Identification of the Product of Growth Arrest-specific Gene 6 as a Common Ligand for Axl, Sky, and Mer Receptor Tyrosine Kinases*

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Axl, Sky, and Mer, members of an Axl/Sky receptor tyrosine kinase subfamily, are typified by the cell adhesion molecule-related extracellular domain. The product of growth arrest-specific gene 6 (Gas6), structurally homologous to the anticoagulant protein S, was recently identified as the ligand for Axl and Sky, but the ligand for Mer remained unknown. We have now obtained evidence that Gas6 can also function as a ligand for Mer. Co-precipitation analysis, using soluble receptors of Axl, Sky, and Mer (Axl-Fc, Sky-Fc, and Mer-Fc) composed of the extracellular domain of receptors fused to the Fc domain of immunoglobulin G1, clearly showed that Gas6, but not protein S, specifically bound to Axl-Fc, Sky-Fc, and Mer-Fc fusion proteins. Quantitative kinetic analyses using a BIAcore biosensor instrument revealed dissociation constants ($K_d$) of the binding of rat Gas6 to Axl-Fc, Sky-Fc, and Mer-Fc are 0.4, 2.7, and 29 nM, respectively. We also found that Gas6 stimulated tyrosine phosphorylation of Axl, Sky, and Mer receptors ectopically expressed in Chinese hamster ovary cells. Taken together, these findings suggest that Gas6 is a common ligand for Axl, Sky, and Mer, all known members of an Axl/Sky receptor subfamily.

Receptor tyrosine kinases play important roles in transducing signals from the extracellular environment, and this leads to a variety of cellular responses, including proliferation, differentiation, and survival (1, 2). Although a number of receptor tyrosine kinases have been identified, some are “orphan” receptors, the ligands of which remain unknown. In view of the pivotal role of receptor tyrosine kinases in cell to cell signaling, further searches are warranted for novel members of receptor tyrosine kinases and the ligands for these receptors need to be identified.

On the basis of sequence similarities, known receptor tyrosine kinases are classified into >10 distinct subfamilies (2). The Axl/Sky receptor subfamily is one of these subfamilies, including Axl (also called Ark and Ufo) (3–5), Sky (also called Rse, Brt, and Tyro3) (6–10), and Mer (a putative mammalian homologue of chicken c-Eyk) (11–13). The members of this subfamily are typified by characteristic extracellular domains. They are composed of two Ig-like motifs and two fibronectin type III motifs, which have similarity to those found in cell adhesion molecules such as neural cell adhesion molecules and receptor tyrosine phosphatases (14, 15). The cDNA for Axl, the first member of this family, was identified as a transforming gene from human myelogenic leukemia cells by DNA transfection/selection assay (3). The cDNA for Sky was isolated by low stringency hybridization screening of a human HepG2 hepatoma cDNA library, using the avian viral oncogene v-sea as a probe (6). The cDNA for chicken c-Eyk was isolated as a proto-oncogene for the avian sarcoma-inducing viral oncogene v-eyk (previously called v-ryk) (11, 16), and the putative mouse and human homologues of c-Eyk, referred to as Mer, were also identified (12, 13). Since the Axl/Sky family receptors have a cell-transforming activity through overexpression (3, 10, 17, 18), they are thought to be involved in tumor progression and in normal cell proliferation, but little is known of the physiological functions of these receptors. The patterns of expression of mRNAs for these receptors differ; Sky mRNA is expressed predominantly in the brain while Axl and Mer mRNAs are expressed in various tissues (3–13). These different patterns of expression suggest distinct roles for these receptors.

