

The product of a *gas6* splice variant allows the release of the domain responsible for Axl tyrosine kinase receptor activation

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Abstract The product of *gas6* (Gas6) is a growth factor with high level of similarity to protein S and was identified as the ligand for Axl family of tyrosine kinase receptors. Gas6 contains an N-terminal γ -carboxylated domain (Gla), four epidermal growth factor like domains and a large C-terminal D region. An alternative Gas6 spliced form (Gas6SV) having an additional 43 amino acids between fourth EGF like and D domain was characterised. Here we show data indicating that Gas6SV is specifically cleaved within the inserted sequence, thereby splitting the D domain from the remaining part of the protein. The resulting two proteolytic products of 36 kDa and 50 kDa were separated and the 50 kDa fragment corresponding to region D was shown to be responsible for Axl receptor activation. Furthermore a deletion mutant of Gas6 containing only the D domain was shown to similarly activate Axl receptor phosphorylation unequivocally demonstrating that D domain can act as a signalling molecule. The possible roles of the proteolytic processing of Gas6SV in the regulation of growth factor availability are discussed.

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Key words: Growth factor; Receptor; Vitamin K

1. Introduction

Gas6 was initially isolated as a product of a gene whose expression is increased at growth arrest [1]. The protein encoded by *gas6* (Gas6) is a 75 kDa protein highly related to protein S (PS), a negative regulator of blood coagulation and can similarly be divided into defined domains [2]. The Gla domain is rich in γ -carboxyglutamic acid (Gla) and is involved in the Ca^{2+} dependent binding of PS to membrane phospholipids [3]. The loop region contains a thrombin cleavage site in PS which is absent in Gas6 and is followed by four EGF like repeats. The C-terminal region of Gas6 is homologous to steroid binding globulin protein (SHBG), and contains tandem globular domains [2].

Human Gas6 was shown to act as the ligand for three members of the Axl family of tyrosine kinases: Axl, Rse and Mer [4–7]. The extracellular domains of the Axl/Rse/Mer include two immunoglobulin like repeats followed by two fibronectin type III repeats reminiscent of the extracellular region of neural cell adhesion molecules [8]. Consistently murine Axl homologue, Ark, has been reported to be involved in cell to cell interaction through the ability of the extracellular portion to undergo homophilic binding [9].

These receptors are expressed in a number of tissues. Relatively high levels of expression have been detected for Axl in heart and skeletal muscle [8], for Mer in human testis, ovary, prostate, lung, kidney [10], and for Rse in the adult brain [11,12].

We have previously reported that Gas6 is able to induce cell cycle re-entry of serum starved NIH3T3 cells and to efficiently prevent cell death by apoptosis induced by complete serum removal [13]. More recently we have demonstrated that Gas6 signalling requires a PI3K activated pathway for both mitogenic and anti-apoptotic effect [14]. An alternative splice form of *gas6* has been described (accompanying manuscript) containing an additional 43 amino acid sequence between fourth EGF and C-terminal D domain. Here we report on the analysis of the product of such alternative *gas6* spliced form (Gas6SV), which is proteolytically cleaved within the inserted peptide. Biochemical separation of the two proteolytic products (36 kDa and 50 kDa) together with the analysis of Gas6 deletion mutants allowed us to show that the 50 kDa C-terminal region of Gas6SV is responsible for Axl activation. This alternative splicing event may therefore uncover a novel form of regulation for the growth factor availability.

