Vitamin K dependent actions of Gas6.

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Abstract

GAS6 (growth arrest-specific gene 6) is the last addition to the family of plasma vitamin K-dependent proteins. Gas6 was cloned and characterized in 1993 and found to be similar to the plasma anticoagulant protein S. Soon after it was recognized as a growth factor-like molecule, as it interacted with receptor tyrosine kinases of the TAM family; Tyro3, Axl and MerTK. Since then, the role of Gas6, protein S and the TAM receptors has been found to be important in inflammation, hemostasis and cancer, making this system an interesting target in biomedicine. Gas6 employs a unique mechanism of action, interacting through its vitamin K-dependent Gla module with phosphatidylserine-containing membranes and through its carboxy-terminal LG domains with the TAM membrane receptors. The fact that these proteins are affected by anti-vitamin K therapy is discussed in detail.

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I. Introduction

The family of plasma vitamin K-dependent proteins was well established after the discovery of γ -carboxylation in 1974 (Stenflo 2006). Plasma vitamin K-dependent proteins included the coagulation factors VII, IX, X and prothrombin and the natural anticoagulants protein C, protein S and protein Z, all of them characterized by a preserved N-terminal Gla domain, which contained the γ -carboxyglutamic residues of the protein of hemostasis.

Therefore, it was a surprise when in a screening for genes which expression was up regulated under conditions of growth arrest in embryonic mouse NIH 3T3 fibroblasts, the group of Dr. Schneider found a gene with a sequence related to the anticoagulant protein S (Schneider et al. 1988; Manfioletti et al. 1993). The gene was named growth arrest-specific gene 6, (*Gas6*; MIM#14456) and its expression was found to be increased around 30 times when the cell entered the G0 phase of the cell cycle. Shortly afterwards, the same group and others cloned the cDNA derived from the ortholog gene of human *PROS1* (MIM#176880) in the mouse (*Pros1*; MIM#19128) and the ortholog of *Gas6* in humans (*GAS6*; MIM#600441), demonstrating that they were two distinct entities, despite more than a 40% identity among protein S and *Gas6* protein sequences. Therefore, the short list of proteins dependent on vitamin K in plasma had a new genuine member.

II. *Gas6* structure

Gas6 is a multimodular protein containing several post-transcriptional modifications (Fig. 1). The main subject of this review is the γ -carboxylation of the N-terminal domain, the Gla module (Hansson & Stenflo 2005). Gla modules of plasma γ -carboxylated factors have a common fold, which is preserved in *Gas6*. Together with protein S and prothrombin, *Gas6* contains a disulfide-bridged thumb loop, although it does not seem to be cleaved by the action of serine proteases, as it is the case of protein S, which is inactivated by thrombin or

factor Xa specific cleavages in this region. From this connecting sequence, four epidermal growth factor-like modules are arranged in tandem, two of them with calcium-binding consensus sequences. The C terminal region contains two domains similar to the globular folds of the laminin A chain (LG domains), a structure that is also found in the sex-hormone binding globulin (Villoutreix et al. 1997; Sasaki et al. 2002). The sequence similarity between protein S and Gas6 is probably reflected in a close general structure, as suggested by modeling experiments using the available structures of Gas6 or protein S fragments (Dahlbäck & Villoutreix 2005).

Despite this similitude, Gas6 and protein S seem to differ in function, as it is reflected by the tissue expression of *GAS6* and *PROS1*. Most plasma vitamin K-dependent factors have a gene expression restricted to liver, especially in the case of factor IX and factor X (Fig. 2). The expression of *PROS1* is not so specific of liver, and comparable levels of transcript can be found in kidney, lungs or gonads. *GAS6* is unique among the genes of the family, because *GAS6* expression in liver is minor compared with other tissues, including heart, kidney and lung (Fig. 2). The presence of Gas6 in plasma has been carefully evaluated. Although its presence can be detected, the concentration is quite small, around 20-50ng/mL, or 0.25 nM (Balogh et al. 2005; Borgel et al. 2006); or even lower according to other reports (Gibot et al. 2007). In any case, the concentration of Gas6 is much lower than the rest of the vitamin K-dependent proteins of plasma, which range from 10 nM for factor VII to 1.5 μ M for prothrombin. The range of Gas6 concentration is also much lower than the for protein S, which increases up to 400 nM during an acute-phase response (Garcia et al. 1994).

III. Cellular effects of Gas6

The function of Gas6 has been debated from its discovery. The fact that it was a cell arrest-regulated gene suggested that it could act as a factor involved in cellular homeostasis.