The ligand for Axl was recently identified as the product of the growth arrest-specific gene 6 (Gas6)1 (19, 20). Gas6 was previously isolated as a gene product inducibly expressed in fibroblasts in response to serum starvation and has a structure similar to that of protein S, a vitamin K-dependent plasma protein with anticoagulant activity with 43% amino acid identity (21, 22). We and others subsequently demonstrated that Gas6 can also bind to Sky and stimulate the tyrosine autophosphorylation of Sky (23, 24). Although Stitt et al. (20) reported that human or bovine protein S bound to murine Sky (Tyro3) (20), intraspecies (human-human) ligand-receptor interactions of protein S and Sky could not be detected (23, 24). Hence, it is “probable” that Gas6, but not protein S, is a ligand for Sky, and Gas6 “may” also be a common ligand for Axl and Sky, two related receptors. However, to provide compelling evidence for this notion, it is important to quantitatively test the binding affinity of Gas6 to these receptors by side by side experiments. More importantly, whether or not Gas6 or protein S can bind to Mer, another member of an Axl/Sky receptor family, needs to be determined since the ligand for Mer has not been identified.

In the present study, we examined the binding ability of Gas6 and protein S to Axl, Sky, and Mer, three members of the Axl/Sky receptor subfamily. We also examined whether Gas6 would stimulate the tyrosine phosphorylation of these receptors.

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1 The abbreviations used are: Gas6, the protein product of growth arrest-specific gene 6; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; RU, resonance unit.
**EXPERIMENTAL PROCEDURES**

Gas6 and Protein S—The cDNA encoding rat Gas6 was cloned as described elsewhere (25). The cDNA for human Gas6 was amplified by polymerase chain reaction, using a cDNA library from human aortic smooth muscle cells as a template. The cDNAs were subcloned into the PUC-SR expression vector, and the constructs were transfected into COS-7 cells using liposome methods as described previously (23). Cells were cultured for 3 days in serum-free Dulbecco’s modified Eagle’s medium with 2 μM vitamin K₃ (Menatetrenone, Eisai, Tokyo). Recombinant rat and human Gas6 were purified using methods described elsewhere (25). Protein S was purified from human plasma with anticoagulant activity was a gift from Drs. H. Tsuda and K. Suzuki (26). The concentrations of the purified Gas6 and protein S were determined by amino acid analyses. The purity and quantity of these proteins were also estimated by SDS-PAGE and silver staining.

Antibodies—Rabbit polyclonal antibodies for Sky, Gas6, and protein S were prepared and purified as described previously (23). Anti-Myc epitope monoclonal antibody (9E10) and antiphosphorynosine monoclonal antibody (PY20) were purchased from Santa Cruz Biotechnology and ICN Biomedicals (Costa Mesa), respectively. Peroxidase-conjugated goat affinity-purified antibody to human IgG-Fc was purchased from Organon Teknika (Durham).

Expression and Purification of Fc Fusion Proteins—Sky- and Met-Fc fusion proteins were expressed in COS-7 cells and purified as described previously (23). The cDNA encoding mouse c-Mer was cloned by long stringency hybridization using as a probe chicken c-Eyk cDNA. The cDNA fragment coding for the extracellular domain of human Axl (amino acid residues 1–438) or mouse Mer (residues 1–498) was ligated in-frame with the polymerase chain reaction-amplified cDNA fragment of the Fc region (residues 216–443) of human IgG1, resulting in the production of the cDNA coding for the Axl- or Mer-Fc fusion protein with a spacer sequence Ser-Ser-Val-Pro-Gly. The fused cDNAs were subcloned into PUC-SR expression vector and transfected into COS-7 cells using the liposome method. The serum-free conditioned media of COS cells were collected for 3 days, and the Fc fusion proteins were purified by protein A-Sepharose (Pharmacia Biotech Inc.) chromatography.

Co-precipitation Binding Assays—Gas6 or protein S was incubated at 4 °C overnight with 5 nm each receptor-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% NaN₃, and 1% bovine serum albumin. After centrifugation, the precipitates were washed four times with cold phosphate-buffered saline, suspended in SDS-sampling buffer, and subjected to 8% SDS-PAGE (23). Immunoblot analysis with anti-Gas6 or anti-protein S antibody was performed as described previously (27, 28).

