Characterization of a high-affinity and specific binding site for Gas6

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Received 1 February 1996; revised version received 5 April 1996

Abstract We have purified a novel growth-potentiating factor and demonstrated that the factor is coded by the *gas6* gene. Moreover, we have suggested the presence of a Gas6 receptor on rat vascular smooth muscle cells. In this study, we further analyzed the binding of Gas6 to its receptor. Tissue and cellular distribution of the binding activity of ^{125}I-labeled Gas6 showed that the binding site existed in many but a limited range of tissues and cell types. Further characterization of the binding of [^{125}I]Gas6 using HOS cells demonstrated that the specific binding was dependent on the presence of Ca^{2+}. Chemical cross-linking of [^{125}I]Gas6 to HOS cells resulted in the formation of a high-molecular-mass complex, suggesting the presence of a high-molecular-weight receptor.

Key words: Gas6; Growth-potentiating factor; Receptor; Ca^{2+} dependence; Chemical cross-linking

1. Introduction

In a previous paper, we demonstrated purification of a γ-carboxyglutamic acid (Gla)-containing growth-potentiating factor from culture medium of rat vascular smooth muscle cells (VSMC) [1]. The factor is encoded by a growth arrest-specific gene, *gas6* [2,3], which is related to protein S, a negative regulator of blood coagulation [4]. Thus, we refer to the factor as Gas6. Gas6 does not induce VSMC proliferation by itself but potentiates VSMC proliferation stimulated with growth factors which activate intracellular signal transduction involving Ca^{2+} mobilization or protein kinase C [1]. Several factors such as thrombin, angiotensin II and lysophosphatidic acid are involved in this type of growth factor. However, Gas6 does not potentiate VSMC proliferation stimulated with growth factors which activate receptor tyrosine kinases involving epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). Therefore, analysis of the Gas6-induced signaling mechanism may provide a key to reveal the difference between the two types of growth-stimulating signaling mechanisms.

In our previous paper, we showed the presence of a specific binding site of Gas6 on VSMC membranes using [^{125}I]Gas6 [1]. Scatchard analysis of the binding of [^{125}I]Gas6 indicated that the K_d value was 0.3 nM, which corresponded well to the EC_{50} value of biological activity of Gas6. This result suggested that the action of Gas6 was mediated by a receptor for it. On the other hand, recent papers have reported that Gas6 is a possible ligand for Axl receptor tyrosine kinase [5,6], whose gene, *axl*, was originally isolated from myeloid leukemia cells as a transforming gene [7,8]. Moreover, it has been demonstrated that another Axl-rclated receptor tyrosine kinase, Sky, also binds Gas6 [9]. These findings suggested that VSMC expresses both a ligand and receptor(s), and Gas6 and Axl/Sky organize a new ligand-receptor system which regulates proliferation of VSMC in an autocrine or paracrine fashion.

In order to obtain further information about Gas6 action from the physiological viewpoint, we examined the tissue and cellular distribution of Gas6 receptor and found that a Gas6 binding site existed in a variety of rat tissues. Furthermore, we studied the biochemical characteristics of Gas6 binding to the receptor and demonstrated that the binding was largely dependent on the presence of divalent cations.

2. Materials and methods

2.1. Preparation of rat Gas6 and [^{125}I]Gas6

Rat Gas6 was prepared from culture medium of rat VSMC as described in our previous paper [1]. [^{125}I]Gas6 (6000 cpm/fmol) was prepared by a chloramine T method as described elsewhere [10].

2.2. Preparation of crude membranes from various tissues

Rat liver, kidney, spleen, lung, heart, brain, and skeletal muscle, and human placenta were isolated and washed with ice-cold buffer A (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 0.4 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin and 0.25 M sucrose). Each tissue was minced and suspended in 5 volumes of buffer A, and then homogenized with Physcotron (NITI-ON, Japan) at 4°C. The homogenate was centrifuged at $600 \times g$ for 5 min, and crude membrane fractions were washed twice with buffer A by centrifuging the supernatant at $105\,000 \times g$ for 60 min at 4°C. The resulting pellet was resuspended in buffer A and stored at −80°C until assay.

