Requirement of γ-carboxyglutamic acid residues for the biological activity of Gas6: contribution of endogenous Gas6 to the proliferation of vascular smooth muscle cells

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Gas6 (encoded by growth-arrest-specific gene 6) is a γ-carboxyglutamic acid (Gla)-containing protein which is released from growth-arrested vascular smooth muscle cells (VSMCs) and potentiates VSMC proliferation induced by Ca^{2+}-mobilizing growth factors, but not that induced by receptor tyrosine kinases. In this study we examined the importance of Gla residues for the biological activities of Gas6 and tried to assess the importance of endogenous Gas6 in VSMC proliferation. We demonstrated that Gla-deficient Gas6 lacked receptor-binding and growth-potentiating activities. Therefore the vitamin K-dependent modification of Gas6 appeared to be essential for its biological activities. Next we used warfarin, an inhibitor of vitamin K-dependent γ-carboxylation, to estimate the contribution of endogenous Gas6 to VSMC proliferation. Warfarin markedly inhibited the thrombin-induced proliferation of VSMC without affecting the mRNA or protein expression of Gas6. Therefore the inhibition seems to be due to prevention of the vitamin K-dependent modification of Gas6. However, warfarin did not affect epidermal growth factor-induced proliferation. A neutralizing antibody against Gas6 gave a similar result, i.e. it inhibited thrombin-induced VSMC proliferation but not that induced by epidermal growth factor. These results indicate that endogenously produced Gas6 is very important for VSMC proliferation induced by Ca^{2+}-mobilizing growth factors.

INTRODUCTION

Cell proliferation is mediated by two distinct intracellular signalling pathways [1–3]. One pathway is activated by receptors that have intrinsic protein-tyrosine kinases. This type of receptor is activated by 'classical' growth factors such as epidermal growth factor (EGF), platelet-derived growth factor and basic fibroblast growth factor. The other signalling pathway is activated by a receptor group that interacts with heterotrimeric G-proteins. Activated G-proteins stimulate phospholipase C, resulting in intracellular Ca^{2+} mobilization and activation of protein kinase C. This type of receptor is activated by several factors, such as thrombin, angiotensin II and lysophosphatidic acid.

We have reported that rat vascular smooth muscle cells (VSMCs) release a protein that specifically potentiates VSMC proliferation stimulated by effectors of the latter signalling pathway, and that this protein is encoded by *gas6*, one of the growth-arrest-specific genes [4]. Gas6, a new member of a protein family possessing γ-carboxyglutamic acid (Gla) residues, shows sequence similarity with protein S, a negative regulator of blood coagulation [4,5]. Similarly to protein S, Gas6 is composed of defined structural motifs: a Gla domain, four EGF-like repeats and a C-terminal domain [4,5]. On the other hand, it has been demonstrated that Gas6 is a ligand for the receptor tyrosine kinases Axl and Sky [6–8]. VSMCs have a high-affinity specific binding site for Gas6 on their membrane [4], suggesting that VSMCs express Axl or Sky on the cell surface. Thus Gas6 and its receptor, Axl and/or Sky, may be a new ligand/receptor system that regulates VSMC proliferation in an autocrine fashion.

Gas6 is the first Gla-containing receptor ligand that has been shown to regulate cell activity. Therefore it is important to analyse the biochemical role of the Gla residues of Gas6. In the present study we prepared Gla-deficient Gas6 in order to examine the importance of Gla residues for the activity of Gas6, and showed that Gla residues are essential for its receptor-binding and growth-potentiating activities. Next, using warfarin, an inhibitor of γ-carboxylation [9], and anti-(rat Gas6) IgG, which neutralizes the activity of rat Gas6, we investigated the contribution of Gas6 to the proliferation of VSMCs. We found that endogenously produced Gas6 is very important for VSMC proliferation induced by thrombin, but not for EGF-induced proliferation.

