interact with HS, but where the protein–HS interactions are not at all as well studied as with FGFs, one cannot exclude that proteins requiring well-defined HS sequences will be discovered.

Heparan sulfate structure and cell signaling
Once a signaling FGF–HS–FR complex has been formed on the extracellular side of the cell membrane, the intracellular parts of receptor molecules phosphorylate each other leading thereby to activation of various signaling pathways. The subsequent cellular response (proliferation, migration etc.) is partly dependent on which pathways get activated. How are differently modified HSs affecting the start and propagation of the signaling pathways?

Our findings (Paper II and III) show that HS has a capacity to affect two main FGF-induced pathways, the Ras-MAPK and PI3K-Akt pathways (others were not investigated). The presence of short and full-length HS variants could prolong the duration of phosphorylated ERK1/2 and Akt signals (Fig. 6A). One possible mechanism for that could be the stabilization of FGF–FR complexes. Increased receptor activation has been suggested to be a way to...
prolong ERK1/2 signal duration (Katz et al., 2007). Another possibly contributing mechanism may be protection of heparin/HS bound FGF molecules from degradation, which has been seen both extra- and intracellularly (Sperinde and Nugent, 1998). Besides extracellular stimulation, intracellular mechanisms also affect the duration of signals, such as phosphatases and positive/negative feedback loops, which are activated in parallel with or by the signaling pathways (Ebishuya et al., 2005; Shaul and Seger, 2007). If and how HS could influence these events is not known. Presence of heparin has been shown to differently affect FR tyrosine phosphorylation (Lundin et al., 2003). HS could also influence the activity of some regulators, for example Sef, which is a negative regulator and a transmembrane protein localized in proximity to FRs (Tsang and Dawid, 2004), being therefore a potential target for HS. Signaling complexes undergo endocytosis and it has been shown that the intracellular fate of FGF–FR and FGF–HS–FR complexes differ, suggesting a role for HS inside the cells as well (Reiland and Rapraeger, 1993).

**Figure 6.** Effects of differently modified HS decasaccharides and full-length chains on ERK1/2 and Akt response. Rectangles represent NS-domains supporting FGF–FR complex formation, whereas black lines are NA/NS- and NA-domains. The diagrams show the effect on ERK1/2 and Akt signals in presence (+HS) and absence (−HS) of HS. (A) Co-application of FGF2 with moderately sulfated decasaccharides (+) or intestine pool I (−) prolonged the signal duration. (B) Co-application of FGF2 with the highest sulfated decasaccharide (++) or liver pool III (−) prolonged and increased the signals.

The highest sulfated decasaccharide (Paper II) and the shortest liver HS chains (Paper III) also increased the levels of phosphorylation (Fig. 6B). At the extracellular level a potential explanation could be formation of a higher number of signaling complexes. Another possibility could be in the complex assembly and its stoichiometry, which may be affected by variation in heparin structure and length (Goodger et al., 2008). Intracellularly, the amplitude of at least ERK1/2 is thought to be mainly regulated by scaffold proteins, which can bind several members of the Ras–MAPK pathway simultaneously
thereby potentiating activation of the downstream molecules and affecting their localization (Ebishuya et al., 2005). During a transient response ERK1/2 remains in the cytosol, whereas a sustained activation results in translocation to the nucleus. The ERK1/2 localization in turn affects ERK1/2 induced gene products and might result in different cellular responses (Whitmarsh, 2007). Considering the HS dependent signal prolongation effect observed in our work, it becomes obvious how crucial that change could be for a cell’s fate. Sustained ERK1/2 phosphorylation as a consequence of FGF2 and HS activation was shown to correlate with mitogenic response, whereas transient phosphorylation (FGF alone) could not induce mitogenesis (Delehedde et al., 2000).

**Oligosaccharides versus full-length heparan sulfate chains**

Most of the work in the FGF–HS field is concentrated on the highly sulfated domains, studied by heparin as HS-analogs, and which are the ones supporting the signaling complex formation. But HS chains found on cell surfaces in vivo are tissue dependent mixtures of highly and low sulfated domains. Have low sulfated domains any effect on FGF–FR complex formation and signaling?

