Review Article

Gas6 and protein S – vitamin K-dependent ligands for the Axl receptor tyrosine kinase subfamily

Sassan Hafizi and Björn Dahlbäck

Lund University, Department of Laboratory Medicine, Section for Clinical Chemistry, Wallenberg Laboratory, University Hospital Malmö, SE-205 02 Malmö, Sweden.

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Correspondence:

Sassan Hafizi

Lund University, Department of Laboratory Medicine, Section for Clinical Chemistry, Wallenberg Laboratory, University Hospital Malmö, SE-205 02 Malmö, Sweden.

Tel: +46 40 337083

Fax: +46 40 337044

E-mail: sassan.hafizi@med.lu.se

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SUMMARY

Gas6 and protein S are two homologous secreted proteins that depend on vitamin K for their execution of a range of biological functions. A discrete subset of these functions is mediated through their binding to and activation of the receptor tyrosine kinases Axl, Sky and Mer. Furthermore, a hallmark of the Gas6/Axl system is the unique ability of Gas6 and protein S to tether their non receptor-binding regions to the negatively-charged membranes of apoptotic cells. Numerous studies have shown the Gas6/Axl system to regulate cell survival, proliferation, migration, adhesion and phagocytosis. Consequently, altered activity/ expression of its components has been detected in a variety of pathologies such as cancer, vascular, autoimmune and kidney disorders. Moreover, Axl overactivation can equally occur without ligand binding, which has implications for tumourigenesis. Further knowledge of this exquisite ligand-receptor system and the circumstances of its activation should provide the basis for development of novel therapies for the above diseases.

INTRODUCTION

Growth factors are key players in the arena of cell biology. The “classical” growth factors such as epidermal growth factor and platelet-derived growth factor are well established as major effectors of cell proliferation, survival, migration and differentiation. These are key processes both for development and the maintenance of homeostasis in the adult, as well as in diseases involving neoplastic growth, such as tissue remodelling after injury, tumourigenesis and vasculoproliferative diseases [1]. Growth factors act on target cells through interactions with receptor tyrosine kinases (RTKs), a large family of transmembrane proteins with diverse extracellular ligand-binding structures, but which all possess a highly conserved domain with intrinsic tyrosine kinase activity [1, 2]. It is this tyrosine kinase domain that triggers signal transduction within the cell after receptor stimulation.

In humans, twenty distinct subfamilies of RTKs exist that are categorised according to their amino acid sequence identities and structural similarities in their extracellular regions [2]. One of these is the subfamily comprising Axl, Sky and Mer (as we shall refer to them hereafter), also referred to as the TAM family (Tyro3, Axl and Mer). This RTK subfamily is defined by a combination of dual immunoglobulin (Ig)-like and dual fibronectin type III (FNIII) domains in the extracellular (N-terminal) region (Fig. 1). Despite considerable diversity in the conformations of the extracellular (ecto-) domains of different RTKs, certain protein modules are however common among some RTK subfamilies. For example, Ig-like domains can be found in PDGF receptors, whilst Eph RTK possesses a FNIII domain (Fig. 1).

The Axl RTKs participate in a signalling axis often referred to as the Gas6/Axl system, Gas6 being the ligand. Since the discovery of Axl in 1991, a tantalising assortment of roles for the Gas6/Axl system has been revealed, involving functions ranging from cell survival to phagocytosis. In this review we shall bring to light a ligand-receptor system that is emerging as a major regulator of cell survival and turnover during apoptosis under certain physiological and pathological scenarios.

Gas6 and protein S – vitamin K-dependent ligands of the Axl RTK subfamily

Upon their identification, the Axl subfamily of RTKs were "orphan" receptors with as yet unknown biological ligands. As well as being activated through overexpression, it was conceivable that in non-transformed cells Axl could be stimulated by an appropriate extracellular signal. In 1995, an Axl-stimulatory factor was purified from conditioned medium of the Wi38 cell line and identified by N-terminal sequencing as Gas6 [3]. Previously, the *gas6* gene had first been detected as one of several genes to be upregulated in NIH 3T3 fibroblasts under serum starvation-induced growth arrest, hence its name *growth arrest specific gene 6* [4]. The 678-amino acid Gas6 protein is the latest addition to the vitamin K-dependent family of proteins. Gas6 shows 43% amino acid sequence identity with protein S, an abundant serum protein and a negative regulator of blood coagulation, acting as a cofactor for activated protein C in the degradation of clotting factors Va and VIIIa [5]. Gas6 has the same domain organisation as protein S, namely an N-

