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Gas6-mediated signaling is dependent on the engagement of its gamma-carboxyglutamic acid domain with phosphatidylserine ☆

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ABSTRACT

Gas6 is a vitamin K-dependent protein containing gamma-carboxyglutamic acid (Gla) at its N-terminus and a receptor binding domain at its C-terminus. Gas6–Axl binding is necessary but not sufficient to support endothelial cell survival as decarboxylated gas6 inhibits the pro-survival function of gas6 by binding and inhibiting Axl, even though decarboxylated gas6 cannot support endothelial cell survival itself. It is hypothesized that interactions between the Gla domain of gas6 and phosphatidylserine (PS), though not required for gas6 binding to Axl, are necessary for gas6–Axl function. In support of this hypothesis are results showing that (1) two specific inhibitors of Gla–PS interactions, namely soluble PS and Annexin V, abrogate gas6-mediated endothelial cell survival and (2) Soluble PS inhibits Akt activation, a downstream intracellular event triggered by gas6–Axl binding. In conclusion, we propose a heretofore unknown function of Gla, where Gla–PS binding on the N-terminus of gas6 is necessary for a gas6 function mediated through its binding to Axl via its C-terminus.

Gamma-carboxylation (Gla) is a post-translational modification whereby a second carboxyl group is added to glutamic acid. To-date, the function of Gla is to allow soluble enzyme and substrates of the blood coagulation cascade bind to phosphatidylserine (PS)-containing phospholipid membranes on activated platelets, where these enzymatic reactions can proceed at physiologically relevant rates to generate a fibrin clot [1]. Its importance is highlighted by the clinical use of warfarin, an inhibitor of gamma-carboxylation and effective oral anticoagulant [2].

Gas6 is a member of the vitamin K-dependent family of Gla proteins. Although homologous to the blood coagulation protein, Protein S, it does not possess any activity in the generation of thrombin [3]. Rather, it is a ligand for a series of receptor tyrosine kinases of the Axl family [4]. The role of gas6 has been studied in many different tissues where it can function as a survival factor [5], stimulate mitogenesis [6], and activate platelets, thereby stabilizing thrombus formation [7].

Gas6 is comprised of six protein domains which include, from N to C terminus, a gamma-carboxyglutamic acid (Gla) containing domain, four epidermal growth factor-like domains and a C-terminal steroid hormone binding globulin-like domain. Like other vitamin K-dependent proteins, gas6 contains several Gla residues at its N-terminus [3]. Gas6 is a soluble ligand for a series of receptor tyrosine kinases of the TAM family that include Axl, Mer, and Rse [8]. The receptor-binding domain of gas6 lies in the C-terminal sex hormone binding-like domain [9]. One proposed function of Gla in gas6 is to bridge PS exposed on apoptotic cells to immune effector cells such as macrophages that contain receptor tyrosine kinases. The experiments described in this paper propose a novel functional role of Gla in gas6-mediated endothelial cell survival.

Materials and methods

Materials. Purified recombinant Annexin V was purchased from BD Biosciences (Mississauga, Canada). Soluble phosphatidylserine was obtained from Sigma (Mississauga, Canada).

Generation of polyclonal anti-Axl extracellular domain antibodies. A polyclonal antibody to the extracellular domain of Axl was produced in rabbits through the Pocono Rabbit Farm (Canadensis, PA) using the keyhole limpet hemocyanin-conjugated peptide (KELADSTQTQVPLGEDEQD). The peptide was synthesized at the Sheldon Biotechnology Center at McGill University (Montreal, Canada).

Production of human recombinant gas6. Recombinant human carboxylated and uncarboxylated gas6 were produced and purified as previously described [10].

Fluorescein-labelled gas6. Recombinant human carboxylated and uncarboxylated gas6 were labelled with fluorescein-5-maleimide (Molecular Probes, Mississauga, Canada). Briefly, approximately 500 μg of protein prepared in 50 mM Hepes, pH 8.0 (BioShop

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Canada, Burlington, Canada), 150 mM NaCl (BioShop Canada) were incubated for 24–48 h at 4 °C with fluorescein-5-maleimide (final concentration of 0.5 mM) and protected from light. The free fluorescein-5-maleimide was removed by gel filtration with a NAP-5 column (GE Healthcare Piscataway, NJ, USA) according to the manufacturer's instructions).