Indeed, Gas6 was identified as a growth factor and a rescue factor from apoptosis on different cell types, specifically in cultured cells deprived from serum (Nakano et al. 1995; Li et al. 1996; Goruppi et al. 1996). Growth factors are major effectors of cell differentiation, duplication and survival. Their effect is mediated by the interaction with specific cell membrane receptors, most of them with a tyrosine kinase activity, (receptor tyrosine kinases or RTKs). RTKs are a large group of genes with a highly conserved intracellular tyrosine kinase domain. Intracellular signaling is triggered upon ligand binding, which induces dimerization/oligomerization of the receptor transducing the signal to the intracellular portion of the molecule and leading to activation of the tyrosine kinase activity. An essential early substrate of tyrosine phosphorylation is the RTK itself. RTKs are classified according to their extracellular domain, generally composed of a modular structure (Fig. 1). Members of a subgroup of RTKs often bind common or similar ligands.

A crucial discovery in the understanding of the biology of Gas6 was the discovery that it was a ligand of a previously orphan RTK, Axl. Axl was part of a three member family of receptors composed of Axl, Tyro3 and MerTK, which had been cloned by different groups and given different names: Axl (ARK, Tyro7, Ufo) Tyro3 (Sky, Rse, Brt, Dtk, Tif, Etk2, Rek), and MerTK (Mer, c-Eyk, Nyk, Tyro12). The architecture of these receptors is preserved, and it is constituted by two immunoglobulin domains followed by two fibronectin type III (Fig. 1). This receptor tyrosine kinase family is named TAM from the first letter of the three components (Tyro3, Axl and MerTK).

From the early studies of Gas6 action on cultured cells, the anti-apoptotic effect of Gas6 has been found in many experimental settings, as well as in different species. This has been observed in almost all cell types and lines tested, including NIH3T3 fibroblasts (Goruppi et al. 1996; Bellosta et al. 1997; Goruppi et al. 1999), VSMCs (Nakano et al. 1996; Melaragno et al. 2004; Konishi et al. 2004), endothelial cells (O'Donnell et al. 1999; Healy et

al. 2001; Hasanbasic et al. 2004), chondrocytes (Loeser et al. 1997), neurons (Allen et al. 1999; Yagami et al. 2003), oligodendrocytes (Shankar et al. 2003), hepatocytic precursors (Couchie et al. 2005), epithelial cells (Valverde et al. 2004), and different types of cancer cells (van Ginkel et al. 2004; Vajkoczy et al. 2006; Sawabu et al. 2007). The anti-apoptotic effect was observed after treatment with different stimuli inducing cell death, including serum deprivation, but also more specific effectors as TNF- α (Bellosta et al. 1997; O'Donnell et al. 1999; Valverde et al. 2004; Shankar et al. 2006), changes in pH (D'Arcangelo et al. 2002), oxidative stress (Konishi et al. 2004; Valverde 2005), soluble phospholipase (Yagami et al. 2003) or the amyloid peptide (Yagami et al. 2002). The anti-apoptotic effect of Gas6 requires protein phosphorylation, as general inhibitors as staurosporine abolished it (Bellosta et al. 1997), and in most cases, the PI3K pathway has been demonstrated to be crucial for this effect (see below). Interestingly, ultraviolet light exposure, which induces apoptosis in a p53 dependent manner, was not affected by Gas6/Axl (Bellosta et al. 1997).

While the anti-apoptotic effect of Gas6 has been repeatedly documented, it has not been so easy to study the mitogenic activity of the molecule. In certain cell types, a mitogenic effect has been described as a potentiating effect on other factors. Indeed, Gas6 was cloned as a growth potentiating factor for prothrombin on vascular smooth muscle cells (VSMCs) (Nakano et al. 1995). Gas6 stimulates also the mitogenic effect of EGF on cardiac fibroblasts (Stenhoff et al. 2004), and has been also reported to stimulate heregulin mitogenic activity on Schwann cells (Li et al. 1996). The mitogenic effect observed in these experiments did not seem to be dependent on PI3K activation, rather it was mediated through activation of the Ras/ERK pathway in most cases (Goruppi et al. 1996; Fridell et al. 1996; Goruppi et al. 1997; Goruppi et al. 1999; Sainaghi et al. 2005). Nevertheless, significant differences have been reported depending on the cell type and context studied (Goruppi et al. 2001), which could reflect differences in the span of Gas6 receptors expressed by the cells studied. Indeed, Gas6

has been shown to induce mesangial cell proliferation in cultured cells in a STAT3 dependent manner that correlates with Axl activation (Doi 2001; Yanagita et al. 2001b). It is possible that in certain cell types or in undifferentiated cancer cells Gas6/Axl would have a more clear mitogenic effect than in other cell types (Sainaghi et al. 2005).

A further cellular action of Gas6 is the induction of chemotaxis of VSMCs (Fridell et al. 1998). An effect on cell migration has been suggested to play a role on gonadotropin releasing hormone (GnRH) neurons migration into the hypothalamus during development (Allen et al. 2002; Nielsen-Preiss et al. 2007). In certain tumor types, migration and invasiveness has been correlated with the expression of Gas6 receptors, in particular Axl (Shieh et al. 2005), and Gas6 dependent signaling through Axl has been shown to promotes invasiveness of glioma cells in animal models (Vajkoczy et al. 2006).