2.3. Preparation of crude membranes from various cells

Cells were harvested with ice-cold buffer A after washing the cells with phosphate buffered saline. The cells were suspended in buffer A, and then sonicated with a Branson Sonifier (model 350; output setting 2, twice 15 s). The cell sonicate was centrifuged at $1000 \times g$ for 10 min to remove nuclei and unbroken cells, and the supernatant was further centrifuged twice at $105\,000 \times g$ for 60 min. The resulting pellet was resuspended in buffer A, and used as a crude membrane fraction.

2.4. Binding experiments

For binding assays with adhering cells, the confluent cells in 24-well dishes were washed twice with washing buffer (1 mM Tris-HCl, pH 7.4, containing 1 mM EDTA), and incubated with 0.3 nM [^{125}I]Gas6 at 4°C in 0.2 ml of binding medium (Hanks' solution, pH 7.4, 0.1% bovine serum albumin). After the reaction was stopped by rapid removal of the medium, the cells were washed three times with ice-cold saline, and then solubilized in 1 N NaOH. The radioactivity was measured with a γ-counter. Specific binding was defined as the difference between binding in the presence and absence of the unlabeled Gas6 (30 nM). The binding assays in the crude membrane of HOS cells were carried out according to the method described previously [1].

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Abbreviations: Gas6, the protein encoded by growth arrest-specific gene 6; Gla, γ-carboxyglutamic acid; VSMC, vascular smooth muscle cell; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; DSS, disuccinimidyl suberate; PDGF, platelet-derived growth factor

PII S0014-5793(96)00394-8

2.5. Affinity cross-linking

Confluent HOS cells grown in 24-well dishes were incubated in 0.2 ml of the binding medium containing 0.3 nM [^{125}I]Gas6 with or without 30 nM unlabeled Gas6 for 2 h at 4°C. After washing three times with ice-cold saline, the cells were resuspended in 0.2 ml of Hanks' solution (pH 7.4), and then treated with 0.15 mM disuccinimidyl suberate (DSS), a homobifunctional cross-linking reagent, for 30 min at 4°C. The reaction was stopped by the addition of Tris-HCl (final concentration 50 mM, pH 7.4). The cells were harvested and lysed with 1% Triton X-100 containing a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 mM EDTA and 20 mg/ml leupeptin). The supernatants obtained by centrifugation at 10000×*g* for 10 min were mixed with Laemmli sampling buffer and heated at 60°C for 5 min, and proteins were separated by SDS-PAGE. After electrophoresis, the gel was dried and exposed to Kodak X-Omat AR X-ray film for 2 days at −80°C with two intensifying screens.

3. Results and discussion

In order to study tissue distribution of Gas6 receptor, we used membrane fractions prepared from several rat and human tissues to analyze [^{125}I]Gas6 binding. As shown in Table 1, specific binding of [^{125}I]Gas6 was detected in the membrane fractions of rat spleen, lung, heart and brain, but not in those of liver, kidney and skeletal muscle. The membrane fractions of human placenta also showed activity to bind Gas6. Next, the cellular distribution of Gas6 receptor was examined using several types of cultured cells (Table 2). In addition to VSMC [1], specific binding of [^{125}I]Gas6 was observed on some types of cells tested. UMR-106 and ROS-17/2.8, rat osteosarcoma cell lines, did not have the activity to specifically bind [^{125}I]Gas6, whereas HOS and MG-63, human osteosarcoma cell lines, had relatively high activity of Gas6 binding. These results indicate that the Gas6 receptor is present in many but a limited range of tissues and cell types.

Since HOS cells had the highest binding capacity among the cell lines listed in Table 2, biochemical analysis of the binding of Gas6 to receptor was performed using HOS cells. Both intact and membrane fractions of HOS cells bound [^{125}I]Gas6 and the specific binding was approximately 80% of the total binding (data not shown).

