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Roles of γ-carboxylation and a sex hormone-binding globulin-like domain in receptor-binding and in biological activities of Gas6

Kazuyo Tanabe[a], Kyoko Nagata[a], Kazumasa Ohashi[a], Toru Nakano[b], Hitoshi Arita[b], Kensaku Mizuno[a,*]

[a] *Department of Biology, Faculty of Science, Kyushu University, Hakozaki, Higashi-ku, Fukuoka 812-81, Japan*
[b] *Discovery Research Laboratory, Shionogi and Co., Ltd., Sagisu, Fukushima-ku, Osaka 553, Japan*

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Abstract Gas6 is a ligand for an Axl/Sky receptor tyrosine kinase subfamily and has a structure composed of a Gla domain, four EGF-like domains and a C-terminal sex hormone-binding globulin (SHBG)-like domain. When examining the role of each domain in receptor-binding and biological activities of Gas6, we found that receptor-binding and mitogenic activities were markedly reduced by inhibiting γ-carboxylation of the Gla domain, while a Gas6 mutant composed of only an SHBG-like domain retained both of these activities. Thus, the SHBG-like domain is apparently an entity indispensable for Gas6 activities, and γ-carboxylation of the Gla domain has a regulatory role in retaining the activity of native Gas6.

Key words: Gas6; Gla domain; γ-carboxylation; Sex hormone-binding globulin

1. Introduction

The protein encoded by growth arrest-specific gene 6 (Gas6) was initially identified as a gene product inducibly expressed in fibroblasts in response to serum starvation [1]. Recent studies from our laboratory and others have demonstrated that Gas6 is a ligand for Axl, Sky and Mer receptor tyrosine kinases [2–5], with the Gas6 binding affinities in the order of Axl > Sky > Mer [5]. These receptors are typified by the cell adhesion molecule-related extracellular ligand-binding domain composed of two immunoglobulin-like motifs and two fibronectin type III motifs [6–10]. Gas6 was recently found to act as a growth-potentiating factor for thrombin-induced proliferation of vascular smooth muscle cells [11]. Gas6 also prevented serum starvation-induced cell death of these cells [12]. Mitogenic and survival activities of Gas6 for serum-starved fibroblasts and Schwann cells have also been described [13,14].

Gas6 has a structure similar to that of protein S, a vitamin K-dependent plasma protein with anticoagulant activity, with 43% amino acid identity [1,15]. Similar to protein S, Gas6 is composed of several defined structural domains; an N-terminal γ-carboxyglutamic acid (Gla) domain, followed by a short loop domain, four tandem repeats of epidermal growth factor (EGF)-like domains and a C-terminal sex hormone-binding globulin (SHBG)-like domain [1]. The Gla domain, which is present in various proteins involved in blood coagulation, is generated by vitamin K-dependent γ-carboxylation of a cluster of Glu residues and is thought to be involved in intramolecular and intermolecular protein–protein or protein–membrane interactions through Ca^{2+}-binding [16,17]. The C-terminal domains of protein S and Gas6 have a sequence similarity with human SHBG and rat androgen-binding protein, and these sequences are also distantly related to basement membrane proteins, such as laminin and merosin [18]. Based on the characteristic domain structure of Gas6 compared with other known ligands for receptor tyrosine kinases, it is interesting to know which domain of Gas6 is essential for its receptor-binding and biological activities and if γ-carboxylation in the Gla domain is involved in these activities. In this respect, Mark et al. recently reported data showing the importance of the C-terminal SHBG-like domain for receptor-binding and receptor-stimulating activities of Gas6 [19], but the biological activity of this domain has not been shown. The role of γ-carboxylation in the Gla domain also remains unknown. We report here that the SHBG-like domain has full mitogenic activity on fibroblasts and γ-carboxylation of the N-terminal Gla domain significantly contributes to bring out the activity of native Gas6.

