Communication

Cell Adhesion to Phosphatidylserine Mediated by a Product of Growth Arrest-specific Gene 6*

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Gas6, a product of a growth arrest-specific gene 6, potentiates proliferation of vascular smooth muscle cells and prevents cell death of vascular smooth muscle cells. It has been also demonstrated that Gas6 is a ligand of receptor tyrosine kinases Axl, Sky, and Mer. Gas6 contains γ-carboxyglutamic acid residues, which are found in some blood coagulation factors and mediate the interaction of the coagulation factors with negatively charged phospholipid. In this study, we clarified that Gas6 specifically bound to phosphatidylserine and the binding was dependent on Ca2⁺.

Materials and Methods

**MATERIALS AND METHODS**

Phospholipids—Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and PS were purchased from Avanti.

Preparation of Recombinant Rat Gas6 and Gla-deficient Gas6—CHO cells were transfected with rat Gas6 expression plasmid. Confluent CHO cells were cultured in protein-free culture medium PM-1000 (Eiken, Japan) in the presence of 4 μM vitamin K₃. Recombinant rat Gas6 was purified from the culture medium as described elsewhere (2). To prepare Gla-deficient Gas6, the CHO cells were cultured in PM-1000 in the presence of 1 μM warfarin. The Gla-deficient Gas6 was prepared as described elsewhere (20).

Preparation of Axl-Fc Fusion Protein—cDNA fragment coding for the extracellular domain of human Axl (amino acid residues 1–438) was ligated in-frame with the polymerase chain reaction-amplified cDNA fragment of the Fc region (residues 216–443) of human IgG₄, with the spacer sequence Ser-Ser-Val-Pro-Gly. The fused cDNA was subcloned into PUC-SRα expression vector and transfected into COS-7 cells, using a liposome method. The serum-free conditioned medium of COS cells was collected for 3 days, and the Axl-Fc was purified with Protein A-Sepharose (Pharmacia Biotech Inc.).

Binding of Gas6 to Phospholipids—The binding of Gas6 to phospholipids was examined by enzyme-linked immunosorbent assay (ELISA)

Gla, γ-carboxyglutamic acid; PS, phosphatidylserine; PC, phosphatidylethanolamine; PE, phosphatidylinositol; PI, phosphatidylinositol; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

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† The abbreviations used are: VSMC, vascular smooth muscle cell;
Phospholipids were dissolved in ice-cold ethanol (2 μg/ml unless otherwise described), and 100 μl of the individual phospholipids was used to coat each well of the ELISA plate (Corning). Wells coated with ethanol alone were used as a control. The wells were kept at 25 °C for 18 h to evaporate the ethanol and blocked by incubation of the phospholipid-coated wells with Tris-buffered saline (TBS) (10 mM Tris-HCl, pH 7.4, 150 mM NaCl containing 3% bovine serum albumin (BSA)) for 1 h at room temperature. Then, the wells were washed with TBS containing 0.05% Tween 20 and incubated with Gas6 or Gla-deficient Gas6 in TBS containing 5 mM CaCl₂ unless otherwise described. The binding of Gas6 or Gla-deficient Gas6 was determined using rabbit anti-rat Gas6 IgG and peroxidase-conjugated anti-rabbit IgG (Chemicon International Inc.). The anti-Gas6 IgG detected Gas6 and Gla-deficient Gas6 equally on ELISA and Western blotting (data not shown).

The binding of Gas6 to PS is very firm. EDTA, PS, or a high concentration of salt, which all inhibited the binding, did not dissociate bound Gas6 from the PS-coated plate. Only a strong solubilizing reagent, such as SDS, guanidine HCl, or urea, dissociated the bound Gas6 (data not shown).

**BIAcore Analysis—Axl-Fc fusion protein (0.7 μg) was immobilized to the carboxymethyl dextran layer of a CM5 sensor chip on a BIAcore instrument (Pharmacia), using procedures described in the manufacturer’s manual. Gas6 was dissolved in TBS containing 0.1% BSA and 2 mM CaCl₂ with or without phospholipids and passed over the immobilized Axl-Fc at a flow rate of 2 μl/min for 20 min. The binding of the ligand with Axl-Fc was monitored in real time by the increase in the relative resonance unit on the sensorgram. The apparent association ligand with Axl-Fc was monitored in real time by the increase in the relative resonance unit on the sensorgram. The apparent association rate constant (ka) and dissociation rate constants (kd) were calculated using the manufacturer’s software. The equilibrium dissociation constant (Kd) was calculated as kd/ka.

