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Matters Arising

Reevaluation of the Roles of Protein S and Gas6 as Ligands for the Receptor Tyrosine Kinase Rse/Tyro 3

Recently, Stitt et al. (1995) reported that protein S (PS), but not Gas6, is a potent ligand for the receptor tyrosine kinase known as Rse, Tyro 3, Brt, Sky, and Tif (hereafter referred to as Rse/Tyro 3). PS is an abundant serum protein previously characterized as an essential anticoagulant. Gas6, which was identified as a gene whose expression is increased by growth arrest, shares 43% amino acid identity and overall domain organization with PS (Manfioletti et al., 1993). Stitt et al. (1995) based their conclusions on experiments describing interspecies interactions of bovine Gas6 with murine Rse/Tyro 3 (mRse/Tyro 3) and on interactions of bovine and human PS (hPS) with mRse/Tyro 3. Consistent with the results of Stitt et al. (1995), we identified bovine PS as a ligand for human Rse/Tyro 3 (hRse/Tyro 3), and we also found that hPS can act as a ligand for mRse/Tyro 3. However, when we analyzed the more relevant intraspecies interactions, we obtained different results. We found that human Gas6 (hGas6), but not hPS, acted as a potent ligand for hRse/Tyro 3 (Figure 1). The hRse/Tyro 3 we have studied is very likely the true homolog of mRse/Tyro 3 since they share 90% amino acid identity and a similar expression pattern (Mark et al., 1994; Lai et al., 1994).

To characterize the Rse/Tyro 3 ligand, we constructed soluble receptor proteins containing the extracellular domain of either hRse/Tyro 3 or mRse/Tyro 3 fused to the Fc portion of human immunoglobulin G1 (hRse–IgG and mRse–IgG). A similar fusion protein (termed Tyro 3–Fc) was utilized by Stitt et al. (1995) to characterize the binding of bovine Gas6 and hPS to mRse/Tyro 3. We first determined whether hRse–IgG or mRse–IgG differed in its ability to bind to hPS or hGas6 containing an epitope tag that allows for side-by-side comparison of the binding properties of the two proteins. Either hRse–IgG or mRse–IgG was incubated with conditioned medium containing putative ligands. Complexes were captured with protein A (specific for the IgG fusion protein) and visualized with an antibody specific for the epitope-tagged putative ligand. While hGas6 was bound by hRse–IgG, it was not efficiently bound by mRse–IgG (Figure 2). The reciprocal result was obtained in analysis of binding of hPS to hRse–IgG and mRse–IgG; hPS was bound by mRse–IgG but not by hRse–IgG. These results are consistent with an apparent difference in affinity of hPS for human as opposed to mRse/Tyro 3 (hPS prefers mRse/Tyro 3), as well as an inverse difference for hGas6 (hGas6 prefers hRse/Tyro 3).

A second method was used to analyze quantitatively the interactions of hRse/Tyro 3 and mRse/Tyro 3 to hGas6 and hPS. We measured the binding of ^{125}I-hRse–IgG or ^{125}I-mRse–IgG to purified hGas6 or hPS using an assay in which complexes are captured with a method specific for the Gla domain present in hGas6 and hPS (barium coprecipitation) and in which these complexes are quantitated by virtue of the radioactivity in Rse–IgG. While hGas6 bound ^{125}I-hRse–IgG efficiently, giving a half-maximal binding at 8 nM hGas6, binding to ^{125}I-mRse–IgG over the same concentration range was minimal (Figure 3A). In contrast, hPS was capable of binding ^{125}I-mRse–IgG, although at considerably higher concentrations (half-maximal concentration of 90 nM), but bound very little ^{125}I-hRse–IgG (Figure 3B). We then compared the ability of increasing unlabeled hRse–IgG or mRse–IgG to compete for binding with fixed levels of ^{125}I-hRse–IgG to hGas6 (Figure 3C). The binding of ^{125}I-hRse–IgG to hGas6 was competed by low concentrations of unlabeled hRse–IgG (IC_{50} of 0.3 nM), but only by high concentrations of unlabeled mRse–IgG (IC_{50} of 37 nM). In contrast, the binding of ^{125}I-mRse–IgG to hPS was competed by mRse–IgG (IC_{50} of 18 nM) while hRse–IgG did not appear to compete at all (Figure 3D). These studies are entirely consistent with those shown in Figure 2. There was a profound difference in the affinities of hGas6 and hPS for the extracellular domains of mRse/Tyro 3 and hRse/Tyro 3, with hGas6 showing a preference for hRse/Tyro 3 and hPS exhibiting a preference for mRse/Tyro 3. Similar results were obtained when the binding of hPS or hGas6 to receptor fusion proteins was analyzed by molecular interaction analysis using a stirred-cell optical sensor system (data not shown).

