

Theme Issue Article

Growth arrest-specific gene 6 (GAS6)

An outline of its role in haemostasis and inflammation

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Summary

GAS6 (growth arrest-specific 6) belongs structurally to the family of plasma vitamin K-dependent proteins. GAS6 has a high structural homology with the natural anticoagulant protein S, sharing the same modular composition and having 40% sequence identity. Despite this, the low concentration of GAS6 in plasma and the pattern of tissue expression of GAS6 suggest a distinct function among vitamin-K dependent proteins. Indeed, GAS6 has growth factor-like properties through its interaction with receptor tyrosine kinases of the TAM family; Tyro3, Axl and MerTK. GAS6 employs a unique mechanism of action, interacting through its vitamin K-dependent GLA (γ -carboxyglutamic acid) module with phosphatidylserine-containing membranes and through its carboxy-terminal LamG domains with the TAM

membrane receptors. During the last years there has been a considerable expansion of our knowledge of the biology of TAM receptors that has led to a clear picture of their importance in inflammation, haemostasis and cancer, making this system an interesting target in biomedicine. The innate immune response and the coagulation cascade have been shown to be interconnected. Mediators of inflammation are essential in the initiation and propagation of the coagulation cascade, while natural anticoagulants have important anti-inflammatory functions. GAS6 represents a new player in this context, while protein S seems to have new functions beyond its anticoagulant role through its interaction with TAM receptors.

Keywords

Vitamin K, growth factors, innate immunity, inflammation, receptor tyrosine kinases, GLA

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Discovery of GAS6 and protein structure

Gas6 was cloned in 1988 in a screening for genes whose mRNA was increased under conditions of growth arrest in embryonic mouse fibroblasts, and sequenced by the same group five years later (1, 2). The gene was named growth arrest-specific gene 6 (*Gas6*; MIM#14456) and its expression was found to be upregulated 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 of *Gas6* in humans (*GAS6*; MIM#600441). Protein S and GAS6 have a sequence identity of 40% in their amino acid sequences, and both belong to the family of vitamin K-dependent proteins found in plasma. Protein S is considered an anticoagulant plasma protein based on its capacity to prolong blood-clotting time measured *in vitro* and in the association of *PROS1* mutations and low protein

S concentration with an increased frequency of venous thrombosis (3). Ortholog genes to *PROS1* and *GAS6* are found in all vertebrates, including fish and amphibians, all producing proteins with the same modular architecture, indicating that the duplication of the ancestral sequence from whom the two genes arised is older than the origin of vertebrates. In contrast, no similar proteins are found in insects or other invertebrate animal models, although proteins with partial homology to GAS6 are found in urochordates (4, 5).

GAS6 is a multimodular protein containing several post-transcriptional modifications (6). Gamma-carboxyglutamic-containing (GLA) modules of plasma γ -carboxylated factors share a similar fold, which is preserved in GAS6. Together with protein S and prothrombin, GAS6 contains a disulfide-bridged loop structure coded by a distinct exon, although in the case of GAS6 it does not seem to be cleaved by the action of serine proteases, as it is the case in protein S, where this region is inacti-

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vated by thrombin or factor Xa-specific cleavages (7, 8). In protein S, this loop is essential for direct contacts with activated protein C, while GAS6 lacks an analogous interaction (9–11). From this connecting sequence, four epidermal growth factor-like modules are arranged in tandem, two of them with calcium-binding consensus sequences (6). In protein S, these modules adopt predominantly an extended conformation, although inter-domain flexibility is suggested to be important in their function (12). The carboxy-terminal region contains two domains with a structure similar to the globular modules of the laminin A chain (LamG domains). LamG domains are found in proteins with binding sites for heparan sulphates, steroids or integrins, usually involved in calcium-mediated interactions. Each LamG domain folds into a curved β -sandwich built from two antiparallel sheets and contains a single disulfide bond near the C terminus, and, in the case of GAS6 and protein S, contain also a short α -helix (13, 14).