Human umbilical vein endothelial cells. Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords as previously described [10] and used between passages three and six.

Flow cytometry. Detection and quantification of apoptosis in HUVECs was done by flow cytometry (Becton Dickinson, Mississauga, Canada) after staining the cells with propidium iodide (Sigma, Mississauga, Canada) and enumerating the cells in the sub-G1 fraction [10]. Binding of fluorescently labelled gas6 to HUVECs was monitored by flow cytometry, after incubation with fluorescein labelled-gas6 and propidium iodide staining as previously described [11].

Western blot analysis. Human umbilical vein endothelial cells were plated at a density of 1×10^6 in 100 mm dishes. Twenty hours later they were washed twice with PBS (Wisent, St.-Bruno, Que., Canada) and placed in fresh EBM-2 (serum deplete) or EGM-2 (serum replete) media (Lonza, Walkersville, MD, USA) as indicated, for 6 h. Cells were then treated for 10 min with 100 ng/ml recombinant human carboxylated gas6, soluble PS (*O*-phospho-l-serine, Sigma), or a combination of both after a 10-min incubation together. Cells were then washed twice with cold PBS, lysed by the addition of 300 μl lysis buffer [50 mM tris–HCl, 150 mM NaCl, 1% Igepal, 10 mM NaF, 0.1 mM EDTA, 1 mM DTT, 1 mM Na_3VO_4, and Complete Protease Inhibitor Cocktail (Roche, Laval, Que., Canada)]. Following a 30-min incubation at 4 °C, cells were scraped and centrifuged at 10,000*g* at 4 °C for 20 min. Samples were assayed for total protein content and 100 μg of protein were separated by SDS–PAGE on a 7.5% gel. The gel was subsequently immunoblotted to nitrocellulose and AKT phosphorylation, as well as total AKT were detected using rabbit polyclonal anti-phospho AKT and anti-AKT antibodies purchased from Cell Signalling technologies, Beverly, MA, USA).

Results

The N-terminal domain of gas6 contains Gla and the receptor binding portion resides in the C-terminal steroid hormone binding globulin-like domain [9]. We have previously shown that decarboxylated gas6 cannot support endothelial cell survival [10] although the mechanism for this loss of function is not clear. To determine whether the Gla domain impacts the binding of gas6 to endothelial cells via the C-terminal receptor (Axl) binding site of gas6, the Gla-domain requirement for binding of gas6 to HUVECS was examined. In the experiment shown in Fig. 1, binding of fluorescein-labelled gas6 and decarboxylated gas6 was determined. As shown, the binding of both forms is similar; apparent affinities of carboxylated and decarboxylated gas6 are 16 nM and 20.2 nM, respectively. However, it is observed that increasing concentrations of decarboxylated gas6 progressively diminish the prosurvival effect of gas6 as shown in Fig. 2. In fact, the reduction in the prosurvival effect seen with 100 ng/ml gas6 is reduced approximately 50% by addition of 100 ng/ml decarboxylated gas6, presumably through direct inhibition of gas6–Axl interactions.

In order to further confirm that Gla binding does not alter gas6 binding to Axl even though it is required for the prosurvival function of gas6, we compared the ability of antibodies to the extracellular ligand-binding domain of Axl (anti-Axl-ECD) to block the binding of decarboxylated compared to carboxylated gas6. HUVECs were first incubated with either pre-immune rabbit serum or anti-

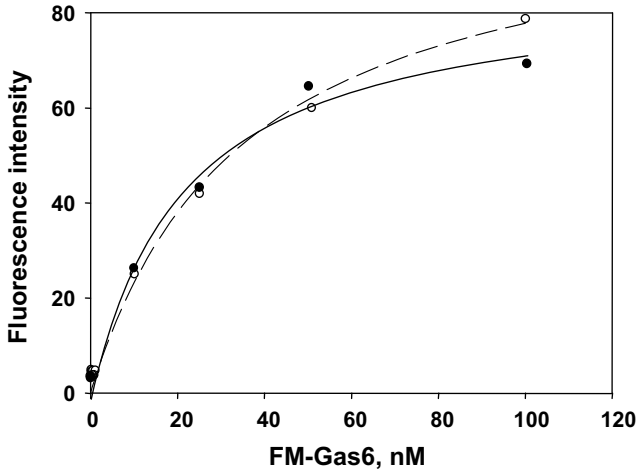

Fig. 1. Binding of fluorescein-labelled gas6 to HUVECs. HUVECs were incubated with increasing amounts of fluorescein-labelled carboxylated gas6 (●) or fluorescein-labelled decarboxylated gas6 (○). The binding was measured by flow cytometry. The data was fitted by non-linear regression analysis to a bi-molecular model and the following dissociation constants were calculated: carboxylated gas6, 16.2 ± 9.5 nM, decarboxylated gas6, 20.2 ± 12.4 nM. Data shown are representative of five independent experiments.

