A novel site contributing to growth-arrest-specific gene 6 binding to its receptors as revealed by a human monoclonal antibody

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Gas6 (growth-arrest-specific gene 6) is a vitamin K-dependent protein known to activate the Axl family of receptor tyrosine kinases. It is an important regulator of thrombosis and many other biological functions. The C-terminus of Gas6 binds to receptors and consists of two laminin-like globular domains LG1 and LG2. It has been reported that a Ca^{2+} -binding site at the junction of LG1 and LG2 domains and a hydrophobic patch at the LG2 domain are important for receptor binding [Sasaki, Knyazev, Cheburkin, Gohring, Tisi, Ullrich, Timpl and Hohenester (2002) *J. Biol. Chem.* **277**, 44164–44170]. In the present study, we developed a neutralizing human monoclonal antibody, named CNTO300, for Gas6. The antibody was generated by immunization of human IgG-expressing transgenic mice with recombinant human Gas6 protein and the anti-Gas6 IgG sequences were rescued from an unstable hybridoma clone. Binding of Gas6 to its receptors was partially inhibited by the CNTO300 antibody in a dose-dependent manner. To characterize further the interaction between Gas6 and this antibody, the binding kinetics of CNTO300 for recombinant Gas6 were compared with independently expressed LG1 and LG2.

The CNTO300 antibody showed comparable binding affinity, yet different dependence on Ca^{2+} , to Gas6 and LG1. No binding to LG2 was detected. In the presence of EDTA, binding of the antibody to Gas6 was disrupted, but no significant effect of EDTA on LG1 binding was evident. Further epitope mapping identified a Gas6 peptide sequence recognized by the CNTO300 antibody. This peptide sequence was found to be located at the LG1 domain distant from the Ca^{2+} -binding site and the hydrophobic patch. Co-interaction of Gas6 with its receptor and CNTO300 antibody was detected by BIAcore analysis, suggesting a second receptor-binding site on the LG1 domain. This hypothesis was further supported by direct binding of Gas6 receptors to an independently expressed LG1 domain. Our results revealed, for the first time, a second binding site for Gas6–receptor interaction.

Key words: Axl, growth-arrest-specific gene 6 (Gas6), laminin-like globular domain, monoclonal antibody, platelet, receptor tyrosine kinase.

INTRODUCTION

Gas6 (growth-arrest-specific gene 6) is a vitamin K-dependent protein that activates the Axl family of receptors, which includes Axl (Ufo/Ark), Sky (Dtk/Tyro3/Rse/Brf/Tif) and Mer (Eyf, Nyk) [1–7]. It is a 70 kDa protein with a structure similar to Protein S [8], a negative regulator of coagulation. Gas6 consists of a Gla domain, four EGF (epidermal growth factor) domains and a C-terminus consisting of two laminin-like globular domains LG1 and LG2. Gas6 shares an approx. 40% sequence similarity with Protein S. However, it lacks the thrombin cleavage site typical of vitamin K-dependent coagulation factors.

Gas6 is widely expressed in terminally differentiated cells of most organs including capillary endothelial cells, VSMCs (vascular smooth-muscle cells) and neurons [8,9]. It is also found in the alpha granules of platelets that are transported to the cell surface on activation [10,11]. Gas6 is generally not detected in the plasma, macrophages, basophils, neutrophils or peripheral lymphocytes. Under pathological conditions, Gas6 is up-regulated at sites of inflammation, vessel injury and in VSMCs of atherosclerotic plaques [12–15].

In accordance with its global distribution, Gas6 is involved in cell survival or proliferation of many cell types including endothelial cells [14], VSMCs [16,17], mesangial cells [18],

osteoclasts [19], chondrocytes [20], Schwann cells [21], epithelial cells [22] and fibroblasts [23]. It is also a chemotactic factor for VSMCs [24]. Studies in Gas6 knockout mice demonstrated that Gas6 is an important platelet amplifier. Gas6-depleted platelets no longer respond to low concentrations of most agonists. As a result, Gas6 knockout mice are protected from challenges of both venous thrombosis and arterial thrombosis [11].

The ability of Gas6 to bind to and activate its receptors requires vitamin K-dependent γ -carboxylation [25,26]. However, previous studies [27,28] also indicated that truncated Gas6 or a splice variant of Gas6 containing only the C-terminal globular repeats is sufficient to activate the receptors. It was postulated that the C-terminal repeats of Gas6 are responsible for its biological activity, whereas the N-terminus modulates its activity through γ -carboxylation. Studies of the crystal structure of this C-terminus have identified a Ca^{2+} -binding site and a hydrophobic patch that are important for Gas6–receptor interaction. Site-directed mutagenesis of several residues within the hydrophobic patch has been shown to diminish receptor binding [29]. It was therefore postulated that the receptor-binding site of Gas6 resides in the hydrophobic patch on the LG2 domain. In the present study, we report the identification of a second receptor-binding site on the LG1 domain as demonstrated by direct binding of the receptors to the LG1 domain. We have also identified a novel peptide sequence

Abbreviations used: Gas6, growth-arrest-specific gene 6; IPTG, isopropyl β -D-thiogalactoside; LG1 domain, laminin-like globular domain 1; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight; VSMC, vascular smooth-muscle cell.