Despite the similarity in protein structure, GAS6 and protein S differ in function, as it is reflected by the different pattern of tissue expression between *GAS6* and *PROS1*, or their murine orthologues *Gas6* and *Pros1*. The gene expression of most plasma vitamin K-dependent factors is restricted to liver, particularly in the case of *F9* and *F10*, and with less specificity also in *F2*, *F7*, *PROC* and *PROZ* (15). The expression of *PROS1* is broader, and its mRNA abundance in liver is comparable to kidney, lungs or gonads. *GAS6* is unique among the genes of the family, because its expression in liver is much lower than in other tissues, including heart, kidney and lung. Soon after its discovery, GAS6 was shown to have growth factor properties. Besides, GAS6 appeared to be capable of rescuing cells from apoptosis on different cell types (16). This antiapoptotic effect has been described in many different cell types and experimental settings (17–24), not only after growth factor deprivation, but also after specific inducers of apoptosis as TNF or β -amyloid peptide (17, 25, 26).

GAS6 binding to cellular receptors

The mechanism of GAS6 function was clarified when human GAS6 and bovine protein S were isolated by affinity chromatography using respectively the extracellular regions of the receptor tyrosine kinases (RTKs) Axl and Tyro3 as baits (27, 28). These proteins are members of the TAM subfamily of RTKs, comprised by Tyro3, Axl and MerTK, the name TAM deriving from the first letter of each receptor. They are single-span transmembrane proteins with an extracellular part composed of two immunoglobulin domains (IG) followed by two fibronectin type III domains (FN3), and an intracellular tyrosine kinase region. Further studies showed that species differences had to be taken into account in order to decipher the physiologically relevant ligands for each TAM receptor. Human protein S was able to bind the murine Tyro3 receptor *in vitro*, but it did not bind the equivalent human Tyro3 extracellular part, while in similar experimental settings GAS6 was able to bind all TAM receptors (29). Furthermore, bovine protein S was able to bind and activate both the human and mouse receptors (29, 30). Despite lacking the capacity of binding to TAM receptors in several experimental settings *in vitro*, human protein S is capable of inducing phosphorylation of human Tyro3 in certain cell types at 200 nM (31). This

concentration, although higher than the concentration needed for human GAS6 or bovine protein S to activate Tyro3, is still in the range of the physiological plasma concentration of protein S, around 350 nM (32). In human plasma almost 60% of protein S is bound to the complement regulatory factor C4BP through the LamG domains (33), the same modules implicated in receptor binding (13, 30). Binding to C4BP abolishes the interaction of bovine protein S with TAM receptors (30), while C4BP binds GAS6 with very low affinity (34). It is important to note that mouse C4BP lacks the protein S-binding subunit, the β -chain, which has become a pseudogene in this species (35). Therefore, protein S in mouse plasma is expected to be in free (active) form. Despite these differences among species, several studies *in vivo* suggest that protein S functions as a ligand of TAM receptors. Protein S was found to be the serum factor responsible for the stimulation of macrophage efferocytosis, the process of clearing apoptotic cells (36, 37). Interestingly, protein S oligomerization seems to be necessary for producing this effect (38), and this observation could be crucial for understanding the lack of binding of purified protein S preparations to TAM receptors observed in initial studies. The importance of the TAM ligand/receptor system in efferocytosis is well illustrated in the retina, where protein S seems to be the relevant ligand in mice (39, 40), while GAS6 could be of equivalent importance in humans (41).

The signaling pathways activated by TAM receptors have been reviewed in detail elsewhere (15, 42), and are summarized in Figure 1. Initial studies showed that activation of the antiapoptotic kinase AKT by GAS6 is essential for increasing cell survival (19, 26, 43–48). Further studies showed that other signals, and specially those related to activation of the mitogen-activated protein kinases (MAPKs) through the Ras/MAPK3 pathway are essential for the observed mitogenic activity of GAS6 (18, 47, 49–51), while being dispensable for the antiapoptotic effect of GAS6 (47). PLC- γ has been shown to interact and be a substrate of TAMs, and its activation is induced in the process of efferocytosis, inducing cytoskeletal rearrangements (52, 53). Finally, the Janus kinase (JAK) pathway, leading to activation of signal transduction and activators of transcription factors (STATs), has been shown to be activated by TAM receptor engagement (54). The activation of STAT3 has been linked to the mitogenic effect of GAS6 on kidney mesangial cells, as reviewed recently (55). Furthermore, STAT function has been shown to play a central role in the regulation of inflammation associated with TAM function (see below).