2. Materials and methods

2.1. Plasmid construction

Gas6 mutants used in this study are schematically shown in Fig. 2A. The 2.8-kb Gas6 cDNA [11] was isolated from a rat brain cDNA library, and subcloned into pBluescript SKII$^-$ (Stratagene) or pGEM7 (Promega) at *Eco*RI site to generate pBS-Gas6 or pGEM-Gas6. To construct pGEM-ΔG, a 280-bp *Eco*RI–*Taq*I fragment of pGEM-Gas6 coding for N-terminal 50 residues of Gas6 was isolated and inserted into *Eco*RI–*Bam*HI site of pBluescript with a synthetic *Eco*RV linker composed of 5′-CGGATATCG-3′ and 5′-GATCCGATATC-3′. The resulting plasmid (pBS-N) was cut with *Cla*I and *Eco*RV and the insert was ligated into the *Cla*I/*Eco*RV-digested pGEM-Gas6. To construct pGEM-ΔE and pGEM-ΔGE, pGEM-Gas6 was digested with *Cla*I, blunt-ended, and ligated with a *Xho*I linker. The resulting plasmid [pGEM(XhoI)-Gas6] was digested with *Xho*I and *Bal*I and ligated with a synthetic linker composed of 5′-TCGAGATATCGTGAGGACATCTTACCGTGTGTGCCCTTCAGCATGG-3′ and 5′-CCATGCTGAAGGGCACACACGGTAAGATGTCCTCACGATATC-3′. The plasmid was then digested with *Xho*I and *Eco*RV, and ligated with a 400-bp *Xho*I–*Eco*RV fragment of pGEM(XhoI)-Gas6 to generate pGEM-ΔE, or was ligated with a 250-bp *Xho*I–*Eco*RV fragment of pBS-N to generate pGEM-ΔGE. To construct pBS-ΔH-Myc, pBS-Gas6 was digested with *Bal*I and *Bam*HI, blunt-ended, and ligated with a *Xba*I linker composed of 5′-CCTCTAGA-3′ and 5′-TCTAGAGG-3′. The resulting plasmid was digested with *Xba*I and *Sac*II, and ligated with a synthetic *Xba*I/*Sac*II linker composed of 5′-CTAGTGAACAAAAGCTTATTTCTGAAGAAGACTTGTGAGCGGCCGCGAATTCCGC-3′ and 5′-GGAA-

*Corresponding author. Fax: (81) 92-642-2645.
E-mail: kmizuscb@mbox.nc.kyushu-u.ac.jp

Abbreviations: BrdU, bromodeoxyuridine; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; Gas6, the protein encoded by growth arrest-specific gene 6; Gla, γ-carboxyglutamic acid; SHBG, sex hormone-binding globulin

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TTCGCGGCCGCTCACAAGTCTTCTTCAGAAATAAGCTTTT-GTTCA-3′ (coding for a C-terminal Myc epitope peptide, SSEQKLI-SEEDL) to generate pBS-ΔH-Myc. To construct plasmids for Myc-tagged mutants (pGEM-Gas6-Myc, pGEM-ΔG-Myc, pGEM-ΔE-Myc and pGEM-ΔGE-Myc), a 820-bp *Bfa*I fragment of pGEM-Gas6 was blunt-ended, and ligated with a *Xba*I linker. The resulting plasmid was digested with *Sal*I and *Xba*I, and the 420-bp fragment was ligated into pBluescript with a *Xba*I/*Sac*II linker described above to generate pBS-C-Myc. To generate pGEM-Gas6-Myc, pBS-C-Myc was digested with *Sal*I and *Not*I and the insert was replaced with a *Sal*I/*Not*I fragment of pGEM-Gas6. pGEM-ΔG-Myc, pGEM-ΔE-Myc and pGEM-ΔGE-Myc were generated, in a similar manner. The authenticity of the expression plasmids was confirmed by nucleotide sequence analysis.

2.2. Preparation of recombinant Gas6 and its mutants

The cDNA constructs were subcloned into the PUC-SRα expression vector [3], and the plasmids were transfected into COS-7 cells, using calcium phosphate methods. Cells were cultured for 3 days in serum-free Dulbecco's modified Eagle's medium (DMEM) with 2 μM vitamin K2 (Menatetrenone, Eisai, Tokyo). Gas6(-Gla), which was deficient in γ-carboxylation of Glu residues, was expressed in serum-free DMEM in the presence of 1 μM Warfarin (a competitive inhibitor of vitamin K, Sigma) in place of vitamin K2. Recombinant Gas6 was purified from conditioned medium, as described elsewhere [11]. Gas6(-Gla) was purified in a similar manner, except that its elution was monitored by dot-blot or Western blot analysis using an anti-Gas6 antibody. The absence of Gla residues in Gas6(-Gla) was confirmed by amino acid analysis and amino acid sequencing [11]. As for Myc-tagged Gas6 mutants, except for ΔH-Myc, the conditioned media of COS cells were used after concentration with Centriprep-10 (Amicon). As ΔH-Myc was not secreted from the cells, the cell lysates were directly used for co-precipitation binding analyses. The concentrations of the purified Gas6 and Gas6(-Gla) were determined by amino acid analysis, and those of Myc-tagged mutants were densitometerically estimated based on immunoblots with 9E10 anti-Myc antibody (Santa Cruz Biotechnology), using Gas6-Myc as a standard.