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20 $\mu\text{l}/\text{min}$, a 160 μl sample solution of 200 nM Axl-Fc, 20 nM Gas6 or 20 nM of Axl-Fc and Gas6 combined. The surface was regenerated with a 15 s pulse of 50 mM NaOH.

CNTO300 binding to Gas6

A solid-phase ELISA was used to detect CNTO300 binding to recombinant human Gas6–FLAG and mouse Gas6 (R & D Systems). Briefly, a plate was coated with recombinant human or mouse Gas6 protein at 1 $\mu\text{g}/\text{ml}$ overnight at 4 °C. The plate was then washed, blocked with SuperBlock (Pierce, Rockford, IL, U.S.A.) and a dose titration of CNTO300 was incubated on the plate for 2 h at room temperature. The plate was washed and incubated with a 1:2000 dilution of goat anti-human IgG Fc-specific antibody (Sigma) for 30 min to detect the bound antibody. The plate was developed using *o*-phenylenediamine dihydrochloride substrate and colour development was halted using equal volumes of 2 M H_2SO_4 . The A_{490} was measured on a multi-well spectrophotometer (Molecular Devices, Sunnyvale, CA, U.S.A.).

Neutralization of Gas6 binding to its receptor by CNTO300

A solid-phase ELISA was used to assess the ability of the CNTO300 monoclonal antibody to neutralize the binding of Gas6 to its receptor Axl. Soluble human receptor Axl-Fc (R & D Systems) was coated at 0.5 $\mu\text{g}/\text{ml}$ on to a 96-well plate and incubated overnight at 4 °C. The plate was then washed, blocked with SuperBlock (Pierce) and, then, 50 ng/ml of recombinant human Gas6–FLAG in the presence of antibody was added to each well. A commercial neutralizing polyclonal antibody to human Gas6 (R & D Systems) was used as a positive control. The plate was incubated at room temperature for 2 h, and anti-FLAG M2 peroxidase conjugate (Sigma) was used as a secondary antibody to detect the bound Gas6 protein. The plate was developed using *o*-phenylenediamine dihydrochloride substrate and colour development was halted using equal volumes of 2 M H_2SO_4 . The A_{490} was measured on a multi-well spectrophotometer (Molecular Devices).

CNTO300 epitope mapping

Immunoprecipitation and tryptic digestion

A mixture of 75 μg of recombinant human Gas6–FLAG protein and 75 μg of CNTO300 monoclonal antibody was incubated at 4 °C overnight, followed by buffer exchange using a 100 000 nominal molecular weight limit filter unit and a digestion buffer (0.1 M Tris/HCl buffer, pH 8.5). Proteolysis was initiated by the addition of 4 μg of trypsin and the reaction was continued for 2 h at 37 °C. After digestion, the remaining antigen–antibody complex was captured by Protein G beads, washed with PBS, and the bound peptides were eluted in 40 μl of elution buffer [0.5 % trifluoroacetic acid and 10 % (v/v) acetonitrile in distilled water].

MALDI–TOF (matrix-assisted laser-desorption ionization–time-of-flight) MS analysis

Eluted peptides from the trypsin-digested complex were desalted by ZipTip C18 and spotted on to a MALDI–TOF chip. MALDI–TOF (DE-STR; Applied Biosystems) was calibrated by Calibration Mixture 2 (Applied Biosystems). The mass spectra were acquired at laser intensity 1650 for the mass range 850–5000 Da. Major mass peaks were further analysed by LC tandem MS (service provided by ProtTech, Norristown, PA, U.S.A.).

Table 1 Binding constants of recombinant human Gas6–FLAG to its receptors

Using a BIAcore 2000 analyser, 50 mM of the soluble receptors Axl-Fc, Dtk-Fc or Mer-Fc were captured by immobilized rabbit anti-human IgG Fc-specific polyclonal antibody. Samples of Gas6 (10 nM for Axl and Dtk and 150 nM for Mer) were then passed over this surface and the data were analysed globally using a simultaneous fit for both dissociation (k_d) and association (k_a). The value for K_d was calculated as k_d/k_a .

	k_d (s^{-1})	k_a ($\text{M}^{-1} \cdot \text{s}^{-1}$)	K_d (pM)
Axl-Fc	4.8×10^{-5}	9.1×10^5	53
Mer-Fc	1.4×10^{-4}	4.6×10^5	304
Dtk-Fc	1.7×10^{-5}	5.4×10^5	31.5

LC tandem MS analysis

The digested protein sample was dried in a SpeedVac (Thermo Electron, Waltham, MA, U.S.A.) and redissolved in 0.1 % trifluoroacetic acid, followed by ZipTip C18 (Millipore, Billerica, MA, U.S.A.) clean-up according to the manufacturer's instructions. The eluate from ZipTip C18 clean-up was then dried and resuspended in 0.1 % trifluoroacetic acid before loading into a C18 capillary column for LC tandem MS analysis.