MATERIALS AND METHODS

Preparation of recombinant rat Gas6 and Gla-deficient Gas6

CHO cells were transfected with a rat Gas6 expression plasmid. Confluent CHO cells were cultured in protein-free culture medium PM-1000 (Eiken) in the presence of 4 μM vitamin K2. Recombinant rat Gas6 was purified from the culture medium as described elsewhere [4]. In order to prepare Gla-deficient Gas6, CHO cells expressing Gas6 were cultured for 2 days in PM-1000 in the presence of 1 μM warfarin. Purification of Gla-deficient Gas6 from the culture medium was carried out as follows. The culture medium was filtered with a 0.2 μm-pore-size filter and concentrated by ultrafiltration using a P0200 ultrafilter membrane (ADVANTEC). Approx. 1.5 litres of culture medium was concentrated to 100 ml, dialysed against 10 mM Tris/HCl, pH 8.3/0.01 % Tween 20 and applied to a column of Q-Sepharose HP (15 ml; Pharmacia) equilibrated with the same buffer. The column was eluted with a gradient of 0–0.5 M NaCl in the same buffer (260 ml) at a flow rate of 1.5 ml/min. Elution of Gla-deficient Gas6 was detected by dot-blotting using anti-Gas6 IgG. Gla-deficient Gas6 was eluted at 0.18 M NaCl (results not shown), while Gas6 containing Gla residues was eluted at 0.32 M NaCl [4]. The fractions containing Gla-deficient Gas6 were dialysed against 10 mM sodium phosphate, pH 6.8/0.01 %

Abbreviations used: EGF, epidermal growth factor; VSMC, vascular smooth muscle cell; Gas6, protein encoded by growth-arrest-specific gene 6; Gla, γ-carboxyglutamic acid; DMEM, Dulbecco's modified Eagle's medium.

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Tween 20 and applied to a hydroxyapatite column (Econo-Pac HTP cartridge, 5 ml; Bio-Rad) equilibrated with the same buffer. The column was eluted with a gradient of 10–400 mM sodium phosphate, pH 6.8/0.01 % Tween 20 (80 ml) at a flow rate of 0.5 ml/min. Gla-deficient Gas6 was eluted at 160 mM sodium phosphate (results not shown). The fractions containing Gla-deficient Gas6 were concentrated with a Centricon-50 concentrator (Amicon). The sample was then applied to two TSK gel G3,000SWXL columns (each 7.8 mm × 30 cm, linked in tandem; Tosoh). The columns were equilibrated with 10 mM sodium phosphate, pH 6.8, 0.4 M NaCl and 0.01 % Tween 20 and eluted at a flow rate of 0.5 ml/min. A major peak eluted at 32 min contained Gla-deficient Gas6 (results not shown). The absence of Gla residues from Gla-deficient Gas6 was confirmed by amino acid analysis and amino acid sequencing [4].

Culture of rat VSMCs and measurement of [^{3}H]thymidine incorporation

Rat VSMCs were isolated from the thoracic aorta of Sprague–Dawley rats and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % (v/v) fetal calf serum. All experiments were conducted using VSMCs of passage number 5–10. Analysis of the expression of smooth muscle α-actin showed that the VSMCs were in the 'synthetic' state (results not shown) [10]. Rat VSMCs were plated in 24-well tissue culture plates and cultured in DMEM containing 10 % fetal calf serum. Confluent rat VSMCs were serum-starved for 2 days in DMEM containing 0.1 % BSA and treated as described in the Figure legends. To analyse the growth-potentiating activity of Gas6, the culture medium was replaced with fresh medium to remove endogenous Gas6. VSMCs do not produce Gla-containing active Gas6 in the fresh medium because of the lack of vitamin K. The cells were then stimulated for 18 h with 0.2 unit/ml thrombin or 0.1 nM EGF, and then labelled with 0.5 μCi/well [^{3}H]thymidine (Amersham) for 2 h. Incorporation of [^{3}H]thymidine was measured as trichloroacetic acid-insoluble radioactivity in the cells.