**BIAcore Analysis**—Purified receptor-Fc fusion protein (0.7 μg) was immobilized to a CM5 sensor chip on a BIAcore instrument (Pharmacia) using procedures described in the manual supplied by the manufacturer. Each 30 μl of Gas6 was dissolved in Hanks’ balanced salt solution containing 20 mM Hepes (pH 7.0), 0.02% NaN₃, and 1% bovine serum albumin and passed over the immobilized Fc fusion protein at a flow rate of 2 μl/min for 25 min. Binding of the ligand with Fc fusion protein was monitored in real time by the increase in the relative resonance unit (RU) in the sensogram. The surface of the sensor chip was regenerated by elution of 15 μl of 0.1 M sodium acetate (pH 2.3), containing 0.15 M NaCl. The rate constants of association (kₐ) and dissociation (kₐ) and dissociation constants (Kₑ) were calculated using the software supplied by the manufacturer and the procedure of Karlsson et al. (29), which is based on the expression dRtdt = kₐCRₘₐₓ − (kₐ + kₐ)Rₜ, where Rₘₐₓ is the maximum RU response at saturation of receptor-Fc with bound analyte, Rₜ is the response at time t, and C is the concentration of injected analyte. A plot of dRtdt versus Rₜ gives a line with slope −(kₐC + kₐ), which is equal to −kₐ. If kₐ is plotted against C, the resulting slope is equal to kₐ. The dissociation rate constant kₐ was given by the first-order rate equation dRₜ/dt = kₐRₜ when the analyte flowing across the flow cell has been replaced with buffer. Integration of the rate equation gives ln(Rₜ/Rₜ₀) = kₐ(t − t₀), and a plot of ln(Rₜ/Rₜ₀) versus t for the initial dissociation rate yields a line with slope of −kₐ.

**Construction of CHO Cell Lines**—The CHO cell line expressing human Sky (B31) was constructed as described previously (23). The cDNA encoding Myc-tagged Axl or Mer receptor (Axl-Myc or Mer-Myc) was constructed to generate the full-length sequence of human Axl or mouse Mer with the Myc epitope peptide (EQKLISEEDL) at the C terminus, using procedures similar to those described elsewhere (30). The cDNA was inserted into the EcoRI or NotI site of the pUCd2SR expression vector containing the neomycin-resistant gene (31). The resulting plasmid was transfected into CHO cells using liposome methods, and the G418-resistant colonies were selected. Cell lines with high expression of Axl-Myc (AM3) or Mer-Myc (MM14), as measured by immunoblot analysis with anti-Myc epitope antibody (9E10), were selected.

**Tyrosine Phosphorylation Assay**—B31, AM3, or MM14 cells were cultured in Ham’s F-12 medium supplemented with 10% fetal calf serum. The cells were serum-starved and treated with rat or human Gas6 for 10 min at 37 °C. The cells were rinsed three times with cold phosphate-buffered saline containing 1 mM orthovanadate and lysed with cold lysis buffer (23). The lysates were immunoprecipitated with anti-Sky (Sky-C) or anti-Myc antibodies (9E10), run on SDS-PAGE, and immunoblotted with anti-phosphotyrosine monoclonal antibody (PY20), as described previously (23).

**RESULTS**

**Binding of Gas6 to Axl, Sky, and Mer-Fc Fusion Proteins, as Measured by Co-precipitation Analysis**—To characterize and compare in parallel experiments the binding potential of Gas6 and protein S to three distinct members (Axl, Sky, and Mer) of an Axl/Sky receptor subfamily, we prepared chimeric soluble receptors (Axl-Fc, Sky-Fc, and Mer-Fc) composed of the extracellular ligand-binding domain of these receptors fused to the Fc region of the human immunoglobulin IgG1 heavy chain. As a control, we also prepared Met-Fc, a chimeric protein composed of the extracellular domain of c-Met (receptor for hepatocyte growth factor) and the Fc region of IgG1. Receptor-Fc fusion proteins were expressed in COS cells and purified from the conditioned media by protein A-Sepharose chromatography. Western blot analyses with anti-Fc antibody indicated that the receptor-Fc fusion proteins were expressed as a disulfide-linked homodimer molecule (data not shown), as previously noted for other IgG fusion proteins (32, 33).