We analyzed the ligand specificity of the Gas6 binding site on HOS cells. Table 3 summarizes the IC_{50} values of several proteins for their ability to displace the specific binding of 0.3 nM [^{125}I]Gas6. Unlabeled Gas6 inhibited the binding of labeled Gas6 with a low IC_{50} value. However, human protein S, which has 43% amino acid residue identity with rat Gas6 [1], did not suppress Gas6 binding even at concentrations higher than 30 nM. Other growth factors such as thrombin, platelet-derived growth factor (PDGF), EGF and bFGF did not affect the binding of [^{125}I]Gas6. Therefore, the Gas6 receptor appears to be highly specific to Gas6.

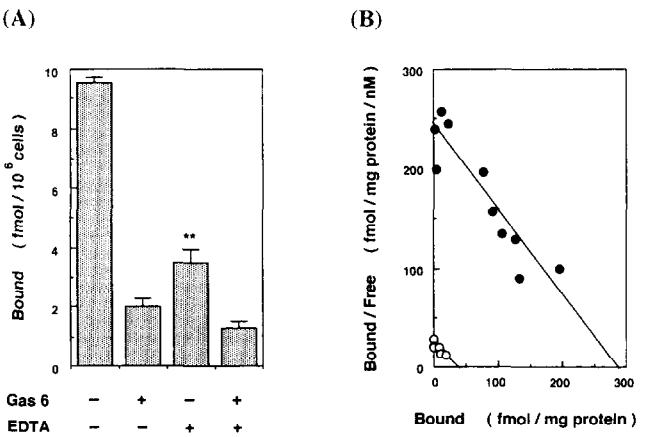

Fig. 1. The Ca^{2+} dependence of the binding of [^{125}I]Gas6 to HOS cells and equilibrium binding of [^{125}I]Gas6. (A) The cells were incubated with 0.3 nM [^{125}I]Gas6 for 2 h at 4°C with or without 30 nM unlabeled Gas6. The assays were carried out in the presence or absence of 10 mM EDTA in the binding medium. $^{**}P<0.05$, compared with −Gas6, −EDTA (Student's *t*-test). (B) Scatchard plot of specific [^{125}I]Gas6 binding. The cells were incubated with various concentrations of [^{125}I]Gas6 alone for 2 h at 4°C (total binding) or in the additional presence of a 20-fold amount of unlabeled Gas6 (non-specific binding). The assays were carried out in the presence (open circles) or absence (closed circles) of 10 mM EDTA in the binding medium. The data are representative of three experiments, and each point represents the mean specific binding for three determinations.

Mature Gas6 has 11–12 Gla residues in the N-terminal region [1]. Gla residues are found in some proteins regulating blood coagulation, which include prothrombin, factor VII, factor IX, factor X, protein C and protein S [11], and interaction with Ca^{2+} has been expected to be one of the functions of Gla residues [12]. Therefore, we examined the effect of Ca^{2+} on the binding activity of Gas6 to HOS cells. Fig. 1A shows the effect of EDTA on the binding of [^{125}I]Gas6 to intact HOS cells. Addition of 10 mM EDTA to the binding medium containing 1.2 mM Ca^{2+} suppressed total binding by approximately 65%. Non-specific binding was also suppressed by EDTA by approximately 35%. Therefore, EDTA inhibited the specific binding by approximately 70%. The effect of EDTA was overcome by further addition of 10 mM Ca^{2+} (data not shown).

In order to examine whether EDTA affected binding affinity

Table 1
Tissue distribution of Gas6 receptor

Tissue		Specific binding (fmol/mg protein)
Rat	liver	<1.0
	kidney	<1.0
	heart	2.3
	lung	4.4
	spleen	8.6
	brain	1.4
	skeletal muscle	<1.0
Human	placenta	4.4

The crude membranes prepared from each tissue (0.2 mg) were incubated with 0.3 nM [^{125}I]Gas6 for 2 h at 4°C in the binding medium, and the specific binding activity was analyzed as described in section 2.