Separation of Gas6 activity on Sephacryl S-300

Confluent rat VSMCs in 15 cm tissue culture dishes were cultured for 2 days in 25 ml of DMEM containing 0.1 % BSA in the presence of 1 μM vitamin K2 (Sigma) or 1 μM warfarin. Conditioned medium (150 ml) was filtered with a 0.2 μm-pore-size filter, concentrated to 2 ml with a Centriprep-30 (Amicon) and separated on a Sephacryl S-300 gel-filtration column (1.5 cm × 47 cm; Pharmacia Biotech Inc.). The column was equilibrated with 20 mM sodium phosphate, pH 7.4/0.5 M NaCl and eluted at a flow rate of 15 ml/h. Each 1 ml fraction was collected, and 30 μl aliquots from each fraction were assayed for growth-potentiating activity. The growth-potentiating activity in the column fractions was inhibited by anti-(rat Gas6) IgG (see below), confirming that the activity was due to Gas6 (results not shown).

Preparation of anti-(rat Gas6) IgG

Rabbit anti-(rat Gas6) IgG against rat Gas6 was obtained as previously described [4], and purified using an Ampure® PA Kit (Amersham).

Western blotting

Confluent VSMCs in 24-well tissue culture plates were cultured for 2 days in DMEM containing 0.1 % BSA in the presence or absence of 1 μM warfarin. Culture medium (1 ml) was concentrated to 0.1 ml using a Centricon-50 concentrator (Amicon). Samples (10 μl) were separated by SDS/PAGE, and immunoblotting with the anti-(rat Gas6) IgG was accomplished using a Blotting detection kit (Amersham).

RNA blotting

Confluent VSMCs in 10 cm tissue culture dishes were cultured for 2 days in DMEM containing 0.1 % BSA in the presence or absence of 1 μM warfarin. Total RNA was prepared from the cells by the method of Chomczynski and Sacchi [11]. The RNA (10 μg) was fractionated on a 1 % agarose gel containing 2.2 M formaldehyde. Hybridization of the RNA transferred to a nylon membrane was performed as described by Church and Gilbert [12], using rat Gas6 cDNA [4] labelled with [α-^{32}P]dCTP (220 TBq/mmol; Du Pont–New England Nuclear) as a probe.

Analysis of ^{125}I-Gas6 binding

^{125}I-labelled Gas6 (6000 c.p.m./fmol) was prepared by a chloramine T method as described elsewhere [13]. Confluent HOS cells in 24-well tissue culture plates were washed twice with washing buffer (1 mM Tris/HCl, pH 7.4, containing 1 mM EDTA). The cells were incubated with 0.3 nM ^{125}I-Gas6 for 2 h at 4 °C in the presence of unlabelled Gas6 or Gla-deficient Gas6 in binding medium (Hank's balanced salt solution, pH 7.4, containing 10 mM $CaCl_2$ and 0.1 % BSA). The cells were washed three times with ice-cold saline, and solubilized with 1 M NaOH. Specific binding was defined as the difference between binding in the presence and absence of 30 nM unlabelled Gas6.

RESULTS

Importance of Gla residues for the growth-potentiating activity of Gas6

In order to clarify the role of Gla residues in the biological activity of Gas6, we prepared Gla-deficient rat Gas6 and analysed the growth-potentiating activity of Gas6 and Gla-deficient Gas6. As shown in Figure 1, Gas6 potentiated thrombin-stimulated [^{3}H]thymidine incorporation with an EC_{50} value of approx.

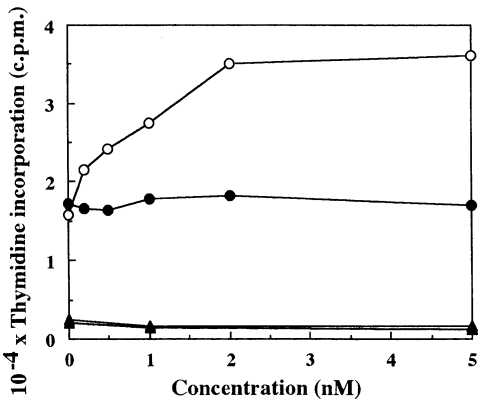

Figure 1 Growth-potentiating activity of Gla-deficient Gas6

The culture medium of serum-starved VSMCs was replaced with fresh DMEM containing 0.1 % BSA. Various concentrations of Gas6 (○, △) or Gla-deficient Gas6 (●, ▲) were added to the cells. The cells were cultured for 18 h with (○, ●) or without (▲, ▲) 0.2 unit/ml thrombin, and then labelled for 2 h with [^{3}H]thymidine.