**Figure 7.** Changes in the ratio of highly versus low modified/sulfated domains and possible effect on FGF–FR complex formation. (A) Sequestration of FGF molecules by the abundant low sulfated domains in liver pool I (−−−−−) leads to the possible consequence to limit FGF–FR complex formation. (B) HS liver pool III chains (−−) having a higher highly/low modified domain ratio can probably assist in formation of more FGF–FR complexes.

Our findings in Paper III with liver and intestine HS illustrate that indeed they may have. The ratio of NA/NS- and NA-domains in a chain was shown to affect the activating potential of liver HS chains. These modestly sulfated domains are good FGF2 binders and probably sequestrate growth factor molecules functioning thereby as an FGF-depot and limiting the number of activated receptors (Fig. 7). These findings imply that the balance between FGF–FR complex supporting sequences versus non-supporting but FGF binding parts in an HS chain is crucial for its effect in cell regulation. The shortest liver chains (pool III) with the highest ratio of highly versus low modified domains act similarly as the highest sulfated decasaccharide. In the
longer chains, less sulfated sequences may efficiently compete with NS-domains for FGF binding, which make these HS chains more alike the moderately sulfated decasaccharides. It is also possible that apart from the different impact on FGF molecules and FGF–FR complexes, the shortest liver chains are more efficient in binding to other cell surface proteins, which affect FGF’s functions. These findings indicate that modification of not only NS-domains, but also of the low sulfated domains, as well as their prevalence in the chain are of importance for a cell’s potential response to FGFs.
Future perspectives

The work presented in this thesis has illuminated some aspects of HS impact in the FGF–FR interplay. Due to the big FGF family size and the variety of events where FGFs are involved, the work left to be done to really understand the role of HS is huge.

Further studies with other FGF–FR combinations under conditions allowing equal evaluation of the different HS modification types are required. This would show what HS structures are required in those cases, how similar or dissimilar they are and also how tolerant other FGF–FR combinations are to changes in structure. It is of great importance that not only highly sulfated oligosaccharides, but also endogenous full-length HS are investigated in order to better understand the impact of domain organization. A way to approach that is to use cells from knockout mice carrying HS chains with disturbed domain organization, which would be a valuable complement to the more common approach with HS-deficient cells and exogenously added HS.

The major challenge is however to connect the HS and signaling fields. FGF signaling and the separate signaling pathways have received a lot of attention, but the role of HS in that aspect is usually ignored. Investigations regarding the role of HS in the induction and regulation of various pathways would probably display new ways of controlling cellular signaling events. That kind of knowledge would also help in explaining the observed phenotypes in knockout animals deficient in HS biosynthetic enzymes.

Finding new ways to affect or control FGF-induced signaling has important clinical applications, since abnormally low and high FGF signaling is the cause of several human disorders.
Proteoglykaner är proteiner med kovalent bundna långa polysackaridkedjor som tillhör familjen glykosaminoglykaner (GAGs). En GAG-kedja är ogrenad och uppbyggd av upprepade disackaridenheter bestående av en hexosamin och en hexuronsyra. Heparansulfat (HS) är en typ av GAG, som man hittar proteinbunden på celler och i den extracellulära matrisen. Under biosyntesen modifieras HS-kedjorna av ett antal olika enzymer vilket leder till ökad negativ laddning av kedjorna. Modifieringsreaktionerna berör dock kedjorna och dess delar i olika omfattning och det resulterar i en domänstruktur med alternerande mer och mindre modifierade delar. HS-kedjornas längd och domänstruktur är cell- och vävnadsspecifika och uppvisar stor mångfald. HS binder till ett stort antal proteiner och påverkar deras stabilitet, tillgänglighet och aktivitet.

Fibroblast tillväxtfaktorer (FGFs) hör till några av de mest studerade HS-bindande proteinerna. FGFs bildar en stor proteinfamilj, där de olika medlemmarna är involverade i en lång rad viktiga moment både under utvecklingen och i den vuxna organismen. De utövar sin verkan genom att aktivera FGF-receptorer (FRs). Dessa finns på cellytor och har en extracellulär del som FGF binder till och en intracellulär del som kan sätta igång flera olika signaleringsvägar och i sin tur resultera i cellens delning, migration, död m.m. HS påverkar bildningen av FGF–FR komplexen och är nödvändig för effektiv receptoraktivering.


Detta arbete belyser betydelsen av HS och dess strukturvariation för FGFs och ökar vår förståelse för faktorer som påverkar cellernas svar på FGF-stimulering.
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References


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)