A Finnigan (ThermoFinnigan, San Jose, CA, U.S.A.) LCQ ion-trap mass spectrometer coupled online with a Beckman gradient HPLC system was used. A C18 capillary column (75 μm inner diameter and 10 cm length) was connected to a NanoSpray device to allow the delivery of stable electrospray in the flow rate range 100–1500 nl/min. Mobile phases were solvent A (2 % acetonitrile, 97.5 % water and 0.1 % formic acid) and solvent B (90 % acetonitrile, 9.5 % water and 0.1 % formic acid). The ion-trap mass spectrometer was set to operate with the automatic gain control on.

For data analysis, the tandem MS data were searched against the most recent non-redundant protein database and then manually analysed and confirmed.

RESULTS

Generation of a human anti-Gas6 monoclonal antibody

Full-length recombinant human Gas6 containing a FLAG epitope at the C-terminus was generated as the antigen to develop anti-Gas6 antibodies. The epitope-tagged Gas6 retained the bioactivity of binding to Axl, Dtk and Mer receptors (Table 1) and inducing receptor activations (results not shown). The binding constants of FLAG-tagged Gas6 are in general agreement with published data using a tag-free Gas6 [36]. Our results suggested that the addition of the FLAG epitope did not alter the ability of Gas6 to bind to the receptors and thus maintained the overall conformation of Gas6.

Human IgG-expressing transgenic mice were used to generate Gas6 antibodies by immunization with recombinant human Gas6–FLAG protein. In general, immunization with Gas6 did not result in robust antibody titres. B-cells from an immunized transgenic mouse that demonstrated a specific human IgG titre of 1:6400 to human Gas6 were fused to murine FO myeloma cells. Only one hybridoma secreting a human antibody specific to human Gas6 was identified. From the first subcloning, only 4.5 % of the clones retained Gas6 reactivity and none of the daughter clones from subcloning had Gas6 reactivity. Subsequent attempts to generate anti-Gas6 hybridoma using different immunization methods also failed. Owing to the difficulty in obtaining a stable hybridoma clone, the parental positive cells were used to rescue the antibody genes by molecular cloning. The DNA sequences of the cloned

Figure 1 Binding of the CNT0300 antibody to recombinant human Gas6 and mouse Gas6

In an ELISA experiment, a dose titration of CNT0300 was added to immobilized recombinant human Gas6 (■) or mouse Gas6 (○) and the binding was detected by a secondary antibody. The data points were obtained from triplicate samples and the experiments were repeated three times. O.D. 490 nm = A_{490} .

Figure 2 Inhibition of Gas6 binding to Axl receptor by the CNT0300 antibody

In an ELISA experiment, human Gas6-FLAG in combination with a dose titration of either the monoclonal antibody CNT0300 (○) or a commercially available polyclonal antibody (■) was added to immobilized soluble human Axl-Fc. Gas6-receptor binding was detected using an anti-FLAG antibody. The data points were obtained from triplicate samples and the experiments were repeated three times. O.D. 490 nm = A_{490} .

antibody variable region genes were determined. As expected, the sequences were found to be derived from the specific human variable region transgenes used in the generation of the transgenic mice. The CNT0300 antibody was expressed and purified from transfected mammalian cells.

In an ELISA, the CNT0300 antibody was capable of binding to immobilized recombinant human and mouse Gas6, with a log higher affinity to the human protein compared with the mouse protein (Figure 1). A similar result was also obtained by BIAcore binding analysis. Given the high degree of homology between the human and mouse Gas6 proteins, the cross-reactivity of CNT0300 antibody to mouse Gas6 was not surprising. The CNT0300 antibody was also found to cross-react, with a lower affinity, with human Protein S.

Neutralization of receptor binding to Gas6 by the CNT0300 antibody

The ability of the CNT0300 antibody to inhibit the binding of Gas6 to the Axl receptor was tested using ELISA. As shown in Figure 2, the CNT0300 antibody dose-dependently inhibited the binding of Gas6 to the immobilized soluble Axl receptor with

Table 2 Binding constants of the CNT0300 antibody for Gas6 and LG1

Using a BIAcore 2000 analyser, the CNT0300 antibody was captured by immobilized rabbit anti-human IgG Fc-specific polyclonal antibody. Samples of 400 nM Gas6, LG1 or LG2 were then passed over this surface. To study the effect of metal on binding, the instrument was equilibrated with a buffer containing 3 mM EDTA and the samples were also prepared using this buffer. The data were analysed globally, using a simultaneous fit for both dissociation (k_d) and association (k_a). NB, no binding detected.

	k_d (s^{-1})	k_a ($M^{-1} \cdot s^{-1}$)	K_d (nM)
Human Gas6-FLAG (– EDTA)	9.60×10^{-4}	1.25×10^5	7.68
Human Gas6-FLAG (+ EDTA)	NB	NB	NB
Human LG1 (– EDTA)	1.42×10^{-4}	1.44×10^4	9.86
Human LG1 (+ EDTA)	1.00×10^{-4}	1.26×10^4	7.94
Human LG2 (– EDTA)	NB	NB	NB

Figure 3 BIAcore sensorgram demonstrating the effect of EDTA on the binding of recombinant human Gas6 and LG1 to CNT0300

The CNT0300 antibody was captured by immobilized rabbit anti-human IgG Fc-specific polyclonal antibody. Samples of 400 nM Gas6 or LG1 were then passed over this surface. (A) Binding of Gas6 without EDTA (●) and with EDTA (△) over captured CNT0300 sensor surface. (B) Binding of Gas6 LG1 without (●) and with EDTA (△). RU, response units.