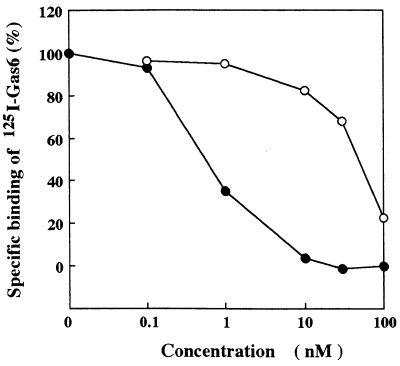

Figure 2 Receptor-binding activity of Gla-deficient Gas6

The specific binding of ^{125}I-Gas6 to HOS cells in the presence of Gas6 (●) or Gla-deficient Gas6 (○) was analysed as described in the Materials and methods section.

1 nM. However, Gla-deficient Gas6 did not have growth-potentiating activity, indicating that Gla residues are required for the growth-potentiating activity of Gas6.

Importance of Gla residues for receptor binding of Gas6

We have reported that HOS cells have the greatest binding capacity for ^{125}I-Gas6 among the cell types tested [14]. We next analysed the receptor-binding ability of Gla-deficient Gas6 using HOS cells. Figure 2 shows that Gas6 inhibited binding of ^{125}I-Gas6 with an IC_{50} value of approx. 0.6 nM, whereas a 100-times higher concentration of Gla-deficient Gas6 was necessary to inhibit binding. These results indicate that Gla residues in Gas6 are very important for both the receptor-binding and growth-potentiation activities of Gas6.

Inhibition of VSMC proliferation by warfarin

We have reported that Gas6 is released from VSMCs and potentiates the proliferation of these cells [4]. However, it is not clear how much VSMC proliferation is dependent on endogenous Gas6. In order to estimate the contribution of Gas6 to VSMC proliferation, we used warfarin, an inhibitor of γ-carboxylation, to block the biological activity of Gas6. Confluent rat VSMCs were serum-starved for 2 days in DMEM containing 0.1 % BSA. During the serum starvation, the cells were treated with various concentrations of warfarin. The cells were then stimulated with 0.2 unit/ml thrombin, and thymidine incorporation was measured. As shown in Figure 3(A), warfarin treatment dose-dependently inhibited thrombin-induced thymidine incorporation. Figure 3(B) shows that the inhibitory effect of warfarin (1 μM) could be overcome by the simultaneous addition of vitamin K, suggesting that the inhibition was due to the specific effect of warfarin on vitamin K-dependent responses.

Suppression of Gas6 activity in VSMC-conditioned medium by warfarin

Next we examined whether warfarin affected the function of VSMCs themselves or the Gas6 activity in the culture medium. When the culture medium of warfarin-treated VSMCs was replaced with a conditioned medium from VSMCs not treated with warfarin, the inhibitory effect of warfarin disappeared (Figure 3A). Moreover, when warfarin was added simultaneously with thrombin to non-warfarin-treated VSMCs, it did not affect thymidine incorporation (results not shown). These results suggest that warfarin does not inhibit the function of VSMCs directly, but suppresses the production of active Gas6 in the VSMC culture medium.