**Adhesion of U937 Cells to PS—ELISA plates were coated with phospholipids (0.5 μg/well) as described above. The wells were blocked with 3% BSA in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.3 mM KH₂PO₄, pH 7.4) for 1 h and washed with PBS containing 0.05% BSA. The wells were filled with PBS, 0.05% BSA containing U937 cells (1 × 10⁵ cells/well) and various concentrations of Gas6 and CaCl₂. The plate was sealed with a transparent film and incubated for 30 min at 37 °C. Then, the plate was inverted and incubated for 15 min. During the incubation, unbound cells came off the plate. The cells on the plate were observed with a microscope, and adherent cells in a field were counted.

**RESULTS AND DISCUSSION**

First, we investigated the interaction of Gas6 with phospholipids. The wells on the ELISA plate were coated with various concentrations of phospholipids, including acidic phospholipids (PS and PI) and neutral phospholipids (PC and PE). The wells were incubated with Gas6, and Gas6 bound to the wells was detected with ELISA using anti-Gas6 IgG. As shown in Fig. 1A, Gas6 interacted with PS but not with PC, PE, or PI. Fig. 1A also demonstrates that the binding was dependent on the concentration of PS, with half-maximal binding occurring at approximately 60 ng/well. Since interaction of some Gla-containing coagulation factors with phospholipids is dependent on Ca²⁺, we examined the Ca²⁺ dependence of the interaction of Gas6 with PS. As shown in Fig. 1B, approximately 2 mM Ca²⁺ was required for maximal binding, whereas Mg²⁺ did not substitute for Ca²⁺.

In the experiment shown in Fig. 1C, the wells coated with PS or those without coating were incubated with various concentrations of Gas6. Specific binding of Gas6 to PS, which was calculated by subtracting the values with non-coated wells from the values with PS-coated wells, was saturable, with half-maximal binding observed at approximately 0.7 μg/ml Gas6.

With some coagulation factors the Gla residues are known to be important for interactions with negatively charged phospholipids (23). We thus investigated the contribution of Gla residues to the binding of Gas6 to PS. As shown in Fig. 1C, no specific binding of Gla-deficient Gas6 to PS was observed, indicating that Gla residues are essential for the binding of Gas6 to PS.

Since the interaction of Gla-containing blood coagulation factors with negatively charged phospholipids remarkably increases the activity of the coagulation factors, we next examined the effect of PS on the binding activity of Gas6 to its receptor Axl. Table I summarizes the effect of PS and PC on kₐ, kₐ, and Kₐ, estimated with BIAcore. The most significant effect of PS was observed on the kₐ value. PS but not PC increased it by 6-fold, whereas PS increased the kₐ value by about 4-fold. As a result, PS decreased the Kₐ value by approximately 30%. On the other hand, PC decreased kₐ and increased kₐ, resulting in an increase of Kₐ by about 10-fold.

The above results demonstrated that Gas6 interacted with PS and the interaction facilitated receptor binding of Gas6. However, the enhancement of receptor binding is not remarkable enough to consider it as a major meaning of the interaction of Gas6 with PS. On the other hand, these findings also implied that Gas6 might be a divalent ligand and raised the possibility of its function as an adhesion molecule. The requirement of Gla residues for the interaction of Gas6 with PS suggests that Gas6 binds to PS via the N-terminal Gla domain. Furthermore, it
has been reported that Gas6 interacts with its receptor via the C-terminal globular domain (24). Therefore, we hypothesized that Gas6 could connect the cells containing Gas6 receptor with the cells expressing PS on their surfaces.