terminal region containing eleven γ-carboxyglutamic acid residues (Gla), a loop region, four epidermal growth factor (EGF)-like repeats, and a C-terminal sex hormone binding globulin (SHBG)-like structure that is composed of two globular laminin G-like (LG) domains, (Fig. 2) [6]. The crystal structure of the SHBG region of Gas6 reveals a V-shaped arrangement of LG domains with a hydrophobic patch and a calcium-binding site at their interface [7] (see also Fig. 3A). The Gla region is the region that is vitamin K-dependent, where glutamate residues are post-translationally modified in the endoplasmic reticulum by gammạ glutamyl carboxylase, an enzyme that requires vitamin K as a cofactor [8]. The negatively charged Gla residues can form complexes with 7-8 calcium ions [9] that can coordinate themselves in a conformationally specific manner with negatively charged membrane phospholipids [10]. The loop region of protein S contains thrombin-sensitive cleavage sites, which are a means of regulating its role in the coagulation system [5]. These sites, however, do not exist in Gas6.

Axl, Sky and Mer

Axl was first isolated in 1991 as the product of a transforming gene from two chronic myelogenous leukaemia patients, and subsequently cloned and termed *axl* from the Greek word 'anexelekto', meaning uncontrolled [11]. The Axl gene is evolutionarily conserved between vertebrate species, and the amino acid sequence of Axl revealed it to be a novel type I transmembrane protein with an intracellular tyrosine kinase domain. Axl is ubiquitously expressed, being detectable in a wide variety of organs and cell lines of epithelial, mesenchymal, and haematopoietic origins, as well as non-transformed cells, although it is absent in lymphocytes and granulocytes [11]. During development, murine *axl* (known as *ark*) expression is detected in a broad spectrum of tissues with a relatively late onset, from day 12.5 [12]. Also significant is that Axl is expressed in endothelial cells, which implicates it in endothelial cell survival under stress conditions, proliferation in tumour angiogenesis, and in vascular remodelling after injury.

In 1994, the full human sequence for a novel Axl-homologous RTK, Sky (gene *TYRO3*), was reported [13], and it has since been variously termed as Brt, Rse, DTK, Tif and Tyro 3. The genomic structure of human

Sky is identical to that of human Axl, demonstrating close conservation within the Axl subfamily. As with Axl, Sky is expressed in many embryonic cell types from day 14 until birth [14]. In particular, Sky expression is predominant in the brain [15], suggesting a special role in CNS development and functions. Sky expression is also high in the adult kidney, testis and ovary [16]. Human pulmonary arterial endothelial cells express Sky [17], also suggesting a role in vascular reactivity or remodelling. Sky appears also to predominate in multinucleated osteoclasts in bone, and appears to stimulate bone resorbing activity [18]. Mer, the third member of the Axl RTK subfamily, was first identified through its protooncogenic chicken orthologue, c-eyk, which was the cellular counterpart of an avian retrovirus [19]. The human protooncogene was cloned and named c-mer (gene *MERTK*) after its mRNA expression pattern (monocytes, epithelial and reproductive tissues) [20]. Mer mRNA is detectable in normal peripheral blood monocytes and bone marrow but not in normal B- and T-lymphocytes, although it is then switched on in neoplastic B- and T-cell lines [20].

Functions of Gas6 as ligand for Axl RTKs

While PROTEIN S is well established as a negative regulator of procoagulant pathways [5], no such role has been found for Gas6. However, Gas6 instead exerts several other functions that belong to the repertoire of growth or survival factors. Firstly, the original observation that Gas6 is upregulated in growth-arrested cells [4] suggested a role in protection from certain cellular stresses, such as apoptosis. Subsequently, many studies demonstrated the ability of Gas6 to either promote cell survival [21, 22] and/or proliferation [23, 24]. Additional growth factor-like properties of Gas6 have also been reported, including stimulation of cell migration [25] and cell-cell adhesion via Axl [26]. Gas6 has also been shown to induce scavenger receptor expression in VSMC, suggesting promotion of foam cell formation in the atherosclerotic process [27]. Furthermore, recent studies have convincingly shown both Gas6 and protein S to be involved in the Mer-mediated phagocytosis of apoptotic cells [28, 29]. Moreover, the inherent affinity of the Gas6 Gla region for negatively charged membrane phospholipids readily implicated Gas6 in the recognition of dying cells.