2. Materials and methods

2.1. Plasmids, DNA deletion and swapping strategy

Human *gas6* and *gas6SV* cDNA were cloned in pGDSV7 eukaryotic expression vector under SV40 virus promoter for constitutive expression and were described elsewhere (and accompanying paper). Gas6 deletion mutants (ΔC , ΔD) were obtained as follows: for ΔC deletion, pBluescript *gas6* [2], (Stratagene), was digested with ApaI and MscI and the resulting vector containing *gas6* ΔC was gel purified, blunted with DNA polymerase Klenow fragment (Pharmacia) and religated; for ΔD deletion, pGDSV7*gas6* was digested with EcoRI and MscI and cloned into pCITE-2b (Novagene, Madison, WI) for reconstitution of the stop codon. Gas6 D domain substitution (ABC *gas6*-D PS swapping) was obtained by splicing overlap extension using pBluescript-*gas6* and pBluescript-PS (kindly provided by B. Dahlback, University of Lund, Sweden) as template in PCR. All Gas6 derivatives were inserted into pGDSV7 vector [15] for constitutive expression as EcoRI-XbaI fragments. *gas6* was cloned into episomal mammalian vector pCEP4 (Invitrogen) for constitutive expression in 293 cells. Restriction enzymes were from New England Biolabs and Promega while Taq polymerase was from Perkin-Elmer.

2.2. Cell culture and transfection of COS-7 cells

COS-7, 293 and A172 cells were grown routinely in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 $\mu\text{g}/\text{ml}$). COS-7 cells were transfected with the DEAE dextran method as described in [13]. Briefly, subconfluent cell cultures growing in 35 mm petri dish were washed twice with PBS and then overlaid with 400 μl of DEAE dextran (50 $\mu\text{g}/\text{ml}$) (Pharmacia) in serum free DMEM pre-mixed with 2 μg of the *pGDSV-7gas6* or *pGDSV-7gas6SV* cDNA.

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Similar amounts were used for each *gas6* mutant deletion derivative. A separate petri dish was mock transfected and used as control. After 30 min at 37°C, 2 ml of DMEM 10% FCS supplemented with 100 μ M chloroquine (Boehringer Mannheim) were added and cells incubated for further 3–5 h. Within 24 h after transfection, the medium was changed with DMEM 0.5% FCS, 5 μ g/ml Vit K1 (Konakion, Roche). Two days later cell supernatants were centrifuged at 2500 $\times$ g and stored at –80°C for subsequent analysis. 293 cells were transfected using a CaPO₄ protocol as previously described [14].

2.3. Antibody production

Production and characterisation of the affinity purified antibody recognising Gas6 D region and Gas6 amino terminal A region were described elsewhere [2,4]. To produce an antibody specific for the insertion peptide of Gas6SV, a peptide spanning amino acids from 296 to 329 within the inserted sequence (GRGAQGSRSEGHIPDRRGPRPWQC) was synthesised according to the Fmoc solid phase peptide synthesis methods [16] and crosslinked to haemocyanin (Boehringer Mannheim) using glutaraldehyde (Sigma). The rabbit was first injected with 200 μ g of the coupled peptide diluted in Freund's complete adjuvant (Sigma) and subsequently injected with the same amount of conjugated peptide in incomplete Freund's adjuvant every 20 days. 5 mg of the same peptide used for immunisation were covalently coupled to SulfoLink resin (Pierce) according to the manufacturer's instructions and the resulting column was used for affinity purification of the specific antibodies.

2.4. Western blotting

Equal amounts of total proteins, as assessed by Bradford analysis, from mock transfected, *gas6*, *gas6SV* or *gas6* mutant deletion transfected COS-7 cell supernatants were separated by 15% SDS-PAGE and blotted on a 0.25 μ nitrocellulose membrane (S and S). The nitrocellulose sheets were saturated for 2 h in 5% non-fat dry milk, 200 mM NaCl, 50 mM Tris pH 7.5 and 0.02% Tween 20, and separately incubated overnight with the specific antibody. After this time bound antibodies were revealed using a goat anti-rabbit horseradish peroxidase conjugated antibody and ECL solutions (Amersham, UK).

Analysis of Axl receptor activation with antiphosphotyrosine antibodies PY20 (Transduction laboratories) was performed on COS-7 cells serum starved (DMEM 0.5% FCS) for 24 h as previously described [13].

