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**A multifaceted study
of the receptor tyrosine kinase Axl
with focus on renal cell carcinoma**

Anna Gustafsson

Doctoral Thesis



LUND UNIVERSITY
Faculty of Medicine

Academic dissertation

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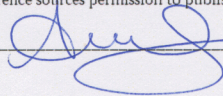
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Abstract The present investigation is a multifaceted study of the receptor tyrosine kinase Axl, whose overexpression and activation is implicated in several human malignancies, with focus on renal cell carcinoma (RCC). At first, our main interest concerned the intracellular Axl interactor named Tensin2 (Tns2) and its family member Tensin3 (Tns3). Tns2 and Tns3 are structural focal adhesion proteins, linking the extracellular matrix and cytoskeleton via integrin and actin binding, and are involved in cellular signaling. Tns2 inhibits the PI3K and Akt/PKB pathway and decreases the level of PtdIns(3,4,5)P3 located at the cellular membrane. Our primary goal was to further characterize the Axl and Tns2 interaction, and thereafter, to explore our hypothesis that Tns2 and Tns3 might act as PTEN-like lipid phosphatases. We verified the Axl and Tns2 interaction to occur through its carboxy-terminal region as originally reported, and excluded other Tns2 domains to take part in the interaction (Paper IV). In addition, we realized that a stretch of 100 amino acids with no known tertiary structure seemed invariant for the interaction. Thereafter, we continued with our exploration of the putative phosphatase activity of Tns2 and Tns3, but despite extensive efforts we were not able to show any direct in vitro activity (Paper III). We continued our Axl exploration concerning the role of Axl and its ligand Gas6 in RCC. RCC is a disease present with poor prognosis and elusive etiology, and with great need for proper molecular prognostic markers. With the opportunity of having a large RCC patient material, we could conclude Axl and Gas6 to correlate with patient survival outcome (Paper I). Furthermore, we found Axl expression in the primary tumor, at time of nephrectomy, to have the potential to be a prognostic marker, where low Axl expression was beneficial for the patient outcome (Paper I). Remaining time until present, we attempted to discover the biological role of Axl and Gas6 in a clear cell RCC (ccRCC) experimental system. Although more can be done, we could see that Gas6 stimulation of Axl in RCC cells had inhibitory effects on migration, and that Gas6 rendered the cells less viable (Paper II). Moreover, Axl expression was inversely correlated to the expression of the tumor suppressor VHL, and also downregulated by Gas6 stimulation (Paper II). Altogether, the results from Paper I and II opens up for further exciting research of the complex role of the Gas6/Axl system in RCC, and for validation of the putative role of Axl as an important prognostic marker in the diagnosis of RCC.		
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**A multifaceted study
of the receptor tyrosine kinase Axl
with focus on renal cell carcinoma**

Anna Gustafsson

Doctoral Thesis



LUND UNIVERSITY

Faculty of Medicine

Division of Clinical Chemistry
Department of Laboratory Medicine, Malmö
Faculty of Medicine
Lund University

2009

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List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

- I. **Gustafsson A.**, Martuszezwska D., Johansson M., Ekman C., Hafizi S., Ljungberg B., and Dahlbäck B. Differential Expression of Axl and Gas6 in Renal Cell Carcinoma Reflecting Tumor Advancement and Survival. *Clin Cancer Res* 2009;15(14) July 15, 2009: 4742-4749.
- II. **Gustafsson A.***, Boström A.*, Ljungberg B., Axelson H., and Dahlbäck B. Gas6 and the receptor tyrosine kinase Axl in clear cell renal cell carcinoma. *PLoS ONE* 4(10): e7575. Doi:10.1371/journal.pone.0007575.
*Authors contributed equally to this work.
- III. **Gustafsson A.**, Oslakovic C., Hafizi S., and Dahlbäck B. No intrinsic lipid phosphatase activity detected in the recombinant PTEN-homology regions of Tensin2 and Tensin3 *in vitro*. Manuscript.
- IV. **Gustafsson A.**, Hafizi S., and Dahlbäck B. A yeast two-hybrid interaction study between various Tensin2 deletion mutants and the Axl receptor tyrosine kinase. Manuscript.

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Abbreviations

ACE	Angiotensin Converting Enzyme
AML	Acute Myeloid Leukemia
APCs	Antigen Presenting Cells
ATP	Adenosine-5'-triphosphate
ccRCC	Clear Cell Renal Cell Carcinoma
chRCC	Chromophobe Renal Cell Carcinoma
CML	Chronic Myeloid Leukemia
DCs	Dendritic Cells
DLC1	Deleted in liver cancer 1
ECs	Endothelial Cells
ECOG PS	Eastern Cooperative Oncology Group performance status
EGF	Epidermal Growth Factor
FAK	Focal adhesion kinase
FNIII	Fibronectin-type III
Gla	γ -carboxyglutamic acid
H ₂ O ₂	Hydrogen peroxide
HIF	Hypoxia Inducible Factor
Ig	Immunoglobulin
IL	Interleukin
IFN	Interferon
LG	Laminin G
MCs	Mesangial Cells
MMP	Matrix Metalloproteinase
NF- κ B	Nuclear Factor kappa B
NK	Natural Killer
PDGF	Platelet-derived growth factor
PKD1	Phosphoinositide dependent protein kinase 1
PI3K	Phosphatidylinositol 3-kinase

pRCC	papillary Renal Cell Carcinoma
PTB	Phosphotyrosine-binding
PtdIns	Phosphatidylinositol
PtdIns(3,4,5)P ₃	Phosphatidylinositol (3,4,5)-trisphosphate
PTEN	Phosphatase and Tensin homolog
RCC	Renal Cell Carcinoma
ROS	Reactive Oxygen Species
RPE	Retinal Pigment Epithelium
RTK	Receptor Tyrosine Kinase
sAxl	soluble Axl
SHBG	Sex Hormone-Binding Globulin
SH2	Src Homology 2
SOCS	Suppressor of Cytokine Signaling
STAT	Signal Transducers and Activators of Transcription
TAM	Tyro3, Axl, and Mer
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor
TNM	Tumor Node Metastasis
Tns1,2,3,4	Tensin 1, 2, 3, and 4 respectively
VEGF	Vascular Endothelial Growth Factor
VHL	von Hippel-Lindau
VSMCs	Vascular Smooth Muscle Cells

Introductory words

Receptor tyrosine kinases are transmembrane proteins that transduce signals from the extracellular environment to the cytoplasm and nucleus. Thereby, they regulate normal cellular processes such as survival, growth, proliferation, differentiation, adhesion, and motility. Sometimes the expression and signaling of receptor tyrosine kinases become abnormal, an event often rendering them able to transform cells. Such oncogenic events are in many circumstances directly implicated in the development of several human cancers. Axl is a receptor tyrosine kinase named from the Greek word “Anexelekto”, meaning the uncontrolled. Axl has in recent years been shown to play important roles in several human malignancies.

This thesis will review the literature field of the receptor tyrosine kinase Axl and its ligand Gas6, the product of the *growth arrest specific gene 6*. Furthermore, it will contribute significant novel findings on both their roles in human renal cell carcinoma. Also, there will be a brief introduction to phosphatases, the tumor suppressor PTEN, and the Tensin family of proteins.

Part I

General background

Receptor tyrosine kinases and the TAM family

Growth factor signaling leading to cellular effects such as growth, proliferation, differentiation, survival, and migration most often involve so called enzyme-linked cell-surface receptors. These receptors are transmembrane proteins constituted by an extracellular ligand-binding domain, at least one transmembrane segment, and an intracellular catalytic domain that either has intrinsic enzymatic activity or associates directly with an enzyme. There are six classes of enzyme-linked receptors: receptor tyrosine kinases (RTKs), tyrosine-kinase-associated receptors, receptor-like tyrosine phosphatases, receptor serine/threonine kinases, receptor guanylyl cyclases, and histidine-kinase-associated receptors.¹

Receptor tyrosine kinases

In the human genome, around 1000 unique protein kinase genes are identified, and about 100 of them are protein tyrosine kinases. Among these tyrosine kinases, 58 are of receptor type, i.e. RTKs, constituting one subfamily of enzyme-linked cell-surface receptors.² RTKs are composed of an extracellular ligand binding region, a transmembrane region, and a cytoplasmic kinase domain^{3,4} classified into 20 structurally different subfamilies². The kinase domain, when activated, transfers a phosphate group from adenosine-5'-triphosphate (ATP) to selected tyrosine residues. The phosphorylation occurs on the receptor itself in an auto-phosphorylation process that increases the activity of the receptor, but also on downstream intracellular signaling proteins that harbor *src* homology 2 (SH2) structural motifs^{5,6} that interacts with the activated receptor.³ The binding of a specific subset of these intracellular signaling molecules is determined by the amino acid sequences surrounding each phosphotyrosine residue, especially the three amino acids carboxy-terminal to the phosphorylated tyrosine⁶. Activation of the kinase domains and further downstream signaling is enabled after receptor-chain oligomerization, in most cases dimerization, as a consequence of ligand binding that brings two receptor-chains together, or through ligand-independent oligomerization processes^{3,4}.

RTKs represent a major class of proto-oncogenes involved in the progression and metastasis of cancer cells⁷.

The TAM family of receptor tyrosine kinases

The TAM family includes the three closely related RTKs Tyro3, Axl and Mer⁸⁻¹⁰.

These single-span transmembrane receptors make up a separate RTK family due to their unique adhesion-molecule-like extracellular region, characterized by two amino-terminal immunoglobulin (Ig)-like motifs, and two fibronectin-type III (FNIII) repeats, similar to neural cell adhesion molecules (NCAMs)^{11,12}, and also due to their conserved carboxy-terminal KW(I/L)A(I/L)ES sequence within their highly conserved intracellular kinase domain¹¹ (Figure 1). Among the known RTKs there is only one RTK family, the Tie receptors, that contains an extracellular domain organization similar to that of the TAM receptors, including both immunoglobulin-like motifs and fibronectin-type III repeats. Despite this similarity, the mesenchymal-epithelial transition factor (MET) family of RTKs is most closely related to the TAM family on the basis of amino acid sequence similarities of their kinase domains.²

Within the TAM family, Axl and Tyro3 seem to have most similar genomic structure sharing the same number and size of exons¹³, however, in contrast to this genomic similarity, Axl and Mer have the most similar tyrosine kinase domain amino acid sequence^{2,14}.

When originally cloned, the TAM receptors were orphans, in that their ligands were unknown^{15,16}. Today, the biological ligand of this subfamily of RTKs is known to be Gas6, the product of *growth arrest-specific gene 6*, a vitamin K-dependent growth factor with significant sequence and structural homology to anti-coagulant protein S^{10,17-19}. Even human protein S can stimulate human Tyro3 and Mer¹⁹⁻²¹ (unpublished observation), although the biological relevance of this interaction has previously been questioned.

Today, protein S as a ligand of TAM receptors, is believed to have a function in mediating phagocytosis of apoptotic cells^{22,23}. For instance, in the mouse retina protein S seems to be the biologically relevant ligand for Mer²⁴. Protein S also plays a role in negatively modulating macrophage function in the uptake of modified lipoproteins via interaction with Mer²¹. Interestingly, it seems that protein S must undergo oxidative oligomerization on the apoptotic cell surface in order to stimulate macrophage phagocytosis through Mer²³.

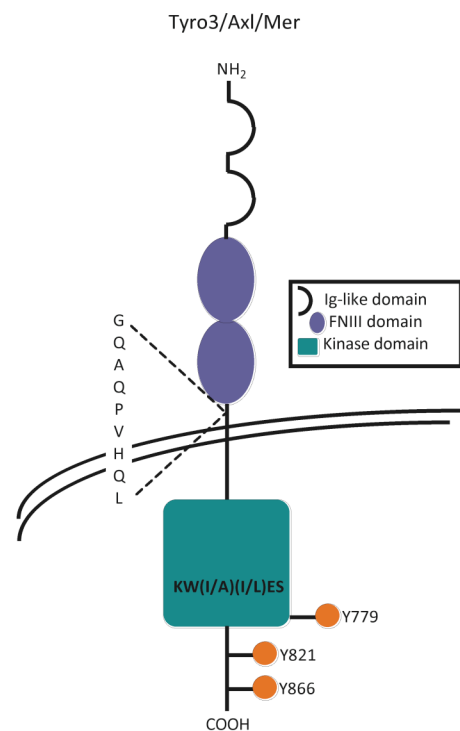


Figure 1. Illustration of the TAM family RTKs

TAM receptors in health and disease

The TAM receptors have a very broad and overlapping tissue distribution¹³.

During development Axl is expressed in various organs including brain oligodendrocytes and neurons²⁵⁻²⁷. In the adult, Axl expression is low but ubiquitous and found in for example hippocampus and cerebellum, monocytes and macrophages, platelets, endothelial cells (ECs), heart, skeletal muscle, liver, kidney, and testis^{11,28-30}.

Tyro3 expression is most abundant in the nervous system and in neural tissue in the brain^{26,31,32}. Tyro3 expression has also been reported in bone osteoclasts, ovary, testis, breast, lung, kidney, and retina, as well as in several human hematopoietic cells including monocytes, macrophages, and platelets¹³.

Mer is for instance expressed in the hematopoietic lineages in monocytes and macrophages, dendritic cells (DCs), natural killer (NK) cells, megakaryocytes, platelets, and B lymphocytes^{8,28,33,34}. Mer is also expressed in the retinal pigment epithelium (RPE) cells of the eye³⁵.

TAM receptors were not believed to be expressed in granulocytes or blood lymphocytes³⁰, however, recently, Mer was found to be expressed on normal B cells³³.

TAM receptors are expressed on cells from the immune system, such as antigen presenting cells (APCs), where they play an evident role in modulating the level of activity and their process of phagocytosis^{26,34,36-39}. They are also expressed by NK cells and their precursor cells, and are essential in the terminal differentiation and functional maturation of NK cells⁴⁰. Moreover, the TAM receptors are suggested to mediate an anti-inflammatory response to ligands found on the apoptotic cells, while simultaneously stimulating phagocytosis³⁹.

Mer is required for phagocytosis of photoreceptor outer segments by RPE cells in the eye³⁵. Mer seem to be the primary phagocytic receptor of the TAM family of receptors in mediating phagocytosis of apoptotic cells by macrophages. Axl and Tyro3, on the other hand, are especially involved in regulating the activation and phagocytic activities in DCs.⁴¹ TAM receptor signaling can attenuate Toll-receptor and cytokine induced activation of DCs in a negative feedback-loop that includes upregulation of Axl expression and a subsequent change from pro-inflammatory to immunomodulatory transcriptional activity⁴². In addition, Mer is also required for apoptotic cell-induced T cell tolerance, a mechanism important in immune homeostasis and self-tolerance⁴³. Unexpectedly, Mer is also required for the loss of B cell tolerance in chronic graft-versus-host disease³³.

Increased expression of all three receptors in human malignancies has been observed. Axl expression and signaling seem to be increased in most human cancers investigated, with profound effects (discussed below). Tyro3 overexpression has been detected in

human myeloid leukemia, and Mer overexpression in mantle cell lymphoma, T-cell leukemia, and in prostate cancer, to give a few examples.¹³

Genetic aberrations of the TAM receptor family

Although expression of TAM receptor mRNA has been observed in embryonic tissues^{14,15,25,44}, single, double, and triple knockouts are viable without obvious signs of developmental defect at birth^{36,45,46} suggesting TAM receptors to be nonessential for embryogenesis. Nevertheless, a role for these receptors in developmental processes, such as in the function of the hematopoietic and nervous systems, and in tumorigenesis has been suggested⁴⁷. Recently, a role for the TAM receptors in regulation of erythropoiesis in mice has been described⁴⁸.