Another cellular effect which has been demonstrated for Gas6 is the targeting of apoptotic cells and induction of phagocytosis by macrophages and other phagocytic cell types (Ishimoto et al. 2000). The induction of phagocytosis has been shown to be specific of apoptotic cell removal or phosphatidylserine containing liposomes, mimicking the situation in the cellular membranes of apoptotic cells (Ishimoto et al. 2000; Hall et al. 2001; Wu et al. 2005; Wu et al. 2006), while Gas6 does not influence the phagocytic capacity of these cells on bacteria, yeast or particulate substrates (Scott et al. 2001; Wu et al. 2006). The mechanism of Gas6 action could be linked to an integrin cooperative recognition of apoptotic cells and subsequent activation of macrophages (Wu et al. 2005). Unraveling the mechanisms and molecules implicated in the phagocytosis of apoptotic cells and the resolution of inflammation has demonstrated that it is a specific process (Wu et al. 2006). The new term efferocytosis has been proposed to designate the specific removal of apoptotic cells by phagocytic cells (Vandivier et al. 2006). Related to the effect on apoptotic clearance, Gas6 has been implicated in the formation of cellular aggregates mediated by Axl. Initially, it was

shown that the mouse Axl receptor was able to induce cellular aggregation in the absence of substrates when expressed as a transgene in *Drosophila* cells (Bellosta et al. 1995).

Afterwards, the formation of aggregates was found to be dependent on the presence of Gas6 in mammalian cells (McCloskey et al. 1997). Nevertheless, recent studies have shown that the soluble form of Axl is able to induce signaling through membrane-bound Axl when coated on plates, indicating that a possible homophilic interaction among Axl molecules independent of ligands is potentially relevant (Budagian et al. 2005a).

Some studies have found a regulatory role of Gas6 on cell activation and differentiation. Cells from innate immunity down regulated the expression of inflammatory cytokines after Gas6 stimulation, a function that has been mainly attributed to the interaction with MerTK (Camenisch et al. 1999; Correll et al. 2004; Sharif et al. 2006; Li et al. 2006). In contrast, MerTK activation seems to mediate IL-8 induction in prostate cancer cells (Wu 2004), inducing its differentiation. Induction of differentiation by Gas6 has also been reported in the maturation process of natural killer cells (Walzer & Vivier 2006; Caraux et al. 2006) and adipocytes (Shugart et al. 1995; Maquoi et al. 2005). Gas6 has shown an activity as modulator of differentiation models on cells of the vessel wall, including the inhibition of osteogenic differentiation of pericytes (Collett et al. 2003) and the differentiation of VSMCs into foam cells, as shown by the induction of the expression of specific markers as the scavenger receptor A (Murao et al. 1999; Ming et al. 2001). This properties could have an important role in the cellular homeostasis of the vasculature after damage (Melaragno et al. 1999).

IV. Interaction with TAM receptor molecules and signal transduction in the Gas6/TAM ligand/receptor system.

The first report proposing Gas6 as a ligand for TAM receptors showed that Gas6 engagement induced auto-phosphorylation of the Axl receptor (Varnum et al. 1995). The active receptor recruits components of several signaling pathways. One of the first molecules to be reported as recruited after Gas6 stimulation of the Axl RTK were PI3K subunits (Goruppi et al. 1997). Furthermore, docking sites in Axl intracellular kinase domain for PI3K subunits, Grb2, c-Src and Lck were localized in phosphorylated tyrosines Y821, Y779 and Y866 (Braunger et al. 1997). Other partners of Axl have been proposed using the yeast two-hybrid system, including SOCS-1, Nck2 and C1-TEN, proteins containing Src homology 2 domains (SH2), apart from confirming the previously found interactions with PI3K components and Grb2 (Hafizi et al. 2002).

To date, almost all studies have found a link between PI3K activation and Gas6/Axl effect on cells. The activation of the PI3K pathway has been shown to be indispensable for the anti-apoptotic effect of Gas6 on different cell types (Goruppi et al. 1999; Allen et al. 1999; Lee et al. 2002; Hasanbasic et al. 2004). The cell survival effect is blocked by pharmacological inhibitors of the PI3K pathway (Goruppi et al. 1997; Goruppi et al. 1999; Allen et al. 1999; Melaragno et al. 2004; Hasanbasic et al. 2004). Axl mutants which are not capable of interacting with PI3K subunits block also the survival effect of Gas6 on cells (Melaragno et al. 2004; Vajkoczy et al. 2006). Several isoforms of PI3K have been shown to interact with Axl, and they could potentially lead to discrete signaling pathways from the receptor (Braunger et al. 1997; Hafizi et al. 2002), and this could have different implications depending on the cell type studied. The activation of PI3K initiates the activation of the anti-apoptotic Akt/PKB pathway, which inhibition downstream of PI3K also blocks the anti-apoptotic effect of Gas6. In this context it is interesting that C1-TEN, a protein phosphatase similar to the PI3K/Akt/PKB inhibitor PTEN was also found to interact with Axl, although the potential regulatory role of the Axl/C1-TEN interaction has not been elucidated (Hafizi et