Figure 4 MS analysis of tryptic Gas6 peptides bound by CNTO300

In an antibody protected protease digestion study, recombinant human Gas6 was incubated with CNTO300 and subjected to tryptic digestion. The tryptic Gas6 peptides bound to CNTO300 were eluted and analysed by MS. **(A)** MALDI MS analysis of the eluted peptides. The Gas6 peptide $^{1403}\text{AVAGDLFQPER}_{414}$ captured by the CNTO300 antibody is indicated. **(B)** Sequence identification of the captured Gas6 peptide, $m/z = 1316.5$, by LC tandem MS.

an IC_{50} value of $15 \mu\text{g/ml}$. Similar inhibition was found using a commercial polyclonal antibody against human Gas6. No inhibition was detected using an irrelevant isotype-matched monoclonal antibody. Consistently, the soluble Axl-Fc competed for Gas6 binding to captured CNTO300 antibody by BIAcore binding analysis (results not shown).

Antibody binding kinetics

BIAcore analyses were performed to determine the binding kinetics of the CNTO300 antibody to Gas6 in comparison with independently expressed LG1 and LG2 domains (Table 2 and Figure 3). The CNTO300 antibody bound to the full-length human Gas6 protein with $K_d = 7.68 \text{ nM}$. This association was disrupted by the addition of EDTA (Figure 3A), consistent with a key role for Ca^{2+} in maintaining Gas6 protein conformation. Further

studies using independently expressed LG1 and LG2 showed that CNTO300 bound to the LG1 domain but not to the LG2 domain. The binding constant of the CNTO300 antibody with LG1 was comparable with the full-length Gas6 protein, yet EDTA had a minimal effect in this case (Table 2 and Figure 3B).

CNTO300 antibody epitope mapping

The binding epitope for CNTO300 was identified by an antibody protected protease digestion method. Briefly, human Gas6 protein was incubated with CNTO300 and then digested by trypsin. A tryptic peptide that remained bound to CNTO300 was eluted and identified by MS. The peptide showed a mass to charge ratio (m/z) of 1316.5 obtained by MALDI MS (Figure 4A). The corresponding peptide sequence, $^{1403}\text{AVAGDLFQPER}_{414}$ located on the LG1 domain, was identified by LC tandem MS (Figure 4B).

Figure 5 Putative CNT0300 antibody-binding site

(A) Amino acid sequence of Gas6. The Gla, EGF, LG1 and LG2 domains are underlined and the expressed LG1 and LG2 fragments are shown by boldface letters. The binding site for CNT0300 is indicated by thick underlining. (B) The crystal structure of the C-terminus of Gas6 was from Sasaki et al. [29]. The Ca²⁺-binding site and the hydrophobic patch are indicated by arrows. The Gas6 tryptic peptide that bound to CNT0300 is highlighted in black.

An independent mapping effort by incubation of CNT0300 with independently expressed LG1 yielded the same peptide. This result is consistent with the BIAcore binding study, demonstrating direct association of the CNT0300 antibody with the LG1 domain. Synthetic peptides containing the epitope sequence, however, bound weakly to the CNT0300 antibody and did not inhibit the binding of the CNT0300 antibody to Gas6. Therefore the epitope is not merely a linear sequence and certain structural features are required for optimal binding to the CNT0300 antibody.

The C-terminal globular repeat region of Gas6, when expressed alone, is known to be sufficient for receptor binding. The crystal structure of the C-terminus reported by Sasaki et al. [29] demonstrated a V-shaped arrangement of LG1 and LG2. This conformation seemed to be strengthened by a Ca²⁺-binding site located at the interface of the LG1 and LG2 domains. A hydrophobic patch connecting the LG1 and LG2 domains was further identified and defined as a receptor-binding site. Several mutations within the hydrophobic patch disrupted Gas6 binding to receptors.

The tryptic peptide captured by the CNT0300 antibody is located at the LG1 domain but is distant from both the Ca²⁺-binding site and the putative receptor-binding hydrophobic patch (Figures 5A and 5B).

Co-binding of Gas6 to its receptors and the CNT0300 antibody

Our previous results demonstrated that binding of the CNT0300 antibody to Gas6 partially disrupted receptor binding. Two hypotheses could be proposed. First, there might be a second receptor-binding site on the LG1 domain, and binding of receptors to this second site is blocked by the CNT0300 antibody. Secondly, binding of the CNT0300 antibody leads to conformational changes on the hydrophobic patch that could no longer support receptor binding. If the first hypothesis is correct, Gas6 should be capable of binding both the antibody and the receptor at the same time. To test this hypothesis, the ability of Gas6 to bind both the CNT0300 antibody and the receptors was tested by BIAcore analysis. The CNT0300 antibody was immobilized on the sensor

Figure 6 BIAcore sensorgrams demonstrating the ability of a complex of Axl-Fc and Gas6 to bind to immobilized CNTO300

△, Axl-Fc binding to the surface of immobilized CNTO300; ○, Gas6 binding to immobilized CNTO300; □, Gas6 and Axl-Fc mixed together and incubated at room temperature for 2 h. RU, response units.