The phenotypes of TAM receptor knockouts are first observed around the onset of sexual maturation at approximately four weeks of age when the spleen and lymph nodes start to aberrantly enlarge⁴⁶. The elevated growth of peripheral lymphoid organs was primarily shown to be due to hyperproliferation of B and T cells. Furthermore, TAM knockout males are infertile due to the degeneration of all germ cells in the testes⁴⁶. Mutations in the Mer gene lead to defective phagocytosis of photoreceptor outer segments by the RPE cells resulting in retinal degeneration³⁵. TAM knockout mice develop blindness as a result of the full degeneration of photoreceptors⁴⁶, and Mer seem to be the responsible receptor for this phenotype⁴⁹.

Among the most dramatic TAM triple knockout phenotypes are those seen in the immune system, where an aberrant proliferation of B and T lymphocytes occurs^{36,46}. Although the levels of both B and T lymphocytes were greatly increased there was a modest enrichment of T cells over B cells and CD4+ cells over CD8+ cells³⁶. Possible mechanisms for the autoimmunity seen in the TAM triple mutants might be due to elevated expression of a number of inflammatory molecules, such as interleukin (IL)-2 receptor, Fas ligand, interferon (IFN)- γ and CD44⁴⁵, and due to lack of intrinsic TAM-mediated feedback inactivation of DCs⁴². But also, Mer knockout mice with defective monocytes express unusually high levels of nuclear factor kappa B (NF- κ B), and are hypersensitive to endotoxic shock and tissue damage⁵⁰. Altogether, TAM knockout mice develop severe autoimmune diseases including lupus and rheumatoid-like syndromes^{36,51}.

In addition, loss of any of the three TAM receptors, or administration of soluble Axl (sAxl) and sequestering of Gas6, protects mice against life-threatening thrombosis⁵².

Part I

Specific background

The Gas6/Axl system

Axl was originally identified as a transforming gene involved in the progression of chronic myeloid leukemia (CML) to acute myeloid leukemia (AML)⁵³, and in chronic myeloproliferative disease⁵⁴. Axl was characterized as a RTK, and given its name from the Greek word “*Anexelekto*” that means the uncontrolled, and its transforming capacity was suggested to be a result of Axl overexpression rather than any activating structural mutation¹¹. Gas6 was purified as an Axl stimulatory compound¹⁰ and identified as the product of the *growth arrest-specific gene 6*^{10,17}.

Gas6

Gas6 was originally cloned in 1988 and identified in fibroblast cells as a gene upregulated about 30 fold during the G₀ phase of the cell cycle^{17,55}, and later on as the ligand of the TAM receptors^{10,18,19,56,57}. Gas6 is a secreted multidomain protein with ~44 % sequence identity to the anticoagulant protein S^{17,58} (Figure 2). Both Gas6 and protein S contain several post-translational modifications, such as γ -carboxylation of glutamic acid residues⁵⁹, and both belong to the family of vitamin K-dependent proteins⁶⁰. Gas6 signaling through the TAM receptors activates several signaling pathways implicated in reversal of cell growth arrest, survival, proliferation, cell adhesion, and hemostasis^{61,62}.

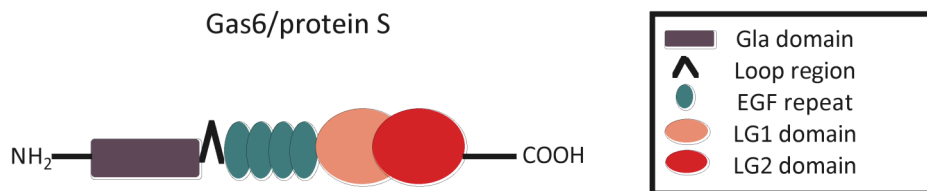


Figure 2. Illustration of the domain organization of Gas6 and protein S.

Gas6 structure

The human Gas6 gene was located to 13q34⁶³. The intron-exon boundaries were further characterized, and eight allelic variants, shown to be due to single nucleotide polymorphisms, were found⁶⁴.

The Gas6 gene encodes a protein product that is composed of 678 amino acids, and the apparent molecular weight is around 75 kDa¹⁷.

Gas6 is expressed with an amino-terminal signal peptide and pro-peptide that eventually is cleaved off¹⁷. The mature Gas6 protein is composed of an amino-terminal γ -carboxyglutamic acid (Gla)-domain posttranslationally generated by γ -carboxylation of glutamic acid residues in the presence of vitamin K⁵⁹. Gas6 binds to phosphatidylserine-containing phospholipids membranes through its Gla-domain as do other vitamin K-dependent proteins⁶⁵⁻⁶⁸. Following the Gla-domain, Gas6 contains a disulfide-bridged loop structure, however, it does not seem to be sensitive to serine-protease cleavage, as protein S, during thrombin-mediated inactivation^{69,70}. From this connecting sequence, Gas6 contains four epidermal growth factor (EGF)-like repeats arranged in tandem, out of which two harbor calcium-binding consensus sequences⁵⁹. The Gas6 carboxy-terminal is comprised of two laminin G (LG)-type domains that form the sex hormone binding globulin (SHBG)-like domain.¹⁷ (Gas6 structure reviewed in^{60,62})

The Gas6 protein can occur as two splice variants, where the alternative form has an additional 43 amino acids insert between the fourth EGF- and the SHBG-like domain⁷¹. The mature alternative Gas6 protein can be cleaved within these additional 43 amino acids by an unknown mechanism releasing the SHBG-like domain⁷².

Though the structural similarity to protein S, no direct anticoagulant activity has so far been found in Gas6, although it nevertheless plays an important role in the vasculature (discussed below).

One shared mechanism of protein S and Gas6 is that they facilitate phagocytosis of apoptotic cells^{22,39,66,73,74}, or for instance senescent red blood cells⁷⁵, in a bridging process where these vitamin K-dependent proteins binds with their Gla-domain to negatively charged surface exposed phospholipids on the target cell, and with their SHBG-like domain to TAM receptors on the APCs^{67,76} (Figure 3). This is a mechanism important in most of all functions of the Gas6 system. Clearance of apoptotic cells can be abrogated by warfarin effect on the Gla-domain⁷⁷, or by sequestering Gas by soluble receptors⁷⁸. Likewise, are non-carboxylated Gas6 unable to mediate most of its effects such as growth, proliferation, and survival effects⁶² (discussed below).

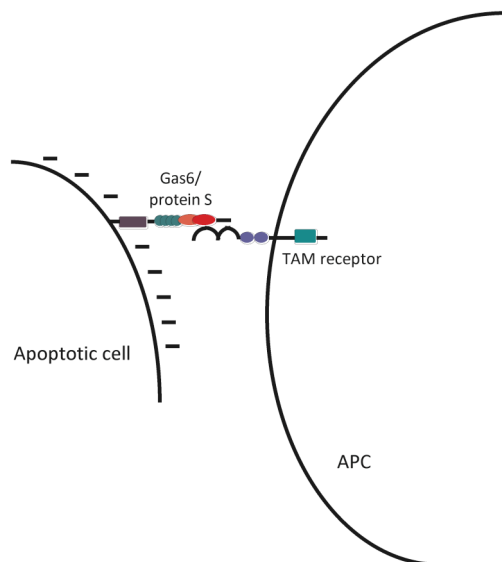


Figure 3. Bridging of apoptotic and phagocytic cell by Gas6/protein S and TAM receptor.

Gas6 expression

Gas6 does not, as most other vitamin K-dependent proteins, have a tissue expression restricted to the liver. In fact, the liver expression of Gas6 is minor compared to the kidney, lung, intestine, and to the vascular endothelium¹⁷, as well as to various cell types, such as smooth muscle cells⁷⁹, bone marrow cells^{80,81}, and in neurons and ECs^{10,19,82}. The Gas6 splice variant shows a similar expression pattern, albeit at lower levels, and no expression is found in placenta and colon⁷¹. In the spleen, the expression level of the splice variant was comparable to Gas6⁷¹.

Furthermore, expression of Gas6 in platelets has been verified in mouse²⁸, and rat⁸³, but so far the expression in human platelets is questionable^{28,84}. Gas6 is also present in mouse and human plasma, where the concentration is measured to vary in between approximately 20-50 ng/mL (< 1nM)⁸⁴⁻⁸⁸.

Compared to protein S levels in serum that can increase up to about 400 nM during an acute-phase response⁸⁹, the Gas6 concentrations are relatively low. However, considering the high protein S concentration, in human circulation about 60 % of protein S is bound to the complement regulatory factor C4b-binding protein (C4BP) through the SHBG domain of protein S, and this binding prevents the protein S binding with TAM receptors⁹⁰. Likewise, under normal conditions most Gas6 is believed to be in complex with sAxl receptor^{91,92}.

Axl

The gene map locus of Axl was localized to 19q13.1⁵⁴, and to 19q13.2¹¹.

The full-length Axl protein contains 894 amino acids, and the predicted molecular weight of Axl is about 98 kDa. However, three apparent molecular weight forms, i.e., 140, 120 and 104 kDa, are occurring that correspond to fully-, partial-, and non-N-glycosylated forms, respectively.^{11,93}

Axl is known to have two splice variants, which products are slightly different in size and molecular weight, resulting from alternative splicing of exon ten^{11,30,94}. Isoform one has nine amino acids more than isoform two. These amino acids are located within the ectodomain outside the transmembrane region on the carboxy-terminal side of the FNIII domains. (Figure 1) Isoform two lacks exon ten coding for these nine amino acids.¹¹

The structure and expression of Axl has already been discussed (see section for the TAM family of receptor tyrosine kinases).

The Gas6/Axl system

Gas6 was identified to be a growth and survival factor that rescued various cells in culture from apoptosis and initiated entry into the S phase of the cell cycle^{79,95,96}. Axl expression and signaling has been suggested to be involved in regulating cell growth and survival, proliferation, cellular adhesion, migration, and invasion as well as in spermatogenesis, immune regulation, and in regulating the dynamics of the blood vessels^{13,62}.

The Gas6/Axl interaction

Gas6 is a ligand of the TAM family receptors^{10,18,56,57,97,98}, and interacts with various affinities to the receptors. It is suggested that Gas6 binds to Axl and Tyro3 with roughly equal affinities in the very low nanomolar range, while Mer affinity for Gas6 is up to 10-fold lower^{18,97,99}.

Gas6 ligation of Axl leads to phosphorylation of the Axl receptor, and the Gas6 function is dependent on a proper Gla-domain in the Gas6 molecule^{65,100,101}. Studies has shown that non γ -carboxylated Gas6 abolish the activation of Axl receptor and its subsequent cellular effects^{10,65}. However, whether decarboxylated Gas6 is able to bind the Axl receptor with similar affinity as carboxylated Gas6 is disputed^{100,102}.

The interaction with the TAM receptors is mediated through the respective SHBG domains of Gas6 and protein S^{72,90,102}, and involves the LG domains^{20,57}. The SHBG domain *per se* could actually bind and activate the receptors, suggesting a regulative role of γ -carboxylation that can affect the binding of the receptor^{57,102}. The crystal structure of the interaction between TAM receptors and the LG-domains of Gas6 has been solved, and involves the two Ig-like domains and the first LG-domain of Gas6^{103,104} (Figure 4). The dimerization between Axl and Gas6 is likely to occur in two steps, where two high-affinity 1:1 complex are assembled first, respectively, and where after a 2:2 stoichiometry complex is believed to form¹⁰⁴. A major and a minor Gas6 binding site is found on the Axl receptor, while only the minor Gas6 binding site is highly conserved in Sky and Mer^{104,105}.

Cellular Gas6/Axl signaling

Signaling through the Axl receptor tyrosine kinase by its ligand Gas6 is primarily involved in cellular transformation of different kinds¹³.

Gas6 activation of Axl results in

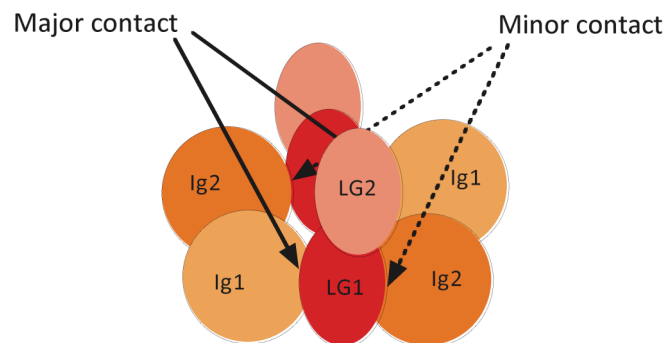


Figure 4. Top/front view of the Axl and Gas6 interaction.

phosphorylation of three known consensus tyrosine residues in the carboxy-terminal kinase region that recruits various signaling molecules: tyrosine 779 (pYALM; phosphatidylinositol 3-kinase (PI3K) binding site), 821 (pYVNM; PLC γ , PI3K, c-Src, Lck, Grb2 binding site), and 866 (pYVLC; PLC γ binding site)^{106,107}. Even though it is suggested, it is not truly clarified that these tyrosine residues are true receptor autophosphorylation sites, and there is still the possibility remaining that they are phosphorylated by recruited kinases. Other Axl binding partners, such as suppressor of cytokine signaling (SOCS)-1, the adaptor protein Nck2, and the tumor suppressor and focal adhesion protein Tensin2 (Tns2; C1-TEN) have also been identified¹⁰⁸.

The PI3K and Akt/PKB pathway, and the Raf and ERK pathway are prominent pathways activated during Gas6/Axl signaling^{13,109}.

The anti-apoptotic effects are the most extensive effects of Gas6 signaling through Axl, and have been detected in almost all cell types tested including fibroblasts^{96,105,110,111}, vascular smooth muscle cells (VSMCs)^{79,112,113}, ECs¹¹⁴, chondrocytes¹¹⁵, neurons⁸², oligodendrocytes^{27,116}, epithelial cells¹¹⁷, and various cancer cells (discussed below). The PI3K pathway involving Akt/PKB is mostly responsible for the survival effects seen of Gas6 signaling^{105,113,114,116-118}, but a simultaneous PI3K and Ras/Erk mediated survival^{82,111,119}, and a Ras/Erk only mediated growth and proliferation has also been reported^{79,95,96,105,120-122}.

It is speculated that Gas6/Axl ligation can generate two context-dependent PI3K signaling pathways; one where PI3K binds directly to Axl resulting in survival, and one where PI3K binds indirectly to Axl through Grb2 leading to proliferation¹⁰⁷.

Chemotaxis or directed migration, has been reported in VSMCs with Gas6 as a chemoattractant¹²³. A chemotactic stimulatory effect on migration has also been reported in gonadotropin releasing hormone neurons, where Gas6 stimulation promoted the development of lamellipodia, membrane ruffles, and chemotaxis^{124,125}. The migration was coordinated by activation of Rac and its downstream effector p38 MAPK. In another study, Gas6/Axl-induced gonadotropin releasing hormone neuronal migration was mediated by the PI3K and Ras pathway upstream of Rac¹²⁶.