2.3. Binding assays

Axl-Fc fusion proteins were expressed in COS-7 cells and purified as described previously [3,5]. Gas6 mutants were incubated at 4°C overnight with 5 nM Axl-Fc fusion protein and Protein A-Sepharose (30 μl of 50% slurry) in 200 μl of Hanks' balanced salt solution containing 20 mM Hepes (pH 7.0), 0.02% NaN_3, and 1% bovine serum albumin. After centrifugation, the precipitates were washed 4 times with cold phosphate-buffered saline (PBS), suspended in SDS sampling buffer, and subjected to SDS-PAGE. Bound proteins were detected by immunoblot analysis with anti-Gas6 antibody [3] or 9E10 anti-Myc epitope antibody. BIAcore binding analysis was carried out, as described previously [5]. Gas6 was passed over the immobilized Axl-Fc fusion protein at a flow rate of 2 μl/min for 25 min. The dissociation constants (K_d) were calculated, using the manufacturer's software (Pharmacia Biotech.).

2.4. Tyrosine phosphorylation assay

CHO cell lines (AM3) expressing Myc-tagged Axl (Axl-Myc) were constructed as described previously [5]. AM3 cells were cultured in Ham's F-12 medium supplemented with 10% fetal calf serum. The cells were serum-starved, treated with Gas6 mutants for 10 min at 37°C, then rinsed 3 times with cold PBS containing 1 mM orthovanadate, followed by lysing with cold lysis buffer (20 mM Hepes (pH 7.2), 1% Nonidet P-40, 10% glycerol, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, and 10 μg/ml leupeptin). The lysates were immunoprecipitated with an anti-Myc antibody (9E10), run on SDS-PAGE, and immunoblotted with anti-phosphotyrosine monoclonal antibody (PY20, ICN Biomedicals), as described previously [3].

2.5. DNA synthesis

NIH3T3 cells were maintained in DMEM supplemented with 10% fetal calf serum, and then serum-starved for 24 h in DMEM with 0.1% BSA and 0.5 nM Axl-Fc. After the addition of 20 nM Gas6 or its mutant, the cells were cultured for 14 h, then labeled with 10 mM bromodeoxyuridine (BrdU) for 3 h. The BrdU incorporation was detected by a BrdU Detection Kit III (Boehringer Mannheim).