To characterize the Gas6 activity further, we separated the Gas6 activity in VSMC culture medium on a column of Sephacryl S-300. As we reported previously, Gas6 activity in the VSMC-conditioned medium, assayed for potentiation of thrombin-induced thymidine incorporation, was eluted before the main

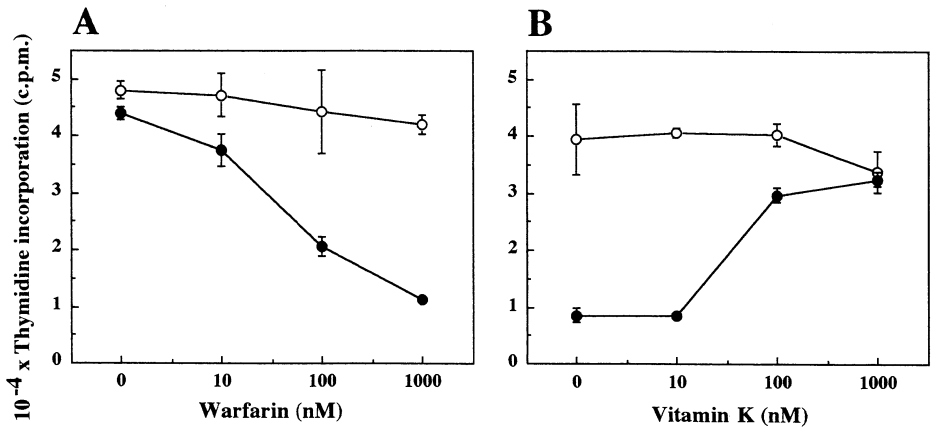

Figure 3 Effects of warfarin and vitamin K on VSMC proliferation

Confluent VSMCs were serum-starved for 2 days in DMEM containing 0.1 % BSA in the presence of (**A**) various concentrations of warfarin (●) or (**B**) various concentrations of vitamin K2 with (●) or without (○) 1 μM warfarin. In (**A**), the culture medium of VSMCs that had been serum-starved in the presence of various concentrations of warfarin was replaced with the culture medium of serum-starved VSMCs not treated with warfarin (○). The cells were stimulated with 0.2 unit/ml thrombin for 18 h and labelled for 2 h with [^{3}H]thymidine. Results are means $\pm$ S.D. ($n = 4$).

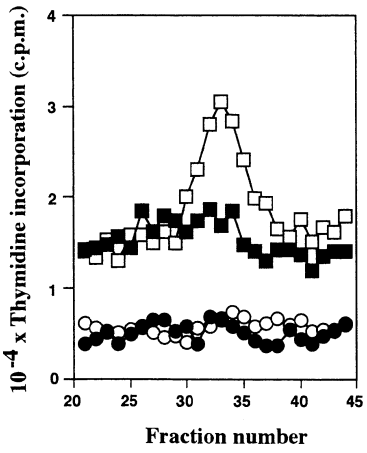

Figure 4 Separation of Gas6 activity in VSMC culture medium

VSMCs were cultured for 2 days in DMEM containing 0.1% BSA in the presence of 1 μM vitamin K2 (○, □) or 1 μM warfarin (●, ■). The culture medium was concentrated and separated on a column of Sephacryl S-300, and the growth-potentiating activity of each fraction was assayed as described in the Materials and methods section. Thymidine incorporation was assayed in the presence (□, ■) or absence (○, ●) of 0.2 unit/ml thrombin.

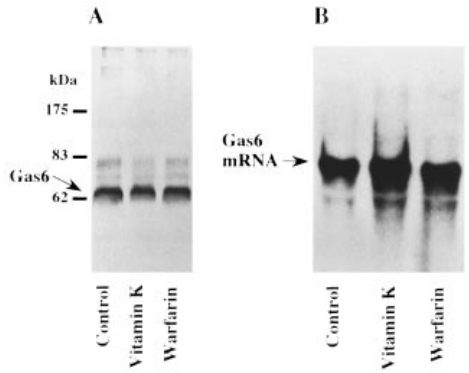

Figure 5 Effects of warfarin and vitamin K on the production of Gas6 protein and mRNA

Confluent VSMCs were cultured for 2 days in DMEM containing 0.1% BSA in the presence of 1 μM warfarin or 1 μM vitamin K2. Gas6 protein in the culture medium (**A**) and Gas6 mRNA in the cells (**B**) were analysed as described in the Materials and methods section.

peak of protein [4]. Figure 4 shows that the growth-potentiating activity in VSMC culture medium was eluted as a peak at fraction no. 33. However, this activity was not detected when the culture medium of warfarin-treated VSMCs was separated on the column (Figure 4). Therefore warfarin-treated VSMCs did not seem to release biologically active Gas6 into the culture medium.