Figure 7 BIAcore sensorgram comparing the bindings of recombinant Gas6, LG1, and LG2 to CNTO300

CNTO300 was captured on to the surface of immobilized rabbit anti-human Fc-specific polyclonal antibody. Samples of 50 nM each were passed over this surface; ○, Gas6; ◆, LG1; □, LG2. RU, response units.

surface and the ability of Gas6, Axl-Fc or Gas6 + Axl-Fc to bind was measured. As expected, Gas6 bound to immobilized CNTO300 antibodies (Figure 6). Although Axl-Fc alone showed no binding, addition of Axl-Fc + Gas6 revealed co-binding of Gas6 to both the antibody and the receptors (Figure 6). This result provided strong support for the two-receptor binding-site theory.

Direct binding of Gas6 receptors to the LG1 domain

To confirm the presence of a second receptor-binding site on the LG1 domain, direct binding of the receptors to independently expressed LG1 domain was measured by BIAcore analysis. The Axl-Fc receptors were captured on the sensor surface and binding of Gas6, LG1 or LG2 was tested. As shown in Figure 7, the Axl-Fc receptor bound to Gas6 and, to a lesser degree, to the LG1 domain. No binding to the independently expressed LG2 domain was detected.

DISCUSSION

The generation of a human monoclonal antibody to Gas6 has allowed further analysis of the Gas6–receptor interaction. The CNTO300 antibody appears to recognize a conformationally sensitive epitope. EDTA disrupted the ability of Gas6 to bind to the antibody, indicating that a Ca^{2+} -sensitive conformation of Gas6 is required for antibody binding. It has been hypothesized that the Ca^{2+} -binding site of Gas6 stabilizes the V-shaped conformation held by the LG1 and LG2 domains [29]. In addition, Ca^{2+} is known to influence the conformation of all γ -carboxylated proteins through interaction with the Gla domain [37]. It is probable that EDTA disrupts the conformation of Gas6 that supports the binding to CNTO300. Furthermore, synthetic peptide derived from the epitope of the CNTO300 antibody, which lacks a rigid structure, bound weakly to the antibody and failed to block binding of the antibody to Gas6.

Gas6 can only bind to its receptors if it is γ -carboxylated as a full protein, yet truncated Gas6 protein lacking the Gla domain clearly retains its biological activities [25–28]. The biological activity of Gas6, therefore, resides on the C-terminal globular repeat region, whereas the N-terminal Gla domain modulates the activity depending on the state of γ -carboxylation. In the present study, we were capable of expressing individual LG1 and LG2 domains in bacteria and showed that the LG1 domain preserved the epitope conformation for CNTO300 antibody binding. In contrast with binding to the full-length protein, EDTA did not disrupt CNTO300 binding to LG1. It is possible that the epitope conformation of Gas6, similar to that of the C-terminal region of the full protein, is modulated by the presence of LG2 in a Ca^{2+} -dependent manner.

Despite the fact that the CNTO300 antibody is capable of partially blocking Gas6 binding to its receptors, both the antibody and the receptors can bind to Gas6 at the same time as demonstrated by BIAcore analysis. In the present study, we hypothesize that there are two receptor-binding sites on Gas6, the hydrophobic patch on LG2 and a novel site on LG1. The CNTO300 antibody binds to LG1 and blocks the novel receptor recognition site on the LG1 domain. This hypothesis is supported by the following data: (i) the CNTO300 antibody did not completely block receptor binding; (ii) the epitope sequence bound by the CNTO300 antibody is located on the LG1 domain distant from the Ca^{2+} -binding site and the putative receptor-binding site at the hydrophobic patch on LG2; (iii) epitope mapping from the CNTO300/Gas6 or CNTO300/LG1 mixture yielded the same antibody-binding sequence, suggesting that the conformation for antibody recognition, and possibly the novel receptor-binding site, is preserved in the expressed LG1 fragment; and (iv) receptors were shown to bind to the expressed LG1 fragment directly. Binding of the CNTO300 antibody to the epitope sequence, therefore, only blocks the novel receptor-binding site on LG1, leaving the other site on LG2 still accessible for receptor binding. Further studies are required to see if the novel receptor-binding site is co-localized at the antibody-binding site.

The difficulty in generating Gas6 antibodies was unexpected. Gas6, although involved in many cellular functions, has not been directly implicated in interfering with the immune system. Yet, we were not able to generate high-titre Gas6 antibodies using the recombinant human Gas6 protein as the immunogen. The following hypotheses could be postulated. First, it is possible that Gas6 protein is not very immunogenic in rodents, especially in the human Ig transgenic mice. Further optimization of the immunization strategy may be required. Secondly, Gas6 antigen may interfere with the humoral immune response to foreign antigen. Although neither Gas6 nor its receptors were found in

peripheral T- or B-cells [38], Axl and Dtk were found in myeloid leukaemic blasts and Mer was found in neoplastic T- and B-cell lines [39–41]. Studies from receptor knockout mice indicated that expression of the Axl family of receptors is required for maintenance of the immune system [42]. Mice lacking all three receptors showed enlarged spleens and developed severe autoimmunity [43]. However, a similar phenotype has not been reported in Gas6 knockout mice. It is possible that Gas6 and other yet to be identified ligand(s) work in concert to mediate the immune function through the Axl family of receptors. Thirdly, Gas6 is a known survival factor for many cell types under serum deprivation. It is possible that Gas6 is important for establishing stable hybridoma cells and anti-Gas6-expressing cells could be depleted during the hybridoma selection process. These hypotheses need to be supported by further studies directed specifically at addressing these issues.