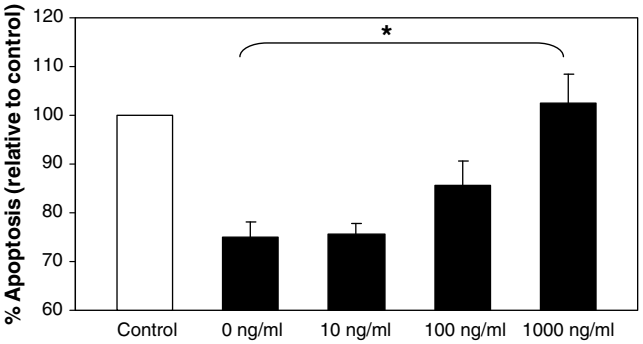

Fig. 2. Decarboxylated gas6 (□) inhibits the protective effect of carboxylated gas6. HUVECs were serum starved in the absence of gas6 or in the presence of 100 ng/ml carboxylated gas6 (■) with the indicated increasing amounts of decarboxylated gas6. The percentage of cells undergoing apoptosis was measured by flow cytometry using sub-G1 analysis. Data shown are mean values ± SEM of three independent experiments ($^*p < 0.02$).

Axl-ECD, and then with 25 nM of either fluorescein-labelled carboxylated gas6 or fluorescein-labelled decarboxylated gas6. As shown in Fig. 3, the decreases in binding of both carboxylated and decarboxylated gas6 to anti-Axl treated HUVECs are inhibited similarly. Overall, these data suggest that the carboxylation state of gas6 does not directly affect the binding of gas6 to Axl and that other mechanisms underlie the inability of decarboxylated gas6 to mediate endothelial cell survival. It is therefore hypothesized that N-terminal Gla-dependent binding to membrane phospholipids impacts the function of gas6.

To determine whether the membrane-bound N-terminal Gla domain of gas6 is required for downstream cell survival events affected by the gas6–Axl interactions, the effect of specifically inhibiting Gla–PS interactions was tested as follows: Surface exposed PS was blocked by the addition of Annexin V, a recently discovered protein that specifically binds PS in a calcium-dependent manner [12]. HUVECs were induced to undergo apoptosis (Fig. 4A) in the absence of gas6, presence of gas6 or presence of gas6 plus annexin V. Results show that, in the presence of annexin V, gas6 is unable to protect endothelial cells from apoptosis, supporting the notion that

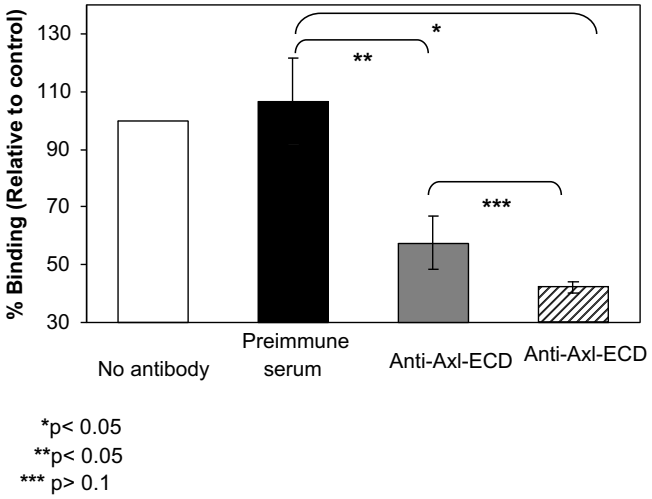

Fig. 3. Binding of carboxylated gas6 and decarboxylated gas6 to HUVECs in the presence of an Axl-ECD antibody. HUVECs were incubated with 25 nM fluorescently labelled carboxylated gas6 in the presence of no antibody (□), preimmune rabbit serum (■), or a polyclonal antibody to the extracellular domain of Axl [Axl-ECD] (▩). Decarboxylated gas6 was incubated with the Axl-ECD antibody (▨). Binding was measured by flow cytometry as described in Material and methods. Data shown are means ± SEM of four independent experiments.