Other downstream signaling molecules involved in Gas6/Axl signaling are, for instance, Gas6 mediated survival via NF- κ B signaling downstream of Akt/PKB with upregulated levels of the anti-apoptotic Bcl-2 and Bcl-x_L molecules, and inhibition of caspase-3 activity^{114,127}. Gas6 also stimulates cell proliferation through the downstream molecule signal transducers and activators of transcription (STAT)-3¹²⁸. Furthermore, the Gas6/Axl system suppresses tumor necrosis factor (TNF)- α inflammatory cytokine production by activation of the Twist repressor¹²⁹.

A summary of molecules involved in Gas6/Axl signaling is illustrated in Figure 5.

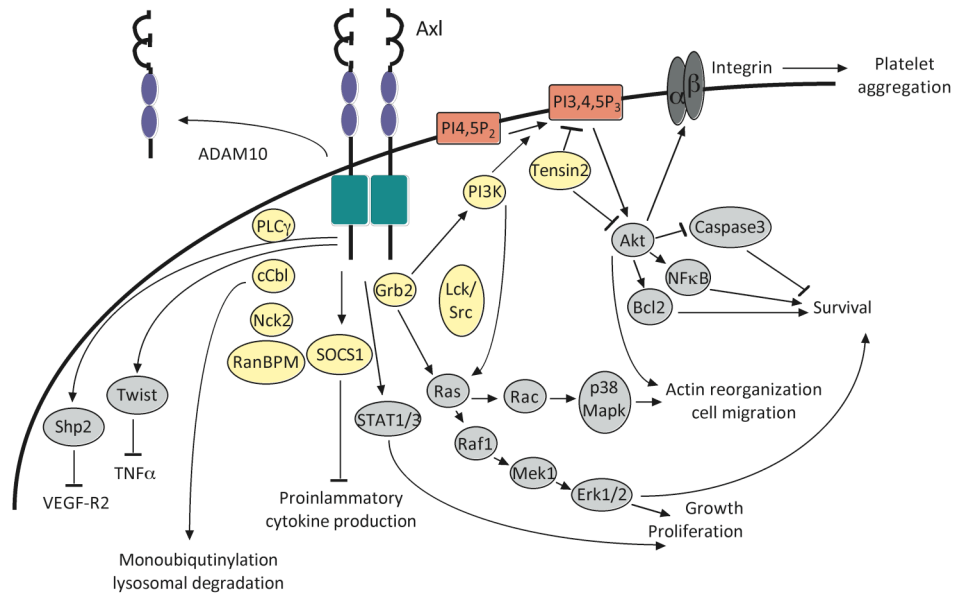


Figure 5. Overview of signaling molecules involved in Axl signaling. Molecules in yellow have been shown to interact with the Axl receptor.

Signaling through RTKs can also occur through atypical activation. In the case of Axl signaling, homophilic interactions of the Axl receptor chains induce adhesion and aggregation, and correlates with increased tyrosine phosphorylation of Axl²⁹. Moreover, the TAM receptors are believed to be capable of heterophilic receptor chain interactions. Recent studies suggest that Gas6-mediated activation of one TAM receptor, in some circumstances, require the presence of one or both of the other TAM receptors^{41,52}.

Another aspect of atypical activation is that of crosstalk between receptor systems. Gas6-independent heterotypic receptor dimerization of Axl with the IL-15 receptor- α chain, and simultaneous trans-activation by IL-15 of Axl, has been reported and leads to downstream activation of PI3K, Akt and ERK¹³⁰. In this study, Axl was indispensable for the IL-15 mediated survival effect. The Gas6/Axl system also interferes with vascular endothelial growth factor (VEGF) signaling in ECs by inducing the dephosphorylation of VEGF activated VEGF receptor 2¹³¹. The TAM receptors are also inhibitors of innate immunity in a process where they take control over the type I IFN receptor and its associated transcription factor STAT-1 pro-inflammatory signaling, in order to provide an intrinsic feedback inhibitor of Toll-like receptor and cytokine-driven immune responses by activation of SOCS-1 and SOCS-3⁴². Finally, Gas6 actions are coupled to integrin signaling and to subsequent activation of macrophages and phagocytosis of apoptotic cells^{74,132,133}.

Regulation and deactivation of the Gas6/Axl system

Several factors have been proven to induce the expression of the Gas6/Axl system.

For instance, Gas6 and Axl expression is upregulated by high glucose stimulation of mesangial cells (MCs) in the kidney^{134,135}. Moreover, in VSMCs exposed to high glucose, Gas6-stimulated ERK signaling is greater than in low glucose with subsequent increase in migration, whilst in low glucose conditions, Gas6-stimulated Akt/PKB signaling is greater than in high glucose with increased cell survival as a result¹³⁶.

Axl activity and expression is regulated by several stress- and redox-stimuli, such as flow-induced laminar shear stress, acidification, and oxidative stress^{112,137,138}. Also, in VSMCs and MCs, Axl and Gas6 expression is induced by a simultaneous angiotensin II and NADPH oxidase dependent mechanism¹³⁹⁻¹⁴¹, further suggesting reactive oxygen species (ROS) to be important in the regulation of the Gas6/Axl system.

Some cytokines have the ability to induce Axl expression. Axl expression is substantially upregulated by type I IFN in oligodendrocytes¹²⁹, and in DCs by type I INF induction following Toll-like receptor activation⁴². IL-15 stimulation of murine fibroblasts induces a significant upregulation of Axl mRNA and protein. Gas6 did not, however, modulate the expression of Axl, but instead Gas6 significantly upregulated the expression of IL-15 receptor- α .¹³⁰

Some drugs confer stability to the Gas6/Axl system. Statins (HMG-CoA reductase inhibitors) inhibits apoptosis by stabilizing Gas6 mRNA and restoration of Gas6/Axl pathway¹⁴². Chemotherapy drugs induce the expression of Axl. Interestingly, in AML was Axl with unmethylated CCWGG sites in the promoter region upregulated to a larger extent than methylated Axl, and only cells with unmethylated Axl promoter conferred resistance to chemotherapy drugs when stimulated with Gas6.¹⁴³

Attenuation of RTK signaling is an important mechanism, since uncontrolled receptor signaling can lead to numerous pathological states including cancer development⁷. Several mechanism have evolved that inhibit aberrant receptor activation including antagonistic ligands, hetero-oligomerization with inactive mutants, phosphorylation of inhibitory residues by other kinases, dephosphorylation of activating residues by phosphatases, and receptor downregulation by ligand-induced endocytosis accompanied ligand dissociation, receptor degradation or both.³

The putative tumor suppressor and lipid phosphatase Tns2 has been shown to bind Axl, and to diminish cell survival, proliferation, and migration^{108,144} (discussed below). Tns2 also harbors a phosphatase and tensin homologue (PTEN) tumor suppressor homology region, and has been shown to decrease the level of phosphatidylinositol-(3,4,5)-trisphosphate (PtdIns(3,4,5)P₃) at the membrane of living cells. Perhaps, Tns2 function as a tumor suppressor that negatively regulates downstream Axl signaling. However, if this inhibitory action is through indirect unknown mechanisms or through phosphatase activity is not known.¹⁴⁵

Evidence exists to support endocytosis as a relevant mechanism for Axl receptor inhibition. Gas6 stimulation has been shown to lead to Axl activation, c-Cbl interaction, and monoubiquitination that is followed by Axl downregulation through lysosomal degradation^{10,146}. Interestingly, it was further shown that also hydrogen peroxide (H₂O₂) also induces Axl activation, although this kind of activation did not lead to Axl and c-Cbl interaction nor Axl ubiquitination and degradation¹⁴⁶.

Proteolytic cleavage by a disintegrin and metallopeptidase (ADAM)-10 release the receptor ectodomain of Axl, and generates sAxl that can be found normally in plasma^{92,93,147} (Figure 5). In human cells, this cleavage of Axl is mapped to a 14-amino acid stretch in the extracellular region, outside the transmembrane region, and carboxy-terminal to the nine extra amino acids in isoform one. The released Axl protein corresponds to a protein with apparent molecular weight around 80 kDa.⁹³ In one study, sAxl was shown to stimulate membrane-bound Axl independent of Gas6, suggesting homophilic interaction among Axl molecules potentially relevant⁹². Even though, most or all of the plasma Gas6 is as mentioned believed to be in complex with sAxl^{91,92}, and sAxl is in general believed to function as a ligand sink to sequester Gas6 ligand, and thereby limit signaling through the full-length Axl receptor¹⁴⁷ (unpublished observation). This notion is strengthened by the observation that releasing sAxl reduces the half-life of the remaining kinase domain to less than half of that of the full-length protein⁹³.

The Gas6/Axl system in important biological contexts

Depending on the type of stimulation and the extracellular microenvironment the Gas6/Axl system can stimulate diverse cellular functions. Mechanisms involved in vascular biology, in regulation of inflammation, and in cellular homeostasis are important aspects of the Gas6/Axl signaling system.¹³

Gas6/Axl in the vasculature

In cardiovascular biology the signal transduction downstream of RTKs has become an increasingly important area of study¹⁴⁸.

One of the first links to Gas6 signaling was that of its role in the vasculature, where Gas6 in VSMCs was found to be a growth-potentiating factor⁷⁹, and where it could prevent serum deprivation-induced apoptosis¹⁴⁹. Later Gas6 was also shown to rescue human umbilical vein endothelial cells from serum deprivation and TNF α induced apoptosis^{114,150}.

In an artery injury model both Axl and Gas6 expression were shown to be upregulated, mainly in the neointima^{139,140}. Later, the same group verified a PI3K and Akt/PKB dependent survival effect in VSMCs with an activated Gas6/Axl signaling system¹¹³.

In vascular biology, the process of acidification of ECs plays an important role. Prevention of apoptosis by acidification, as a response to for instance laminar shear stress on ECs, is mediated by sustained Axl expression and Gas6-dependent activation^{137,138}.

The Gas6/Axl system is involved in vascular remodeling in the context of chemotactic migration of VSMCs after vascular injury and oxidative stress^{112,123}. Oxidative stress that often occurs in vascular injury, results in Axl phosphorylation and induction of the Akt/PKB survival pathway¹¹². Axl deficient mice show a decreased neointima formation with more apoptotic cells, less infiltrating macrophages and neutrophils in flow-dependent remodeling, further suggesting an important role of Axl in vascular apoptosis and vascular inflammation¹⁵¹. Axl has furthermore been proven to affect multiple cellular behaviors required for neovascularization *in vitro* such as proliferation, migration, and survival of ECs, tube formation, and to regulate angiogenesis *in vivo*¹⁵².

In human atherosclerotic lesions, Gas6 is expressed in macrophages, VSMCs, and in ECs. Furthermore, Gas6 is especially highly enriched in the foam cell-rich lesions of the intima, and is suggested to decrease the stability of and increase the inflammation in atherosclerotic plaques.¹⁵³ In relation to this, it has also been shown that Gas6 signaling mediates expression on ECs of the scavenger receptor A that mediates the uptake of low-density lipoproteins¹⁵⁴. Recently, Gas6 was determined to mediate activation of ECs, to mediate platelet and leukocyte endothelial adhesion, and leukocyte extravasation¹⁵⁵. However, Gas6 has also been reported to inhibit granulocyte adhesion to ECs activated with thrombin, IL-1 and TNF- α , but not with IL-8¹⁵⁶.

Gas6 and its receptors modify platelet activation and aggregation^{28,52,157,158}.

In mice, Gas6 is a platelet response amplifier that plays a role in pathological thrombosis, and where Gas6 neutralizing antibodies inhibit platelet aggregation *in vitro*, and prevent fatal thrombosis *in vivo*²⁸. Gas6 was found to be located in α -granules of the platelets and released during platelet activation^{28,83}. When the Gas6 receptors are blocked or inactivated, initial platelet aggregation occurs, but the stabilization of platelet aggregates is impaired and mice are protected against thrombosis. One explanation is that Gas6 stimulates PI3K and Akt/PKB activation and phosphorylation of the β 3 integrin, amplifying the "outside-in" signaling that is needed for full platelet activation.⁵²

Blockade of Gas6, or any of the TAM receptors, reduces thrombus formation without an increase in bleeding^{28,158}. Erythroblasts from acute anemic mice, stimulated with Gas6, had subsequent activation of the Akt/PKB pathway and obtained normalized hematocrit levels⁷⁵.

Gas6 is, as mentioned, present in human plasma at a concentration of about 20 – 50 ng/mL, where most of the Gas6 probably is in complex with sAxl, although the biological relevance of this is debatable^{84-86,88}. Whether expression of Gas6 occurs in human platelets is a controversial issue. Even though Gas6 mRNA and protein have been detected in human platelets in one study²⁸, Gas6 could not be detected in another study using a highly sensitive ELISA method⁸⁴. This suggests that if Gas6 is involved in human primary hemostasis it is originating from Gas6 in circulation. It should be noted that in a recent study no effect could be found of physiological human plasma Gas6 levels on platelet aggregation¹⁵⁹.

Gas6/Axl signaling in cancer

Several so called “hallmarks of cancer” which normal cells acquire during oncogenesis have been proposed; self-sufficiency in growth signals, insensitivity to antigrowth signals, limitless replicative potential, tissue invasion and metastasis, sustained angiogenesis, and evasion of apoptosis. RTKs play important roles in many of these oncogenic events.¹⁶⁰

Axl is a transforming gene originally cloned from patients with CML¹¹, and chronic myeloproliferative disorder⁵⁴. Axl is a proto-oncogene whose overexpression can transform cells even in the absence of ligand, and it is believed that Axl transforming capacity is due to activation and overexpression of the receptor, whilst no activating mutations have been found^{11,54,161}. Axl activation in tumor cells has been suggested to follow an autocrine/paracrine pathway¹⁶², and Axl transforming capacity also seem to be cell-specific¹⁶¹.

Many studies have shown that Axl is overexpressed in a variety of human tumor cells or in several types of human malignancies, such as in AML¹⁴³, breast cancer¹⁶³⁻¹⁶⁵, colon cancer¹⁶⁶, gastric cancer¹⁶⁷, lung adenocarcinoma¹⁶⁸, melanoma¹⁶⁹, osteosarcoma¹⁷⁰, renal cell carcinoma (RCC)^{88,171}, squamous cell skin cancer¹⁷², and thyroid cancer¹⁷³.

Simultaneous overexpression of both Axl and Gas6 have also been shown in human malignancies, such as in human gliomas^{174,175}, gastric cancer¹⁷⁶, and in ovarian cancers¹⁷⁷.

In AML, Axl expression is associated with worse progression-free and overall survival¹⁷⁸. Axl expression in AML is also induced by chemotherapy drugs, and confers drug resistance to the tumor cells¹⁴³. In RCC, both Axl and Gas6 correlate to advancement of the tumor, and to patient survival. Furthermore, tumor Axl mRNA remains an independent factor when assessed against tumor stage and grade, wherein low Axl levels is associated with much longer patient survival⁸⁸ (thesis Paper I).

In human breast cancer, Gas6 expression correlated inversely with tumor markers such as tumor size and lymph node metastases¹⁷⁹. The Gas6 locus has been described as a target for amplification in breast cancer¹⁸⁰, to be an estrogen gene target¹⁸², and to be

upregulated 23-fold in breast cancer cells by progesterone acting through the progesterone receptor B¹⁸¹.