In conclusion, we have identified a second receptor-binding site on the LG1 domain of Gas6. The interaction between Gas6 and its receptors is associated with a variety of pathological conditions in the brain, heart, kidney and other vital organs [11,13,19,44–48]. Gas6 and its receptors thus represent a new class of therapeutic targets. Understanding the nature of the Gas6–receptor interaction would ultimately help the development of novel small-molecule drugs or neutralizing monoclonal antibodies for therapeutic applications for the detrimental diseases where the interaction between Gas6 and its receptors contributes to the disease progression or pathology.

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REFERENCES

- Ohashi, K., Mizuno, K., Kuma, K., Miyata, T. and Nakamura, T. (1994) Cloning of the cDNA for a novel receptor tyrosine kinase, Sky, predominantly expressed in brain. *Oncogene* **9**, 699–705.
- Godowski, P. J., Mark, M. R., Chen, J., Sadick, M. D., Raab, H. and Hammonds, R. G. (1995) Reevaluation of the roles of protein S and Gas6 as ligands for the receptor tyrosine kinase Rse/Tyro 3. *Cell* (Cambridge, Mass.) **82**, 355–358.
- Ohashi, K., Nagata, K., Tushima, J., Nakano, T., Arita, H., Tsuda, H., Suzuki, K. and Mizuno, K. (1995) Stimulation of sky receptor tyrosine kinase by the product of growth arrest-specific gene 6. *J. Biol. Chem.* **270**, 22681–22684.
- Stiitt, T. N., Conn, G., Gore, M., Lai, C., Bruno, J., Radziejewski, C., Mattsson, K., Fisher, J., Gies, D. R., Jones, P. F. et al. (1995) The anticoagulation factor protein S and its relative, Gas6, are ligands for the Tyro 3/Axl family of receptor tyrosine kinases. *Cell* (Cambridge, Mass.) **80**, 661–670.
- Varnum, B. C., Young, C., Elliott, G., Garcia, A., Bartley, T. D., Fridell, Y. W., Hunt, R. W., Trail, G., Clogston, C., Toso, R. J. et al. (1995) Axl receptor tyrosine kinase stimulated by the vitamin K-dependent protein encoded by growth-arrest-specific gene 6. *Nature* (London) **373**, 623–626.
- Nagata, K., Ohashi, K., Nakano, T., Arita, H., Zong, C., Hanafusa, H. and Mizuno, K. (1996) Identification of the product of growth arrest-specific gene 6 as a common ligand for Axl, Sky, and Mer receptor tyrosine kinases. *J. Biol. Chem.* **271**, 30022–30027.
- Crosier, K. E. and Crosier, P. S. (1997) New insights into the control of cell growth; the role of the Axl family. *Pathology* **29**, 131–135.
- Manfioletti, G., Brancolini, C., Avanzi, G. and Schneider, C. (1993) The protein encoded by a growth arrest-specific gene (gas6) is a new member of the vitamin K-dependent proteins related to protein S, a negative coregulator in the blood coagulation cascade. *Mol. Cell. Biol.* **13**, 4976–4985.
- Prieto, A. L., Weber, J. L., Tracy, S., Heeb, M. J. and Lai, C. (1999) Gas6, a ligand for the receptor protein-tyrosine kinase Tyro-3, is widely expressed in the central nervous system. *Brain Res.* **816**, 646–661.
- Ishimoto, Y. and Nakano, T. (2000) Release of a product of growth arrest-specific gene 6 from rat platelets. *FEBS Lett.* **466**, 197–199.
- Angelillo-Scherrer, A., de Frutos, P., Aparicio, C., Melis, E., Savi, P., Lupu, F., Arnout, J., Dewerchin, M., Hoylaerts, M., Herbert, J. et al. (2001) Deficiency or inhibition of Gas6 causes platelet dysfunction and protects mice against thrombosis. *Nat. Med.* (N.Y.) **7**, 215–221.
- Melaragno, M. G., Wuthrich, D. A., Poppa, V., Gill, D., Lindner, V., Berk, B. C. and Corson, M. A. (1998) Increased expression of Axl tyrosine kinase after vascular injury and regulation by G protein-coupled receptor agonists in rats. *Circ. Res.* **83**, 697–704.
- Melaragno, M. G., Fridell, Y. W. and Berk, B. C. (1999) The Gas6/Axl system: a novel regulator of vascular cell function. *Trends Cardiovasc. Med.* **9**, 250–253.
- O'Donnell, K., Harkes, I. C., Dougherty, L. and Wicks, I. P. (1999) Expression of receptor tyrosine kinase Axl and its ligand Gas6 in rheumatoid arthritis: evidence for a novel endothelial cell survival pathway. *Am. J. Pathol.* **154**, 1171–1180.
- Carmeliet, P. and Luttun, A. (2001) Genetic studies on the role of proteinases and growth factors in atherosclerosis and aneurysm formation. *Ann. N.Y. Acad. Sci.* **947**, 124–133.