Gla domain–PS interactions are necessary for gas6-mediated survival of endothelium. In another series of experiments, a soluble form of PS was used to specifically block the Gla domain of gas6. Soluble PS comprises the PS head group with a short six carbon acyl chain, is soluble and can support blood coagulation reactions in vitro [13,14]. HUVECs were induced to undergo apoptosis in the presence or absence of carboxylated gas6 in the absence and presence of soluble PS. As shown in Fig. 4B, gas6 is unable to exert its protective effect when Gla domain–PS interactions are blocked using soluble PS.

To gain insight into the question of whether Gla–PS interactions are required for, at least, initial downstream signalling events that underlie the protection of endothelial cells from apoptosis mediated by gas6-receptor binding, we examined Akt activation by gas6. Akt is an intracellular signalling mediator commonly phosphorylated/activated during cell survival and, as we showed previously, Akt becomes phosphorylated following gas6 treatment [5]. Therefore, we examined Akt phosphorylation during gas6 rescue of apoptotic cells. This experiment (Fig. 4C) was carried out in the absence and in the presence of soluble PS. As shown, soluble PS inhibits Akt phosphorylation providing support for the conclusion that Gla–PS interactions are required for proper gas6-mediated signalling in endothelial cells.

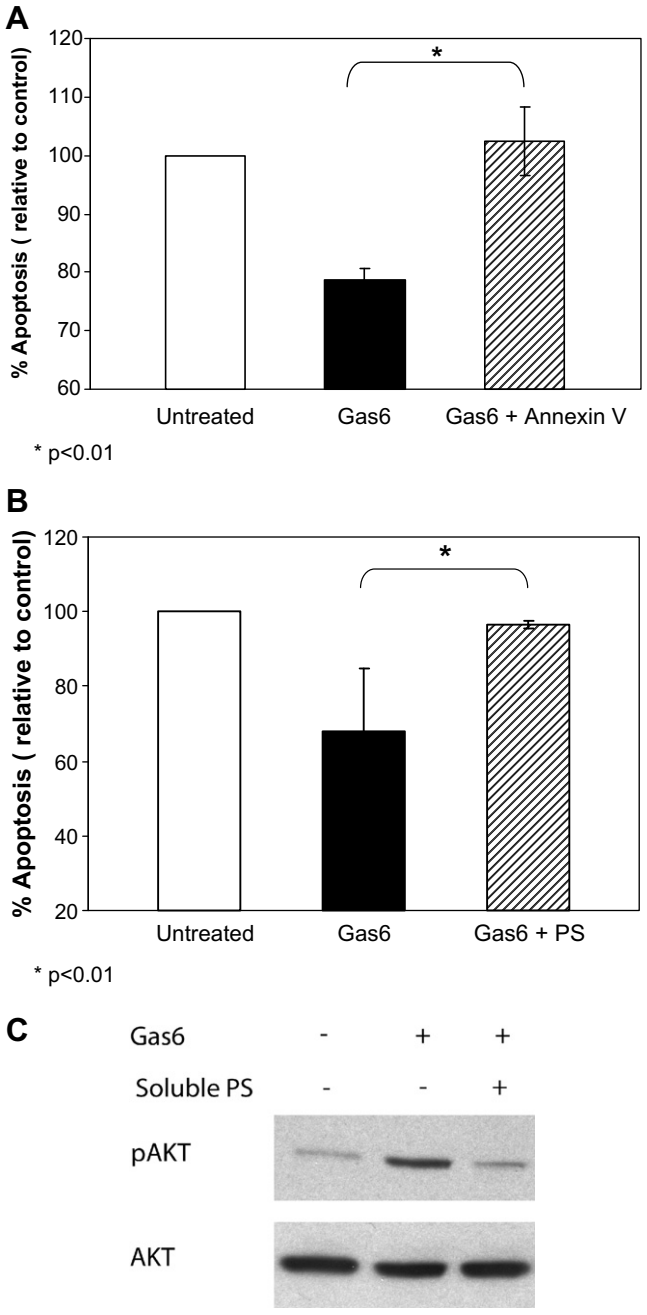

Fig. 4. The survival effect of gas6 is abrogated by specifically inhibiting Gla–PS interactions. (A) HUVECs were induced to undergo apoptosis by serum starvation in the absence (□) or presence of 100 ng/ml gas6 (■) or in the presence of 100 ng/ml gas6 and 5 μg/ml annexin V (▩). The percentage of cells undergoing apoptosis was measured by flow cytometry. Data shown are means ± SEM of four independent experiments. ($^{*}p < 0.01$). (B) HUVECs were induced to undergo apoptosis by serum starvation in the absence (□) or presence (■) of 100 ng/ml gas6 or in the presence of 100 ng/ml gas6 and 500 μM soluble L-PS (▨). The percentage of cells undergoing apoptosis was measured by flow cytometry. Data shown are means ± SEM of three independent experiments ($^{*}p < 0.01$). (C) HUVECs were serum starved for 6 h followed by a 10-min treatment with carboxylated gas6 with or without soluble PS as indicated in the Methods. Cell lysates were subjected to SDS–PAGE followed by Western blotting with antibodies to phosphoAkt and total Akt. Figure shown is representative of three separate experiments.

Discussion

Gamma-carboxylation is a post-translational modification that has primarily been described in soluble blood coagulation proteins. This modification renders the side chain of glutamic acid residues highly negatively charged and capable of binding divalent cations such as calcium [15]. Properly gamma-carboxylated blood coagulation proteins bind to negatively charged phospholipid membranes containing phosphatidylserine (PS), usually on platelets, where they can generate fibrin at physiologically relevant rates [16]. However, the role of gamma-carboxylation in proteins not involved in blood coagulation has not been completely elucidated.