al. 2002). Downstream substrates of Akt/PKB shown to be modulated by Gas6 include the anti-apoptotic phosphorylation of Bad (Goruppi et al. 1999; Lee et al. 2002). Further, the PI3K/Akt/PKB pathway activates NF κ B transcriptional activity, increasing the concentration of Bcl-2, among other effects (Demarchi et al. 2001; Hasanbasic et al. 2004). Another effector of the Akt/PKB pathway in the context of Gas6 action could be glycogen synthase kinase-3 (GSK3), which has been shown to mediate contact inhibition by interacting with the Wnt pathway (Goruppi et al. 2001).

Interaction of the Axl kinase domain with the adaptor protein Grb2 (Fridell et al. 1996; Braunger et al. 1997) seems to be the necessary link with Ras mediated activation of the mitogen activated protein kinase (MAPK) pathway of the extracellular regulated kinases (ERK). In this context, it is interesting to note that early studies found an Axl dependent phosphorylation of the ribosomal protein S6 kinase (RPS6KB1), which activity leads to an increase in protein synthesis and cell proliferation (Goruppi et al. 1997). As mentioned before, the activation of the Ras/ERK pathway is essential for the observed mitogenic activity of Gas6 (Goruppi et al. 1996; Fridell et al. 1996; Stenhoff et al. 2004). A related adapter protein containing SH2 binding domains, Nck2, was also found to interact with Axl in the two hybrid system (Hafizi et al. 2002). This interaction is interesting, as it could provide a link of Axl activation with integrin-linked kinases, affecting in this way the cytoskeletal dynamics through focal adhesions and providing a cooperative pathway with integrin signaling (Hafizi & Dahlbäck 2006), as has been shown for MerTK (Wu et al. 2005; Nandrot & Finnemann 2006; Finnemann & Nandrot 2006). This process is probably mediated by interaction with Vav1 and small GTPases of the Rho family (Mahajan & Earp 2003). The activation of another phosphatase, SHP-2, has been shown to be essential for the effect of Gas6/Axl on inhibition of vascular endothelial growth factor receptor 2 (VEGFR2, FLK1, KDR) signaling on endothelial cells (Gallicchio et al. 2005).

The cross-talk between Gas6/Axl and KDR illustrates the way in which cells integrate signals from different stimuli, thereby coordinating and refining complex responses. An interesting example of this cooperation has been provided by the study of IL-15 anti-apoptotic effect. In this case, a direct interaction of the Axl receptor with the receptor for IL-15 has been demonstrated and shown to be relevant for the signaling cascades of both receptors. IL-15 is able to transactivate Axl through its specific receptor IL-15R (Budagian et al. 2005b). In an Axl dependent manner, IL-15 is able to activate the Akt/PKB pathway and induce survival to TNF- α in Axl transfected cells. Differences among the cells studied suggest that specific mechanisms related to putative partners of the Gas6/Axl system could be acting in different cell types leading to relevant changes in the response to Gas6. Furthermore, these studies demonstrate that differences in the extracellular part of the receptors could contribute not only to ligand recognition, but also to partner recruitment.

Other signaling pathways activated by Gas6/Axl have been studied. Some studies found a transient activation of other MAPKs, including JNK/SAPK and p38 (Bellosta et al. 1997; Goruppi et al. 1999). The later activation has been shown to be important in the migration of GnRH neurons (Allen et al. 2002; Nielsen-Preiss et al. 2007). Finally, the Janus kinase (JAK) pathway is also affected by Gas6/Axl, leading to activation of signal transduction and activators of transcription factors (STATs). As mentioned earlier, the mitogenic effect of Gas6 on mesangial cells was shown to be mediated by activation of STAT3 through stimulation of Axl (Yanagita et al. 2001b). This effect has been shown to be important in the development of certain models of renal disease (Yanagita et al. 2002; Yanagita 2004).

The signaling pathways initiated by Tyro3 have been comparatively less studied to those initiated by Axl or MerTK. Apart from autophosphorylation of the receptors, Tyro3 and MerTK have been found to bind and activate Src kinase (Toshima et al. 1995; Wu et al.