Affinity adsorption and co-precipitation analysis using receptor-Fc fusion proteins in the presence of protein A-Sepharose clearly showed that both Axl- and Sky-Fc co-precipitated rat Gas6 protein (Fig. 1A). Binding of Gas6 to Mer-Fc was faintly detected after a longer exposure, but binding to Met-Fc was not detected. To demonstrate more clearly the specific binding of Gas6 to Mer-Fc, higher concentrations of Gas6 were incubated with Met-Fc, and the binding to Met-Fc was examined in comparison with the binding to control Met-Fc. As shown in Fig. 1B, Mer-Fc, but not Met-Fc, specifically co-precipitated Gas6 at 40 nM. Thus, it appeared that Gas6 could specifically bind to Axl-, Sky-, and Mer-Fc fusion proteins, but not to Met-Fc. Based on the intensity of the immunoreactive Gas6 co-precipitated with Fc fusion proteins, the Gas6 binding affinities of these receptors were suspected to be in the order of Axl>Sky>Mer. Under similar conditions, protein S did not co-precipitate with any of the receptor-Fc fusion proteins examined (Fig. 1C).

**Binding Analyses by the Use of BIAcore**—To further define the specific binding of Gas6 to Axl, Sky, and Mer receptors, we analyzed binding interactions between them using a BIAcore Biosensor instrument. Receptor-Fc fusion proteins were immobilized on the surface of a BIAcore sensor chip, and then Gas6 or protein S was passed over the sensor chip. Binding was measured as the increase in RUs. As shown in Fig. 2A, lane 1, rat Gas6 (20 nM) bound to Axl-Fc, Sky-Fc, and Mer-Fc at 4448, 1815, 100 RUs, respectively, but not to control Met-Fc. When a higher concentration (100 nM) of rat Gas6 was used, specific binding to Mer-Fc, but not to Met-Fc, was evident (Fig. 2B, lane 1). We next examined the binding potential of human Gas6 to these receptor-Fc fusion proteins. Human Gas6 bound to human Axl-Fc and human Sky-Fc at 1813 and 473 RUs, respectively, but specific binding to either mouse Mer-Fc or control Met-Fc was not observed (Fig. 2, lane 2). Thus, in spite of species coincidence, human Gas6 rather than rat Gas6 bound
less tightly to human Axl-Fc and human Sky-Fc. No specific binding of human protein S was detected for Axl-, Sky-, or Mer-Fc fusion protein (Fig. 2, lane 3). When the concentrated conditioned media of COS cells transfected with rat protein S cDNA (containing about 120 nM rat protein S) were passed over the surface immobilized with mouse Mer-Fc, an increase in RUs was never evident (data not shown).

To quantitate binding affinities of Gas6 to these receptors, various concentrations of rat or human Gas6 were passed over the sensor chip immobilized with each receptor-Fc fusion protein. As shown in Fig. 3, the amounts of Gas6 bound to the Fc fusion protein were monitored in real time, as an increase in RUs in the sensorgram. Table I summarizes $k_d$ and $k_a$ values as well as calculated dissociation constants ($K_d$) for the binding interactions between Gas6 and receptor-Fc fusion proteins. Correlating with the results given above, Axl-Fc bound rat Gas6 with the highest binding affinity, the $K_d$ value being 0.4 nM. The affinity of Sky-Fc was intermediate with a $K_d$ value of 2.7 nM, and Mer-Fc bound with the lowest affinity with a $K_d$ of 29.0 nM. The binding affinity of human Gas6 to human Axl-Fc or human Sky-Fc was lower than that of the rat counterpart, and specific binding of human Gas6 to mouse Mer-Fc was not observed (Table I).

Stimulation of Tyrosine Phosphorylation of Sky, Axl, and Mer—We recently reported that rat Gas6 stimulated tyrosine phosphorylation of Sky stably expressed in CHO cells (23). In the present study, we constructed CHO cell lines ectopically expressing the C-terminal direction Myc epitope-tagged Axl (Axl-Myc) and Mer (Mer-Myc) and examined whether Gas6 would stimulate tyrosine phosphorylation of these receptors. The tyrosine phosphorylation was assessed by immunoblotting with an anti-phosphotyrosine antibody after immunoprecipitation of the cell lysates with anti-Sky or anti-Myc antibody. As shown in Fig. 4, tyrosine phosphorylation of human Sky and Axl-Myc was induced when the cells were treated with rat or human Gas6, and tyrosine phosphorylation of mouse Mer-Myc