The Gas6/Axl system is involved in several human malignancies contributing to the disease, although the exact role of this signaling system in cancer is not elucidated.

As mentioned, several lines of evidence suggest that the Gas6/Axl system plays an important role in survival pathways, and is linked to regulation of the actin cytoskeleton in both normal and cancerous cells.¹³ For instance, overexpression of murine Axl in insect cells results in cell aggregation by homophilic binding²⁹. Interestingly, overexpression of Axl in myeloid progenitor cells is not sufficient to induce cell aggregation. Rather, attachment of Gas6 to the plasma membrane is required to induce cell aggregation, and this effect is blocked by excess sAxl.¹⁸³ In addition, Axl expression in human lung cancer is correlated with cellular adhesion¹⁸⁴. Interestingly, Axl expression in non-small cell lung carcinoma cell lines augments drug resistance and invasiveness, and is accompanied by activated NF- κ B signaling¹⁸⁵. Elevated Axl expression correlated with adherence, motility, and invasiveness of osteosarcoma cells selected for their high metastatic ability in an *in vivo* model of lung metastasis¹⁷⁰. Axl expression drives motility and an invasive phenotype of breast cancer cells¹⁶⁵.

The role of Axl RTK in glioma and breast cancer tumor formation, and its role in angiogenesis have been studied in somewhat more detail.

Inhibition of Axl signaling suppresses glioma growth and prolongs survival in a xenograft mouse model.¹⁷⁴ The authors indicate a role for Axl in mediating glioma cell proliferation, migration, and invasion. In human gliomas, Axl and Gas6 are frequently overexpressed in glioma cells and vascular cells of tumor vessels, and predict poor prognosis in glioblastoma multiforme patients¹⁷⁵.

Angiogenesis occurs aberrantly in hypoxic tumors, securing a blood supply for the tumor to facilitate tumor growth and metastasis, and proliferation and migration of VSMCs are key events required during angiogenesis¹⁴⁸. As mentioned, proliferation and migration of VSMCs are promoted by signaling through the Gas6/Axl system^{79,123}. Recently, it was shown that Axl signaling contribute a great deal to neovascularization *in vitro* and *in vivo* by regulating growth, survival, proliferation, migration, and tube formation of vascular ECs, and also mediates tumor growth in an *in vivo* breast cancer model¹⁵². As nothing is simple, the Gas6/Axl system has also been shown to antagonize the angiogenic program driven by VEGF, one of the most prominent initiator of angiogenic sprouting of newly formed vessels, through inhibition of the VEGF receptor-2^{131,148}.

Finally, Axl can also promote cell invasion independently of Gas6 where the stimulatory invasion process is mediated through induction of the matrix metalloproteinase (MMP)-9 enzyme¹⁸⁶.

The Gas6/Axl system for potential therapeutic applications

Protein kinases are important in tumor development and progression, and a variety of them serve as targets for therapeutic intervention in cancer¹⁸⁷.

The Gas6/Axl system clearly appears to play a role in the progression of multiple cancers and in the development of metastasis. There are validations of the therapeutic potential of targeting this system. As mentioned, inhibition of Axl signaling reduces glioblastoma growth and invasiveness¹⁷⁴, and downregulation of Axl expression restricted tumor angiogenesis and diminished growth of breast carcinoma tumors¹⁵².

A more detailed study of the potential of Axl as a target for therapeutic intervention has been evaluated in a model of breast cancer progression¹⁶⁵. The use of polyclonal antibodies specific towards Axl ectodomain or knockdown of Axl expression inhibited motility and invasivity of breast cancer cells. Moreover, the use of 3-quinolinecarbonitrile small molecular inhibitory compounds was shown to inhibit Axl phosphorylation after Gas6 stimulation, and the use of the Src and Abl kinase inhibitor SKI-606, in addition, strongly interfered with the motility and invasivity of invasive breast cancer cells.¹⁶⁵

Another small molecule inhibitor designed to inhibit Axl receptor function has been reported, namely MP470. It is a potential Axl inhibitor that also blocks other tyrosine kinases, such as c-Kit and platelet-derived growth factor (PDGF) receptors, and reduces the metabolic activity of an Axl expressing, drug-resistant, gastrointestinal stromal tumor cell line¹⁸⁸.

The use of small molecule inhibitors that specifically target the ATP-binding site of the Axl kinase domain would be suitable for drug use in order to abolish Axl signaling in general. However, Axl signaling is complex, being dependent on Gas6 stimulation in many cases, but also independent of Gas6 in other, and being mediated through homophilic and heterophilic interactions^{29,130,147,186}. Therefore, in the future, when more is known about each kind of Axl signaling, perhaps it would be desirable to design inhibitors that target the extracellular domain of Axl, and specifically abrogate the present interaction inducing the kinase activity, but not Axl enzymatic activity in general.

Another approach that could be appreciated in some circumstances would be to soak up available ligand. Indeed, treatment with secreted sAxl ectodomains abrogates Gas6-induced stimulation and cell proliferation of prostate carcinoma cells¹²², and the use of an anti-Gas6 antibody prevented fatal thrombosis *in vivo*²⁸.

Most likely there will be benefits from targeting the Gas6/Axl system specifically. This is highlighted by the example of some gastrointestinal stromal tumors undergoing a kinase switch from c-Kit mediated signaling to Axl tyrosine kinase signaling, conferring Imatinib drug resistance¹⁸⁸. Furthermore, Axl activation is not influenced by the tyrosine kinase inhibitor Sorafenib (unpublished observation), which is an inhibitor commonly used in treatment of RCC, where Axl expression correlates with much poorer prognosis⁸⁸ (thesis Paper I). Thus, other, more specific therapies targeting Axl may be more effective in relevant Axl overexpressing RCC tumors.

Part II

General background

The kidney and the Gas6/Axl system

The Gas6/Axl pathway appears to be important for growth control of MCs in the kidney, and to play a fundamental role in the progression of various inflammatory renal diseases. And inhibition of the Gas6/Axl system with low-dose warfarin has been suggested to be a potent therapeutic intervention in treatment of human kidney inflammatory diseases.¹³⁴

This thesis presents evidence that the Gas6/Axl system also might play a role in RCC. A low Axl mRNA expression in the tumor correlate independently with prolonged survival, and inverse, high Gas6 mRNA expression in the tumor is associated with better prognosis⁸⁸ (thesis Paper I). Moreover, in clear cell RCC cells Gas6 exerts inhibitory effects on migration and viability (thesis Paper II).

Hence, the biology of Gas6/Axl is complex, and there is a great need for better understanding of the role of this receptor system in the kidney.

The kidneys

The kidneys convey several important biological functions in addition to the main one of producing urine, and these functions are for instance regulation of electrolytes, acid-base balance, and blood pressure. Furthermore, glucose and amino acids reabsorption, and hormone production, including vitamin D and erythropoietin, are also important mechanisms.¹⁸⁹

Blood enters the kidneys through the renal arteries at the renal hilum at the concave surface of the kidney. This is also where the renal vein and ureter leaves. The renal capsule, which is a fibrous tissue that surrounds the kidney, is covered by perinephric fat, the renal fascia of Gerota, and paranephric fat. Superficially within the kidney substance lays the renal cortex and deep is the renal medulla.¹⁸⁹

The functional units of the kidneys are the nephrons that span the cortex and medulla. A nephron is constituted by a renal corpuscle that is composed of a glomerulus enclosed in a Bowman's capsule, the basic filtration unit of the kidney that filtrate circulating blood. Within the glomerulus, cells, proteins, and other large molecules are filtrated out, leaving an ultrafiltrate to enter Bowman's space and to pass through the proximal tubule, the loop of Henle, the distal convoluted tubule, and a series of collecting ducts to form urine.¹⁸⁹ (Figure 6)

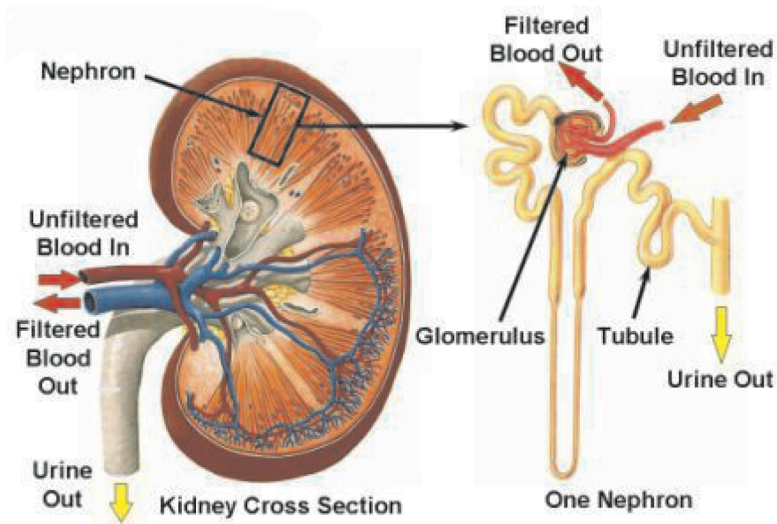


Figure 6. Illustration of a cross-section of a kidney (left) and a nephron (right).
Used with permission from the UNC Kidney Center

MCs are specialized cells lining blood vessels of the kidney, located mainly in the mesangium, the inner layer of the glomerulus (Figure 7). These cells are usually divided into two types, intraglomerular and extraglomerular MCs, each having a very distinct function and location. MCs are phagocytic cells, whose main function is that of providing structural support in part by secretion and maintenance of the extracellular matrix.¹⁸⁹

Glomerular disease and mesangial cell proliferation

Renal failure can occur gradually or with a sudden onset and eventually leading to loss of kidney function. Acute and chronic kidney failures are terms used when some kidney function remains. Total loss of kidney function is termed end-stage renal disease and indicates permanent loss of kidney function.¹⁸⁹

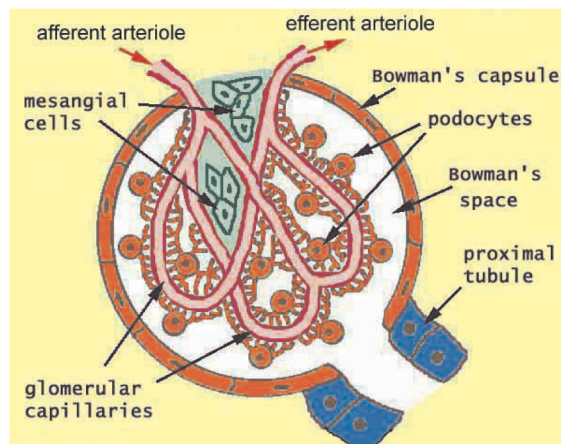


Figure 7. Illustration of a cross-section of a glomerulus.
*Image copyright by David King 2009, used with permission
www.siumed.edu/%7Edking2/crr/rnquide.htm*

Many diseases affect kidney function by attacking the glomeruli, and several factors can cause glomerular damage, for instance, infections, drugs toxic to the kidneys, and diseases such as diabetes, lupus, or cancer. Glomerular diseases fall into two major categories, which are glomerulonephritis – inflammation of the membrane tissues in the kidney, and glomerulosclerosis – the scarring and hardening of the tiny blood vessels within the kidney. Typical symptoms of glomerular disease include proteinuria, hematuria, reduced glomerular filtration rate, hypoproteinemia, and edema.¹⁸⁹

Proliferation of MCs and glomerular hypertrophy is occurring in many types of glomerular disease, and is usually associated with matrix expansion leading to the development of glomerulosclerosis and end-stage renal failure¹⁹⁰. Therefore, in order to identify potential targets for therapeutic intervention in various kidney diseases it is important to understand the role of MCs in the kidney. The activity of MCs is primarily controlled by cytokines such as IL-1, PDGF and transforming growth factor (TGF)- β ¹⁹⁰.

An important regulator of renal disease is angiotensin II. Currently, interference with the renin-angiotensin-aldosterone system is the main action used in clinical treatments of nephropathy.¹⁹¹ However, it has been hypothesized that also warfarin treatment improves the prognosis of renal disease, and that warfarin might prevent the deposition of fibrin within and around the glomeruli¹⁹². However, the exact mechanism of action of warfarin in kidney disease remains elusive unless the fact that warfarin seem to work through mechanisms not directly related to the coagulation pathway¹⁹².

Axl and Gas6 in the kidney

MCs express Axl and Gas6^{193,194}, and Gas6 can be detected in the culture medium of MCs¹⁹³. Axl and Gas6 expression is also found in the glomeruli with a mesangial pattern, in podocytes, small vessel media and intima, and in tubular epithelial cells¹⁴¹ (unpublished observation).

Gas6 stimulation of MCs leads to Axl upregulation, phosphorylation, and cell proliferation in an autocrine manner. This is an effect inhibited by warfarin treatment at concentrations significantly lower than the doses used for therapeutic warfarin treatment.¹⁹³ The Gas6/Axl system plays a key role in proliferation of MCs *in vivo* acting through the STAT-3 pathway, and is a prominent mediator in the development of experimental glomerulonephritis^{128,194-196}. Additionally, in an *in vivo* model of a progressive form of glomerulonephritis (nephrotoxic nephritis), leading to glomerular sclerosis and end-stage renal disease, similar results were found. Gas6 knockout mice displayed a reduction in mortality and proteinuria, a reduced glomerular cell proliferation, and less sclerosis etc.¹⁹⁵

One of the most common causes of end-state renal disease is diabetic nephropathy¹⁹⁷. In diabetic nephropathy, both Gas6 and Axl expression is upregulated, for instance, in

the media of small vessels, in the glomeruli, and in the renal tubule cells with a mesangial and endothelial staining pattern^{134,135,141}. Furthermore, Gas6 induces hypertrophy of MCs by activating the Akt/PKB pathway in the pathogenesis of diabetic nephropathy^{134,135}. The authors also lay evidence that warfarin treatment of diabetic rats reduce both mesangial and glomerular hypertrophy, and improves glomerular filtration rate.

Hence, the Gas6/Axl system is a player in progressive and chronic renal disease specifically as a mediator of abnormal proliferation of MCs^{135,194,196}. However, the story of Gas6 and Axl in the kidney is far from complete.

A simultaneous angiotensin II and NADPH oxidase dependent expression of Axl and Gas6 was shown to occur in MCs¹⁴¹. Interestingly, Gas6/Axl mediated cell survival probably depends on activation of NF- κ B¹²⁷, a transcription factor that is involved in inflammatory glomerular and vascular diseases¹⁹⁸, and being out of several mechanisms especially activated by ROS¹⁹⁹. To add complexity to the story, an increase of Gas6 and Axl has also been detected in renal tubules following administration of an angiotensin converting enzyme (ACE) inhibitor²⁰⁰. The consequences of this ACE inhibitor-dependent upregulation of Axl and Gas6 is not known, however, it is very interesting in this respect that one adverse side effect of ACE inhibitors are renal impairment, for which the reason is still unknown.