2005), similarly as was observed for Axl (Braunger et al. 1997). The activation of regulatory subunits of PI3K has also been demonstrated for Tyro 3 (Lan et al. 2000). In osteoclasts, Gas6 has been shown to stimulate Tyro3 through a Ras/ERK mediated mechanism (Katagiri et al. 2001). Interestingly, in this cell type no activation of alternative MAPK pathways was reported (Katagiri et al. 2001). Constitutively active forms of MerTK have been shown to autophosphorylate residues Y749, Y753 and Y754 and, upon activation, phosphorylate PLC- γ , PI3K, Shc, Grb2, Raf-1, and ERK downstream the receptor (Ling et al. 1996). In a second study, the binding to Grb2 was shown to be dependent on Y872, and it is essential in the PI3K activation leading to an anti-apoptotic signal through activation of the NF κ B pathway (Georgescu et al. 1999). In contrast to the mitogenic activity observed with Axl activation, MerTK kinase domain rescued cells from apoptosis, but did not induce proliferation, despite activating ERK, PI3K and p38 pathways (Guttridge et al. 2002). PLC- γ has been shown to interact and be a substrate of MerTK, is essential in phagocytosis of apoptotic cells (Todt et al. 2004). Again, PLC- γ has also been shown to bind Axl, where it could be implicated also in a pathway affecting the dynamics of the cytoskeleton (Braunger et al. 1997; Nielsen-Preiss et al. 2007).

As was shown for Axl, MerTK has been shown to activate the Jak/STAT pathway leading to increased activation of Stat3 and increased transformation efficiency (Besser et al. 1999). In this context, it is interesting to note that another partner of Axl found by the yeast two-hybrid assay was SOCS-1, a negative regulator of cytokine signaling acting through the inhibition of Jak phosphorylation, and therefore inhibiting the Jak/STAT pathway (Hafizi & Dahlbäck 2006). Although not studied, it is possible that a similar interaction is provided by MerTK. From these studies, the general view shows that due to the high conservation in the intracellular sequence of the TAM receptors; most interacting partners are common, although

each receptor in the family show marked differences in the biological function of the three receptors.

V. Role of the Gas6/TAM system in vascular biology.

One of the first discoveries linking Gas6 with the biology of vascular vessels was the already mentioned cloning of a growth potentiating factor of VSMCs, shown to be Gas6 (Nakano et al. 1995). Axl was also cloned from the rat carotid artery after mechanical damage. Both Gas6 and Axl increased their expression in this model, mainly in the developing neointima up to 14 days after damage, the increase in Gas6 preceding that of the receptor (Melaragno et al. 1998; Melaragno et al. 1999). Given the earlier studies showing an anti-apoptotic and mitogenic effect of the Gas6/Axl system in cultured cells, it was supposed that proliferating cells would need the signaling through Gas6/Axl in order to migrate and/or proliferation of VSMCs. The clear effect observed in VSMCs migration in cell culture (Fridell et al. 1998) indicates that Gas6 could also play a role in these systems in vivo, affecting neointima migration as well as the organization of the atherosclerotic plaque (Lutgens et al. 2000). Indeed, Axl deficient mice show a decreased neointima formation after mechanical injury (Konishi et al. 2004; Korshunov et al. 2006). Nevertheless, the Axl knockouts showed not only a decreased number of proliferating muscular cells, and an increase in apoptosis, but also less macrophages and neutrophils (Korshunov et al. 2006). A parallel situation has been reported in atherosclerosis of Gas6^{-/-} animals, where a lower content of lymphocytes and macrophages has been found (Lutgens et al. 2000).

Apart from the effect on VSMCs, a role of Gas6 on the endothelium has been suggested from its anti-apoptotic effect in culture (O'Donnell et al. 1999; Healy et al. 2001; D'Arcangelo et al. 2002; Hasanbasic et al. 2004). In the one hand, the decreased content in

macrophages and neutrophils in *Axl*^{-/-} mice during intimal thickening suggests differences in cell recruitment, a process mediated by endothelial/leukocyte interactions (Korshunov et al. 2006). On the other hand, the addition of Gas6 has been shown to inhibit adhesion of granulocytes to endothelial cells (Avanzi et al. 1998). These studies suggest an effect of the Gas6/TAM system on endothelial function beyond the anti-apoptotic effect observed in cultured endothelial cells.

Recently, an important function of Gas6 has been described in relation to the differentiation of pericytes. Vascular pericytes undergo osteogenic differentiation *in vivo* which may be implicated in diseases involving ectopic calcification and osteogenesis, a process that was inhibited by Gas6/*Axl* signaling (Collett et al. 2003). This effect is not completely surprising considering several reports implicating the Gas6/TAM system in osteoclast function (Nakamura et al. 1998; Katagiri et al. 2001; Kawaguchi et al. 2004). It has recently been reported that the induction of calcification of cultured VSMCs is inhibited by *Axl* (Collett et al. 2007), and the reversion of the process by statins was also mediated by activation of the Gas6/*Axl* pathway (Son et al. 2006; Son et al. 2007). In this context, the linkage between genetic variants in *GAS6* and stroke could be due to the effect of Gas6 on vascular calcification, a process clearly associated with the risk of stroke (Munoz et al. 2004).