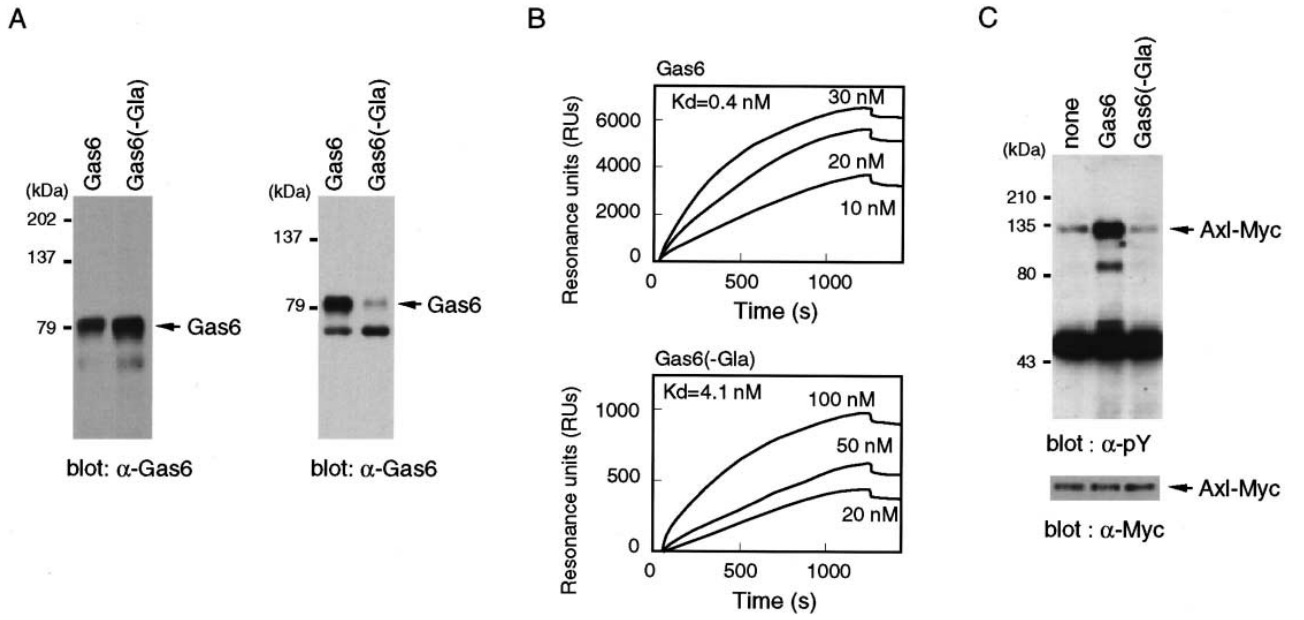


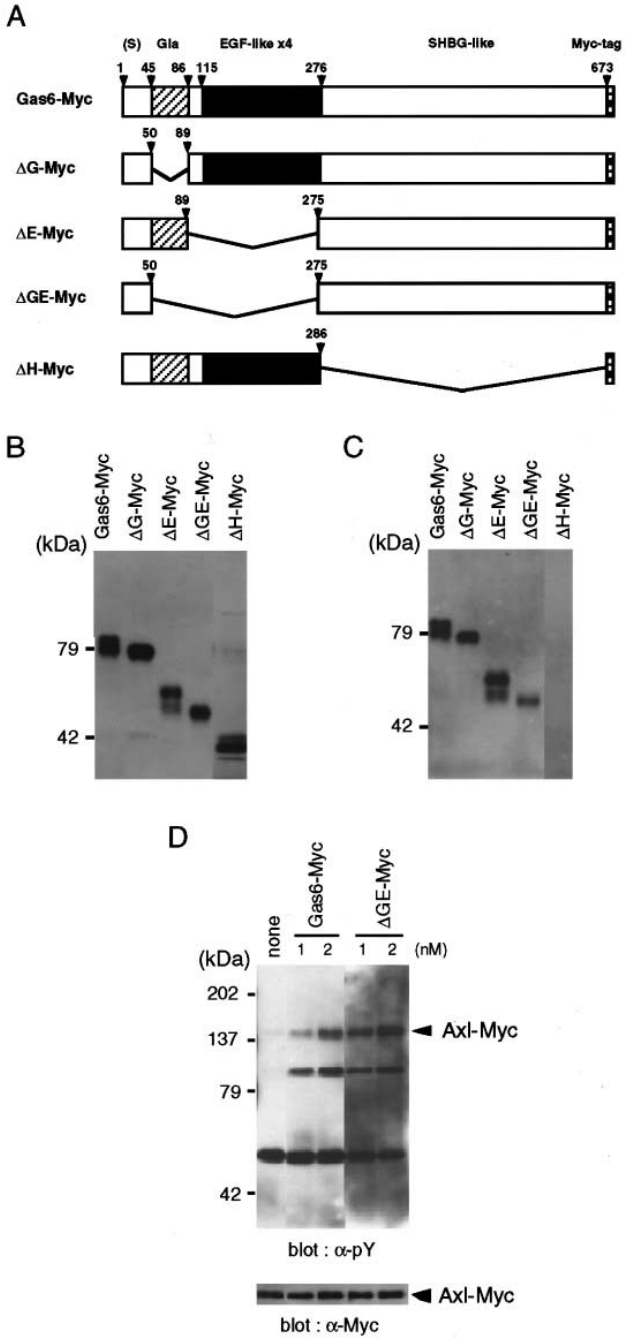
Fig. 1. Requirement of vitamin K-dependent γ-carboxylation for receptor-binding and receptor-stimulating activities of Gas6. A (left): Immunoblot analysis of purified Gas6 and Gas6(-Gla), prepared in the presence and absence of vitamin K, respectively. A (right): Co-precipitation binding assay. Gas6 or Gas6(-Gla) (each 2 nM) was incubated with 5 nM Axl-Fc and Protein A-Sepharose. After centrifugation, the precipitates were solubilized, run on SDS-PAGE and the bound Gas6 was visualized by immunoblot analysis with use of an anti-Gas6 antibody. Arrows indicate the elution position of Gas6. Molecular sizes (kDa) of marker proteins are indicated on the left. B: BIAcore binding analyses. Each panel shows the sensorgram (RUs vs. time) for Gas6 or Gas6(-Gla) passed over the surface immobilized with Axl-Fc on a BIAcore instrument. The concentrations of Gas6 or Gas6(-Gla) injected are indicated. C: Axl tyrosine-phosphorylation activity. AM3 cells stably expressing Myc-tagged Axl (Axl-Myc) were treated for 10 min with 2 nM Gas6 or Gas6(-Gla). Cell lysates were immunoprecipitated with anti-Myc antibody, run on SDS-PAGE, and immunoblotted with anti-phosphotyrosine antibody (upper panel) or anti-Myc antibody (lower panel). Arrows indicate the elution position of Axl-Myc. Molecular sizes (kDa) of marker proteins are indicated on the left.

