Stimulation of Sky Receptor Tyrosine Kinase by the Product of Growth Arrest-specific Gene 6*

(Received for publication, July 11, 1995)

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Sky (also called Rse, Brt, and Tyro3) is a member of a subfamily of related receptor tyrosine kinases, including Axl/Ufo/Ark and c-Eyk/Mer. We obtained evidence that Gas6 (the product of growth arrest-specific gene 6) is a ligand of the Sky receptor tyrosine kinase. Gas6, but not protein S (an anticoagulant protein structurally similar to Gas6), specifically bound to the soluble form of Sky (Sky-Fc), composed of the extracellular domain of Sky fused to the Fc domain of human immunoglobulin G1. The native and recombinant Gas6, but not protein S, stimulated tyrosine phosphorylation of Sky ectopically expressed in Chinese hamster ovary cells. Stimulation of Sky in response to Gas6 was inhibited by Sky-Fc. The half-maximal concentration of Gas6 that stimulated Sky was about 1 nM. Thus, Gas6 as a ligand for Sky specifically binds to and stimulates Sky receptor tyrosine kinase.

Receptor tyrosine kinases play a central role in transducing the external signals across cell membranes into intracellular signaling systems and these signals lead to cell proliferation, differentiation, and other responses (1). Based on similarities of the sequences of kinase catalytic domains and the structural motifs in the extracellular domains, these receptors can be classified into subfamilies (1). The members of an Axl/Sky receptor subfamily, which include Axl (also called Ufo and Ark) (2–4), Sky (also called Rse, Brt, and Tyro3) (5–9), and c-Eyk (also called Mer) (10, 11), contain the characteristic extracellular ligand-binding domain composed of two immunoglobulin-like motifs and two fibronectin type III motifs. Axl was originally identified as the oncogene for human myelogenic leukemia (2, 3), and the gene for c-Eyk was isolated as a proto-oncogene for the avian viral oncogene v-eyk (10). Overexpression of Axl and Sky led to cell transformation (2, 9, 12). As the Axl/Sky family receptors have an oncogenic potential, they may be involved in tumor progression and in normal cell proliferation. Northern blot analysis revealed that the Sky transcripts are predominantly expressed in the brain (5–9), while those for Axl and c-Eyk are more widely distributed in various tissues (2–4, 10, 11).

The functional roles of the Axl/Sky subfamily of receptor tyrosine kinases have been given much attention, especially the identification of ligands. The ligands for Axl and Sky were recently reported to be the product of growth arrest-specific gene 6 (Gas6) (13, 14). Protein S is a vitamin K-dependent plasma glycoprotein that has anticoagulant activity by acting as a cofactor of activated protein C (APC)-catalyzed inactivation of coagulation factors Va and VIIIa (15). Gas6, originally identified as a gene product expressed in response to growth arrest, has structural similarity to protein S with 42–43% identity (16) and was seen to function as a potentiation factor for thrombin-induced proliferation of vascular smooth muscle cells (VSMC) (17).

Prior to investigating the physiological functions of Sky and its reported ligand protein S, we examined the potential of protein S to bind to Sky and to stimulate tyrosine phosphorylation of Sky. In contrast to an earlier report (14), we found no specific binding of protein S to Sky or stimulation of Sky tyrosine phosphorylation by protein S. Gas6, not protein S, did show potent activity to bind to Sky and to induce the phosphorylation of Sky. We describe here our evidence that Gas6 is the ligand for Sky.

EXPERIMENTAL PROCEDURES

Purification of Gas6 and Protein S—Gas6 was purified from the conditioned medium of rat VSMC (17). The purity of Gas6 was confirmed by SDS-polyacrylamide gel electrophoresis (PAGE) analysis (17). Protein S was purified from normal human plasma by the reported procedures (18, 19). Based on the SDS-PAGE analysis, under reduced conditions, the preparations of protein S used in this study contained almost equal amounts of thrombin-cleaved and uncleaved protein S (18). The purified protein S had APC cofactor activity and also inhibited the activity of the platelet prothrombinase complex (20).