Platelets are key players in the cardiovascular system, not only providing primary hemostasis, but also mediating inflammatory responses and cellular interactions. Gas6 is found in platelets from rats and mice (Ishimoto & Nakano 2000; Angelillo-Scherrer et al. 2001), and animals deficient in Gas6 are protected in thrombosis models and their platelets are less prone to activation (Angelillo-Scherrer et al. 2001). Both MerTK and *Axl* and to a lesser extent Tyro3, have been proposed as mediators of this function using knockout mice and antibody inhibition (Angelillo-Scherrer et al. 2001; Chen et al. 2004; Gould et al. 2005;

Angelillo-Scherrer et al. 2005). However, further studies showed that the concentration of Gas6 in human platelets was lower than the concentration found in plasma, and did not affect platelet response (Balogh et al. 2005; Clauser et al. 2006). Still, Gas6 could affect antiplatelet drug responsiveness (Burnier et al. 2006). Recently, the extracellular part of MerTK has been shown to be able to inhibit platelet aggregation and pulmonary embolism, further emphasizing that, at least in mice, the Gas6/TAM pathway is involved in hemostasis (Sather et al. 2007).

VI. Gas6 in innate immunity: a modulator of the inflammatory response

Cells from the immune system express TAM receptors and their ligands. TAMs are detected on antigen presenting cells from both peripheral blood or spleen, thymus and lymph nodes either with specific antibodies after cell sorting or by in situ hybridization (Neubauer et al. 1994; Lu & Lemke 2001; Behrens et al. 2003; Mahajan & Earp 2003; Sen et al. 2007). In contrast, TAMs are not expressed in granulocytes or blood lymphocytes (Neubauer et al. 1994; Graham et al. 1995). It is likely that most monocytic cells express more than one TAM receptor, although it cannot be excluded that certain populations express only specific receptors (Lu et al. 1999; Lemke & Lu 2003a). The function of TAM receptors in the immune system is dramatically illustrated in animals deficient in all three receptors (Lu et al. 1999, Lu et al. 2001). TAM deficient animals survive and do not present defects during early life. Peripheral lymphoid organs are normal, indicating a broadly normal hematopoiesis, but from one month of age, spleens and lymph nodes grow disproportionately, reaching 10 times the weight of these organs in wild type mice. While all three mutations are involved in this phenotype, the MerTK mutation produces the largest effect (Lu & Lemke 2001; Scott et al. 2001). Animals deficient in MerTK present defects in monocyte function, including an enhanced sensitivity to endotoxic shock (Camenisch et al. 1999), a delayed clearance of

apoptotic cells and splenomegaly (Scott et al. 2001; Cohen et al. 2002). TAM or MerTK deficient animals show signs of autoimmunity, with features resembling certain human autoimmune pathologies including serum autoantibodies against DNA, collagen and antiphospholipid antibodies (i.e. anticardiolipin antibodies) and lymphocyte activation and hyperproliferation (Lu et al. 1999; Lu & Lemke 2001). The fact that lymphocytes become activated in TAM deficient mice, despite not expressing the TAM receptors, indicates that autoimmunity in these animals is mediated through a cell-dependent mechanism. Probably the lack of an inhibitory signal arising from Gas6/TAM lowers the activation state of antigen-presenting cells subsequent to an initial immune response. Multiple observations, made in both the MerTK single mutants and the triple TAM mutants, are consistent with this suggestion. First, macrophages from MerTK mutants and TAM deficient animals, when challenged with bacterial lipopolysaccharide (LPS), express unusually high levels of activated NF κ B, and the mice are hypersensitive to LPS-induced endotoxic shock, tissue damage and death as a result of the excessive production of TNF- α (Lu et al. 1999; Camenisch et al. 1999; Scott et al. 2001; Cohen et al. 2002; Sen et al. 2007). Markers of antigen-presenting cell activation, including cell-surface expression of MHC class II and the B7 co-receptors, are constitutively activated in the TAM mice in comparison to wild type mice and are consistently hyperactivated in response to LPS challenge (Lu & Lemke 2001).

In addition to lymphocyte hyper-responsiveness, the second key feature displayed by TAM mutants that almost certainly contributes to their development of autoimmune disease is that macrophages fail to clear apoptotic cells. This phenomenon has been most thoroughly analyzed in the MerTK single mutants (Scott et al. 2001; Cohen et al. 2002; Li et al. 2006; Sen et al. 2007). The delayed clearance of apoptotic cells is dependent on the ability of the vitamin K-dependent protein S and Gas6 to bind to phosphatidylserine and acidic phospholipids through their amino-terminal Gla domains (Nakano et al. 1997a; Scott et al.

2001; Cohen et al. 2002; Wu et al. 2006; Sen et al. 2007). Probably, the lack of efferocytosis is enhanced in the TAM deficient animals by an increased cell death due to the lack of an anti-apoptotic rescue signal, as has been shown for Gas6 in cell cultures. In vivo, an increased apoptotic cell death has been observed in retina, brain, gonads and other organs, suggesting that TAMs play a role in maintaining the cellular homeostasis of organs beyond their function in the immune response (Lu et al. 1999; Lu & Lemke 2001; Lemke & Lu 2003b).