Effects of GAS6 on leukocyte biology

Antigen presenting cells express TAM receptors and their ligands, both in lymphoid tissues and peripheral blood (56–59), but TAMs are not present on granulocytes or blood lymphocytes. It is likely that most monocytic cells express more than one TAM receptor; for instance dendritic cells and macrophages use different sets of TAM receptors (60). The function of TAM receptors in the immune system is dramatically illustrated in animals deficient in all three receptors and, with a less severe phenotype, in MerTK-deficient mice (56, 57, 61, 62). In these knockout mice, peripheral lymphoid organs are enlarged and the animals develop an autoimmune reaction, with features resembling certain

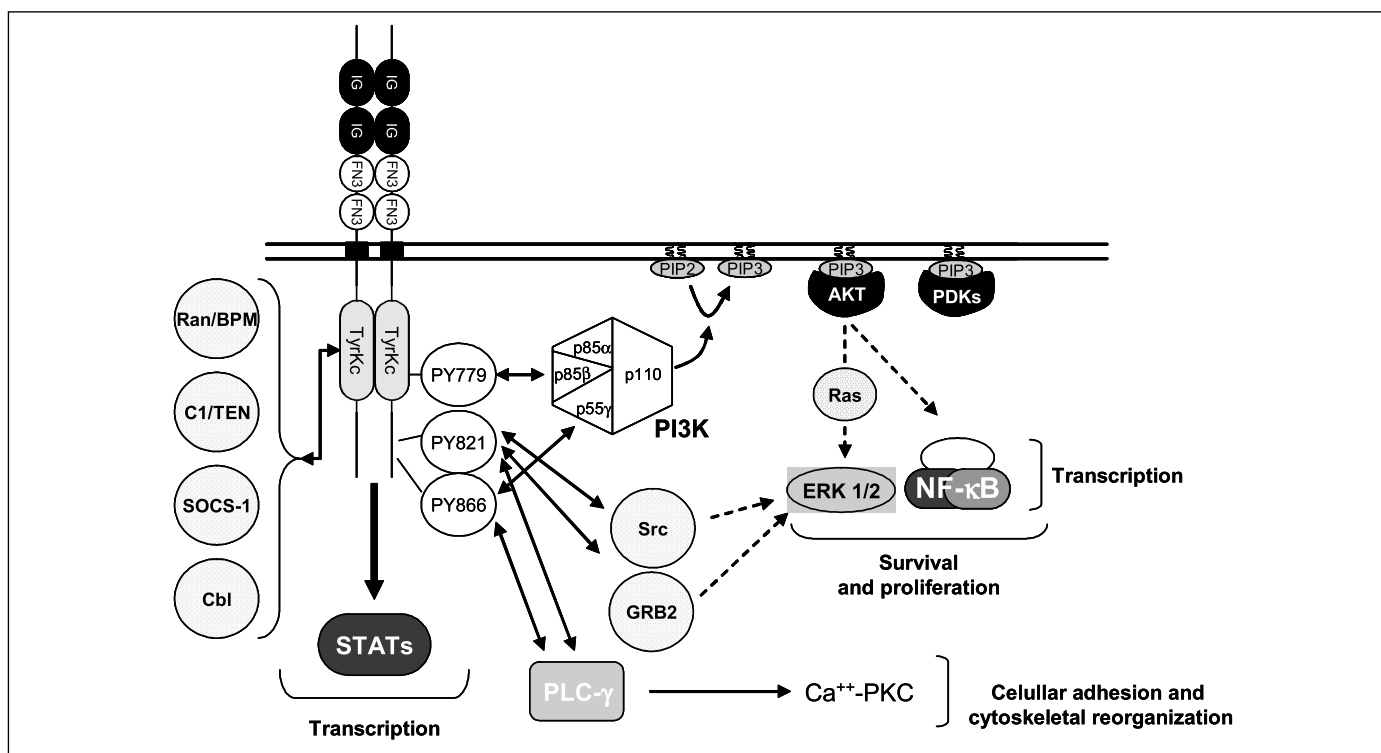


Figure 1: Effect of GAS6 interaction with Axl. The main pathways activated by Axl and their cellular effects are indicated. Components of the signal transduction machinery that have been shown to interact directly with Axl are shown on the left.

human autoimmune pathologies including serum autoantibodies and lymphocyte activation and hyperproliferation. The fact that lymphocytes become activated in TAM-deficient mice, despite not expressing TAM receptors, indicates that autoimmunity in these animals is mediated through a cell-dependent mechanism. It was proposed that the lack of an inhibitory signal arising from GAS6/TAM lowers the activation threshold of antigen-presenting cells subsequent to an initial trigger of the immune response. Multiple observations made both in mice deficient in MerTK or in the triple TAM mutants, are consistent with this hypothesis. Macrophages from MerTK mutants and TAM-deficient animals, when challenged with bacterial lipopolysaccharide (LPS), activate the NF- κ B pathway more than the wild-type animals. As a consequence, MerTK-deficient mice are hypersensitive to LPS-induced endotoxic shock, tissue damage and death as a result of the excessive production of TNF (57, 59, 62–64). The fact that MerTK-deficient animals are more severely affected than Axl- or Tyro3-deficient animals, and results on double gene mutants, support the view of MerTK as the essential receptor in the context of innate immunity (4), and suggest that, in the absence of MerTK, the other TAM receptors could “rescue” the function of MerTK to a certain extent (57).

The initial trigger of the inflammatory response in these murine models is possibly the deficient clearance of apoptotic cells by efferocytosis. This process contributes to the exacerbation of the inflammatory response in TAM-deficient animals. Most of the effect of activating efferocytosis seems to be mediated by MerTK (37, 60, 62, 63, 65, 66). GAS6, as well as protein S, have been shown to bind to the phosphatidylserine-rich membranes of cells undergoing apoptosis through the GLA domains (67), and