3. Results

3.1. Requirement of γ-carboxylation for receptor-binding and -stimulating activities of Gas6

To examine the role of vitamin K-dependent γ-carboxylation for Gas6 activity, Gla-deficient Gas6 [Gas6(-Gla)] was prepared under vitamin K-depleted conditions. The absence of Gla residues was confirmed by amino acid analysis and amino acid sequencing [11]. The receptor-binding potential of Gas6(-Gla) was assessed by co-precipitation assay, using a chimeric soluble receptor (Axl-Fc) composed of the extracellular ligand-binding domain of Axl fused to the Fc region of human immunoglobulin IgG1 [5]. As shown in Fig. 1A, the binding ability to Axl-Fc was significantly decreased in Gas6(-Gla), when compared with Gas6 prepared in the presence of the vitamin K. Quantitative binding analyses using a BIAcore instrument revealed a significant decrease in the binding affinity of Gas6(-Gla) to Axl-Fc and dissociation constants (K_d) of Gas6 and Gas6(-Gla) to Axl-Fc were calculated to be 0.4 nM and 4.1 nM, respectively (Fig. 1B).

As Gas6 was earlier shown to stimulate tyrosine-phosphorylation of Axl, Sky and Mer receptors [2–5], we examined the effect of γ-carboxylation of Gas6 on the activity to stimulate tyrosine-phosphorylation of Myc epitope-tagged Axl (Axl-Myc) expressed on CHO cells. As shown in Fig. 1C, tyrosine-phosphorylation of Axl-Myc was induced by treatment with Gla-containing Gas6, but not with Gla-deficient Gas6(-Gla). Taken together these results suggest that vitamin K-dependent γ-carboxylation is required for Gas6 to exhibit full activity to bind to and to stimulate Axl.



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3.2. An SHBG-like domain is sufficient for receptor-binding and -stimulating activities of Gas6

To locate the functional domain(s) of Gas6 essential for its receptor-binding and biological activities, we constructed expression plasmids coding for a set of deletion mutants of Gas6, as shown in Fig. 2A. Each mutant was tagged with a Myc epitope peptide at the C-terminus, the objective being to detect and quantitate expression products with anti-Myc monoclonal antibody (9E10). Immunoblot analysis confirmed the expression of proteins of predicted molecular sizes (Fig. 2B). Co-precipitation binding analyses revealed that Gas6-Myc and its mutants, ΔG-Myc (deletion of the Gla domain), ΔE-Myc (deletion of four EGF-like domains) and ΔGE-Myc (deletion of Gla and four EGF-like domains), bound to Axl-Fc, but ΔH-Myc (deletion of the C-terminal SHBG-like domain) did not (Fig. 2C). These observations, in accord with those of Mark et al. [19], indicate that the C-terminal SHBG-like domain is necessary and sufficient for the receptor-binding activity of Gas6.