Warfarin does not inhibit Gas6 protein production

We analysed the production of Gas6 protein by Western blotting using anti-Gas6 IgG. Although Gas6 activity in the culture medium of warfarin-treated VSMCs was suppressed, the protein level of Gas6 in the culture medium was not affected by warfarin treatment (Figure 5A). Figure 5(B) shows that warfarin treatment also did not affect the Gas6 mRNA level in VSMCs. These results demonstrate that warfarin does not inhibit the production of Gas6 protein, although Gas6 activity in the culture medium of warfarin-treated VSMCs is suppressed; the latter effect is probably due to insufficient γ-carboxylation of Gas6.

Specificity of the inhibitory effect of warfarin

Since Gas6 specifically potentiates VSMC proliferation induced by Ca^{2+}-mobilizing growth factors such as thrombin, angiotensin II and lysophosphatidic acid [4], we examined the specificity of warfarin, using thrombin and EGF as representatives of the two types of growth factors for VSMC proliferation. Figure 6 shows the dose–response curves for VSMC proliferation induced by thrombin and EGF. Treatment of the cells with 1 μM warfarin suppressed thrombin-induced thymidine incorporation to a substantial degree without shifting the curve (Figure 6A), but did not affect the EGF-induced response (Figure 6B). Proliferation induced by angiotensin II or lysophosphatidic acid was also inhibited by warfarin (results not shown). Therefore the inhibitory effect of warfarin appears to be specific to VSMC proliferation induced by Ca^{2+}-mobilizing growth factors.

Effect of purified Gas6 on the inhibitory effect of warfarin

When 3 nM Gas6, a concentration giving the maximal response for Gas6 [4], was added to warfarin-treated cells simultaneously with thrombin, the inhibitory effect of warfarin was abolished (Figure 6A), suggesting that the inhibition was mainly due to the suppression of Gas6 activity in the culture medium. On the other hand, VSMC proliferation induced by EGF was not affected by Gas6 (Figure 6B).

Specificity of the inhibitory effect of the anti-Gas6 IgG

As a second approach to examining the contribution of Gas6 to the proliferation of VSMCs, we used a neutralizing antibody against rat Gas6. Figure 7 shows that rabbit anti-(rat Gas6) IgG inhibited the activity of Gas6, i.e. the potentiation of thrombin-induced thymidine incorporation, while normal rabbit IgG did not (results not shown). On the other hand, anti-Gas6 IgG did not inhibit thrombin-induced thymidine incorporation in the absence of Gas6 or thymidine incorporation in the absence of thrombin. These results indicate that the anti-Gas6 IgG specifically inhibited the activity of Gas6. In order to estimate the contribution of endogenous Gas6 to VSMC proliferation, serum-starved VSMCs were stimulated with various concentrations of thrombin (Figure 8A) or EGF (Figure 8B) in the presence or absence of 6 μg/ml anti-Gas6 IgG. Anti-Gas6 IgG markedly suppressed thrombin-induced VSMC proliferation; however, EGF-induced proliferation was unaffected.

DISCUSSION

We purified Gas6 from the culture medium of VSMCs as a growth-potentiating factor for these cells [4]. Thus Gas6 is thought to possess some autocrine role in VSMC proliferation, but the extent of its importance has not been clear. One of the purposes of the present study was to clarify the biological importance of Gas6 in VSMC proliferation. Using warfarin and anti-Gas6 IgG, we demonstrated that endogenous Gas6 is almost essential for the proliferation of VSMCs stimulated by Ca^{2+}-mobilizing growth factors such as thrombin. However, since warfarin or anti-Gas6 IgG did not inhibit EGF-induced VSMC proliferation, Gas6 does not appear to be necessary for VSMC proliferation induced by growth factors that activate receptor tyrosine kinases.