2.5. Protein purification and activity determination

293 cell supernatant from cells transfected with a *gas6SV*-pCEP4 (Invitrogen) was adjusted to 2 mM EDTA and loaded onto a Q-Sepharose column equilibrated with 150 mM NaCl, 2 mM EDTA, 1 mM Benzamidine (Sigma), 20 mM Tris pH 7.4. The flow through material containing the D region was reserved for further analysis. The material which bound to the column was eluted first with 10 mM CaCl₂ and then with a gradient of NaCl (from 150 mM to 500 mM). Fractions were analysed by Western blotting for the presence of the full length *Gas6SV* or the N-terminal 36 kDa region. Appropriate fractions were buffer exchanged by repeated centrifugations on Centricon concentrators (Amicon) and subsequent dilution with DMEM. The activity of these fractions was determined by receptor phosphorylation assay [4].

3. Results and discussion

From the original screening for the isolation of human *gas6* homologue, we isolated a *gas6* alternative spliced form (*gas6SV*) containing an additional open reading frame coding for 43 amino acids between the fourth EGF domain and the C-terminal D domain. In order to produce *Gas6SV* recombinant protein, Cos cells were transfected with the *gas6SV* cDNA and the production of *Gas6SV* in the supernatant was assayed by Western blot. In parallel experiments Cos cells were mock or transfected with *gas6* and used as control. Western blot analysis was carried out with three different antibodies recognising the N-terminus (A region), the C-terminus (D region) or the specific peptide sequence within the insertion

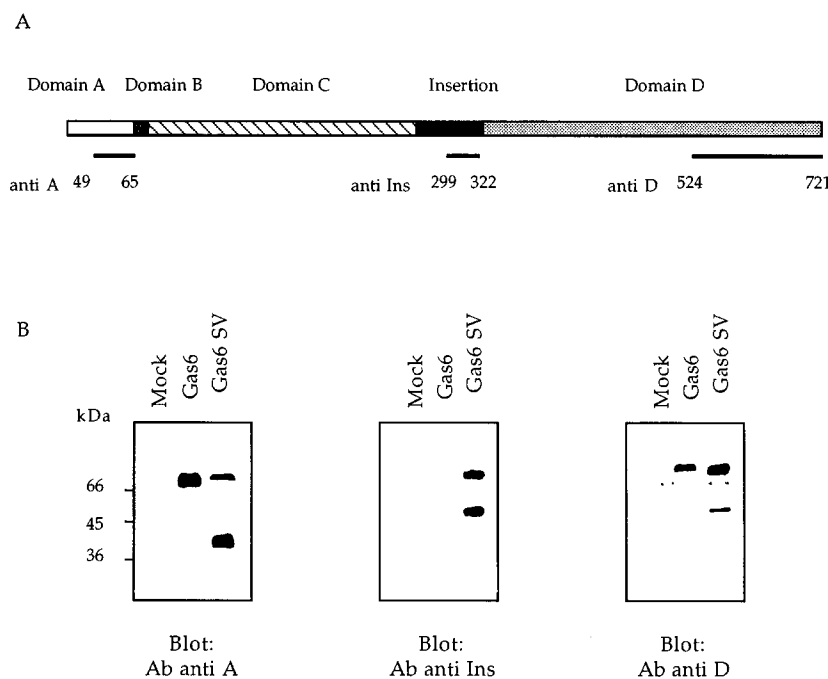


Fig. 1. *Gas6SV* is proteolytically cleaved. A: *Gas6SV* protein organisation after Manfioletti et al., 1993. The white box indicates *Gas6SV* γ -carboxylated domain A which is followed by the short linker region B (black box) and the four EGF repeats containing domain C (shaded box). The black box indicates the 43 amino acid sequence of the *Gas6SV* insertion, while the spotted box represent the large C-terminal D domain. The bars under *Gas6SV* regions indicate the localisation of amino acid sequences used for antibodies production. B: Western blot analysis of mock, *gas6* and *gas6SV* transfected COS-7 cells as assayed with antibodies recognising separate *Gas6SV* regions. Equal amounts of total proteins, as assessed by Bradford analysis, from the supernatant of mock transfected, *gas6* or *gas6SV* transfected COS-7 cells were separated by 15% SDS-PAGE. Nitrocellulose membranes were separately incubated with antibodies specific for N-terminal region (Ab anti-A), or insertion (Ab anti-Ins) or C-terminal region (Ab anti-D).