Part II

Specific background

Renal cell carcinoma

A common feature associated with an expansion in size of the kidney is the presence of kidney cysts, which are quite common and occur in about 50 % of all adults above 50 years of age. Other tumors of the kidney that are differentially diagnosed when RCC is suspected are renal pelvis carcinoma, oncocytoma, angiomyolipoma, and Wilms tumor. However, RCC is the most common tumor of the renal parenchyma.^{201,202}

Renal cell carcinoma

RCC is the most lethal cancer of the urological cancers, with more than 40 % of patients dying. The only curative treatment today is removal of the primary tumor. However, about 20 – 30 % of the patients display metastatic disease at time of nephrectomy, and about the same number of patients will develop distant metastases or local recurrence. Metastatic RCC present with very poor prognosis.²⁰³

Epidemiology

RCC is the most common cancer of the adult kidney, and accounts for about 3 % of all human malignancies worldwide. In 2009, the number of estimated new cases of RCC and disease-specific deaths in the United States is 57 760 (35 430 male and 22 330 female), and 12 980 (8160 male and 4820 female), respectively.²⁰⁴

In RCC, the prevalence is skewed between men and women (1.5:1), peaks at 60 – 70 years of age, and the incidence has increased over the last 20 years²⁰⁵. Globally, there is a trend for 2 % per year increase in RCC incidence, however, in Sweden and Denmark, a decreased incidence rate of 2 % has been observed during the last two decades²⁰⁶. The increased global rate of RCC incidence might be a reflection of the improved and more available diagnostic tools, such as ultrasonography, computerized tomography (CT), and magnetic resonance imaging (MRI) that have resulted in earlier detection of the tumor²⁰⁷.

The reason behind the opposite trend in the two Nordic countries is unclear. Known established risk factors for RCC are cigarette smoking that is believed to account for about 30 % of all sporadic RCCs, obesity, hypertension, and acquired cystic kidney disease occurring in end-stage renal disease²⁰¹. Indeed, the smoking habits in the Nordic countries are different from the rest of the world in general.

Etiology and histological subtypes

RCC arises from the renal epithelium, but is not a single tumor type. Instead, RCC comprise a group of tumors with partially unknown etiology originating from different parts of the kidney with varying genetic bases, morphological features, and clinical behaviors²⁰⁸. The major malignant RCC tumors are sub-divided according to histology, genetics, and their putative cell of origin into clear cell RCC (ccRCC; conventional RCC), papillary RCC (pRCC), chromophobe RCC (chRCC), collecting duct carcinoma, and unclassified RCC²⁰⁹. In about 4 % of the cases, benign tumors such as oncocytoma, thought to arise from type A intercalated cells of the collecting duct, is differentially diagnosed when malignant RCC is suspected. Genetic predisposition is rare and only about 2 – 4 % of all RCCs are associated with inherited syndromes.^{202,203}

The major RCC type is ccRCC occurring in about 80 % of the cases and is believed to be of proximal tubular origin²⁰⁹. Out of these tumors, about 60 % are characterized by sporadic loss of function of the *von Hippel-Lindau* (VHL) tumor suppressor protein resulting from mutations in one or both copies of the VHL gene. Silencing of the remaining allele by epigenetic events, such as hypermethylation, may explain some of the remaining cases of sporadic ccRCC. Rarely, the ccRCC cases are due to underlying hereditary genetic abnormalities, such as the autosomal VHL syndrome, an inherited disorder that manifest in tumor formation in multiple organs. Patients with VHL syndrome have a greater than 70 % risk of developing ccRCC by age of 60. A few percentages of hereditary ccRCC without any detectable VHL inactivation have also been described.^{201,203}

The VHL protein function as an ubiquitin ligase and targets the hypoxia inducible transcription factors (HIF)-1 α and -2 α , when prolyl hydroxylated in the presence of oxygen, for proteasomal degradation (Figure 8). During hypoxia these transcription factors are protected from degradation. One of the two isoforms then translocates to the nucleus and dimerizes with the constitutively expressed HIF- β . In the nucleus the formed complex binds to hypoxia response elements (HREs), and drives the expression of proteins such as TGF α , VEGF, and the glucose transporter Glut1. Loss of VHL function leads to a pseudo-hypoxic response in ccRCC that conveys much of the disease progression.²¹⁰

The second most common form of RCC is pRCC, believed to originate from the distal tubule cells, occurs in about 10 – 15 % of the cases²⁰⁹. Localized pRCC metastasize less frequently than ccRCC and present with better prognosis. However, the survival rate of metastatic pRCC is worse than ccRCC.²⁰³ pRCC is further subdivided into a lower grade type 1 tumor, with a favorable prognosis, and a higher grade type 2 tumor with poorer prognosis²¹¹. Type 1 pRCC most often displays duplication of the MET proto-oncogene, an event that together with activating mutations in the c-Met protein are associated with hereditary pRCC. These patients have increased risk of developing type 1 pRCC.

Patients with the hereditary leiomyomatosis syndrome are associated with mutations in the fumarate hydratase gene, and are at risk for developing aggressive type 2 pRCC.^{201,203}

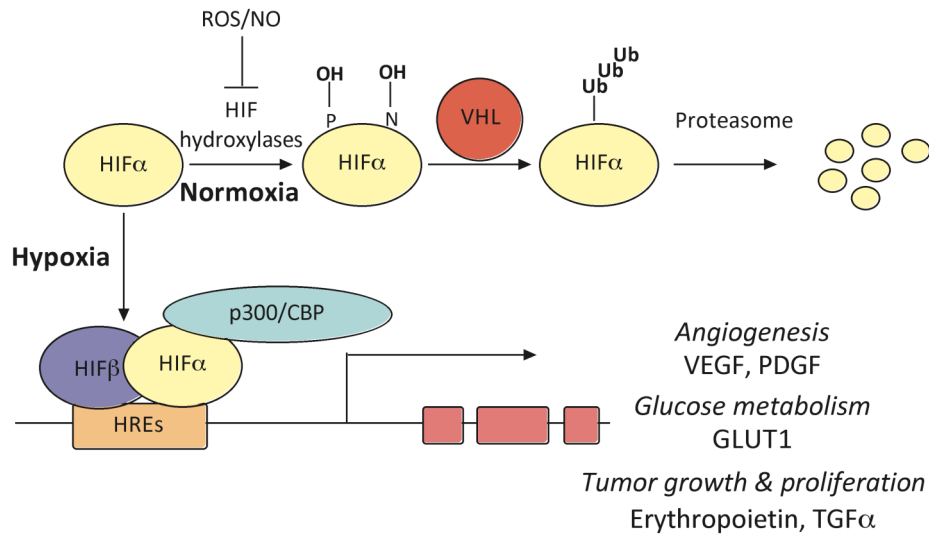


Figure 8. Simplistic view of HIF regulation and transcriptional activity.

Around 5 % of the RCC cases are of chRCC subtype that is believed to arise from the type B intercalated cells of the collecting ducts. Patients with the Birt-Hogg-Dubé cancer syndrome, characterized by lung cysts, cutaneous fibrofolliculomas, and multifocal renal tumors, will in about half of the cases develop chRCC-like tumors, and are associated with abnormalities in the *Birt-Hogg-Dubé* gene that encodes a tumor suppressor protein.²⁰³

Collecting duct carcinomas are rare tumors with an aggressive clinical course arising in about 1 % of the RCC cases²⁰³.

Unclassified RCC accounts for about 4 – 5 % of the RCC cases. This classification is used when a RCC tumor does not fit into one of the others categories, and contains tumors with a variety of appearances and genetic lesions.²⁰⁹

Sarcomatoid degeneration may occur in any of the RCC subtypes, and is associated with elevated aggressiveness of the disease^{209,212}.

Prognostic factors

Prognostic factors are those that differentiate patients based on risk for a particular outcome independently of therapy and other markers. Prognostic markers in RCC include, histologic, clinical, and molecular factors. However, to date the most important single variable in prognostic validation of RCC patient survival outcome is the tumor-node-metastasis (TNM) staging system proposed by the Union Internationale Contre le Cancer (UICC) and the American Joint Committee on Cancer (AJCC).²¹³

The TNM system is taking into account the primary tumor size and extension beyond the renal capsule, involvement of tumor material in the renal vein and/or vena cava, regional lymph node involvement, and distant metastasis. The TNM system has undergone several systematic revisions since the original proposal 1974, and the sixth and currently used version is the TNM 2002 edition²¹⁴ (Table 1). Despite the usefulness of the TNM staging system, further modifications are justified to enhance its predictive value^{213,215}.

Table 1. The 2002 TNM staging system and stage grouping for RCC²¹⁴.

Primary tumor (T)	
T1a	Tumor 4 cm or less in greatest dimension, limited to the kidney
T1b	Tumor more than 4 cm but not more than 7 cm in greatest dimension, limited to the kidney
T2	Tumor more than 7 cm in greatest dimension, limited to the kidney
T3a	Tumor directly invades adrenal gland or perirenal and/or renal sinus fat but not beyond Gerota's fascia
T3b	Tumor grossly extends into the renal vein or its segmental (muscle –containing) branches, or vena cava below the diaphragm
T3c	Tumor grossly extends into vena cava above diaphragm or invades the wall of the vena cava
T4	Tumor invades beyond Gerota's fascia
Regional lymph nodes (N)	
N0	No regional lymph node metastases
N1	Metastases in a single regional lymph node
N2	Metastasis in more than one regional lymph node
Distant metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis

Table 1 (cont.). The 2002 TNM staging system and stage grouping for RCC²¹⁴.

Stage grouping
Stage I: T1N0M0
Stage II: T2N0M0
Stage III: T1-2N1 or T3N0-1
Stage IV: T4 (any N or M) or N2 (any T or M) or M1

Other validated prognostic factors are the tumor grade according to Skinner²¹⁶ and Fuhrman²¹⁷, and the Eastern Cooperative Oncology Group performance status (ECOG PS) of the patients²¹⁸. Since the presentation of the Fuhrman grading it has become the most widely used and accepted nuclear grading system²⁰⁵. Despite the evidence that high-grade tumors have poorer prognosis than those of low-grade, there are considerable ongoing debates regarding its predictive value, especially for tumors classified into the intermediate grade²¹⁵, and in several studies Fuhrman grading did not remain independent when assessed against TNM stage²¹⁹.

Histological subtype is also associated with RCC patient survival, where pRCC is believed to have better prognosis than ccRCC, and chRCC better prognosis than both ccRCC and pRCC^{209,218}. However, when assessed against TNM stage, Fuhrman grade, and ECOG performance status, in multivariate analysis, histology did not remain independent and should not be regarded as a prognostic marker²¹⁸. The collecting duct tumors are highly aggressive and develop metastases rapidly²⁰³. Sarcomatoid differentiation is also associated with worse survival prognosis²¹².

A large number of molecular biomarkers have also been associated with cancer-specific survival in RCC patients. However, out of them, several are not independent predictors of disease progression after nephrectomy for pathologically localized tumors, and so far, only a few of the independent ones have undergone external validations. Also, to date, many described biomarkers require histopathologic assessment of the tumor specimen.²²⁰ Several combinations of known prognostic variables into algorithm or nomograms have been made in an attempts to improve the precision of the outcome prediction, and also to identify subsets of patients responding to a particular treatment^{220,221}.

Symptoms and diagnosis

The majority of the RCC tumors remain asymptomatic until large enough to displace or invade other organs. The classical manifestations of RCC are hematuria, flank pain, and abdominal mass. However, today with modern imaging techniques the tumors usually are incidentally detected before these symptoms arise. The lungs, bone, liver, and brain are the most common site of metastasis, in that order, and some patients suffer from metastasis-associated symptoms, such as bone pain and coughing. Paraneoplastic symptoms, such as hypertension, anemia, abnormal liver function, elevated erythrocyte

sedimentation rate (ESR), amyloidosis, and serum C-reactive protein (CRP) levels also take place in RCC.^{201,205}

Treatment and survival

The only curative treatment of RCC is surgery^{213,222}. Open radical nephrectomy has so far been the standard choice of treatment for localized RCC. However today, smaller and less advanced RCC tumors are detected by improved imaging techniques, and open or laparoscopic nephron-sparing surgery of tumors < 4 cm in diameter is an alternative. Laparoscopic nephrectomy presents with lower morbidity when compared with open surgery, and is believed to become a standard care of patients with RCC tumors limited to the kidney.^{201,205}

However, as mentioned, a substantial number of patients present with metastatic disease at time of diagnosis or will develop metastatic RCC after nephrectomy. The outcome of metastatic RCC is poor and mortality remains very high with a median cancer-specific survival of 21 months^{220,223}. Treatment of metastatic RCC with conventional chemotherapy is largely ineffective, and the majority of patients do not benefit from this treatment. Therefore, treatment of metastatic RCC, in addition to removal of primary tumor and resection of metastases, is largely palliative, and includes systemic immunotherapy approaches, such as e.g. high-dose IL-2 and IFN- α treatment.^{205,224}

Recently, the development of a number of exciting new therapeutic approaches has emerged. There are a number of promising new tyrosine kinase inhibitors that have received approval from the Food and Drug Administration (FDA) for treatment of metastatic RCC, such as anti-angiogenic drugs that targets the VEGF and PDGF pathways.^{224,225}

Sunitinib malate (Sutent) targets VEGF and PDGF receptors, and the stem cell factor receptor c-Kit. Sunitinib has been reported to be well tolerated, and to increase progression-free survival in comparison to traditional IFN- α treatment.²²⁶ Due to the improvement in progression-free survival and its tolerability, Sunitinib has become a new standard of first-line treatment of good- and intermediate-risk metastatic ccRCC²²⁴.

Sorafenib tosylate (Nexavar), another multitarget kinase inhibitor, interferes with Raf-1 kinases, VEGF and PDGF receptors, and c-Kit. Like Sunitinib, Sorafenib is well tolerated, and increases progression-free survival.²²⁷

Temsirolimus (Torisel) and Everolimus are immunosuppressive drugs targeting the mTOR molecule, and are among the most recent drugs tested in clinical trials for treatment of metastatic RCC. Clinical efficiencies of these drugs are promising, with positive effects on progression-free and overall survival. Temsirolimus has been proven

to increase median overall survival in advanced RCC patients²²⁸, and is suggested to be considered a standard in poor-risk metastatic ccRCC.²²⁴

Bevacizumab (Avastin), a monoclonal antibody targeting the VEGF molecule that thereby prevents its ligation of the VEGF receptor, has proven to increase progression-free survival of metastatic RCC patients in several trials²²⁴.

Part III

General background

Phosphatases and Lipid signaling and PTEN

The fundamental process of intracellular phosphorylation of protein and lipids is carefully counterbalanced by regulated dephosphorylation by a large superfamily of enzymes called phosphatases.²²⁹

Lipids are important mediators of intracellular signaling and are regulated by lipases, lipid kinases, and lipid phosphatases among which the dual-specificity and lipid phosphatase PTEN is a crucial member²³⁰⁻²³².

Phosphatases

Phosphatases are abundantly occurring and highly specific enzymes for phosphorylated substrates. The majority of phosphatases are classical cysteine-based phosphatases, including protein tyrosine phosphatases, and the dual-specificity phosphatases with phospho-serine and phospho-threonine as additional substrates, and the lipid phosphatases.²²⁹

These enzymes share structurally a highly conserved phosphatase domain with an invariant HCX₅R consensus active site sequence located within a catalytic pocket that is needed for their hydrolytic activity. Despite their general structural similarity of the phosphatase domain, small modifications of the appearance of the catalytic cleft enables binding of deeper tyrosine residues, more superficial serine and threonine residues, and/or bulky acidic phospho-lipid headgroups.²³³

In addition, phosphatases most often contain extra targeting and interaction domains that contribute to their specificity²³⁴.