- Nakano, T., Higashino, K., Kikuchi, N., Kishino, J., Nomura, K., Fujita, H., Ohara, O. and Arita, H. (1995) Vascular smooth muscle cell-derived, Gla-containing growth-potentiating factor for Ca(2+)-mobilizing growth factors. *J. Biol. Chem.* **270**, 5702–5705.
- Nakano, T., Kawamoto, K., Higashino, K. and Arita, H. (1996) Prevention of growth arrest-induced cell death of vascular smooth muscle cells by a product of growth arrest-specific gene, gas6. *FEBS Lett.* **387**, 78–80.
- Yanagita, M., Arai, H., Ishii, K., Nakano, T., Ohashi, K., Mizuno, K., Varnum, B., Fukatsu, A., Doi, T. and Kita, T. (2001) Gas6 regulates mesangial cell proliferation through Axl in experimental glomerulonephritis. *Am. J. Pathol.* **158**, 1423–1432.
- Katagiri, M., Hakeda, Y., Chikazu, D., Ogasawara, T., Takato, T., Kumegawa, M., Nakamura, K. and Kawaguchi, H. (2001) Mechanism of stimulation of osteoclastic bone resorption through Gas6/Tyro 3, a receptor tyrosine kinase signaling, in mouse osteoclasts. *J. Biol. Chem.* **276**, 7376–7382.
- Loeser, R. F., Varnum, B. C., Carlson, C. S., Goldring, M. B., Liu, E. T., Sadiev, S., Kute, T. E. and Wallin, R. (1997) Human chondrocyte expression of growth-arrest-specific gene 6 and the tyrosine kinase receptor axl: potential role in autocrine signaling in cartilage. *Arthritis Rheum.* **40**, 1455–1465.
- Li, R., Chen, J., Hammonds, G., Phillips, H., Armanini, M., Wood, P., Bunge, R., Godowski, P. J., Sliwkowski, M. X. and Mather, J. P. (1996) Identification of Gas6 as a growth factor for human Schwann cells. *J. Neurosci.* **16**, 2012–2019.
- Hall, M. O., Prieto, A. L., Obin, M. S., Abrams, T. A., Burgess, B. L., Heeb, M. J. and Agnew, B. J. (2001) Outer segment phagocytosis by cultured retinal pigment epithelial cells requires Gas6. *Exp. Eye Res.* **73**, 509–520.
- Goruppi, S., Ruaro, E. and Schneider, C. (1996) Gas6, the ligand of Axl tyrosine kinase receptor, has mitogenic and survival activities for serum starved NIH3T3 fibroblasts. *Oncogene* **12**, 471–480.
- Fridell, Y. W., Villa, Jr, J., Attar, E. C. and Liu, E. T. (1998) GAS6 induces Axl-mediated chemotaxis of vascular smooth muscle cells. *J. Biol. Chem.* **273**, 7123–7126.
- Nakano, T., Kawamoto, K., Kishino, J., Nomura, K., Higashino, K. and Arita, H. (1997) Requirement of gamma-carboxyglutamic acid residues for the biological activity of Gas6: contribution of endogenous Gas6 to the proliferation of vascular smooth muscle cells. *Biochem. J.* **323**, 387–392.
- Tanabe, K., Nagata, K., Ohashi, K., Nakano, T., Arita, H. and Mizuno, K. (1997) Roles of gamma-carboxylation and a sex hormone-binding globulin-like domain in receptor-binding and in biological activities of Gas6. *FEBS Lett.* **408**, 306–310.
- Mark, M. R., Chen, J., Hammonds, R. G., Sadick, M. and Godowski, P. J. (1996) Characterization of Gas6, a member of the superfamily of G domain-containing proteins, as a ligand for Rse and Axl. *J. Biol. Chem.* **271**, 9785–9789.
- Goruppi, S., Yamane, H., Marcandalli, P., Garcia, A., Clogston, C., Gostissa, M., Varnum, B. and Schneider, C. (1997) The product of a gas6 splice variant allows the release of the domain responsible for Axl tyrosine kinase receptor activation. *FEBS Lett.* **415**, 59–63.
- Sasaki, T., Knyazev, P. G., Cheburkin, Y., Gohring, W., Tisi, D., Ullrich, A., Timpl, R. and Hohenester, E. (2002) Crystal structure of a C-terminal fragment of growth arrest-specific protein Gas6. Receptor tyrosine kinase activation by laminin G-like domains. *J. Biol. Chem.* **277**, 44164–44170.
- Bruggemann, M., Caskey, H. M., Teale, C., Waldmann, H., Williams, G. T., Surani, M. A. and Neuberger, M. S. (1989) A repertoire of monoclonal antibodies with human heavy chains from transgenic mice. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6709–6713.
- Taylor, L. D., Carmack, C. E., Huszar, D., Higgins, K. M., Mashayekh, R., Sequar, G., Schramm, S. R., Kuo, C.-C., O'Donnell, S. L., Kay, R. M. et al. (1993) Human immunoglobulin transgenes undergo rearrangement, somatic mutation and class switching in mice that lack endogenous IgM. *Int. Immunol.* **6**, 579–591.
- Lonberg, N., Taylor, L. D., Harding, F. A., Trounstein, M., Higgins, K. M., Schramm, S. R., Kuo, C.-C., Mashayekh, R., Wymore, K., McCabe, J. G. et al. (1994) Antigen-specific human antibodies from mice comprising four distinct genetic modifications. *Nature* (London) **368**, 856–859.