VII. Further functions of Gas6/TAM receptors in cellular homeostasis.

The role of Gas6 on cellular homeostasis, related to its mitogenic and anti-apoptotic properties, has been implicated in several organs and models of disease. Considering the tissue distribution of both ligands and receptors, it was expected that the Gas6-Protein S/TAM system would have implications in neural tissues. Tyro3 single mutants exhibit compromised central nervous system (CNS) function (diminished hippocampal long-term potentiation [LTP]) as young adults and, when aged, they exhibit neural degeneration accompanied by seizures and hind limb paralysis (Lu et al. 1999). This phenotype is clearly more severe in the animals deficient in all TAM receptors (Lu et al. 1999). Gas6 has also been implicated in the cellular homeostasis of the kidney after certain damages, including nephrotoxic nephritis and diabetic glomerulopathies (Yanagita et al. 1999; Yanagita et al. 2001a; Yanagita et al. 2002; Nagai et al. 2005). The mechanism of action seems to be mediated by the mitogenic effect of Gas6 on mesangial cells. Mesangial cell proliferation is a general characteristic of a variety of glomerular diseases (Yanagita et al. 1999; Yanagita et al. 2001a; Yanagita et al. 2001b). A third system where Gas6 function has been implicated is in the development of germinal cells (Matsubara et al. 1996). Most TAM deficient animals are infertile due to the degeneration of all germ cells in the gonads (Lu et al. 1999). These observations support the view that Gas6 could act as a factor maintaining at least certain stem

cells alive and in their undifferentiated state (Matsubara et al. 1996; Li et al. 1996; Nakamura et al. 1998; Dormady et al. 2000; Player et al. 2006).

VIII. Overlapping functions of Gas6 and protein S.

From very early after the discovery of the Gas6/Axl ligand/receptor interaction (Varnum et al. 1995), some researchers discovered an interaction of protein S with receptors of the TAM family (Stitt et al. 1995). In this study, it was proposed that human protein S was able to interact with Tyro3 in a parallel way to Gas6 interaction with Axl. Nevertheless, it was also found that species differences were distinctly important in the affinity of the different ligands with the TAM receptors (Godowski et al. 1995; Nagata et al. 1996; Mark et al. 1996). Indeed, purified human protein S was not able to bind to the extracellular domains of human Tyro3 or the other TAM receptors in the same assays where it was able to bind the extracellular domain of mouse Tyro3. Interestingly enough, bovine protein S seems to be able to bind and activate both the human and mouse receptors (Godowski et al. 1995; Nyberg et al. 1997). Despite this lack of binding activity, human protein S is capable of inducing phosphorylation of human Tyro3 in certain cell types at higher concentrations than human Gas6 or bovine protein S (around 200 nM), but still lower than the concentration of protein S found in plasma (Evenas et al. 2000). In plasma, protein S has a concentration of 350 nM, but almost 60% is bound to the complement regulatory factor C4BP (Giri et al. 2002). Binding to C4BP abolishes protein S binding to TAM receptors (Nyberg et al. 1997), but C4BP does not bind Gas6 with a very low affinity (Evenas et al. 1999). One of the problems that makes it more difficult to evaluate the results published so far is the capacity of purified protein S of multimerizing in high molecular weight complexes that have specific properties, different from protein S monomers (Heeb et al. 2006). This effect has been studied regarding the anticoagulant properties of protein S, but has not been taken into account in relation to their

putative effects on receptor binding. Furthermore, purified Gas6 preparations show a similar behavior, and this could affect the interpretation of the results obtained in purified systems (Stenhoff, Hafizi and Dahlbäck, personal communication).

Despite these discrepancies on the putative effect of protein S on TAM receptors, several studies *in vivo* suggest that protein S, and in particular human protein S, has a function as a ligand of TAM receptors. In the one hand, protein S was found to be the serum factor responsible of the stimulation of the phagocytosis/efferocytosis (Anderson et al. 2003). Protein S-depleted serum or antibodies against protein S could block the effect of serum on clearance of apoptotic cells by macrophages. Furthermore, the effect of purified protein S was also specific for apoptotic cells, not affecting other forms of phagocytosis. This finding is indicative that the Gla-dependent interaction with negatively charged phospholipids membranes was necessary for the effect of protein S (Anderson et al. 2003). A second system where the effect of protein S as a TAM ligand has been demonstrated is the retina. MerTK-deficient animals were found to be blind as a result of the complete degeneration of photoreceptors during the first month of life (Hall et al. 2001; Collett et al. 2003; Hall et al. 2005; Prasad et al. 2006; Nandrot & Finnemann 2006; Finnemann & Nandrot 2006). Interestingly, a parallel line of research found that the Royal College of Surgeons (RCS) rat, a decades-old animal model of retinitis pigmentosa, had a mutated MerTK gene (Nandrot et al. 2000), and mutations in the human MerTK gene have been detected in patients with retinitis pigmentosa (Gal et al. 2000). Several lines of evidence suggest that, at least in mice, the protein S/MerTK pair is the most relevant system in the retina, including the presence of protein S in the retina, the lack of phenotype in Gas6 deficient animals (Hall et al. 2005; Prasad et al. 2006) and the ability of mouse protein S to activate MerTK in extracts of retina (Prasad et al. 2006).