Expression of Recombinant Gas6 and Protein S—The cDNA encoding rat Gas6 was obtained as previously reported (17). The cDNA encoding human protein S was provided by Dr. B. Dahlbäck (21). The cDNAs were subcloned into the PUC-SRα expression plasmid (22), and the constructs were transfected into COS-7 cells using liposome methods (23). Cells were cultured for 3 days in serum-free Dulbecco’s modified Eagle’s medium with 10 μM sodium menadione bisulfite. Levels of proteins expressed in the conditioned media (100–1000 ng/ml) were determined by immunoblotting with anti-Gas6 or anti-protein S antibodies.

Antibodies—Rabbit anti-Sky polyclonal antibody (Sky-C) raised against the C-terminal peptide of human Sky was prepared and purified as described previously (24). Rabbit anti-protein S polyclonal antibody was raised against the purified human protein S, as described elsewhere (25). Rabbit anti-Gas6 antiserum was raised against the purified Gas6. Anti-phosphotyrosine monoclonal antibody (PY20) was specific for the phosphorylated tyrosine residues of the proteins expressed in the conditioned media (100–1000 ng/ml) were determined by immunoblotting with anti-Gas6 or anti-protein S antibodies.

*This work was supported in part by a research grant from the Ministry of Education, Science and Culture of Japan and by research grants from the Ryōichi Naitou Foundation and the Mochida Memorial Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: Gas6, the protein encoded by growth arrest-specific gene 6; APC, activated protein C; CHO, Chinese hamster ovary; EGF, epidermal growth factor; FGF, fibroblast growth factor; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline, VSMC, vascular smooth muscle cells.
sine kinase. Immunoblot analysis using the anti-Sky antibody
CHO cells stably expressing the full-length Sky receptor tyro-
stimulate tyrosine phosphorylation of Sky, we constructed
protein S can specifically bind to the extracellular domain of
). These observations suggest that Gas6 but not
Met-Fc (Fig. 1). In contrast, protein S was not co-precipitated by either Sky-Fc or
specifically co-precipitated Gas6 protein (Fig. 1). The precipitates were washed four times with cold phosphate-buffered saline (PBS), suspended in SDS sampling buffer (25 mM Tris-HCl, pH 6.5, 5% glycerol, 1% SDS, 144 mM 2-mercaptoethanol, 0.05% bromphenol blue), and subjected to 8% SDS-PAGE. The gels were then transferred to poly-
vinyldiene difluoride membrane (Bio-Rad) and the membrane was
blocked with 3% nonfat dry milk in PBS containing 0.05% Tween 20 and incubated for 1 h at room temperature with anti-Gas6 or anti-protein S antibody diluted in PBS containing 1% nonfat dry milk and 0.05%
Tween 20. After washing the membrane with PBS, it was incubated with the horseradish peroxidase-conjugated anti-rabbit IgG (Amer-
sham Corp.) and immunoreactive bands were visualized using ECL chemiluminescence reagent (Amer sham).

Construction of CHO Cell Lines Expressing Sky—A 3.8-kb human sky
cDNA (S) was subcloned into the NotI site of the expression vector pRc/RSV (Invitrogen) containing the neomycin-resistant gene. The re-
sulting plasmid was transfected into CHO cells using liposome methods, and the G418-resistant colonies were selected. One of the cell lines (B31) with high Sky expression, as measured by immunoblot analysis, was selected.

Tyrosine Phosphorylation Assay—B31 cells were plated on 60-mm dishes at a density of 2.5 × 10^6 cells/cm² and cultured in Ham’s F-12 medium supplemented with 10% fetal calf serum. After 16 h, the cells were serum-starved for 3 h and treated with Gas6 or protein S for 10 min at 37 °C, as indicated. Cells were then lysed once with cold PBS containing 1 mM orthovanadate and lysed with cold lysis buffer (20 mM Hepes, pH 7.2, 1% Nonidet P-40, 10% glycerol, 1 mM phenylmethyl-
sulfonyl fluoride, 1 mM orthovanadate, and 10 μg/ml leupeptin). The lysates were immunoprecipitated with anti-Sky antibody, run on SDS-
PAGE, and immunoblotted with anti-Sky antibody or anti-phospho-
rysin monoclonal antibody, as described previously (24).