promote efferocytosis by macrophages (38, 53, 62, 63, 65, 68). Apoptotic cells stimulate the production of larger amounts of cytokines in MerTK-deficient animals than in control macrophages (69). Therefore, GAS6 (and protein S) would not only stimulate efferocytosis, but also hold back the activation of excessive inflammatory response during this process. The induction of efferocytosis has been shown to be specific of apoptotic cell removal or phosphatidylserine containing liposomes, while does not seem to influence the capacity to phagocyte bacteria, yeast or particulate substrates (37, 63, 65). The intracellular mechanism of GAS6 action seems to be linked to the cooperative recognition of apoptotic cells by MerTK through GAS6 and/or protein S and integrin activation. This has been demonstrated in the photoreceptor outer segment phagocytosis by retinal pigment epithelium, where the interaction of signals arising from the integrin $\alpha\beta 5$ and from MerTK leads to focal adhesion kinase phosphorylation, increased calcium concentration and cytoskeletal rearrangement (41, 66, 70, 71).

Dendritic cells and macrophages use TAM receptors to regulate the inflammatory reaction started by Toll-like receptors. The intracellular molecular mechanism implicated in the inhibitory signal beginning from TAM receptors on antigen presenting cells has been elucidated recently (72). Ligand engagement of the TAM receptors is able to inhibit direct Toll-like receptor (TLR) and cytokine receptor cascades through the interaction with type I interferon receptors. Axl expression is upregulated as a consequence of TLR/cytokine response and interacts with the interferon α and β receptor (IFNAR1). This complex produces the sequestration and activation of STAT1 to induce the expression of cytokine and TLR inhibitors from the suppressors of cytokine sig-

naling (SOCS) family. This negative feedback loop requires that STAT1 changes from a proinflammatory transcription factor to an immunomodulatory function, acting on a different set of promoters. Although the exact mechanism for this change has not been fully elucidated, repressors of transcription as Twist are putative candidates to mediate this regulation (73). SOCS1 was already found in a two-hybrid screening as a molecule with direct interactions with Axl (74), and it is possible that the regulatory mechanism of TAM receptors implicates a multiprotein complex in which TAM and cytokine receptors are interacting. In this context, Axl has also been shown to physically interact with the IL15 receptor in murine fibroblasts cells (75), again down-regulating the effect of a cytokine, in this case TNF.