- 33 Fishwild, D. M., O'Donnell, S. L., Bengoechea, T., Hudson, D. V., Harding, F., Bernhard, S. L., Jones, D., Kay, R. M., Higgins, K. M., Schramm, S. R. et al. (1996) High-avidity human IgG1 monoclonal antibodies from a novel strain of minilocus transgenic mice. *Nat. Biotechnol.* **14**, 845–851
- 34 Neuberger, M. (1996) Generating high-avidity human Mabs in mice. *Nat. Biotechnol.* **14**, 826
- 35 Mendez, M. J., Green, L. L., Corvalan, J. R. F., Jia, X.-C., Maynard-Currie, C. E., Yang, X.-D., Gallo, M. L., Louie, D. M., Lee, D. V., Erickson, K. L. et al. (1997) Functional transplant of megabase human immunoglobulin loci recapitulates human antibody response in mice. *Nat. Genet.* **14**, 146–156
- 36 Chen, J., Carey, K. and Godowski, P. J. (1997) Identification of Gas6 as a ligand for Mer, a neural cell adhesion molecule related receptor tyrosine kinase implicated in cellular transformation. *Oncogene* **14**, 2033–2039
- 37 Yan, S. B. (1996) Review of conformation-specific affinity purification methods for plasma vitamin K-dependent proteins. *J. Mol. Recognit.* **9**, 211–218
- 38 Lu, Q. and Lemke, G. (2001) Homeostatic regulation of the immune system by receptor tyrosine kinases of the Tyro 3 family. *Science* **293**, 306–311
- 39 Graham, D. K., Dawson, T. L., Mullaney, D. L., Snodgrass, H. R. and Earp, H. S. (1994) Cloning and mRNA expression analysis of a novel human protooncogene, c-mer. *Cell Growth Differ.* **5**, 647–657
- 40 Neubauer, A., Fiebeler, A., Graham, D. K., O'Bryan, J. P., Schmidt, C. A., Barckow, P., Serke, S., Siegert, W., Snodgrass, H. R., Huhn, D. et al. (1994) Expression of axl, a transforming receptor tyrosine kinase, in normal and malignant hematopoiesis. *Blood* **84**, 1931–1941
- 41 Crosier, P. S., Hall, L. R., Vitas, M. R., Lewis, P. M. and Crosier, K. E. (1995) Identification of a novel receptor tyrosine kinase expressed in acute myeloid leukemic blasts. *Leuk. Lymphoma* **18**, 443–449
- 42 Schwartzberg, P. L. (2001) Immunology. Tampering with the immune system. *Science* **293**, 228–229
- 43 Lu, Q., Gore, M., Zhang, Q., Camenisch, T., Boast, S., Casagrande, F., Lai, C., Skinner, M. K., Klein, R., Matsushima, G. K. et al. (1999) Tyro-3 family receptors are essential regulators of mammalian spermatogenesis. *Nature (London)* **398**, 723–728
- 44 Neubauer, A., Burchert, A., Maiwald, C., Gruss, H. J., Serke, S., Huhn, D., Wittig, B. and Liu, E. (1997) Recent progress on the role of Axl, a receptor tyrosine kinase, in malignant transformation of myeloid leukemias. *Leuk. Lymphoma* **25**, 91–96
- 45 Nagai, K., Arai, H., Yanagita, M., Matsubara, T., Kanamori, H., Nakano, T., Iehara, N., Fukatsu, A., Kita, T. and Doi, T. (2003) Growth arrest-specific gene 6 is involved in glomerular hypertrophy in the early stage of diabetic nephropathy. *J. Biol. Chem.* **278**, 18229–18234
- 46 Yin, J. L., Hambly, B. D., Bao, S. S., Painter, D., Bishop, G. A. and Eris, J. M. (2003) Expression of growth arrest-specific gene 6 and its receptors in dysfunctional human renal allografts. *Transpl. Int.* **16**, 681–688
- 47 Fiebeler, A., Park, J. K., Muller, D. N., Lindschau, C., Mengel, M., Merkel, S., Banas, B., Luft, F. C. and Haller, H. (2004) Growth arrest specific protein 6/Axl signaling in human inflammatory renal diseases. *Am. J. Kidney Dis.* **43**, 286–295
- 48 Munoz, X., Sumoy, L., Ramirez-Lorca, R., Villar, J., de Frutos, P. G. and Sala, N. (2004) Human vitamin K-dependent GAS6: gene structure, allelic variation, and association with stroke. *Hum. Mutat.* **23**, 506–512

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