In whole tissues or animals, increased expression of both Gas6 and Axl has been observed in the rat arterial neointima after experimental injury [30]. In the kidney, increased glomerular expression of Gas6 and Axl has been detected in animal models of kidney disease [31]. Significantly, warfarin administration at sub-clinical doses inhibits these increases, further supporting the involvement of vitamin K (and Gas6) in the disease aetiology. In addition, Gas6 knockout mice were less susceptible to developing accelerated nephrotoxic nephritis relative to wild type animals [32]. Gas6 upregulation was also reported in conjunction with allograft rejection in a rat kidney transplant rejection model [33] as well as in dysfunctional human renal allografts [34].

In a separate study of Gas6 knockout mice, we observed that these animals were protected from both venous and arterial thrombosis [35]. This protection was apparently afforded through absence of Gas6 in platelets, indicating that it may function as a secondary signal amplifier in platelets. Likewise, mice genetically lacking each one of the three receptors are also protected against thrombosis, mainly due to impaired stabilisation of platelet aggregates [36]. However, the situation appears to be quite different in humans. Using a sensitive ELISA method, we could measure Gas6 in human plasma in the subnanomolar range (0.16-0.28 nM) although we could not detect Gas6 in human platelets [37]. Furthermore, RT-PCR analysis by one group could only show expression of Mer in human platelets [38]. Therefore in humans, the role of Gas6 in the thrombotic process is by no means established, and if at all relevant, might involve Gas6 from sources other than platelets.

A fascinating insight into the physical and evolutionary link between Gas6 and Axl was provided by the identification of an apparent chimeric gene in the tunicate *Halocynthia roretzi* [39]. This gene encodes a transmembrane protein that has an Axl-like intracellular domain whilst possessing an extracellular region housing a Gla domain that is highly homologous to that of Gas6. The existence of this invertebrate gene and the fact that its transcription is restricted to oogenesis points to a major role in growth and development. It also suggests a gradual fine-tuning process during evolution, where the molecule separates into two molecules that have to interact as part of a more sophisticated regulation of the same function.

Functions of Protein as ligand for Axl RTKs

Surprisingly, only a handful of studies to date have reported functional effects of protein S that appear to be independent of its anticoagulant function. This paucity of information may reflect a lack of interest due to the uncertain status of protein S as a receptor ligand. It is noteworthy that the concentration of free protein S in human plasma is high (131 nM [40]), which is in stark contrast to the subnanomolar levels of plasma Gas6 [37]. Nevertheless, protein S could like Gas6 function through local overexpression in cells and tissues and thereby act as a growth/survival/phagocytic factor in an autocrine or paracrine manner. We have previously shown protein S to be expressed in multiple organs in rabbits other than the liver, the main site of production of protein S and other coagulation factors [41].

Prior to its receptor being identified, human protein S was shown to be a potent mitogen for human VSMC, and a protein S-receptor complex was identified by chemical crosslinking [42]. In contrast, protein S inhibited the proliferation of rat astrocytes after injury, nevertheless suggesting a direct effect on cells [43]. Later, protein S was demonstrated to promote bone resorbing activity in osteoclasts via Sky RTK [18]. Also, a novel neuroprotective effect was revealed for protein S in a mouse study of stroke, where administration of protein S protected ischaemic neurons both in vivo and in vitro [44]. Furthermore, in conjunction with the discovery of Mer-mediated phagocytosis of apoptotic cells, protein S was implicated as an even more significant player in this process than Gas6 [28, 29]. This was further strengthened by the recent observation that mouse protein S directly stimulated mouse Sky and Mer, both of which are present in the eye, and this was coupled to the potential for protein S to mediate phagocytosis of rod outer segments by retinal pigment epithelial cells [45]. A similar apoptotic cell clearance function for protein S may indeed also occur in the testis, as Leydig cells express protein S [46] and they are taken up by Sertoli cells, which express Axl and Sky [47]. Other tantalising clues to a contra-immune response function for protein S include its upregulation by in primary T cells by interleukin (IL)-4, which could be part of the mechanism behind which IL-4 antagonises cell-mediated immunity [48].

Therefore, one cannot exclude the possibility that protein S plays a significant biological role as a ligand for the Axl RTKs, despite its apparent lower affinity than Gas6 from in vitro studies. Clearly, roles for protein S in regulating cell turnover on and preventing autoimmunity are becoming increasingly apparent. All this is notwithstanding the potential for both ligands to be dispensible in situations where sole overexpression of Axl/Sky/Mer is the main effector of the phenotype.