From these studies, the effect of the TAM ligand/receptor system would act as a broad inhibitor of inflammation (72), acting both at the level of initial phase on antigen presenting cells and later, avoiding the autoimmune responses that could be generated by reactions towards apoptotic cells not efficiently phagocytosed by macrophages.

Effects of GAS6 on endothelial cells

The inflammatory reaction could be considered a vascular response to harmful stimuli, where the regulation of cell traffic through the vessel wall is a crucial regulatory step. GAS6 has been shown to play an important role in this part of the inflammatory response. The importance of GAS6 in the vessel wall was already suggested in 1995, when it was cloned as a growth-potentiating factor for vascular smooth muscle cells (16). The activity of GAS6 in this context is most likely mediated by its interaction with Axl (19, 76). Axl-deficient mice show a decreased neointima formation after mechanical injury (77–79). Interestingly, situations potentially harmful for the vascular homeostasis, as for instance changes in pH, hyperglycemia, hypertension or atherosclerosis, all seem to be affected by Axl and/or GAS6 expression (78–83). Axl-deficient mice show a decreased number of proliferating vascular smooth muscle cells (VSMCs) after vessel injury and an increased number of apoptotic cells in the media and intima, but also less infiltration of neutrophils and macrophages (79). Similarly, GAS6-deficient mice have lower content of macrophages in atherosclerotic plaques (84).

The process of vascular calcification, a dysfunction of the vascular wall induced by calcium and phosphate excess and uraemia and a major risk factor of cardiovascular disease, has also been related to GAS6. GAS6 signaling through Axl inhibits mineral deposition by cultured VSMCs (85, 86). Statins, which were known to inhibit the process of calcification, need a functional Axl pathway to mediate this action on the vessel wall, although it is not clear by which mechanism statins activate Axl signaling.

All these studies imply a correlation between classical risk factors of vascular disease and signaling through Axl. The effect of the GAS6/Axl pathway could include beneficial effects, as for instance reduced calcification, but also potentially harmful ones, as mediating vascular response towards hyperglycemia or hypertension and inducing restenosis (78). In this context, it is not surprising that genetic variants in *GAS6* have been associated with stroke and other vascular diseases (87, 88). Until now, the effect

of the different haplotypes or variants on the function of the gene has not been elucidated. Therefore it is not clear if the genetic association with cardiovascular disease is related to GAS6 or reflects a linkage to other genes in the same chromosomal region.

Several studies suggest a function of GAS6/TAM in endothelial function, both in the regulation of cellular homeostasis as well as in its function under inflammatory conditions. For instance, the regulation of the vascular tone in hypertension models was defective in Axl-deficient mice (81). Endothelial cells in culture express and release GAS6 (89), and it has a potent anti-apoptotic effect (17, 43, 82, 90). Under inflammatory conditions, the addition of GAS6 has been shown to inhibit adhesion of granulocytes to endothelial cells in culture (91). While this observation seems to imply an anti-inflammatory role of GAS6 by decreasing cellular extravasation, results from Axl- and GAS6-deficient animals indicate an opposite effect. During intimal thickening, Axl^{-/-} deficient mice have less leukocyte recruitment (79). GAS6-deficient animals had diminished endothelial cell/leukocyte interactions *in vivo* after endothelial cell stimulation, and decreased leukocyte infiltration in models of organ transplantation and rejection (89). The differences observed were due, at least in part, to an impaired stimulation of the endothelial cells by proinflammatory cytokines. Differences with previous studies could be due to differences in the experimental settings. *In vivo*, several processes contributing to leukocyte/endothelial cell adhesion and extravasation could be affected by the lack of GAS6 under inflammatory conditions. For instance, the formation of platelet-leukocyte conjugates was impaired in *Gas6*^{-/-} mice, after systemic injection of LPS.