To further define the role of an SHBG-like domain in Gas6 activity, we examined if the ΔGE mutant composed of almost only the SHBG-like domain would stimulate tyrosine-phos-

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Fig. 2. A: C-terminal SHBG-like domain is sufficient for receptor-binding and receptor-stimulation activities of Gas6. A: Diagrams showing structures of rat Gas6 and variously deleted mutants used in this study. The hatched boxes represent Gla domains; black boxes, four EGF-like repeats; gray boxes, SHBG-like domains; and horizontally striped boxes, Myc-tag peptides. S, signal sequence. The numbers on top of the diagram of Gas6-Myc indicate amino acid residues flanking each domain and numbers on deleted mutants indicate the residues flanking deleted domains. B: Immunoblot analyses of Gas6 mutant proteins. The serum-free conditioned media of COS cells transfected with plasmids coding for Gas6 mutants (except for ΔH-Myc) were concentrated, run on SDS-PAGE, and immunoblotted with anti-Myc antibody. In the case of ΔH-Myc, cell lysates were used. C: Co-precipitation binding assay with Axl-Fc. Gas6 mutants (each 5 nM) were incubated with 5 nM Axl-Fc and Protein A-Sepharose. After centrifugation, the precipitates were solubilized, run on SDS-PAGE and the bound protein was visualized by immunoblotting using an anti-Myc antibody. Molecular sizes (kDa) of marker proteins are indicated on the left. D: Axl tyrosine-phosphorylation activity of Gas6-Myc and ΔGE-Myc. CHO cells stably expressing Axl-Myc were treated for 10 min with 1–2 nM of Gas6-Myc or ΔGE-Myc. Cell lysates were immunoprecipitated with anti-Myc antibody, run on SDS-PAGE, and immunoblotted with anti-phosphotyrosine antibody (upper panel) or anti-Myc antibody (lower panel). Arrows indicate the elution position of Axl-Myc. Molecular sizes (kDa) of marker proteins are indicated on the left.

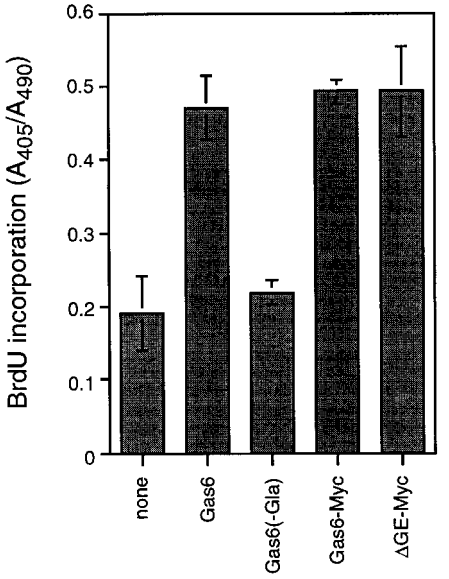

Fig. 3. Effects of Gas6 and its mutants on DNA synthesis of NIH3T3 cells. DNA synthesis was measured by BrdU incorporation, as described in Section 2. Each value represents the mean ± SE of triplicate measurements.

phorylation of Axl receptor expressed on CHO cells. As shown in Fig. 2D, tyrosine-phosphorylation of Axl-Myc on AM3 cells was induced by ΔGE-Myc as well as Gas6-Myc, in a dose-dependent manner. ΔGE-Myc and Gas6-Myc also stimulated tyrosine-phosphorylation of Sky expressed on CHO cells (data not shown).

3.3. *Roles of γ-carboxylation and an SHBG-like domain in mitogenic activity of Gas6*

We next examined the effects of Gas6 and Gas6 mutants on DNA synthesis of NIH3T3 cells. Gas6 was previously shown to be secreted from serum-starved NIH3T3 cells [1]. To exclude the effect of endogenous Gas6, the cells were cultured in the presence of Axl-Fc during serum starvation, an approach which reduced the background level of BrdU incorporation. As shown in Fig. 3, Gas6 significantly stimulated the DNA synthesis of NIH3T3 cells, but Gla-deficient Gas6(-Gla) did not. ΔGE-Myc also stimulated the DNA synthesis of NIH3T3 cells, with a potency similar to that of Gas6-Myc. These results suggest that γ-carboxylation of the Gla domain is required for the mitogenic activity of full-length Gas6, while only the C-terminal SHBG-like domain is sufficient for this activity.