In general, a strong suggestion of a role of protein S as a ligand to TAM receptors derives from comparing different knockout models. If Gas6 would be the only and essential ligand of all three TAM receptors, one would expect a similar phenotype of TAM deficient and Gas6 deficient animals. This is not the case, as Gas6 deficient animals do not have a similar spontaneous phenotype to the TAM triple knockouts (Lu et al. 1999; Angelillo-Scherrer et al. 2001; Yanagita et al. 2002), not showing signs of autoimmune disease, infertility or seizures. This clearly indicates the presence of alternative mechanisms of TAM receptor activation, and most likely protein S could be a rescue factor of some of these phenotypes in Gas6 deficient animals. Other ligands, as IL-15 or soluble or cell-bound Axl cannot be excluded (Budagian et al. 2005a; Budagian et al. 2005b).

IX. The implications of vitamin K in Gas6 function.

In most studies, the presence of a properly γ -carboxylated Gla module in Gas6 has been found to be essential for Gas6 function. This was evident from the first report describing the interaction of Gas6 with Axl. The authors already noticed that producing Gas6 under warfarin-induced inhibition of vitamin K-dependent γ -carboxylation abolished completely the Axl receptor auto-phosphorylation (Varnum et al. 1995). This lack of activity of Gas6 produced on warfarin was extended to in vitro binding studies, where Gas6 lacking γ -carboxylated residues was unable to bind Axl (Tanabe et al. 1997). This suggested that the Gla domain could affect the binding site of the receptor, although the LG domains in Gas6 seemed to contain the binding site for TAM receptors (Mark et al. 1996; Evenas et al. 2000; Fisher et al. 2005). Gas6 binds to phosphatidylserine-containing phospholipid membranes in an analogous manner to the rest of the proteins of the family, dependent on the Gla module and therefore of vitamin K action (Nakano et al. 1997a; Ishimoto et al. 2000; Hasanbasic et al. 2005). Warfarin abrogates Gas6-mediated protection of fibroblasts, VSMCs and

endothelial cells from serum starvation-induced apoptosis (Nakano et al. 1997b; Stenhoff et al. 2004; Konishi et al. 2004; Hasanbasic et al. 2005). Proliferation of certain cells could also be abolished by warfarin through a Gas6 dependent mechanism, as in neointima formation (Konishi et al. 2004) and mesangial cell proliferation (Yanagita et al. 1999; Yanagita et al. 2001a). Finally, macrophage-mediated clearance of apoptotic cells is also inhibited by warfarin effect on Gas6 Gla domain (Hall et al. 2002).

This cellular effects have been translated to in vivo models, using oral anticoagulation to test the effect of inhibition of γ -carboxylation of Gas6 in different contexts. In the retina and other models of efferocytosis, Gas6 needs to be γ -carboxylated in order to be functional (Ishimoto et al. 2000; Hall et al. 2002). Mesangial cell proliferation is also inhibited by warfarin through its action on Gas6 (Yanagita et al. 2001a). The concentration of oral anticoagulants as warfarin necessary to inhibit Gas6-dependent effects were comparable or lower than therapeutic concentrations, indicating that the effects observed in animal models could translate to effects in humans (Yanagita et al. 2001a). Interestingly, Gas6 in plasma has been shown to be affected by oral anticoagulants (Balogh et al. 2005). The decrease in Gas6 concentration in anticoagulated patients compared to a control group was similar to that observed in protein S (Zöller et al. 1995). Although the concentration of Gas6 in plasma could not be as crucial as the local production of the ligand, it could still reflect a systemic effect of oral anticoagulants on the Gas6/Axl system. Therefore, it would be interesting to evaluate if patients in chronic anticoagulant therapy could present side-effects derived from the decrease in Gas6 function.

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Figure 1. Schematic representation of Gas6, protein S and the three members of the TAM receptor family, Tyro3, Axl and MerTK. The domains were predicted with the Simple Modular Architecture Research Tool (SMART) program (<http://smart.embl.de/>) and represented using pfam graphical view of domain structure, from the Sanger institute (<http://www.sanger.ac.uk/>). The tyrosine kinase active sites are indicated ().

Figure 2. Expression of selected plasma vitamin K-dependent proteins in a subset of human (red bars) and mouse (blue bars) organs. Data were obtained from the Symatlas microarray expression database and represent the relative value of expression of each gene (<http://symatlas.gnf.org/>; Su et al. 2002).