Molecular features of ligand-receptor interaction in the Gas6/Axl system

Several studies, utilising either site-specific blocking antibodies, or partial protein constructs, have established the SHBG region of both Gas6 and protein S as being the receptor-binding site. More detailed molecular studies revealed the necessity of the first LG domain in the Gas6 SHBG region for Axl binding [49]. More recently, the publication of the crystal structure of a minimal Gas6-Axl complex has provided for the first time a detailed view of the regions within Gas6 and Axl involved in their interaction [50]. In this complex, the two Ig-like domains of an Axl monomer are crosslinked by the first LG domain of a Gas6 molecule in a first high affinity interaction. Lateral diffusion of such 1:1 complexes then results in dimerisation to form a circular 2:2 assembly (Fig. 3A). Two different sites of Gas6-Axl contact were revealed, one major and one minor, with only the minor one being conserved within the Axl subfamily. No direct Axl-Axl or Gas6-Gas6 contacts were apparent in the complex. In the major contact site, several charged residues were identified in both Axl and Gas6 that form part of polar β-sheet surfaces interacting with each other. It is interesting that protein S does not possess such a distribution of charged residues as which occurs in Gas6, which may explain its inability to bind to Axl. Alternatively, clues may be provided as to the regions within protein S mediating its interaction with both Sky and Mer.

It is noteworthy that roughly 30% of protein S normally exists in human plasma in free form, whilst the remainder is in a high affinity complex, via its SHBG domain, to C4b-binding protein (C4BP), a negative regulator of complement activation [51]. Therefore, it is this free protein S that is available to bind to the receptor, and indirect support for this comes from our observation of a functional distinction between the

type of protein S that is bound to apoptotic cells. Specifically, both the free and C4BP-bound forms of protein S can bind to apoptotic cells via the protein S Gla region [52]. However, only free protein S provided a stimulatory effect on the engulfment of apoptotic cells by primary human macrophages [53], indicating that only free protein S tethered to an apoptotic cell via its Gla region is able to activate the receptor to promote ingestion.

For the protein S-Sky interaction, there are inter-species differences in the interaction's affinity. For example, human and bovine protein S share 82% amino acid sequence identity but exhibit distinct affinities for Sky from different species, with only the bovine variant clearly activating human Sky [54]. In this regard, we have utilised domain swapping and mutational approaches to advantage, to show similar receptor-binding features as for Gas6-Axl [54, 55]. Indeed, a considerable array of inter-species variations in ligand receptor-affinities within the whole Gas6/Axl system has been reported (Table 1).

Alongside the fact that the SHBG domain binds directly to Axl [50], a supporting role for the Gla region in the functional effects of Gas6 is also apparent. For example, a requirement for fully γ-carboxylated Gas6 has been demonstrated for the cell growth/survival functions of Gas6 [23, 56]. This was observed through a lack of effect of Gas6 produced in the presence of warfarin, an antagonist of the vitamin K-dependent γ-carboxylation reaction. We also demonstrated a facilitating function for the Gla domain in that antibodies directed against the Gla domain of bovine protein S blunted its activation of human Sky [55]. One can thus propose a model in which uncarboxylated and calcium-free Gas6 that is free of the membrane has a conformation that sterically hinders the interaction of the C terminal region with the receptor. Conversely, a fully modified Gla region is able to juxtapose itself against and interact with the membrane, thus enabling the SHBG domain to bind to the receptor on either the same or another cell.

Axl, Sky and Mer in cancer

The transforming activity of Axl under experimental conditions attests to its oncogenic potential, the driving force being the intracellular tyrosine kinase domain. Indeed, a partial Axl construct beginning 33 amino acids downstream of the transmembrane region is sufficient to induce tumours in nude mice [57]. Axl appears to be the principal oncogene of its subfamily, being overexpressed in a variety of human cancers (Table 2). Much less is known about the status of Sky and Mer in cancer, although they too have transforming abilities. The greater reported prevalence of Axl in cancers may reflect its wider expression pattern, or simply that it has been targeted for analysis more often.