Table 2
Cellular distribution of Gas6 receptor

Cells		Specific binding (fmol/10^5 cells)
Rat	VSMC	0.57
	A7r5	0.28
	renal mesangial cell	0.31
	ROS-17/2.8	<0.02
	UMR-106	<0.02
Human	HUVEC	0.33
	HOS	1.33
	MG-63	1.08

Cells were incubated with 0.3 nM [^{125}I]Gas6 for 2 h at 4°C in the binding medium, and the specific binding activity was analyzed as described in section 2.

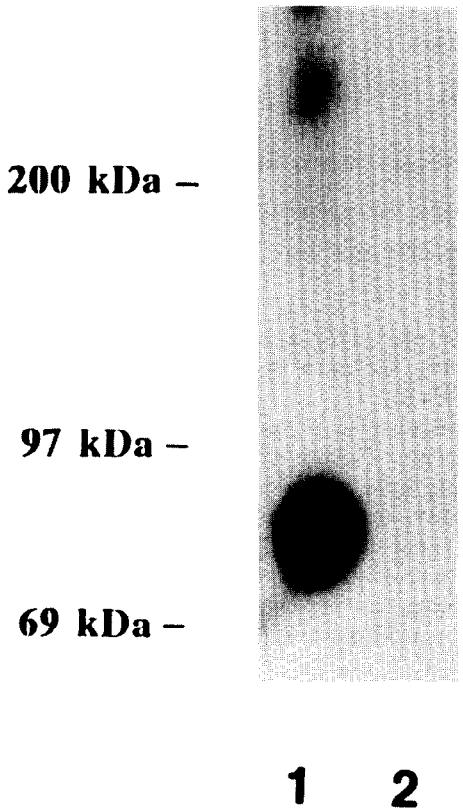

Fig. 2. Characterization of the Gas6 receptor by affinity cross-linking experiment. Binding assay was carried out at 4°C for 2 h by incubating HOS cells with 0.3 nM [^{125}I]Gas6 in the absence (lane 1) or presence (lane 2) of 30 nM unlabeled Gas6. After the incubation, the cells were washed and treated with 0.15 mM DSS for 30 min at 4°C, and then analyzed as described in section 2.

or binding capacity, Scatchard analysis was performed using membrane fractions of HOS cells in the presence or absence of 10 mM EDTA. As shown in Fig. 1B, addition of EDTA reduced the number of binding sites from 280 fmol/mg protein to 35 fmol/mg protein, while EDTA had little effect on the binding affinity. The K_d values in the presence and absence of EDTA were 1.2 nM and 1.5 nM, respectively. This result indicated that divalent cations were almost essential for binding of Gas6 to its receptor.

Identification of the binding proteins responsible for [^{125}I]Gas6 binding was performed by affinity labeling experiments. After [^{125}I]Gas6 was incubated with HOS cells for 2 h, the cells were treated with DSS and labeled proteins were analyzed by SDS-PAGE. As shown in Fig. 2, a band with an apparent molecular weight of approximately 300–400 kDa appeared after treatment with DSS (lane 1), whereas the formation of the cross-linked complex was blocked by the presence of excess unlabeled Gas6 (lane 2). The high-molecular-mass complex was not observed in the absence of DSS (data not shown). The molecular weight of the cross-linked complex agreed well with that of the Gas6/Axl complex described by Varnum et al. [5]. Therefore, the specific binding site of Gas6 on HOS cells may be Axl or Sky, or another Axl-related molecule such as Mer/Eyk [6,9,13].