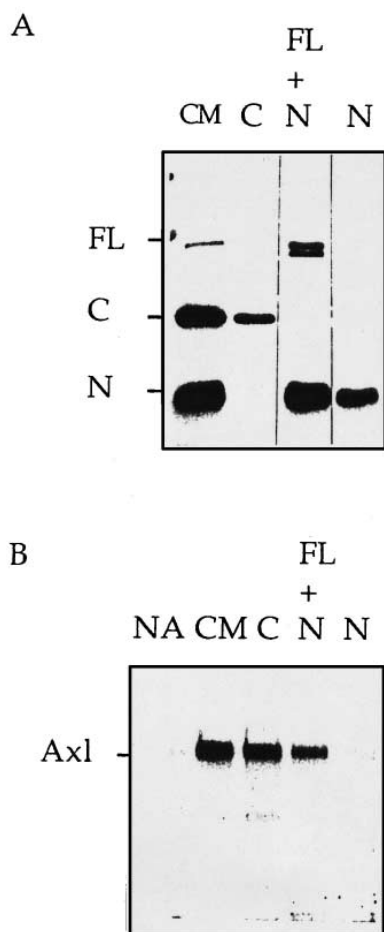


Fig. 2. Biochemical fractionation of the processed Gas6SV products and identification of the Axl activation domain. A: Gas6SV protein fractionation. 293 cell supernatant from cells transfected with a *gas6SV*-pCEP4 was loaded onto a Q-Sepharose column (CM). The flow through contained only the C-terminal D region of Gas6SV (C). The bound Gas6SV proteins were eluted first with 10 mM CaCl_2 (N) and subsequently with a NaCl gradient (FL+N). The fractions were separated on SDS-PAGE gel and analysed by Western blotting for the presence of the full length Gas6SV or the N-terminal 36 kDa region. B: Analysis of Gas6SV purified fractions for Axl receptor autophosphorylation. Gas6SV fractions purified as described were tested in receptor autophosphorylation assay [4]. Western blot analysis with antiphosphotyrosine antibodies of Axl receptors immunoprecipitated from A172 cells before (NA) and after stimulation either with the 293 conditioned medium (CM), or the flow through containing the D domain (C), or the proteins eluted with the NaCl gradient (FL+N) and CaCl_2 addition (N).

(Ins). As expected *gas6SV* transient expression yielded a product which was slightly larger than the Gas6 protein (Gas6 as compared to Gas6SV, Fig. 1, panel A and B). Interestingly, the majority of Gas6SV protein in the supernatant was cleaved into a 36 kDa product (N-terminal region) and a 50 kDa product (C-terminal region). Such proteolytic processing most likely took place in the extracellular compartment, since only the full length protein could be detected in the cellular fraction (not shown). The split products were also found in the supernatant from 293 cells and murine NIH3T3 fibroblasts similarly transfected with *gas6SV* thus suggesting that processing of Gas6SV involves a protease expressed by different cell types. Moreover, since anti-Ins antibodies recognise

the 50 kDa form in Western blot (Fig. 1, panel B), the site recognised by such protease may be contained in the C-terminal region of the inserted peptide sequence.

Biochemical separation of the two proteolytic products from conditioned medium of stable transfected 293 cells (CM) was performed by Q Sepharose chromatography, based on the presence of several negative charges in the Gla domain. Different fractions containing respectively the C-terminal 50 kDa product (C), the N-terminal 36 kDa product (N) and a mixture of full length and the 36 kDa (N+FL) (Fig. 2, panel A) were obtained. The purified fractions as well as the starting material from 293 supernatants were tested for their ability to stimulate Axl phosphorylation in A172 cells as previously reported [4]. As shown in Fig. 2, panel B, stimulation of Axl activity, detected as increased tyrosine phosphorylation, was associated with the fractions containing the 50 kDa C-terminal D domain alone or both p50 and full length Gas6SV. No Axl phosphorylation was detected when the N-terminal portion of the protein containing the EGF repeats was tested (Fig. 2, panel B). In addition, activation of Axl receptor tyrosine kinase activity by the purified C-terminal region was similar to that obtained with the conditioned medium containing the full length Gas6SV, further suggesting that Gas6 D domain is responsible for Axl stimulation. Thus, the EGF repeats are apparently not essential for receptor activation and may therefore be involved in additional Gas6 (and Gas6SV) interactions.