RESULTS AND DISCUSSION

To determine the binding potential of protein S and Gas6 to Sky, we prepared a chimeric protein (Sky-Fc) that contains the extracellular ligand-binding domain of Sky fused to the Fc region of human immunoglobulin IgG1 heavy chain. Affinity adsorption and precipitation analysis using Fc fusion proteins in the presence of Protein A-Sepharose showed that Sky-Fc, but not Met-Fc (a control chimeric protein composed of the extracellular domain of c-Met and the Fc region of human IgG1), specifically co-precipitated Gas6 protein (Fig. 1A). On the other hand, protein S was not co-precipitated by either Sky-Fc or Met-Fc (Fig. 1B). These observations suggest that Gas6 but not protein S can specifically bind to the extracellular domain of Sky.

Ligands for receptor tyrosine kinases bind to their cognate receptors, then rapidly stimulate tyrosine phosphorylation of these receptors. To determine if Gas6 and/or protein S could stimulate tyrosine phosphorylation of Sky, we constructed CHO cells stably expressing the full-length Sky receptor tyrosine kinase. Immunoblot analysis using the anti-Sky antibody

raised against the C-terminal peptide of Sky revealed that the Sky protein with an apparent molecular mass of 140 kDa was detected in Sky-transfected (B31) cells, while the parental CHO cells showed no detectable immunoreactive band (Fig. 2A). Cell surface biotinylation experiments revealed that a 140-kDa protein immunoprecipitated with anti-Sky antibody was exposed at the cell surface (data not shown). The tyrosine
phosphorylation of Sky expressed on B31 cells was assessed by immunoblotting with anti-phosphotyrosine antibody after immunoprecipitation of the cell lysates with anti-Sky antibody. As shown in Fig. 2B, phosphorylation of Sky was induced when the B31 cells were treated with Gas6 (compare lanes 1 and 2), but not when treated with protein S (lane 4), while the amount of Sky in the immunoprecipitates remained unchanged. No immunoreactive band with anti-phosphotyrosine antibody was detected in the anti-Sky immunoprecipitates of the parental CHO cells treated with Gas6 (Fig. 2B, lane 5). These results clearly show that Gas6 but not protein S can stimulate tyrosine phosphorylation of the Sky receptor tyrosine kinase expressed in B31 cells. Additionally, phosphorylation of Sky in response to Gas6 was almost completely blocked in the presence of 10 μM EGTA (lane 2 versus lane 3). This means that Sky phosphorylation was induced by direct interaction between Gas6 and Sky and not by indirect cross-phosphorylation of Sky by other receptor tyrosine kinases.

To exclude the possibility that the Gas6 preparation purified from VSMC used in this study might be contaminated by the protein activating Sky, recombinant Gas6 was expressed in COS cells and examined to observe if it would stimulate Sky phosphorylation. Recombinant protein S was also expressed and examined, in parallel experiments. Expression of Gas6 and protein S in culture supernatants of COS cells transfected with each expression plasmid was confirmed by immunoblot analysis (Fig. 3A). As shown in Fig. 3B, the culture supernatants of COS cells expressing recombinant Gas6 induced tyrosine phosphorylation of Sky, whereas the supernatants of mock-transfected COS cells and the supernatants of COS cells expressing recombinant protein S had no detectable activity. These observations also show that Gas6 but not protein S stimulates Sky tyrosine phosphorylation.

Protein S contains 11 γ-carboxyglutamic acid (Gla) residues in the N-terminal Gla domain, one β-hydroxyaspartic acid residue in the first epidermal growth factor (EGF)-like domain, and three β-hydroxyasparagine residues in three other EGF-like domains (15). These modified residues appear to be involved in Ca2+ binding (15). As these residues are conserved in the sequence of Gas6, Gas6 may also bind Ca2+ ions through these residues (16, 17). To examine the effects of Ca2+ ion on Sky-stimulating activity of Gas6, recombinant Gas6 was treated with EGTA and lost all activity (Fig. 3C). Therefore, Ca2+ ion binding is essential for the conformation and activity of Gas6 to bind to and stimulate Sky.

Recombinant Gas6 purified to apparent homogeneity from the culture medium of COS cells transfected with Gas6 expression plasmid stimulated Sky phosphorylation in a dose-dependent manner (Fig. 4). Tyrosine phosphorylation of Sky was detectable at 0.5 nM of recombinant Gas6 and the half-maximal stimulation was obtained at approximately 1 nM. The concentration of Gas6 required for stimulation of Sky is comparable to the Ki value (0.3 nM) of the binding of Gas6 to membranes of VSMC, for which Gas6 has growth potentiating activity (17).