4. Discussion

We obtained evidence that γ-carboxylation of the Gla domain is required for receptor-binding and mitogenic activity of Gas6. We also showed that only the C-terminal SHBG-like domain of Gas6 has the potential to bind to Axl-Fc and to stimulate receptor tyrosine-phosphorylation and DNA synthesis of fibroblasts. These results, together with recent report by Mark et al. [19], suggest that the C-terminal SHBG-like domain represents the entity essential for receptor-binding and biological activities of Gas6 while the N-terminal Gla domain plays a role to modulate the activity. It is presumed that the Gla-deficient (i.e. Glu) form of a Gla domain inhibits the receptor-binding and biological activities of Gas6 by intra- (or inter-) molecularly interfering with receptor-binding functions of the SHBG-like domain (Fig. 4). The γ-carboxylation of the Gla domain and subsequent Ca^{2+}-binding to Gla residues probably release this negative regulation. Ca^{2+}-binding to Gla residues seems to be involved in this function of the Gla domain, since depletion of Ca^{2+} by treatment with EGTA or EDTA significantly reduced receptor-binding and stimulating activities of Gas6 [3,20]. Studies of Furie et al. on the Gla domain of the vitamin K-dependent blood clotting proteins indicated that two or three Gla residues bind a single Ca^{2+} to form a rigid conformation of the Gla domain, with intramolecular bridges [16,21]. As shown in Fig. 4, such conformational changes mediated by Ca^{2+} may lead to exposure of a receptor-binding surface in the SHBG-like domain. In addition, as the Ca^{2+}-bound Gla domain is thought to be involved in protein–membrane interactions through association with phospholipids [16,17], Gla residues in Gas6 may have a promoting effect on ligand–receptor interactions on target cell membranes. Although vitamin K-dependent γ-carboxylation has been implicated in regulating blood coagulating systems, our evidence for requirement of γ-carboxylation for Gas6 activity suggests that vitamin K also has a physiologically im-

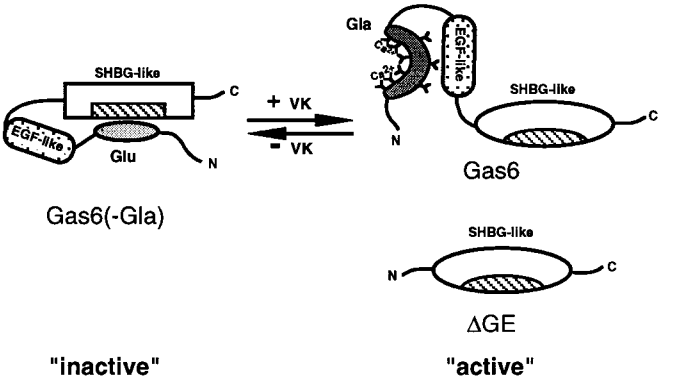

Fig. 4. A model for regulation of Gas6 activity. In Gas6(-Gla), the Gla-deficient (Glu) form of a Gla domain negatively regulates the receptor-binding and biological activities of Gas6 probably by masking the receptor-binding surface (indicated by a hatched area) within the SHBG-like domain. The vitamin K-dependent γ-carboxylation induces binding of Ca^{2+} and conformational changes, which lead to the 'active' conformation of Gas6 by exposing the receptor-binding surface of the SHBG-like domain. ΔGE mutant composed of almost only the SHBG-like domain retains receptor-binding and biological activities of Gas6.

portant role in the regulation of cell growth, survival and differentiation, through actions of Gas6. Each of four EGF-like domains in Gas6 contains a consensus sequence for β-hydroxylation of Asp and Asn residues [22], the presence of which is correlated with a high-affinity Ca^{2+}-binding site and seems to be involved in protein–protein interactions [23]. Thus, the EGF-like domains in Gas6 may play a role in stabilizing an active conformation of Gas6 or modulating its activity.

We reported that Gas6 binds to and stimulates Axl, Sky and Mer receptors [5]. The binding affinity of Mer to Gas6 was relatively low, which means that Gas6-related protein(s) may function as preferable ligands for Mer. Identification of the C-terminal SHBG-like domain, as an essential domain for Gas6 activity, provides basic information needed for studies on Gas6-related proteins.

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