The intracellular signal transduction pathways coupled to activation of Axl, Sky and Mer are reviewed in detail elsewhere [58]. Briefly, activation of the phosphatidylinositol 3-kinase (PI3K) pathway appears to be a pivotal event in Axl signalling, mediating cell survival, proliferation and migration [21]. A more novel aspect to Axl signalling is its constitutive interaction with IL-15 receptor α, the latter transactivating Axl (Fig. 3B) [59]. This novel cross-talk mechanism expands the boundaries of signalling mediators, and it is therefore not unlikely that Sky and Mer could be involved in heterotypic interactions. Little is currently known about Sky RTK signalling, whilst Mer signalling has been shown to affect cytoskeletal dynamics (described below).

Adhesive functions of Axl RTKs

Due to its domain organisation, the Axl ectodomain resembles that of adhesion molecules, suggesting that overexpression of Axl RTKs might confer adhesive properties onto cells. This may occur through ligand-independent homophilic interactions between receptor molecules on neighbouring cells, and the structural prerequisites for such an interaction have been described for Sky [60]. Cell adhesion is indeed a feature of experimental overexpression of Axl, featuring formation of cell aggregates, accompanied by receptor activation [61] (Fig. 3B). Furthermore, the adhesiveness of Axl *per se* appears to be independent of intracellular kinase activity, since cells expressing a receptor lacking the intracellular domain entirely still undergo aggregation [61]. Axl expression could be correlated with a greater adhesiveness in non small cell

lung cancer cell lines [62] and in human osteosarcoma cells [63]. This adhesion could contribute to increased metastasic properties of tumour cells [64]. Therefore, when Axl is overexpressed in cancer, its mediation of increased cell-cell adhesion may be at least as significant as activation of intracellular signalling.

It will be of interest to directly compare Axl, Sky and Mer with each other in assessing their effects on cell adhesion and aggregation, since they all possess similar structural elements. Moreover, the potential for heterophilic interactions between the sister receptors has yet to be explored. For example, revelation of an interaction between Axl and IL-15 receptor α represents a striking deviation from the current repertoire of Axl interactions [59]. Interestingly, the extracellular portion of Axl was essential for this interaction whilst Axl kinase activity *per se* was not. This heterotypic interaction appears to be a novel mechanism for transactivation of the Axl receptor, utilising IL-15 as ligand. Axl activation in turn leads to IL-15 receptor phosphorylation. Clearly, this finding opens up a fascinating new area of investigation, where there exists a previously unsuspected promiscuity amongst diverse cell surface receptors.

Soluble extracellular forms of Axl RTKs

The extracellular regions of several transmembrane proteins such as adhesion molecules and growth factor and cytokine receptors, have been found in circulating forms in plasma [65]. These soluble ectodomains are shed from the full-length protein and may thereby limit the accessibility of the cell-bound receptor to the ligand. They therefore represent an important post-translational mechanism for controlling ligand efficacy under certain clinical conditions. Soluble Axl ectodomain released as a result of proteolytic cleavage has been detected in conditioned medium of various cell lines [62, 66]. A mouse Axl ectodomain was detected in tumor cell and dendritic cell medium and in serum, and the disintegrin-like metalloproteinase ADAM 10 was suggested to be involved in its generation through proteolytic cleavage [67]. Furthermore, a significant amount of this soluble Axl, but not Sky or Mer, in mouse serum was found to be in complex with Gas6 [67].

These observations indicate the value in investigating the presence of soluble Axl ectodomain in human plasma. Detection and quantitation of plasma Axl may reflect altered regulation of Gas6/Axl system components under various clinical conditions, and may therefore be of diagnostic value.

Mer RTK – a novel phagocytic receptor

Several recent studies have uncovered a discrete function for Mer that apparently sets it apart from its sister RTKs. Mer appears to be required for uptake of apoptotic cells by professional phagocytes such as monocytes/macrophages, retinal pigment epithelial (RPE) cells and dendritic cells (Fig. 4). The process that led to this discovery began with the Royal College of Surgeons (RCS) rat, which is a classic model of hereditary retinal degeneration first described in 1938. The RCS model is characterised by an inability of eye RPE cells to fulfill their nomal function of phagocytosing shed outer segments (OS) of bleached photoreceptors. It was not until 2000 that the underlying genetic cause for the RCS rat retinal dystrophy phenotype was finally pinpointed to the Mer gene. The dystrophy locus was localised by positional cloning to within a 0.3-cM interval on rat chromosome 3, where there was a DNA deletion, resulting in a much shortened transcript for Mer [68]. Confirmation of Mer as the culprit was demonstrated through experimental correction of the RCS phenotype by retinal gene transfer of *MERTK* [69], and the observation that Mer knockout mice exhibited an identical RCS rat-like phenotype [70]. In humans, therefore, alterations in the *MERTK* gene were implicated in clinical cases of retinitis pigmentosa, which is a heterogeneous group of retinal dystrophies. This was indeed shown to be the case when MERTK mutations were found in 3 unrelated individuals from a screen of patients with retinitis pigmentosa [71]. In addition, an R844C mutation in a young retinal dystrophy patient was functionally characterised and shown to be less stable and active than wildtype Mer [72].