**Fig. 1.** Co-precipitation binding assay of Gas6 and protein S to Axl-, Sky-, and Mer-Fc fusion proteins. A, recombinant rat Gas6 (r Gas6) (10 nM) was incubated with protein A-Sepharose in the absence (lane 6) or presence each of 5 nM Sky-Fc (lane 2), Axl-Fc (lane 3), Mer-Fc (lane 4), or Met-Fc (lane 5) fusion protein. After centrifugation, the precipitates were run on SDS-PAGE, and the bound Gas6 was visualized by immunoblotting with anti-Gas6 antibody (α-Gas6). Lane 1 shows the immunoblot of the recombinant Gas6 used in this assay. B, recombinant rat Gas6 at the indicated concentration was incubated with protein A-Sepharose in the absence (lane 6) or presence each of 5 nM Mer-Fc (lanes 2–4) or Met-Fc (lane 5) and treated as in A. Lane 1, recombinant Gas6 used in this assay. C, human protein S (h PS) (10 nM) purified from plasma was treated as in A, and the precipitates were immunoblotted with anti-protein S antibody (α-PS). Elution positions of rat Gas6 and human protein S are indicated by arrows. The upper and lower bands of protein S correspond to the intact and thrombin-cleaved form, respectively (26). Molecular sizes (kDa) of marker proteins are indicated on the left.

**Fig. 2.** Binding activity of Gas6 and protein S to receptor-Fc fusion proteins, as measured using a BLAcore instrument. Recombinant rat Gas6 (lanes 1), recombinant human Gas6 (lanes 2), or human plasma protein S (lanes 3) (each 20 nM in A or 100 nM in B) was passed over a BLAcore sensor chip immobilized with either Axl-Fc, Sky-Fc, Mer-Fc, or Met-Fc fusion protein. Binding activity was measured as RUs at 25 min after sample injection. The level of nonspecific binding was less than 70 RUs.
was induced by the treatment with rat Gas6. No phosphorylation of mouse Mer-Myc was detected by human Gas6 even at 100 nM (data not shown). Correlating with the binding affinities determined by BIAcore analyses, higher concentrations of rat Gas6 were needed for activation of Mer, and rat Gas6 has more potent stimulating activity to human Sky and human Axl than does human Gas6.

No Homophilic Binding Activity of Axl-, Sky-, and Mer-Fc Fusion Proteins—To determine whether or not extracellular domains of Axl, Sky, and Mer have homophilic binding ability, as in the case of neural cell adhesion molecules and receptor protein tyrosine phosphatases (14, 34), the receptor-Fc fusion protein was immobilized to the surface of the BIAcore sensor chip, and solution containing any of the receptor-Fc fusion proteins at 50 nM was passed over the sensor chip. There was, however, no increase seen for any of the combinations of receptor-Fc fusion proteins, indicating that they had no homophilic or heterophilic binding activity, at least under the conditions of our experimental setup (data not shown).

**DISCUSSION**

Various groups of researchers reported ligands for the two related receptor tyrosine kinases, Axl and Sky, but conclusions as to the ligand-receptor relationships in these reports were inconsistent. Gas6 was first identified as the ligand for Axl, based on intraspecies (human-human) ligand-receptor interactions (19). Stitt et al. (20) reported that protein S, but not Gas6, is a ligand for Sky (Tyro3), based on observations of interspecies (human-mouse) ligand-receptor interactions (20). In contrast to this report, we and others subsequently reported that human protein S was unable to bind to human Sky, and alternatively, Gas6 bound to Sky (23, 24). The inability of intraspecies (human-human) ligand-receptor interactions between protein S and Sky suggested that protein S could not function as a natural ligand for Sky. Thus, Gas6, but not protein S, seems to be the ligand for the two related receptors, Axl and Sky.