Concept of mechanism

The catalytic cysteine within the classical phosphatase active site motif has a low pK_a and is unprotonated at physiological pH, and exists as a thiolate anion. The thiolate acts as a nucleophile, attacking the phosphorus center of the substrate that leads to a substrate-phosphoryl-cysteine intermediate. Following substrate binding to the catalytic pocket, an important conformational change occurs bringing a critical aspartic acid in proximity to the intermediate. The aspartic acid serves at first as a general acid by protonating the leaving phenolic oxygen group from the substrate (P-O bond),

releasing the dephosphorylated substrate. The same aspartate residue is then postulated to serve as a general base by reacting with water that will attack the remaining phosphoryl-cysteine (Cys-PO₃) intermediate and this will liberate free phosphate and regenerate the enzyme to an active state.²³⁵

Importantly, the active site cysteine is a target of oxidation by for instance cellular H₂O₂. This redox regulation is a mean of regulating the phosphatase activity in cellular signaling, and yields a pro-phosphorylation balance enabling for instance RTK signaling to proceed and extend.^{236,237}

Lipid signaling and PTEN

Lipid molecules are important mediators of intracellular signaling, and imbalances in major lipid signaling pathways contribute to disease.²³⁸

Phosphatidylinositol (PtdIns; PI) is a membrane phospholipid capable of being phosphorylated by lipid kinases on the 3, 4, and 5 position of its inositol ring to form seven unique lipid signaling molecules, PtdIns3P, PtdIns4P, PtdIns5P, PtdIns(3,4)P₂, PtdIns(3,5)P₂, PtdIns(4,5)P₂, and PtdIns(3,4,5)P₃, respectively (Figure 9). Together, all derivatives play important roles as lipid messengers in several cellular events such as vesicular sorting, adhesion, migration, proliferation, and survival.^{239,240}

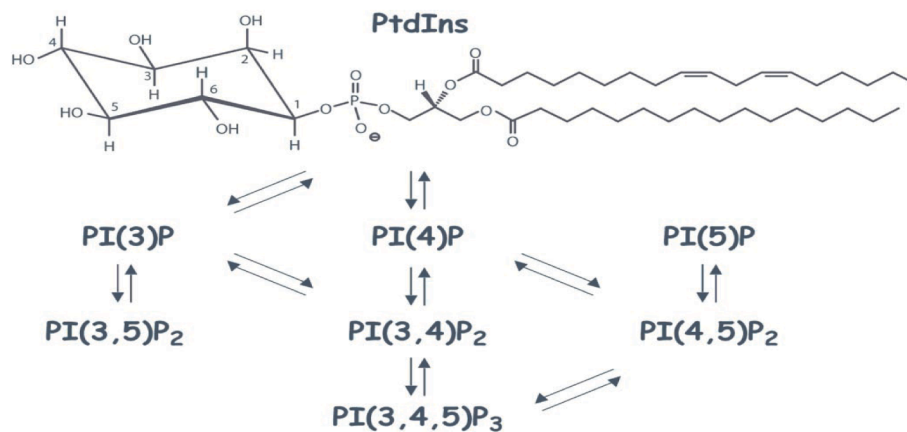


Figure 9. Drawing of phosphatidylinositol (PtdIns) and its seven phospho-derivatives.

PI3K and the Akt/PKB signaling pathway

The PI3K and Akt/PKB signaling pathway is one of the most important driver of cell proliferation and cell survival (Figure 10).²⁴¹

PI3K is an enzyme capable of phosphorylate PtdIns in response to extracellular stimuli specifically at the 3 position of the inositol ring ultimately resulting in production of the signaling molecule PtdIns(3,4,5)P₃. In turn, PtdIns(3,4,5)P₃ recruits phosphatidylinositol-dependent kinase 1 (PDK1) and the serine and threonine kinase Akt/PKB to the membrane, and serve as binding sites for both proteins through their pleckstrin homology (PH) domains. Once bound Akt/PKB is activated by PDK1-mediated threonine-phosphorylation and through serine-phosphorylation by another kinase that until recently has been unknown, but now is suggested to be the mTOR complex 2²⁴² (mTORC2; Rictor). Activated Akt/PKB in turn phosphorylates several downstream target proteins resulting in versatile cellular responses such as survival, growth, proliferation, and migration.²⁴¹

However, the level of PtdIns(3,4,5)P₃ is normally low in resting cells due to phosphatase activity of the dual-specificity and lipid phosphatase PTEN.²⁴³

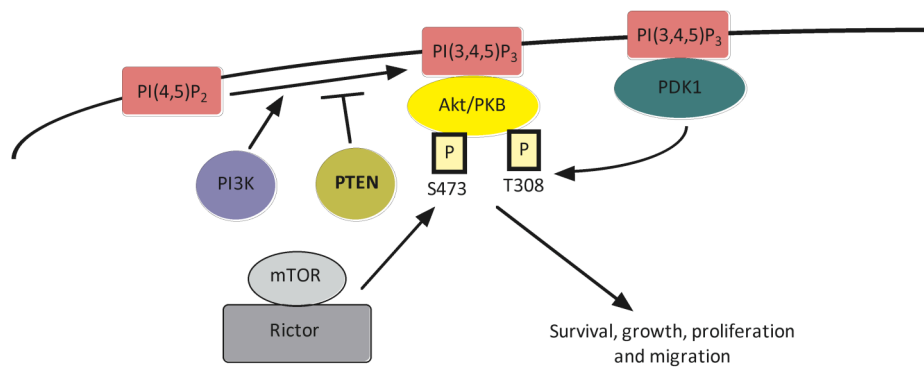


Figure 10. Illustration of key events in the PtdIns – PI3K – Akt/PKB signaling pathway.

PTEN

PTEN is a tumor suppressor protein, frequently mutated in human malignancies, that negatively controls cell-growth, cell-cycle progression, proliferation, induces apoptosis and that is involved in cell spreading and directional migration.

PTEN is one of the most highly mutated tumor suppressor genes in the post p53 era.²⁴⁴

Being a dual-specificity phosphatase with both protein and lipid phosphatase activity PTEN dephosphorylates tyrosine, serine, and threonine residues, and plays an important role in regulation of focal adhesion kinase (FAK) activity, and in autoregulation by protein phosphatase activity²⁴⁵. However, the major substrate for

PTEN in its role as a tumor suppressor is PtdIns(3,4,5)P₃, which PTEN dephosphorylates specifically the 3-position of the inositol ring and thereby counteracts the work of PI3K.²⁴⁴

Interestingly, PTEN displays punctuate cytoplasmic distribution and a substantial presence in the nucleus. This subcellular localization might be one way of regulating the specificity of PTEN activity, besides PTEN redox-regulation^{246, 247}. It has been suggested that nuclear PTEN mediates growth suppression and cell cycle arrest, and that cytoplasmic PTEN induces apoptosis²⁴⁸. Additionally, in migrating cells PTEN is believed to be located in the rear of the cell, and together with PI3K placed in the front of the cell, PTEN creates a PtdIns(3,4,5)P₃ gradient that governs migratory directionality with protrusions of the front and retraction of the rear.^{249,250}

PTEN is in large built up by an amino-terminal classical cysteine-based phosphatase domain with dual-specificity and lipid phosphatase activity (Figure 11). Tightly associated to the phosphatase domain is a C2 domain similar to those in Ca²⁺-independent protein kinase C (PKC) isoforms.²⁵¹ The crystal structure of PTEN elucidated the structural basis for PTEN catalytic activity. Unique for PTEN is a four amino acid insertion in a loop embracing the catalytic pocket resulting in a wider pocket enabling binding of the bulky headgroup of PIP₃. Another distinctive feature in the PTEN structure is the presence of specific positively charged amino acid residues that accounts for the PTEN preference for highly acidic PIP₃ headgroups.²⁵¹

PTEN is believed to interact with the membrane primarily through electrostatic interactions, but a putative PtdIns(4,5)P₂ binding site that is located in the PTEN amino-terminal and the C2 domain might also contribute to lipid binding^{252,253}.

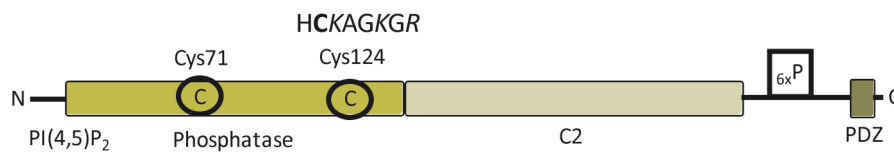


Figure 11. Representative view of PTEN domain organization with essential information depicted; amino-terminal putative PI(4,5)P₂ binding site, redox-sensitive cysteine residue 71 and 124 (bold; within catalytic motif), catalytic motif, carboxy-terminal phosphorylation sites and PDZ-binding motif. Invariant catalytic motif basic residues highlighted in italics.

Part III

Specific background

The Tensin family

The Tensin family proteins are focal adhesion proteins that play important roles in focal complex formation by linking integrins with the actin filaments and that mediate signal transduction occurring at the adhesive complex²⁵⁴.

Furthermore, they are relatively recently identified proteins, where three out of four family members harbor a PTEN homology region, due to which they are also classified as members of the PTEN family of phosphatases²²⁹. Although, no phosphatase activity so far has been detected their role as tumor suppressors are emerging.

The Tensin family

The Tensin family consists of four protein members; Tensin1 (Tns1), Tensin2 (Tns2; C1-TEN), Tensin3 (Tns3), and Tensin4 (Tns4; cten)²⁵⁵ (Figure 12).

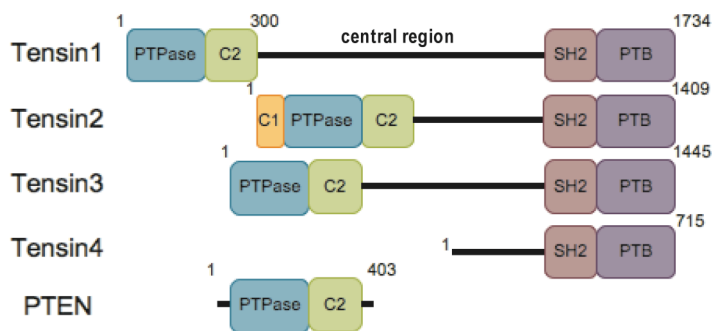


Figure 12. Illustration of the domain organization of the Tensin family proteins in comparison to PTEN.

Tensin structure

Tns1 through Tns3 have high similarity in their respective amino-terminal and carboxy-terminal ends, but are unique in their individual central regions. Tns4 is much shorter and is only similar in its carboxy-terminal end.²⁵⁵ Tns1 through Tns3 shares an amino-terminal focal adhesion binding site and an actin-binding domain that can cross-link actin filaments. All tensins have in common a carboxy-terminal SH2 and PTB domain

located in tandem that constitute another focal-adhesion binding site. The divergent central region of Tns1 through Tns3 have no known conserved domain structure, however Tns1 has been shown to be able to cap barbed ends of actin within this region. The central region in Tns2 is also highly proline-rich predicting a large rigid, stretched and disordered region (unpublished observations). The tensins bind integrins through their carboxy-terminal PTB domains, by which they also are suggested to bind PI3K and FAK.²⁵⁴ Moreover, tensin family members have also been shown to bind the tumor suppressor deleted in liver cancer 1 (DLC1) through their SH2 and/or PTB domains²⁵⁶⁻²⁵⁸.

Interestingly, Tns1 through Tns3 shares, within their amino-terminal end a conserved classical phosphatase domain and C2 domain, altogether with high structural similarity to almost the entire PTEN protein. Sequence analysis reveals that Tns2 and Tns3, but not Tns1, harbor a putative catalytically active cysteine at the same position as PTEN, and that Tns3 contains a conserved HCX₅R motif (Figure 13). A more detailed structural view of the catalytic pocket suggests Tns2 yet to be, and Tns3 not to be, structurally more likely of accommodate the bulky and acidic PtdIns(3,4,5)P₃ headgroup. Moreover, both Tns2 and Tns3 putative catalytic motifs contain basic amino acids in place to help bind and position acidic phosphorylated PtdIns substrates.²⁵⁹

The structure of the Tns1 PTB was recently solved and found to be able to bind PtdIns(4,5)P₂ as PTEN²⁶⁰.

		Catalytic motif
Tensin1	108	VVVLH HNKGNRGRIG
Tensin2	226	VVVL YCKGNKGLG
Tensin3	102	VV VIHCRGGKGRIG
PTEN	119	V AAIHCKAGKGR TG

Figure 13. Sequence alignment of the Tns1 through Tns3 region corresponding to the PTEN catalytic motif (grey box).

The biological role of Tensin

Tns2 was found in our group as an interactor to the cytoplasmic region of the Axl RTK¹⁰⁸, and when overexpressed in cells to negatively regulate PI3K signaling by decreasing the level of activated Akt/PKB, inhibiting proliferation and migration, and by inducing apoptosis¹⁴⁴. Furthermore, more sophisticated confocal microscopy live imaging revealed that overexpression of Tns2 wild type, but not Tns2 mutant with the putative catalytic cysteine residue changed to a serine residue, or Tns3 wild type, reduced the level of PtdIns(3,4,5)P₃ at the cellular membrane of living cells²⁵⁹.

It should be emphasized that Tns2 comes in three different isoforms. Not much is known about variant one, however it seems as if variant two and three has opposing effects. Tns2 variant two inhibits migration (above), and Tns2 variant three is shown to stimulate migration²⁶¹, and to be upregulated in and associated with progression of hepatocellular carcinoma²⁶². (The Tns2 variant number is written if known)

Tensin family members Tns1 and Tsn2 seem to play a role in the kidney.

Tns1 expression is found in the kidney glomerular mesangium, tubular epithelial cells, and parietal epithelial cells of Bowman's capsule²⁶³, is increased in MCs of immunoglobulin A and diabetic nephropathy patients²⁶⁴, and Tns1 knockout mice develop cystic kidneys and eventually die from renal failure²⁶⁵.

Tns2 expression is found in glomerular podocytes and tubular epithelial cells, and an eight nucleotide deletion of Tns2, resulting in premature translation termination, is believed to lie behind the phenotype of the ICGN mouse with nephrotic syndrome and renal failure²⁶⁶. Recently, Tns2 was suggested to induce the expression of nephrin by podocytes, a function that when impaired possible can explain the ICGN mouse phenotype²⁶⁷.

Tns3 knockout mice display growth retardation with incomplete small intestine, lung, and bone and postnatal lethality²⁶⁸. The role of Tns3 in stabilization of focal complex formation and actin reorganization has been clearly shown. Tns3 links integrins and actin filaments, but when the expression of Tns4 is upregulated as a consequence of EGF stimulation Tns3 is displaced for interaction with the EGF receptor by Tns4, and since Tns4 does not harbor an actin binding site the integrin-actin linkage is disrupted. Displacement of Tns3 with Tns4 is followed by loss of actin stress-fibers and focal complex disruption and enhanced cell migration.^{269,270}

Finally, the tensins family members are widely expressed in human tissues, and have been shown to be downregulated in human malignancies, for instance in RCC²⁷¹. However, also upregulation of Tns4 has been correlated to cancer²⁷².