Interestingly, endothelial cells, platelets and macrophages seem to express distinct sets of TAM receptors and ligands. When the expression of these genes is measured in the same conditions, endothelial cells contain more *Axl* mRNA than macrophages, while *MerTK* was predominantly expressed in macrophages (unpublished data). This expression pattern suggests that in the vasculature, the Axl receptor would provide most of the response to GAS6 and/or protein S; while in macrophages, MerTK would be the main receptor. Platelets contained considerable amounts of *Pros1* mRNA, but much less *Gas6* or any of the TAM receptors compared to the other cell types.

Functions of GAS6 in haemostasis

The presence of GAS6 in plasma has been carefully evaluated by several authors. Although its presence can be detected, the concentration is low, about 20–60 ng/ml, approximately 0.25 nM (92–94); or even lower according to other reports (95). The range of GAS6 concentration is much lower than the concentration of protein S, which increases up to 400 nM during an acute-phase response (97). The concentration of GAS6 seems to be around a 10–15% higher in women, and is decreased by oral contraceptives (96), resembling the effect observed on protein S. 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 mM for prothrombin.

The protection against prothrombotic stimuli observed in *Gas6*^{-/-} mice suggested a function of GAS6 on platelet biology. Indeed, GAS6 is found in platelets from rats and mice (67, 98),

and platelets from animals deficient in GAS6 are activated less efficiently (98). Both MerTK and Axl, and to a lesser extent Tyro3, have been proposed as mediators of this function using knockout mice and antibody inhibition (98–102). The initial studies suggested that deficiency or inhibition of TAM signaling would not produce a bleeding tendency (98), although a recent study has shown that combined deficiencies of at least two TAM receptors would produce a mild but significant bleeding tendency in mice (103). Recently, the extracellular part of MerTK has been shown to inhibit platelet aggregation and pulmonary embolism, further emphasizing that, at least in mice, the GAS6/TAM pathway is involved in haemostasis (68). In humans, platelets seem to contain a lower concentration of GAS6 than the concentration found in plasma, and addition of purified GAS6 to platelet preparations does not affect their response (92, 96, 104). Still, GAS6 could affect antiplatelet drug responsiveness (105).

The proposed mechanism of action of TAM receptors on platelets has been related with stabilization of the thrombus, as opposed to the initial activation of platelets (99). Most events leading to stabilization of platelet aggregates take place in the close space between platelet membranes once aggregation begins. These contacts are comparable to those found between immunitary cells or neurons, and therefore the term platelet synapse has been coined to describe this structure (106). Proteins that have been implicated in the process of platelet stabilization include immunoglobulin domain-containing proteins such as

PECAM-1, junctional adhesion molecules (JAMs), and CD2/SLAM. All these proteins are thought to act through homophilic cell-cell adhesion interactions, bridging membranes of neighboring platelets. A second group of proteins important for platelet synapse interactions include receptors and their ligands, e.g. CD40/CD40L, semaphorins, as well as ephrin and its receptors. These receptor/ligand systems would interact not only with their membrane-bound counterparts, but also with specifically shed soluble receptors. The GAS6/TAM system would be included among this later class of molecules (99).

Conclusion

The last years have seen a blooming of research on GAS6/TAM function that has provided essential clues on the biological implications of this system. Through careful regulation of cell/cell interactions and cellular activation, GAS6 has shown a crucial role in innate immunity and haemostasis. Interestingly, links between this two systems of response to damage have been known for several years (107). In this context, GAS6 and protein S contain in their structures unique sensors for activated cell surfaces, their GLA domains, linked to a RTK binding domains, capable of initiating intracellular responses leading to cell (in)activation. The importance of these processes in human pathology suggest that modulators of the GAS6/TAM system could provide interesting pharmacological targets.

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