Recently, Gas6 was identified as a ligand for the receptor tyrosine kinases Axl and Sky [6–8]. Members of the Axl/Sky

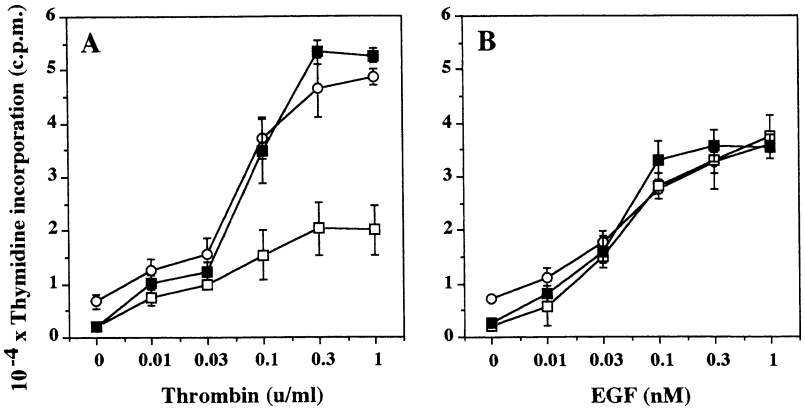

Figure 6 Specific effect of warfarin on thrombin-induced VSMC proliferation

Confluent VSMCs were cultured for 2 days in DMEM containing 0.1% BSA in the presence (○, □) or absence (■) of 1 μM warfarin. The cells were stimulated for 18 h with various concentrations of thrombin (units/ml) (**A**) or EGF (**B**) in the presence (○) or absence (□, ■) of 3 nM Gas6, and then labelled for 2 h with [^{3}H]thymidine. Results are means ± S.D. ($n = 4$).

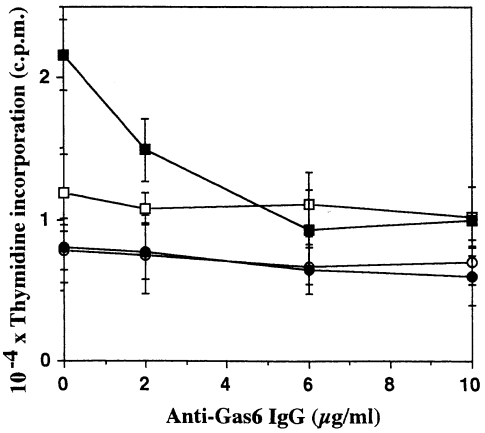

Figure 7 Neutralization of Gas6 activity with anti-Gas6 IgG

Confluent VSMCs were cultured for 2 days in DMEM containing 0.1% BSA. Various concentrations of anti-Gas6 IgG were added to the cells. The cells were stimulated for 18 h with (□, ■) or without (○, ●) 0.2 unit/ml thrombin in the presence (■, ●) or absence (□, ○) of 3 nM Gas6. Results are means ± S.D. ($n = 4$).

receptor subfamily, including Axl (also called Ufo and Ark) [15–17], Sky (Rse, Brt and Tyro3) [18–22] and c-Eyk (Mer) [23,24], contain a characteristic extracellular ligand-binding domain composed of two immunoglobulin-like motifs and two fibronectin type III motifs. It has been reported that Sky transcripts are predominantly expressed in the brain [18–22], while Axl mRNA is nearly ubiquitously expressed [15–17]. Goruppi et al. [25] reported the interaction of Gas6 with Axl on NIH-3T3 fibroblasts. We detected Axl mRNA in rat VSMCs (T. Nakano, J. Kishino and K. Nomura, unpublished work), suggesting that Gas6 also activates the tyrosine kinase Axl in VSMCs.