Table 3
Inhibition of [^{125}I]Gas6 specific binding to HOS cells by protein S and other growth factors

Compound	IC_{50} (nM)
Gas6	1.3
Protein S	>30
Thrombin	>50
PDGF	>100
EGF	>100
bFGF	>100

Cells were incubated with 0.3 nM [^{125}I]Gas6 in the presence of various concentrations of agents for 2 h at 4°C in the binding medium. The IC_{50} values were evaluated from the displacement curves as the concentration that inhibits half of the [^{125}I]Gas6 specific binding.

We first isolated Gas6 from the culture medium of rat VSMC and implicated the physiological role of Gas6 in the proliferation of VSMC via the specific receptor [1]. However, as illustrated in Table 1, a specific binding site for Gas6 was also found in a variety of rat tissues, such as heart, lung, spleen and brain. Moreover, expression of Gas6 mRNA in these tissues has been reported [3]. Therefore, Gas6 may also have biological importance in tissues other than the aorta. Neubauer et al. [8] have reported the expression of Axl mRNA in normal and malignant hematopoietic tissues and suggested Axl kinase may be operative in hematopoiesis. Nevertheless, potentiation of VSMC proliferation is the only biological activity of Gas6 clearly demonstrated so far [1]. We are now conducting further studies on Gas6-induced biological responses of other tissues and cell types for a better understanding of its biological relevance.

Acknowledgements: We thank Ayako Terawaki and Miho Okuyama for excellent technical assistance.

References

[1] Nakano, T., Higashino, K., Kikuchi, N., Kishino, J., Nomura, K., Fujita, H., Ohara, O. and Arita, H. (1995) J. Biol. Chem. 270, 5702–5705.
[2] Schneider, C., King, R.M. and Philipson, L. (1988) Cell 54, 787–793.
[3] Manfioletti, G., Brancolini, C., Avanzi, G. and Schneider, C. (1993) Mol. Cell Biol. 13, 4976–4985.
[4] Esmon, C.T. (1987) Science 235, 1348–1352.
[5] Varnum, B.C., Young, C., Elliot, G., Garcia, A., Bartley, T.D., Fridell, Y., Hunt, R.W., Trail, G., Clogston, C., Toso, R.J., Yanagihara, D., Bennet, L., Sylber, M., Merewether, L.A., Tseng, A., Escobar, E., Liu, E.T. and Yamane, H.K. (1995) Nature 373, 623–626.
[6] Stitt, T.N., Conn, G., Gore, M., Lai, C., Bruno, J., Radziejewski, C., Mattsson, K., Fisher, J., Gies, D.R., Jones, P.F., Masiakowski, P., Ryam, T.E., Tobkes, N.J., Chen, D.H., DiStefano, P.S., Long, G.L., Basilico, C., Goldfarb, M.P., Lemke, G., Glass, D.J. and Yancopoulos, G.D. (1995) Cell 80, 661–670.
[7] O'Bryan, J., Frye, R.A., Cogswell, P.C., Neubauer, A., Kitch, B., Prokop, C., Espinosa, III R., Le Beau, M.M., Earp, H.S. and Liu, E.T. (1991) Mol. Cell Biol. 11, 5016–5031.
[8] Neubauer, A., Fiebeler, A., Graham, D.K., O'Bryan, J.P., Schmidt, C.A., Barckow, P., Serke, S., Siegert, W., Snodgrass, H.R., Huhn, D. and Liu, E.T. (1994) Blood 84, 1931–1941.
[9] Ohashi, K., Nagata, K., Toshima, J., Nakano, T., Arita, H., Tsuda, H., Suzuki, K. and Mizuno, K. (1995) J. Biol. Chem. (in press).
[10] Arita, H., Hanasaki, K., Nakano, T., Oka, S., Teraoka, H. and Matsumoto, K. (1991) J. Biol. Chem. 266, 19139–19141.
[11] Davie, E.W. (1986) Chem. Scripta 26B, 241–245.
[12] Borowski, M., Furie, B.C., Bauminger, S. and Furie, B. (1986) J. Biol. Chem. 261, 14969–14975.
[13] Jia, R. and Hanafusa, H. (1994) J. Biol. Chem. 269, 1839–1844.