The role of Mer as a phagocytic receptor extends beyond the eye. In mice genetically lacking the kinase domain of Mer, macrophages show impaired clearance of apoptotic thymocytes [73]. These mice also have an increased number of circulating nuclear autoantibodies, suggesting an autoimmune response to a defective homeostatic mechanism that allows a buildup of cellular debris. Increased numbers of apoptotic cells have also been shown in Axl/Sky/Mer triple knockout mice, which also developed autoimmunity as well as being blind and the males being sterile [74]. These phenotypes highlight an essential role for the Axl RTKs in regulating uptake and clearance of apoptotic cells in distinct organs. An interesting additional feature of the triple knockout mice was that they exhibited grossly enlarged spleens and lymph nodes in adulthood, mainly due to hyperproliferation of B and T cells. This lymphoproliferation was likely enabled through the absence of the three receptors on antigen-presenting cells (macrophages and dendritic cells) and their consequent hyperactivation, which otherwise normally express them and are in a baseline state. Furthermore, loss of only Mer was also shown to be sufficient to induce the autoimmune phenotype in a study of single Mer knockout mice, shedding light on its particular importance in immune homeostasis [75]. Moreover, Mer has been shown to be involved in discrete signalling interactions for cytoskeletal dynamics, described in detail elsewhere [58].

CONCLUSION

The Gas6/Axl system is now making its presence felt amongst the several growth factor–receptor pairings that are well established as role players in both development and disease. In particular, it appears that discrete functional outcomes can arise from a particular ligand-receptor combination on a particular cell type (Fig. 4). Axl overexpression and activation appears to feature in many different types of cancer. Similarly, Axl activation, both with and without Gas6 stimulation, controls cell plastic processes typical for many growth factors. Gas6 itself is a growth, survival and chemotactic factor and, along with protein S, also a possible recognition bridge between phagocytes and apoptotic cells. Mer and possibly Sky have emerged as novel phagocyte receptors that signal for the engulfment process, and impairment of this system has been

linked to autoimmune-like disorders. Furthermore, a recent study by Sharif et al. demonstrated that both Gas6 and protein S could stimulate Axl RTK to actually suppress inflammation, through downregulation of TNF alpha expression, achieved through induction of the Twist transcriptional repressor [76]. Moreover, an additional novel role for the Gas6/Axl was recently uncovered pertaining to natural killer (NK) cell differentiation [77]. It was shown that expression of all three receptors on NK precursor cells and their stimulation by bone marrow stromal cell-derived Gas6/protein S, were essential for NK cell functional maturation. Therefore, novel roles for the Gas6/Axl system in immune homeostasis on several levels is becoming increasingly apparent. Our current level of knowledge should stimulate future research efforts aimed at further elucidating this unique molecular grouping, which appears to be more important with every finding.

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TABLE 1. Affinities of Gas6 and protein S for Axl, Sky and Mer RTKs within and across different species[1].

The equilibrium dissociation constant, K_d (nM), is given where calculated for positive interactions. √ denotes reported positive interactions without a K_d value. X signifies reports of an absence of binding. References are provided beside each entry. nd - interaction was not determined.

	Human Axl	Mouse Axl	Rat Axl	Human Sky	Mouse Sky	Rat Sky	Human Mer	Mouse Mer	Rat Mer
Human Gas6	4 nM [3] 1 nM [78] 1.6 nM [79] 0.18 nM [62] 4-5 nM (Gas6 SHBG) [7, 50] 0.05 nM [49] √[22, 25, 80, 81]	√[23, 36, 82]	√[25]	4.2 nM [83] 10.8 nM [78] 3.6 nM [79] 0.03 nM [49] √[84, 85] (Gas6 SHBG) [60]	X[84, 86] √[36]	nd	9.7 nM [79] 0.3 nM [49]	X[78] √[29, 36]	nd
Rat Gas6	0.4 nM [78]	nd	√[87]	2.7 nM [78] √[88]	nd	nd	nd	29 nM [78]	nd
Bovine Gas6	√[28]	√[89]	nd	nd	nd	nd	nd	nd	X[28]
Human protein S	nd	nd	nd	X[60, 84, 88] √ (weak) [54]	√[84, 89]	√[90]	X[79]	nd	nd
Mouse protein S	nd	nd	nd	nd	√[45]	nd	nd	√[45]	nd
Rat protein S	nd	nd	nd	nd	nd	nd	nd	nd	√[28]
Bovine protein S	X[28]	nd	nd	√[55, 60]	√[89]	nd	nd	nd	√[28]