One interesting observation in relation to the role of tensin proteins in cancer, that also might explain parts of their contribution to tumor suppression despite the putative phosphatase function of Tns2 and Tns3, is that they interacts with the DLC1 tumor suppressor protein, a Rho family GTPase activating protein (GAP), and that this interaction to tensin within the focal complex seem to necessary for the tumor suppressor function of DLC1²⁵⁶.

Part IV

The Present Investigation

Paper I – IV and future perspectives

The present investigation is a multifaceted study of the receptor tyrosine kinase Axl with focus on RCC.

At first, our main interest concerned the intracellular Axl interactor Tns2. Tns2 was originally found in our lab to bind the Axl cytoplasmic region in a human heart library yeast two-hybrid screen¹⁰⁸. Tns2 is a focal adhesion protein involved in cellular signaling, where it inhibits the Akt/PKB pathway, and decreases the level of PtdIns(3,4,5)P₃ located at the cellular membrane^{144,145,255,261}. Our primary goal was to characterize the Axl and Tns2 interaction. Due to the presence of a PTEN homology region with a putative catalytic cysteine in Tns2 and the negative effects of Tns2 on PI3K and Akt/PKB signaling, we also wanted to explore the hypothesis that Tns2 might negatively regulate Axl signaling by functioning as a PTEN-like lipid phosphatase.

We verified the Axl and Tns2 interaction to occur through the carboxy-terminal end of Tns2, as originally detected, and excluded other Tns2 domains to be likely to take part in the interaction (Paper IV). Despite extensive efforts, we were not able to show any direct *in vitro* lipid phosphatase activity of Tns2 or Tns3, another family member with a conserved catalytic motif (Paper III).

We continued our Axl exploration concerning the role of Axl and its ligand Gas6 in RCC, a disease that presents with poor prognosis and elusive etiology, and with a great need for proper molecular prognostic markers²⁰¹. We thought it could be possible to reveal an aberrant Gas6/Axl system, since it has been shown to be involved in progression of several human malignancies and to contribute to tumor growth and metastasis.^{13,273}

With the opportunity of access to a large and unique RCC patient material we measured the level of expression of Axl and Gas6 mRNA in the patient primary tumors and protein in serum and tumor. We could determine that Axl and Gas6 correlated with patient survival outcome (Paper I). In more detail, we found Axl mRNA expression in the primary tumor, at time of nephrectomy, to have the potential to be a prognostic marker, where low Axl expression is beneficial for the patient (Paper I). Gas6 primary tumor mRNA levels could, in the patient group with low Axl levels and better prognosis, further define a sub-population with even longer survival (Paper I). In serum, increased levels of both Axl and Gas6 protein correlated with severity of disease (Paper I).

Remaining time until present we attempted to uncover the biological role of Axl and Gas6 in a ccRCC experimental system. Although more is to be done, we could see that Gas6 stimulation of Axl in ccRCC cells had inhibitory effects on migration, and that Gas6 activation of Axl rendered the cells less viable (Paper II). Moreover, Axl protein expression was inversely correlated to the presence of the tumor suppressor protein VHL (Paper II). Axl protein was also downregulated by prolonged Gas6 stimulation (Paper II).

Altogether, the results from Paper I and II open up further exciting avenues of research into the complex role of the Gas6/Axl system in RCC, and for validation of the putative role of Axl as an important prognostic marker in the diagnosis of RCC.

Paper I – Differential Expression of Axl and Gas6 in Renal Cell Carcinoma Reflecting Tumor Advancement and Survival

Prior knowledge

Axl overexpression and activation is implicated in a broad spectrum of human malignancies, and Axl has in many been shown to mediate growth and survival¹³. Axl mediates glioma cell proliferation, migration, and invasion¹⁷⁴, and in human gliomas both Axl and its ligand Gas6 are frequently overexpressed and predict poor prognosis¹⁷⁵. Axl affects multiple cellular behaviors required for neovascularization^{152,273}. Moreover, Axl is directly linked to the progression of cancer metastasis²⁷³. In the human kidney, Axl and Gas6 expression are increased in inflammatory renal disease¹⁴¹, and in RCC, Axl mRNA level has likewise been suggested to be increased¹⁷¹.

RCC is a disease with poor prognosis due to inadequate therapy of metastatic disease, but despite an increased understanding of the underlying oncogenic events in RCC, where several molecular markers have been associated with increased survival, no molecular markers are currently validated for estimation of prognosis^{201,213}.

Results/Significance

Axl and Gas6 were differentially expressed in RCC tumor types as measured by the mRNA level in the primary tumor and at the protein level in serum of patients. The expression of both was implicated in RCC tumor advancement and correlated to patient survival. Especially, tumor Axl mRNA levels at time of nephrectomy was an independent factor in multivariate analysis when assessed against RCC tumor stage and grade, where low tumor Axl mRNA associated with longer patient survival. High Gas6 mRNA levels in the same samples further defined a sub-population of patients with even longer survival.

The survival advantage with low Axl mRNA expression in the tumor is in agreement with the literature field that implies Axl expression and signaling in several malignancies and tumorigenic events^{13,273}. However, the fact that there was no correlation between Axl mRNA level in the primary tumor and Axl protein in tumor tissue is intriguing, and raises the question whether Axl protein expression and signaling directly contributes to tumor progression or if Axl *per se* does not have any direct pathological impact and that the Axl mRNA level perhaps instead correlates with an to us unknown disease-contributing event. Yet, the level of Gas6 mRNA in the tumor constitutes another layer of prognostic information, and therefore strengthens the idea that there is a direct contribution to disease from this ligand/receptor system. However, the study does not elucidate the underlying molecular mechanisms that could explain the results. Serum protein levels of Axl and Gas6 were decreased and increased respectively in RCC patients compared to healthy controls. This tendency gives a balance of Axl and Gas6 levels that might allow some Gas6 to be free in serum. Although the changed Axl and Gas6 levels in RCC serum, detected in all tumor types, probably is a reflection and not a cause of disease, free Gas6 might contribute to the more pro-thrombotic phenotype seen in many RCC patients. It is difficult to explain that an increased level of both Axl and Gas6 protein in serum correlated with advanced disease.

Nevertheless, the results altogether imply that Axl and Gas6 are potential molecular primary tumor and serum markers in RCC. Axl expression levels in the tumor at time of nephrectomy might in the future be used as a prognostic marker to interpret patient survival, to stratify patient treatment groups, and perhaps even as a therapeutic target.

Paper II – Gas6 and the receptor tyrosine kinase Axl in clear cell renal cell carcinoma

Prior knowledge

The receptor tyrosine kinase Axl and its ligand Gas6 are expressed in the tubular epithelial cells and MCs of the kidney. Furthermore, it has been shown that Gas6 functions as an autocrine growth and survival factor for MCs in the kidney mediated via Axl, a function generally believed to be the outcome of Gas6/Axl signaling.^{13,134,141}

However, in RCC that is a disease originating from the epithelial cells of the kidney parenchyma, the expression of Axl correlates positively and the expression of Gas6 negatively to survival^{88,209} (Paper I). Largely, low Axl mRNA levels in the tumor at time of nephrectomy independently correlate with substantially longer patient survival, and patients with low, but not high, Axl and high Gas6 mRNA levels as a combination display even longer survival. This suggests Gas6 protein expression to be beneficial for RCC patients, although Axl expression is superior, in contrast to what could be expected from the general view of Gas6 regarded as a growth and survival factor.

Thus, the molecular biology of the Gas6/Axl system seem to be compound, and it would be of great interest to uncover its role in RCC and assumed contribution to disease progression.

Results/Significance

Axl was highly expressed in ccRCC cells deficient in functional VHL protein. VHL is a tumor suppressor gene often inactivated in ccRCC²¹⁰. VHL reconstituted cells expressed reduced levels of Axl protein, but not Axl mRNA, suggesting VHL to regulate Axl protein expression. Furthermore, Gas6-mediated activation of Axl in ccRCC cells resulted in Axl phosphorylation, receptor downregulation, decreased cell viability and migratory capacity. No effects of the Gas6/Axl system could be detected on invasion.

The Gas6 inhibitory effect on migration was specific for Axl activation, and the Axl molecule *per se* did not contribute to migration as perhaps one could speculate due to the adhesion-molecule-like extracellular domain and ability of homophilic interaction^{11,12,29}. Gas6 inhibition of migration and viability are two explanatory variables for the assumed beneficial condition of having low Axl and high Gas6 levels in the RCC tumor mass, but also the Gas6-dependent long-term Axl downregulation might be advantageous, since the presence of Axl *per se* seem to be superior to the level of Gas6 in respect of survival outcomes (Paper I). Interestingly, in respect of the current discussion, Gas6-mediated Axl activation has also been shown to inhibit the VEGF-driven angiogenic program by inhibition of ligand-dependent migration and vascularization by inducing dephosphorylation and inactivation of the VEGF receptor 2¹³¹. Furthermore, we detected a dip in total Akt levels in Gas6 stimulated ccRCC cells, an event that we currently cannot explain. However, it is interesting to note that inhibition of VEGF receptor signaling leads to downregulation of total Akt protein levels by protein degradation²⁷⁴. The various outcomes of the Gas6/Axl signaling suggest that the biology of this ligand/receptor system is complex and probably context specific, and furthermore, not always add with growth and survival.

Altogether, the results from Paper I and II imply that Axl contributes to disease by an undefined Gas6-independent mechanism, a process antagonized by Gas6 stimulation by either subsequent Axl receptor downregulation and/or signal-specific induction of putative inhibitory pathways. How Axl on it own possibly contributes to disease-progression in RCC is not revealed by these experiments. Here, Axl knockdown had no effect on migration, and did not influence invasion, a job otherwise attributed to Axl in several contexts^{13,186}. It is known that Axl signaling is in crosstalk with other signaling systems, for instance in promoting survival of apoptosis-induced fibroblasts in conjunction with IL-15 receptor signaling independently of Gas6^{42,130,131}. Perhaps could Axl tumor promoting competence evolve in liaison with other receptor systems? The observation that VHL might downregulate Axl protein expression is of great interest, since VHL is a tumor suppressor protein inactivated in the majority of ccRCC cases²¹⁰.

Finally, in ccRCC patient tumor tissues, both Axl and Gas6 proteins were expressed at high levels, and Axl was phosphorylated and Gas6 γ -carboxylated, allowing both proteins to be functionally active *in vivo*. Interestingly, Axl phosphorylation levels varied from almost undetectable to abundant. If Gas6 was expressed and active in the samples where Axl was phosphorylated is not revealed by these experiments, and any interpretation from known Gas6 mRNA levels cannot be done due to lack of correlation between mRNA and protein expression (Paper I).

In summary, Axl and Gas6 have the potential to be functionally active players in ccRCC. These results suggest a role of Gas6 in the kidney apart from being a growth and survival factor, and are in line with the results from paper I showing that higher Gas6 levels in the tumor are beneficial in respect of survival.

Paper III – No intrinsic lipid phosphatase activity detected in the recombinant PTEN-homology regions of Tensin2 and Tensin3 *in vitro*

Prior knowledge

Tns2 and Tns3 are members of the Tensin-family of intracellular focal adhesion proteins connecting the extracellular matrix to the actin cytoskeleton via integrins²⁵⁵. These proteins are also classified as members of the PTEN family of lipid phosphatases due to the presence of a PTEN homology region, including a classical phosphatase domain in tight conjunction with a C2 lipid-binding domain^{229,251}. Similar to the tumor suppressor PTEN they also contain a conserved archetypal cysteine in a position required for enzymatic activity^{235,275}. Tns3 additionally harbors a complete conserved active site HCX₅R motif, and both Tns2 and Tns3 share unique basic residues known to aid in preference and positioning of the acidic PTEN PtdIns(3,4,5)P₃ substrate. Yet, based upon sophisticated molecular modeling analysis, the catalytic pocket of Tns2 phosphatase domain appeared structurally more similar to that of PTEN, and more likely to accommodate the bulky headgroup of PtdIns(3,4,5)P₃ substrate.²⁵⁹ Furthermore, overexpression of Tns2 but not Tns3 is shown to reduce the level of PtdIns(3,4,5)P₃ at the surface membranes of living cells²⁵⁹, and to inhibit the subsequent activation of Akt/PKB yielding anti-survival and anti-migratory effects¹⁴⁴. However, whether the Tns2 inhibitory effects of the PtdIns(3,4,5)P₃ and Akt/PKB pathway is mediated through intrinsic lipid-phosphatase activity is not known.

Results/Significance

A sensitive lipid phosphatase activity assay was developed. In house PTEN was generated and used to verify functionality and specificity of the assay. PTEN displayed a

ten-fold higher activity than previously reported towards its main lipid substrate PtdIns(3,4,5)P₃²⁵¹. We expressed and purified the PTEN homology region of Tns2 and Tns3, including the phosphatase and C2 domain, but despite extensive efforts no *in vitro* intrinsic lipid phosphatase activity could be found in these recombinant proteins.

The main advantage of our lipid phosphatase assay was the introduction of ultracentrifugation and subsequent reduction of background absorbance prior analysis of hydrolyzed free phosphate that to our knowledge was not used in this context at the time. Clearance of samples and the use of absorbance spectrum measurements enabled detection of weak activities that otherwise would have been overlooked. The reason behind the higher specific activity by our generated PTEN compared to the reported activity of PTEN is not obvious, however Lee *et al.* used an extensive purification system during which some of the redox-sensitive PTEN protein activity could have been lost²⁵¹. Moreover, the introduction of bovine serum albumin (BSA) in our reaction buffer increased the activity of PTEN about two to three fold. As reported the PTEN activity was dependent on a reduced environment, and furthermore the activity increased with the amount of negatively charged phosphatidylserine lipids in the carrier vesicles, probably due to enhanced membrane interaction^{276,277}. The weak phosphatase activity of the Tns2 and Tns3 samples displayed against PtdIns(3,4,5)P₃, PtdIns(3,5)P₂, and PtdIns(4,5)P₂ in decreasing activity order was not intrinsic to the recombinant proteins. Instead these activities most certainly could be traced to a co-purified contaminating bacterial phosphatase, since Tns2 and Tns3 cysteine to serine mutants, where the putative catalytic activity should be abrogated, and unrelated proteins purified in the same way displayed similar activities as the wild type Tns2 and Tns3 samples. Other proteins purified with a different system did not display any activity.

Altogether, our results argue against the suggested function of Tns2 as a PTEN-like lipid phosphatase, since no *in vitro* catalytic activity was found in the isolated recombinant Tns2 and Tns3 phosphatase domains, respectively. However, the results do not exclude the possibility that full-length Tns2 and Tns3 are functional phosphatases *in vivo*.

Paper IV – A yeast two-hybrid interaction study between various Tnsin2 deletion mutants and the Axl receptor tyrosine kinase

Prior knowledge

Tns2 is a multidomain focal adhesion and signaling protein known to bridge the extracellular matrix and cellular cytoskeleton via integrin and actin binding^{255,261}. The precise biological functions of Tns2 are largely unknown, as are most of the intracellular molecules important for Tns2 signaling, except for the known negative regulation of the PI3K and Akt/PKB pathway, and the interaction with the DLC1 protein, invariant for its

tumor suppressor function^{144,256}.