We have previously shown that proper gamma-carboxylation of gas6 is necessary for its anti-apoptotic effects. We have also previously demonstrated that PS-containing liposomes bind to gas6 through its Gla domain [10]. Nakano et al. describe the requirement of the Gla domain of gas6 for the binding of U937 cells to PS-coated ELISA plates [17] and Ishimoto et al. demonstrated that uptake of PS liposomes by macrophages is mediated by gas6 [18]. In addition, there are also suggestions that gamma-carboxylation

plays a role in the functional properties of gas6. Tanabe et al. have demonstrated that decarboxylated gas6 cannot stimulate CHO cells expressing Axl [19]. In addition, the Gla domain of gas6 is indispensable for its mitogenic activity in mesangial cells [20,21] and for cardiac fibroblast proliferation [22]. Finally, the mitogenic effect in mesangial cells can be inhibited by warfarin, an inhibitor of gamma-carboxylation [20]. The experiments described in this study further confirm the importance of gamma-carboxylation for the anti-apoptotic function of gas6 and specifically define the requirement for Gla–PS interactions for the functional survival properties of gas6 itself.

What is not known, however, is how the Gla domain imparts functionally relevant prosurvival properties. The molecular detail of Gla domains in blood coagulation proteins has been well characterized and describes a highly ordered compact tertiary structure in the presence of calcium ions [15,23]. In the present study, both carboxylated and decarboxylated gas6 bind similarly to Axl on endothelium (Fig. 2) implying that the Gla domain per se does not influence gas6–Axl binding. Two mechanisms may account for the lack of activation. First, receptor dimerization, a common signaling motif for receptor tyrosine kinase activation [24], may not occur as a disordered Gla domain could prevent the apposition of two receptors. Alternatively, the disordered Gla domain within gas6 may itself prevent activation. Regardless of the mechanism, the present findings clearly identify Gla domain–PS interactions as being necessary for the biologic effects of gas6.

PS usually resides in the inner membrane of cellular membranes and has been shown to be a docking site and activator for protein kinase C [25–27], an important regulator of different intracellular signalling pathways. The role of PS on the outer membrane leaflet is not clear, especially in the binding of ligands to transmembrane receptors. The data in this paper suggest that translocation of PS to the outer membrane, where it can bind soluble ligands such as gas6, has a role in mediating gas6's prosurvival effects. Ongoing experiments in our laboratory are being carried out to address this aspect of gas6-membrane interactions.

In conclusion, we show that Gla–PS interactions comprise an essential step in the cellular events that lead to cell survival. This observation is significant for two reasons. First, it provides new insights into the structural basis of important functionally productive receptor–ligand interactions and, second, suggests a possible use of the commonly prescribed anticoagulant, warfarin, as a modulator of cell growth and survival.

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