In order to confirm the ability of the D domain to activate Axl receptor, we produced Gas6 mutant deletions lacking the D domain (Gas6 Δ D) or lacking the region containing the four EGF (Gas6 Δ C). An additional construct was obtained by replacing the Gas6 D region with the corresponding D region of protein S (Gas6-DPS) [5].

Cos cells were transfected with *gas6*, *gas6SV*, *gas6 Δ C*, *gas6 Δ D* and *gas6-DPS* as described above and the recombinant proteins were allowed to accumulate in the supernatant for 48 h. Equal amounts of cell supernatant were separated by 15% SDS-PAGE, blotted on membranes and revealed using antibodies recognising the amino terminus of Gas6. Cos cells were also mock transfected and used as control. Gas6, Gas6SV and all the mutant deletion proteins were secreted and were found in the supernatant of transfected cells (Fig. 3, panel B). The ability of such recombinant proteins to induce Axl receptor tyrosine phosphorylation in Cos cells was further assayed. Equal amounts of the respective chimeric constructs as assessed by Western blot were added to Cos cells for 10 min at 37°C. Analysis of receptor autophosphorylation was carried out as previously described [13]. Gas6 as well as Gas6SV were able to induce Axl receptor phosphorylation. According to the previous results, only Gas6 Δ C was able to activate Axl receptor, while Gas6 Δ D and Gas6-DPS, both lacking the D domain of Gas6, did not show activity (Fig. 3, panel B). These results therefore confirm the requirement of D domain for Axl receptor activation and show that this region is still able to activate the receptor when separated from the EGF repeats. Our evidence points to the potential role of the processing of Gas6SV in regulating ligand availability (Fig. 4). Indeed the processed p50 product retains receptor stimulating activity but is no longer associated with the N-terminal p36 region. By analogy with the Gla domain of protein S and other vitamin K dependent proteins, the N-terminal region, containing the Gla domain of Gas6,