Another objective of the present study was to clarify the importance of Gla residues for the functioning of Gas6. Gla residues are found in some proteins that regulate blood coagulation, such as factors VII, IX and X, prothrombin, protein C and protein S. It has been demonstrated that the importance of the Gla domain in these proteins lies in its ability to interact with Ca^{2+} and phospholipids [26–30]. Since Gas6 is the first receptor ligand shown to contain Gla residues, it is important to clarify the role of these Gla residues in the receptor-binding activity of Gas6. We have reported that the interaction of Gas6 with its receptor requires Ca^{2+}, suggesting the involvement of Gla residues in Gas6 receptor binding [14]. To obtain direct evidence demonstrating the importance of the Gla residues, we prepared Gla-deficient Gas6 by inhibiting vitamin K-dependent γ-carboxylation. As shown in Figures 1 and 2, Gla-deficient Gas6 showed poor receptor-binding activity and growth-potentiating activity. Therefore we concluded that Gla residues are very important for the function of Gas6. However, Mark et al. [31] recently reported that a deletion variant of Gas6 lacking the Gla domain bound and activated Sky and Axl, suggesting that the receptor-binding site is in the C-terminal domain. In conclusion, these observations suggest that the absence of the entire Gla domain fixes the conformation of the receptor-binding site in the active form [31], that the presence of a 'Gla domain' lacking γ-carboxylation, as in the present study, fixes the conformation in the inactive form, and that the presence of a γ-carboxylated Gla domain changes the conformation of the binding site to the active form in the presence of Ca^{2+} and to the inactive form in the absence of Ca^{2+} [14]. Therefore the Gla domain in Gas6 may act as a regulatory domain.

Both our group [32] and Goruppi et al. [25] have reported that Gas6 also possesses an activity that prevents the growth-arrest-induced cell death of VSMCs and fibroblasts. When endogenous Gas6 was removed, growth-arrested VSMCs died, while addition of Gas6 prevented cell death [32]. Therefore Gas6 may be important for both the maintenance of growth-arrested VSMCs and the proliferation of growing VSMCs. The possible involvement of Gas6 in the *in vitro* activity of VSMCs suggests that it contributes to the development and maintenance of the vascular wall, as well as to the process of vascular disease. Further *in vivo* studies are needed to clarify the physiological importance of Gas6. It is also noteworthy that vitamin K is necessary for Gas6 to exert these activities. Such a contribution to the maintenance and proliferation of VSMCs may be a new biological function of vitamin K, which is another subject that needs to be studied *in vivo*.

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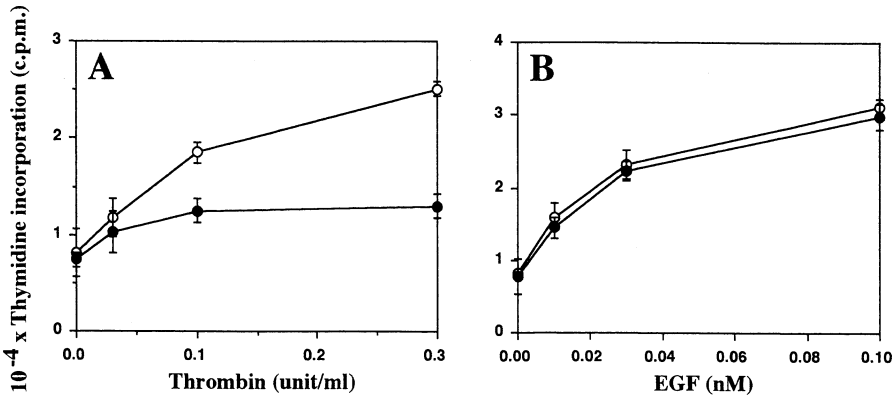

Figure 8 Specific effect of anti-Gas6 IgG on thrombin-induced VSMC proliferation

Confluent VSMCs were cultured for 2 days in DMEM containing 0.1% BSA. The cells were stimulated for 18 h with various concentrations of thrombin (**A**) or EGF (**B**) in the presence (●) or absence (○) of 6 μg/ml anti-Gas6 IgG, and then labelled for 2 h with [^{3}H]thymidine. Results are means ± S.D. ($n = 4$).

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