In light of all these findings, we propose that Gas6, but not protein S, is a ligand for Sky, specifically binding to Sky and stimulating tyrosine phosphorylation. Our observations differ from those of Stitt et al. (14). They reported that protein S but not Gas6 efficiently binds to and stimulates mouse Sky (Tyro3). Although the Sky receptor they used was of a different species (mouse Sky used by Stitt et al. (14) versus human Sky used in our study), the species difference would not likely explain the different results in ligand specificity, because sequences of human and mouse Sky are highly homologous (85% identity within their extracellular domains). If all results are compiled, human protein S does bind to mouse Sky (as described by Stitt et al.), but does not bind to human Sky (as described here). As the purified human protein S used in our study retained both APC cofactor activity and inhibitory activity of the prothrombinase complex activity (20), it is unlikely that the purified protein S we used was structurally damaged. Thus, at present we have no valid explanation for the discrepancy between the results obtained in this study and those by Stitt et al. (14).

Two different research groups reported that Gas6 is the ligand for Axl, a receptor closely related to Sky (13, 14). The effective dose of Gas6 to Sky shown in this study is comparable to the reported value for Gas6-Axl interaction (13). Thus, Gas6 may be a common ligand for the two related receptors, Sky and Axl. The binding of a ligand to two distinct members of a receptor subfamily is also seen for fibroblast growth factor (FGF) family ligands (acidic FGF and basic FGF), both of which bind to two members of an FGF receptor subfamily, Flg and Bek, with similar affinity constants (28), and the ligands for Eph family receptors (B61 and EHK1-L), which bind to two distinct members of the Eph family.
Gas6 as a Ligand of Sky Receptor

members of an Eph receptor subfamily, Eck and EHK-1, with similar affinity constants (29). Although neither the functions of Gas6 nor the physiological significance of receptor redundancy are well understood, Gas6 may exhibit diverse functions in a cell and tissue-dependent manner through two distinct receptors, Sky and Axl, whose expression patterns differ significantly.

Identification of the ligand for Sky should pave the way to initiate research on the functional roles of Sky and its ligand Gas6. As Sky is expressed predominantly in neurons in restricted regions of the brain (6), future study will focus on the biological function of Gas6 on neurons. As Sky is also expressed in some extents in other tissues, such as testis, ovary and kidney, and in certain types of cells (5–9, 12), the functional roles of Gas6 to these tissues and cells may be identified. Gas6 potentiates cell proliferation of VSMC stimulated by Ca\(^{2+}\)-mobilizing growth factors, such as thrombin, and may be involved in intimal thickening of the vascular wall accompanying atherosclerosis or restenosis (17). To develop antagonists for Gas6 that will aid in overcoming these vascular diseases, it is also important to clearly define which receptor, Sky or Axl, mediates the action of Gas6 on VSMC. In addition, it will be interesting to search for other members of the protein S-related protein family, which may function as ligands for Sky-related receptors such as c-Eyk.

Acknowledgments—We thank Drs. Y. Fujiki, T. Nakamura, S. Iwanaga, and M. Ohara for advice and helpful comments, Dr. G. F. Vande Woude for the human c-Met cDNA, and Dr. B. Dahlbäck for the human protein S cDNA.

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Fig. 4. Dose-dependent tyrosine phosphorylation of Sky in response to purified recombinant rat Gas6. Recombinant Gas6 was purified from the culture supernatants of COS-7 cells transfected with rat Gas6 expression plasmid. Serum-starved B31 cells were treated for 10 min at 37°C with the indicated concentrations of purified recombinant Gas6. Cell lysates were immunoprecipitated and immunoblotted with α-PY (upper panel) or α-Sky antibody (lower panel), as described in Fig. 2. Elution position of Sky is indicated by an arrow. Molecular sizes (kDa) of marker proteins are indicated on the left. B, relative levels of tyrosine phosphorylation of Sky were plotted as a function of the concentration of Gas6 added. The levels of tyrosine phosphorylation of Sky were calculated by dividing the density of anti-phosphotyrosine immunoreactive band by the density of anti-Sky immunoreactive band. The density was evaluated from data in a NIH image software. The level of tyrosine phosphorylation of Sky when treated with 10 nM purified rat Gas6 was taken as 100%.
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J. Biol. Chem. 1995, 270:22681-22684.
doi: 10.1074/jbc.270.39.22681

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