To assess this possibility, we examined the interaction of monoblastic cells U937 with PS-coated on ELISA plates. It has been reported that U937 cells express Axl, a receptor for Gas6 (25). When U937 cells were incubated with an ELISA plate coated with PS, they did not bind to the plate (Fig. 2A). However, when they were incubated in the presence of Gas6, U937 cells adhered to the plate (Fig. 2B). In the experiment in Fig. 2, U937 cells and Gas6 were simultaneously incubated in PS-coated wells. On the other hand, when PS-coated wells were first incubated with Gas6, washed, and then incubated with U937 cells, the cells also adhered to the wells (Fig. 2C). Furthermore, Gas6 stimulated the binding of U937 cells to PS when the incubation was carried out at 4 °C instead of at 37 °C (data not shown). From these results, it is suggested that Gas6 interacts with PS and Axl at the different sites in Gas6 molecule and mediates adhesion of Axl-expressing cells to PS-expressing surfaces. As shown in Fig. 3, binding of U937 cells to PS required Ca\(^{2+}\), which is also required for the interaction of Gas6 with PS (Fig. 1B) or receptor (20). Fig. 3 also shows that Gla-deficient Gas6, which does not bind to PS (Fig. 1C) or receptor (20), did not stimulate the binding of U937 cells to PS-coated plate.

Fig. 4 shows the binding of U937 cells to the plates coated with several phospholipids. Binding of U937 cells to PS definitely required both Ca\(^{2+}\) and Gas6. U937 cells also adhered to the PI-coated plate, whereas the adhesion did not require Ca\(^{2+}\) or Gas6. U937 cells weakly adhered to the plates coated with PC or PE. However, the addition of Ca\(^{2+}\) decreased the binding to PC or PE and Gas6 did not affect the binding.

PS is normally almost totally confined to the inner leaflet of the plasma membrane but has been reported to be exposed on apoptotic cells, senescent red blood cells, or activated platelets (26–28). Therefore, PS is supposed to be one of the markers for phagocytic macrophages to identify dying cells. Our results demonstrate that binding of monoblastic cells U937 to PS is dependent on Gas6. Thus, Gas6-mediated adhesion may be one of the mechanisms for phagocytic cells to recognize dying cells.

**TABLE I**

| Binding of Gas6 to Axl | Control | +PS | +PC |
|------------------------|---------|-----|-----|
| $k_1$                  | $3.8 \times 10^4$ | $21.8 \times 10^4$ | $2.0 \times 10^4$ |
| $k_2$                  | $1.5 \times 10^{-5}$ | $5.8 \times 10^{-5}$ | $9.8 \times 10^{-5}$ |
| $K_0$                  | $3.8 \times 10^{-10}$ | $2.7 \times 10^{-10}$ | $4.9 \times 10^{-9}$ |

**Fig. 2. Adhesion of U937 cells to PS.** U937 cells in PBS containing 5 mM CaCl\(_2\) were incubated in PS-coated wells on ELISA plate in the absence (A) or presence (B) of 10 nM Gas6. C, PS-coated wells were first incubated with 10 nM Gas6, washed, and then incubated with U937 cells.

**Fig. 3. Requirement of Gla residues of Gas6 and Ca\(^{2+}\) for Gas6-dependent adhesion of U937 cells to PS.** U937 cells in PBS containing various concentrations of CaCl\(_2\) were incubated in PS-coated wells on ELISA plate in the presence of 10 nM Gas6 (●) or Gla-deficient Gas6 (○).

**Fig. 4. Phospholipid specificity of Gas6-dependent cell adhesion.** U937 cells in PBS with or without 5 mM CaCl\(_2\) were incubated in wells coated with PS, PI, PC, PI, or ethanol alone, in the presence or absence of 10 nM Gas6. Data are mean ± S.D. (n = 3).

which express PS on their surfaces. It has been reported that classes A and B macrophage scavenger receptors may be involved in phagocytosis of apoptotic cells (29, 30). As shown in Fig. 4, adhesion of U937 cells to PI is not dependent on Gas6 or Ca\(^{2+}\). Therefore, adhesion to PI, another negatively charged phospholipid, may be mediated by other mechanisms including those scavenger receptors.

In this study, we demonstrated that Gas6 links Axl-expressing cells to the PS-expressing surface and suggested that Gas6-dependent adhesion may be involved in the recognition of dying cells by phagocytic cells. The Gas6-mediated adhesion is unique since it also stimulates tyrosine phosphorylation in receptor-expressing cells. The phosphorylation may stimulate phagocytic activity of the cells. However, further *in vivo* studies are necessary to clarify the physiological importance of the Gas6-mediated cell adhesion mechanism.

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