In a yeast two-hybrid screen of a human heart library using the intracellular cytoplasmic domain of Axl RTK as bait, it was previously shown that Tns2 interacts with Axl through its carboxy-terminal region including the tandemly located and conserved SH2 and PTB domains. This interaction seemed reasonable since the yeast two-hybrid screen presented with several already known, and a few novel putative Axl interactors amongst Tns2. Although, a natural occurring biological interaction has not been proven, the interaction was further verified in a mammalian two-hybrid system and by co-immunoprecipitation of Tns2 and Axl ectopically expressed in mammalian cells.¹⁰⁸ It was not shown whether Tns2 interacts with Axl through any of its other conserved domain structures, and also if the presence of another region would influence the affinity of the present interaction.

Results/Significance

Tns2 binds to Axl cytoplasmic region through the originally identified carboxy-terminal SH2 and PTB domain pair, and not likely through any other region. What was not known from before is that the interaction seems to be increased affinity-wise several-fold by and dependent on the presence of a stretch of 100 amino acids adjacently and amino-terminally located to the SH2 domain. Without these additional amino acids hardly no binding could be detected. Interestingly, our results also proposed a large Tns2 central proline-rich region with no known tertiary structure to display intrinsic DNA transcriptional activity.

It was surprising to find that the interaction between Axl and Tns2 very much relied on the extra amino-terminal amino acids, since that part of the protein does not to our knowledge contain any conserved interaction domain, rather instead probably is constituted by an extended rigid polypeptide chain, together with the known function of PTB and SH2 domains to bind tyrosine and phospho-tyrosine residues. Even though the yeast two-hybrid method is stated to generate very low amounts of false positive interactions, due to the presence of four reporter genes that are under the control of totally three different heterologous promoter regions, we experienced an untrue interaction in case of the Tns2 central region. Unlikely, the Tns2 central region could activate all reporter genes by itself if brought close enough to the DNA by a DNA binding domain, meaning that this region of Tns2 might have intrinsic DNA transcriptional activity. From the results of the study it cannot be concluded whether or not this activity has any biological relevance or simply is an artifact due to isolation of that particular region. Another drawback with the yeast two-hybrid system is the frequently occurring cases of false negatives, i.e. the lack of detection of a biological relevant interaction that normally would occur. However, we tried to circumvent this problem by changing vector directionality of the bait and target proteins. It is not as likely that the lack of interaction will occur in both situations, however one cannot rule it out.

On the whole, our results confirmed the previously found interaction between the Tns2 carboxy-terminal region and the intracellular region of Axl. Furthermore, the study opens up for interesting research regarding putative intrinsic Tns2 transcriptional activity.

Future perspectives

The Gas6/Axl system seems to be important in RCC, and the mechanistic role of these two proteins and their contribution to the disease should be explored further.

Especially, it is of interest when taking into account the putative role of Axl as an independent prognostic marker that might be used to interpret patient survival outcome, to define patient treatment groups, and also perhaps be used as an attractive drug target. The use of Axl as a prognostic RCC marker should therefore be validated in large independent studies. Furthermore, it should be manifested to what extent Axl, as an active molecule, plays a role in the progression of the disease, in other words, whether Axl is an interesting target for directed therapy or not. Hence, the molecular events taking place in RCC cells in respect to Axl should be clarified. For instance, if playing a direct Gas6-independent role in RCC pathogenesis, it would be interesting to clarify whether the Axl extracellular domain *per se* plays a role and/or if the activity of the intracellular kinase domain contributes to the progression of the disease. In relation to this, it would also be of interest to investigate whether Axl work in liaison with other signaling systems in RCC. And also, what are the contributions of the Gas6/Axl system in other supporting cell types present in the RCC tumor mass? For instance, Axl is one of the genes that seem to be highly expressed in so called “kidney stem cells” of the normal kidney (personal communication). And also, the potential impact of Axl on neovascularization of the growing RCC tumor is a research field not covered in the present investigation, however an area of great interest that could be further explored. It would also be of relevance to learn more about the role of Gas6 protein in serum of RCC patients, where Gas6 might be free in circulation, and thereby perhaps contribute to tumor-thrombus formation.

Other questions arising in respect of the role of Axl and Gas6 in RCC include whether Axl in ccRCC cells is a direct target of the ubiquitin ligase and tumor suppressor protein VHL. Furthermore, the extent of Gas6 protective roles in RCC and the underlying mechanisms should be clarified, the latter a question partly addressed by this thesis. The fact that Gas6 functions as a growth and survival factor in MCs, but have inhibitory effects on migration and reduces viability in ccRCC cells, suggests the role of the Gas6/Axl system to be cell and context specific in the kidney, a realization that certainly needs further investigation. Perhaps, established Gas6 knockout and wildtype mice could be used in an experimental RCC model with Axl wildtype and various knockout xenografts to explore the functional role of this receptor system *in vivo*.

If not constrained by time, we would like to have completed our work with Tns2 and Tns3 to be able to accept or discard our hypothesis that Tns2 in particular might function as a PTEN-like lipid phosphatase. Since we could not abandon the possibility that correctly folded PTEN homology region of Tns2 and/or full-length Tns2 protein in its correct microenvironment harbors the ascribed activity, it would be appreciated, for instance, to perform *in vitro* translation experiments of the full-length protein to investigate the enzyme activity using our generated *in vitro* lipid phosphatase assay. It is interesting to note that Tns2 is reported to be a RCC tumor suppressor²⁷¹, and that the Tns2 mRNA expression in RCC tumors correlates with the expression of Axl and Gas6 (unpublished observation).

With respect to Paper IV, we believe that the impact of the scope is not strong enough to continue the study. But if so, it would be necessary to compare the strength of the interaction of the SH2-PTB_{100C} and SH2-PTB constructs to Axl, respectively, in a direct quantitative manner, to confirm the protein expression in yeast of all constructs, and to repeat and verify the experimental set-up in mammalian cells by for instance co-immunoprecipitation analysis.

Of pure curiosity it would be of interest to set up a new study to explore the transcriptional activity of the central region, especially in the context of recent findings that Tns2 deletion constructs harboring the C1-domain localize to the nucleus²⁷⁸.

Part IV

The present investigation Populärvetenskaplig sammanfattning

Den aktuella studien utgör ett omfattande och mångfacetterat forskningsarbete som i huvudsak kretsar kring en specifik cellmolekyl som kallas för Axl.

Axl är en så kallad receptor tyrosin kinas, en molekyl som sitter inorporerad i cellens yttre membran där den kan ta emot signaler från utsidan av cellen och transportera dem över cellmembranet till cellens insida. Axl utgör endast en av ett flertal kända receptor tyrosin kinaser. Extracellulärt består Axl av en strukturell del som kan binda in till sin ligand Gas6. Gas6 i sin tur är en extracellulär löslig signalmolekyl och tillväxtfaktor som produceras och utsöndras av bland annat celler som är försatta i tillväxtarrest. Ligandbinding leder till en konformationsförändring av Axl molekylen som möjliggör aktivering av den intracellulära funktionella delen av Axl med så kallad kinas aktivitet. Aktivering av kinasdomänen aktiverar i sin tur nedströms intracellulära signalmolekyler och leder slutligen till reglering på transkriptionell nivå. Med andra ord bidrar aktivitet av Gas6/Axl systemet till huruvida en specifik gen ska uttryckas till effektor protein eller inte.

Den första tiden av arbetet ägnades åt att studera interaktionen mellan den intracellulära delen av Axl och en intracellulär signalmolekyl som kallas för Tensin2, och vidare den funktionella aktiviteten av Tensin2.

Tensin2 var tidigare identifierat av vår grupp som en Axl interaktör och är ett protein lokaliserat till celladhesions strukturer. Där medverkar Tensin2 till att fästa cellen till sin extracellulära omgivning, och till att mediera signaler från den extracellulära omgivningen in i cellen. Dessa adhesions strukturer är nödvändiga för bland annat att cellen ska kunna förflytta sig i en process som kallas för migrering, och för att transmittera överlevnadssignaler in till cellen. Utan överlevnadssignaler genomgår cellen apoptos - programmerad celledöd. Migrering och apoptos är normala cellulära mekanismer, men vilka är onormalt reglerade i cancer och därmed bidrar till metastasering och överdriven överlevnad av cancer celler.

Tensin2 har tillskrivits en potentiell tumörsuppressorer roll, där Tensin2 bland annat minskar aktiveringen av en intracellulär överlevnadsfaktor som kallas för Akt/PKB, och vidare minskar mängden celler som migrerar och förökar sig. Det har också visat sig att Tensin2 minskar mängden av en intracellulär lipidmolekyl, nödvändig för nedströms aktivering av Akt/PKB, som kallas för fosfatidylinositol-3,4,5-trisfosfat (PtdIns(3,4,5)P₃). Tensin2 är delvis strukturellt homologt med ett annat tumörsuppressorer protein som

kallas för PTEN, vilket minskar mängden PtdIns(3,4,5)P₃ genom en defosforyleringsprocess där en fosfatgrupp tas bort i en enzymatisk reaktion. Detta genererar PtdIns(4,5)P₂ som inte kan verka för aktivering av Akt/PKB på samma sätt som PtdIns(3,4,5)P₃.

Utifrån den här kunskapen formulerade vi hypotesen att Tensin2 potentiellt kunde verka som ett PTEN-liknande enzym, vilket defosforylerar PtdIns(3,4,5)P₃ som en del av Axl signalering.

Axl - Tensin2 interaktionsstudien (Paper IV) bekräftade tidigare fynd att Tensin2 interagerar med Axl genom två av sina domäner, som kallas för SH2 och PTB. Vidare såg vi att interaktionen var av mycket större affinitet när 100 aminosyror lokaliserade direkt uppströms om SH2 domänen var inkluderade. Ett oväntat fynd från den här studien var att den centrala delen av Tensin2, utan någon känd tredimensionell struktur, kunde aktivera DNA transkription på egen hand.

Den funktionella studien av Tensin2 (Paper III) baserades på vår hypotes att Tensin2 kunde verka som ett PTEN-liknande tumörsuppressordomän enzym. I detta syfte lyckades vi generera en specifik och känslig metod för evaluering av den här typen av aktivitet. Funktionalitet av metoden verifierades med hjälp av PTEN enzym som vi också lyckades att producera artificiellt i vårt labb. Från studien kunde vi dra slutsatsen att artificiellt uttryckta PTEN homologiregioner av Tensin2 och Tensin3 (ett liknande protein från samma protein familj som Tensin2) inte hade någon inneboende enzymaktivitet. Vår hypotes är dock inte motbevisad då dessa proteiner fortfarande har möjligheten att vara funktionellt aktiva när de är uttryckta i sin helhet och befinner sig *in vivo* – i sin normala cellulära miljö, med tillgång till eventuella okända, men nödvändiga faktorer som hjälpmedel till den enzymatiska processen.

Den senare delen av tiden ägnades åt att studera Axl och dess ligand Gas6 i ett mer biologiskt perspektiv. Vi studerade rollen av detta ligand/receptor system i renal cell cancer. Renal cell cancer är den mest förekommande maligna tumören i njurparenkymet (den funktionella delen av njuren). Förekomsten av renal cell cancer är ungefär 2 % av all cancer i Sverige och medianåldern vid diagnos är cirka 63 år. Vidare är dödligheten av renal cell cancer hög, och för metastaserande sjukdom finns inga kurerande behandlingsmetoder utan endast palliativ vård. Tyvärr finns det ännu inga pålitliga validerade molekyllära markörer för prognos av sjukdomen, utan tumörstadium är i nuläget den mest betydelsefulla prognostiska faktorn när det gäller överlevnad. Uppkomsten av fler molekyllära markörer som kan bidra till en bättre prognos av patientens överlevnad och till en bättre indelning av behandlingsgrupper är därmed av stor vikt.

Vi hade förmodligen kunnat arbeta med ett stort njurcancer patient material, vilket generöst var givet oss tillhanda av professor Börje Ljungberg vid Umeå

Universitetssjukhus. Med hjälp av detta material av biopsier insamlade sedan början av 80-talet av professor Ljungberg studerade vi mängden Axl och Gas6 mRNA i tumören vid tiden för nefrektomi och vidare mängden Axl och Gas6 protein i serum från patienter vid samma tillfälle (Paper I). Dessa data kunde sedan analyseras i förhållande till respektive patients överlevnad, tumörstadium samt en mängd andra tumörvariabler. Från den här studien kunde vi dra slutsatsen att både Axl och Gas6 korrelerade med hur avancerad tumören var vid diagnostillfället (tid för nefrektomi) och med patientens överlevnad vid uppföljning. Mer specifikt så visade det sig att lågt Axl uttryck (de patienter med de lägsta 25 % av Axl uttrycks nivå) i tumören vid diagnostillfället korrelerade med mycket bättre överlevnadsprognos än resterande patienter med högre Axl nivåer. Ett stort fynd visade sig vara att Axl tumörnivå stod kvar som en oberoende prognostisk variabel i multivariabel analys genomförd gentemot bland annat tumörstadium. Intressant var att hög Gas6 nivå (de patienter med de högsta 50 % av Gas6 uttrycks nivå) vidare kunde definiera en patientgrupp med ytterligare bättre överlevnad utifrån de patienter med låg Axl tumör nivå och redan relativt bättre prognos.

Möjligtvis kan Axl komma att utgöra en prognostisk faktor för uppskattningen av renal cell cancer patienters överlevnadsprognos, och vidare möjligtvis för indelning av mer korrekta behandlingsgrupper, och till sist möjligen vara en ny målmolekyl för framtida behandling. För detta krävs ytterligare omfattande externa oberoende valideringsstudier av Axl i renal cell cancer.

Den sista tiden av avhandlingsarbetet lades på att studera och försöka klarlägga den biologiska roll som Gas6/Axl systemet spelar i renal cell cancer. Därmed studerade vi Gas6 och Axl i ett cellbaserat *in vitro* system – laborativ experimentell modell – med hjälp av en specifik typ av renal cell cancer celler, så kallad klarcellig renal cell cancer (Paper II). Vi fann att Axl, men inte Gas6, var uttryckt på hög nivå i dessa celler, och vidare att Axl proteinnivåer korrelerade negativt med uttrycket av ett annat tumörsuppressor protein som kallas VHL, vilket ofta är ofunktionellt i just klarcellig form av renal cell cancer. Detta var intressant då VHL fungerar genom att markera vissa cellulära proteiner för nedbrytning, och i klarcellig njurcancer med ofunktionellt VHL bryts vissa proteiner som kan bidra till tumörformationen inte ned i den utsträckning som de borde. Kanske VHL fungerar just på detta sätt gentemot Axl proteinet också? Vidare fann vi att stimulering av dessa klarcelliga renala cell cancer celler med Gas6, som tillförts exogent, dvs. utifrån, kunde aktivera Axl och att det som resultat ledde till att Axl receptorn nedreglerades och till att cellerna migrerade mindre och var mindre viabla än obehandlade celler.

Delvis förklarar resultaten från Paper II hur Gas6/Axl systemet bidrar till en mer aggressiv tumörcellfenotyp. Dock återstår bland annat frågan hur och om Axl allena bidrar till progression av renal cell cancer. Är det kanske så att Axl genom samarbete med något annat signaleringssystem kan bidra till tumörväxt, eller är det så att Axl endast korrelerar med någon ännu okänd molekylär mekanism som i sin tur har inflytande över patientens överlevnad. Den molekylära mekanismen av Gas6/Axl

systemet bör vidare undersökas i ett biologiskt modellsystem som kan återge den komplexa situation som utgörs av tumörtillväxt och metastasering i människokroppen.

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