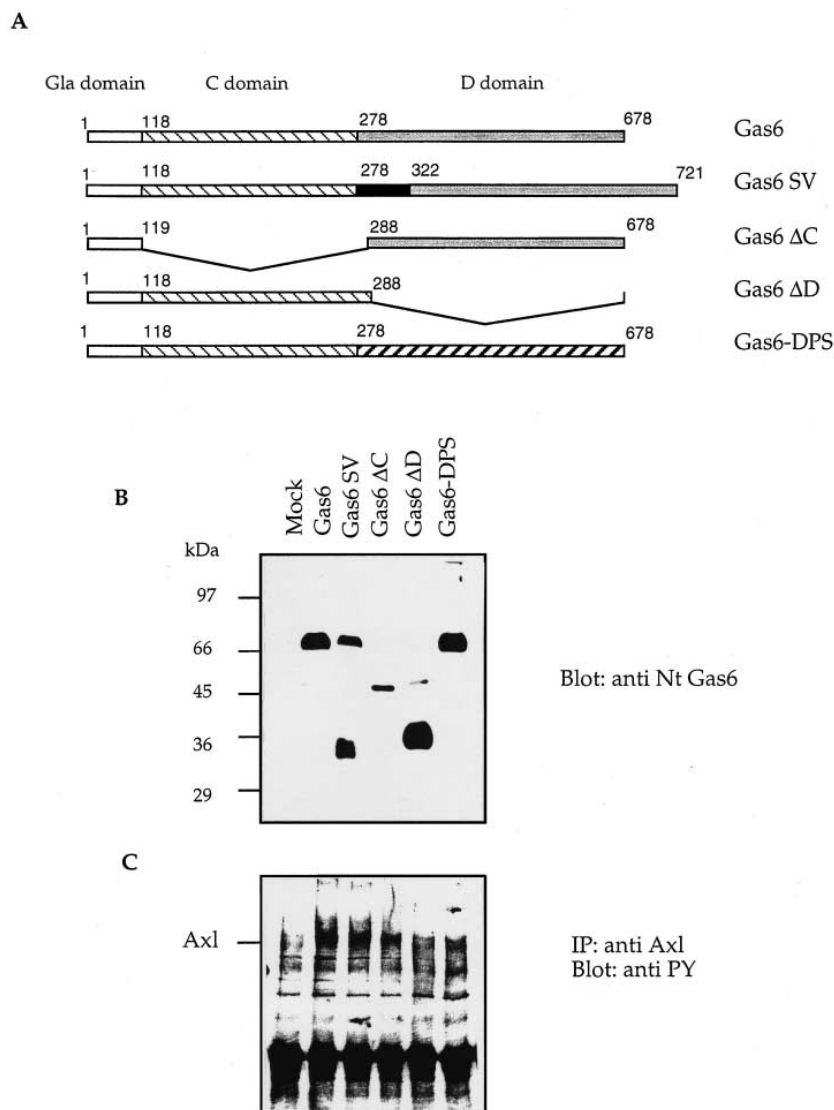


Fig. 3. Deletion mutant analysis of Gas6 domain involved in Axl activation. A: Scheme of human Gas6 and Gas6SV domains organisation and position of human Gas6 deletions mutants (Gas6ΔC and Gas6ΔD) analysed by receptor autophosphorylation assay. The shaded area represent the four EGF like repeats (C domain) while grey boxes indicates the large C-terminal D domain. The darker shaded box indicates the D domain of human protein S that was inserted in Gas6 (Gas6-DPS). The blackened box is the 43 amino acid peptide of the Gas6SV form. B: Western blot analysis of supernatant from COS-7 cells after transfection with the cDNAs described in A. Equal amounts of conditioned medium were loaded on SDS-PAGE gels and analysed by Western blot using an antibody recognising N-terminal region of Gas6. A petri dish was mock transfected and used as control. C: Western blot analysis with antiphosphotyrosine antibodies of Axl receptors immunoprecipitated from COS-7 cells. Supernatants described in B were analysed for their ability to induce Axl receptor phosphorylation. The result shown here is representative of three separate experiments.

has the potential to localise Gas6 to the membrane compartment [17]. Thus Gas6 may be sequestered on the surface of cells at, or very near, the site of synthesis. Processing of the splice variant protein could therefore serve as a means of separating the receptor activating region from the region responsible for interaction to membranes, or potential ECM binding sites, thereby releasing it from this local environment. As a consequence the released D domain would become available for activating receptors at sites remote from that of synthesis. Our findings are in agreement with a recent report suggesting that SHBG like repeats present on Gas6 D domain are responsible for its biological activity, while the N-terminus modulates its activity [18]. The expression of an alternatively spliced form that allows the release of the receptor activation region from the whole molecule may therefore represent an

additional and novel mechanism of growth factor availability control.

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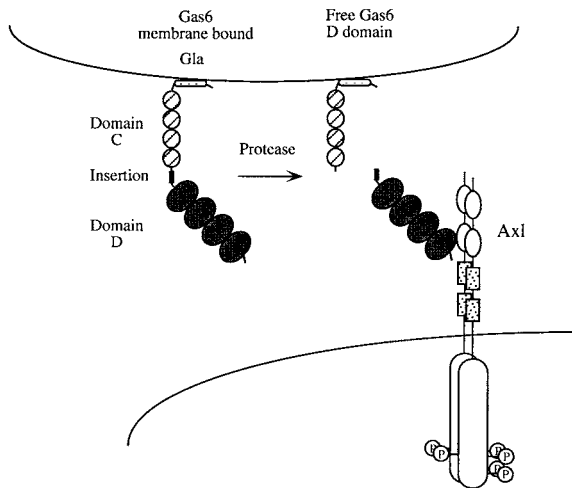


Fig. 4. Model for regulation of Gas6SV availability. Gas6SV is sequestered in the sites of synthesis by the interaction with the cellular membrane through its Gla domain. The presence of the peptide sequence in Gas6SV introduces a target site for a protease thus separating the receptor activatory D domain from the Gas6 protein.

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