In the present work, we characterized in side by side experiments, the binding potential of rat and human Gas6 and protein S to three members of an Axl/Sky receptor family, human Axl, human Sky, and mouse Mer. Human Gas6 bound to human Axl and human Sky, and rat Gas6 (which is closely related to mouse Gas6, with 94% amino acid identity) bound to mouse Mer. Based on observations of binding of Gas6 to Axl, Sky, and Mer within the same or closely related species, together with the activity of Gas6 to stimulate tyrosine phosphorylation of these receptors, we concluded that Gas6 is a ligand for these three receptors. On the other hand, human protein S had no detectable binding ability to either human Axl-Fc or human Sky-Fc, and rat protein S had no binding ability to mouse Mer-Fc. Therefore, it is unlikely that protein S is a ligand for any of these receptors.

Rat Gas6 appears to be a better ligand than human Gas6 for human Axl and Sky receptors, the reason of which remains unknown. Since the concentrations of rat and human Gas6 were accurately determined by amino acid analyses, this observation is not due to the mis-estimation of their concentrations. It is probable that some of the residues characteristic to “rat” Gas6 contribute to its high-affinity binding to these receptors.

Quantitative analysis using the BIAcore instrument re-

**FIG. 3.** Binding analyses of Gas6 to Axl-Fc (A), Sky-Fc (B), and Mer-Fc (C) fusion proteins using a BIAcore instrument. Each panel shows the sensorgram (RU versus time) for rat Gas6 passed over the surface immobilized with the receptor-Fc protein on a BIAcore instrument. The concentrations of rat Gas6 injected are indicated.

**TABLE 1**

| Receptor-Fc       | Ligand      | \(k_a\) \(\text{m}^{-1}\text{s}^{-1}\) | \(k_d\) \(\text{s}^{-1}\) | \(K_d\) \(\text{nM}\) |
|-------------------|-------------|--------------------------------------|-----------------------------|--------------------------|
| Human Axl-Fc      | Rat Gas6    | \(7.5 \times 10^4\)                 | \(3.4 \times 10^{-5}\)     | 0.4                      |
|                   | Human Gas6  | \(1.5 \times 10^5\)                 | \(1.6 \times 10^{-4}\)     | 1.0                      |
| Human Sky-Fc      | Rat Gas6    | \(2.0 \times 10^6\)                 | \(5.6 \times 10^{-4}\)     | 2.7                      |
|                   | Human Gas6  | \(2.3 \times 10^7\)                 | \(2.5 \times 10^{-3}\)     | 10.8                     |
| Mouse Mer-Fc      | Rat Gas6    | \(3.2 \times 10^7\)                 | \(9.3 \times 10^{-4}\)     | 29.0                     |
|                   | Human Gas6  | ND\(^a\)                              | ND\(^a\)                   | ND\(^a\)                |

\(^a\) Specific binding was not detected when 100 nM of human Gas6 was passed over the surface immobilized with mouse Mer-Fc.
Gas6 as the Ligand for Axl, Sky, and Mer

Gas6 was previously identified in NIH3T3 cells as being one of the genes induced by serum starvation, but little is known of its biological functions. Gas6 was recently isolated from conditioned media of rat vascular smooth muscle cells and was found to be a growth-potentiating factor for thrombin-induced proliferation of vascular smooth muscle cells (25). Gas6 in itself has no mitogenic activity to these cells but does enhance the mitogenic activity of calcium ion-mobilizing growth factors such as thrombin or lysophosphatidic acid. Goruppi et al. (37) have just reported that Gas6 has mitogenic activity to NIH3T3 cells, when these cells are cultured in the presence of 0.5% fetal calf serum, and survival activity was apparent under completely serum-starved conditions (37). Such being the case, Gas6 may support or potentiate the mitogenic activity of other growth factors and/or protect the cells from apoptotic death, under conditions of absence of growth factors. In light of the findings that receptor tyrosine kinases can transduce various types of signals such as differentiation, survival, and neuronal axon guidance as well as proliferation, Gas6 may function not only as a growth regulator but also as a regulator for cell differentiation or cell type-specific functions.

In summary, we report here clear evidence for ligand-receptor relationships between two members of a protein S/Gas6 family and three members of an Axl/Sky receptor family. Identification of Axl, Sky, and Mer, as receptors for Gas6, provides a framework for future studies to elucidate the physiological functions of Gas6.

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