[1]Both rat Gas6 and human protein S were shown to stimulate rabbit Sky [17].

TABLE 2. Increased expression of Axl, Sky and Mer in human cancers

Axl	Sky	Mer	References
Experimental tumorigenesis (mouse)	Experimental - cell transformation	Experimental – cell transformation	[57, 90-92]
Myeloid and erythro-megakaryocytic leukaemias	Experimental - haematopoietic cell expansion	Neoplastic B- and T-cell lines	[20, 93-96]
Oesophagus	Acute myeloid leukaemia	Mantle cell lymphoma	[97-99]
Gastric	Myeloma cells	ACTH-secreting adenomas, but UNDER-expressed in PRL-secreting adenomas	[100-102]
Colon	Breast (mouse)		[103-105]
Thyroid	Lung		[62, 106-1]
Liver	DOWNregulated in diffuse astrocytomas		[109-111]
Prostatic carcinoma cell line DU145			[112]
Melanoma			[113]
Breast			[114, 115]
Lung			[116, 117]
Kidney			[118]
Osteosarcoma			[63]
Ocular melanoma			[22]
Endometriotic endometria, uterine leiomyoma, ovarian carcinoma			[119-123]
Glioma			[124]

FIGURE LEGENDS

Fig. 1. Extracellular domain organisations of RTKs. In this schematic are shown domain similarities between the Axl RTK subfamily and PDGFR (Ig domains) and EphA (FNIII and Ig domains) RTKs. No similarity is shared between Axl and EGFR, which possesses cysteine-rich domains (Cys).

Fig. 2. Domain organisation of Gas6 and protein S. Both proteins are composed of: an N-terminal region containing multiple γ-carboxyglutamic acid residues (Gla), four EGF-like repeats, and a C-terminal region made up of two globular LG domains (also known as SHBG).

Fig. 3. A. Crystal complex of Gas6 LG domains with the Ig domains of Axl. Gas6 LG domains are in cyan (N-terminal segment and LG1) and green (LG2), whilst Axl Ig domains are in yellow (IG1) and brown (IG2); a calcium ion in the LG1–LG2 interface is shown as a pink sphere, and the Gas6/Axl contact sites are labelled. Reprinted by permission from Macmillan Publishers Ltd: The EMBO Journal ([50]), copyright (2006).

B. Models for extracellular activation of Axl RTKs. (1) Direct, ligand-independent homo- or heterophilic interaction between two Axl/Sky monomers. (2) Ligand–induced dimerisation of Axl monomers from two 1:1 (ligand–receptor) complexes to one 2:2 (2 x ligand-receptor) complex. (3) Heterotypic interaction between one Axl monomer and one monomer of IL-15 receptor alpha. (4) Hypothetical model for interaction between two Axl monomers on neighbouring cells.

Fig. 4. Distinct roles of Axl subfamily RTKs in cell survival and uptake of apoptotic cells and immune regulation. (1) Gas6/protein S-Axl interaction on the surface of several mesenchymal-derived cell types leads to signalling for cell survival and possibly growth. In addition, soluble Axl ectodomain can be generated by extracellular protease action, leading to formation of a soluble Gas6-Axl complex that blocks Gas6 ligand action. (2) Gas6/protein S acts as a bridging molecule between apoptotic cells and Mer RTK,

causing cytoskeletal alterations that drive ingestion of the bound apoptotic cell. The apoptotic cell is decorated with negatively charged phospholipids on its outer surface, which interacts with the Gla domain of Gas6/protein S. Sky RTK may also be involved in mediating both of the above processes. In addition, a role for Axl subfamily RTKs has also been implicated in anti-inflammatory processes, whereby they inhibit induction of pro-inflammatory cytokines such as TNF-α.