hPS was reported to induce Rse/Tyro 3–mediated responses in NIH 3T3 cells. We directly compared the ability of hPS or hGas6 to induce phosphorylation of hRse/Tyro 3 or mRse/Tyro 3 expressed in NIH 3T3 cells. As noted

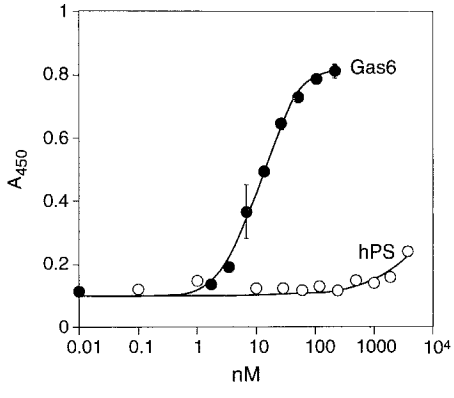

Figure 1. Comparison of Purified hGas6 and Purified Plasma-Derived hPS as Ligands for hRse/Tyro 3

The indicated concentrations of purified recombinant hGas6 (closed circles) or plasma-derived hPS (open circles) (Enzyme Research Laboratories) were tested for ability to induce phosphorylation of hRse/Tyro 3 expressed in CHO cells using an ELISA-based KIRA assay (see Experimental Procedures). Identical results were observed when hRse/Tyro 3 phosphorylation was analyzed by immunoprecipitation using anti-Rse/Tyro 3 antibodies and Western blotting with anti-phosphotyrosine antibodies.

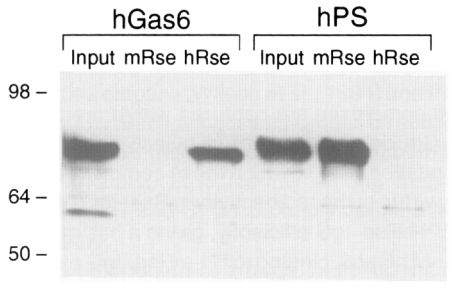

Figure 2. Species-Specific Binding of Epitope-Tagged hGas6 and hPS by hRse–IgG and mRse–IgG

Either hRse–IgG or mRse–IgG was incubated with unfractionated conditioned medium containing epitope-tagged hGas6 or hPS. Complexes were captured with protein A (specific for the IgG fusion protein) and fractionated by SDS–PAGE followed by Western blotting. Bound proteins were detected using the anti-gD antibody specific for the epitope-tagged putative ligand to compare directly the input and bound amounts of recombinant hPS and hGas6. The unfractionated (input) lanes represent 20% of the material used for the precipitation assay. Molecular sizes are indicated on the left (in kilodaltons).

previously (Stitt et al., 1995; our unpublished data), phosphorylation of hRse/Tyro 3 and mRse/Tyro 3 is observed upon treatment of cells with fetal bovine serum (FBS), indicating that both hRse/Tyro 3 and mRse/Tyro 3 expressed in NIH 3T3 cells are functional (Figure 4). Phosphorylation of hRse/Tyro 3 was not observed in response to high levels of either purified hPS or conditioned medium containing recombinant hPS, but was activated by conditioned medium containing hGas6. In contrast, mRse/Tyro 3 was activated by purified hPS and by conditioned medium containing recombinant hPS, but not by medium containing hGas6. The level of hPS required to activate mRse/Tyro 3 in our assays was significantly higher than that reported by Stitt et al. (1995) to activate mRse/Tyro 3 expressed in Rat2 cells. We note that in the studies reported here and by Stitt et al., the levels of recombinant hPS were estimated by comparison to purified hPS on Western blots. The concentration of hPS required to activate mRse/Tyro 3 in the phosphorylation assay reported here is consistent

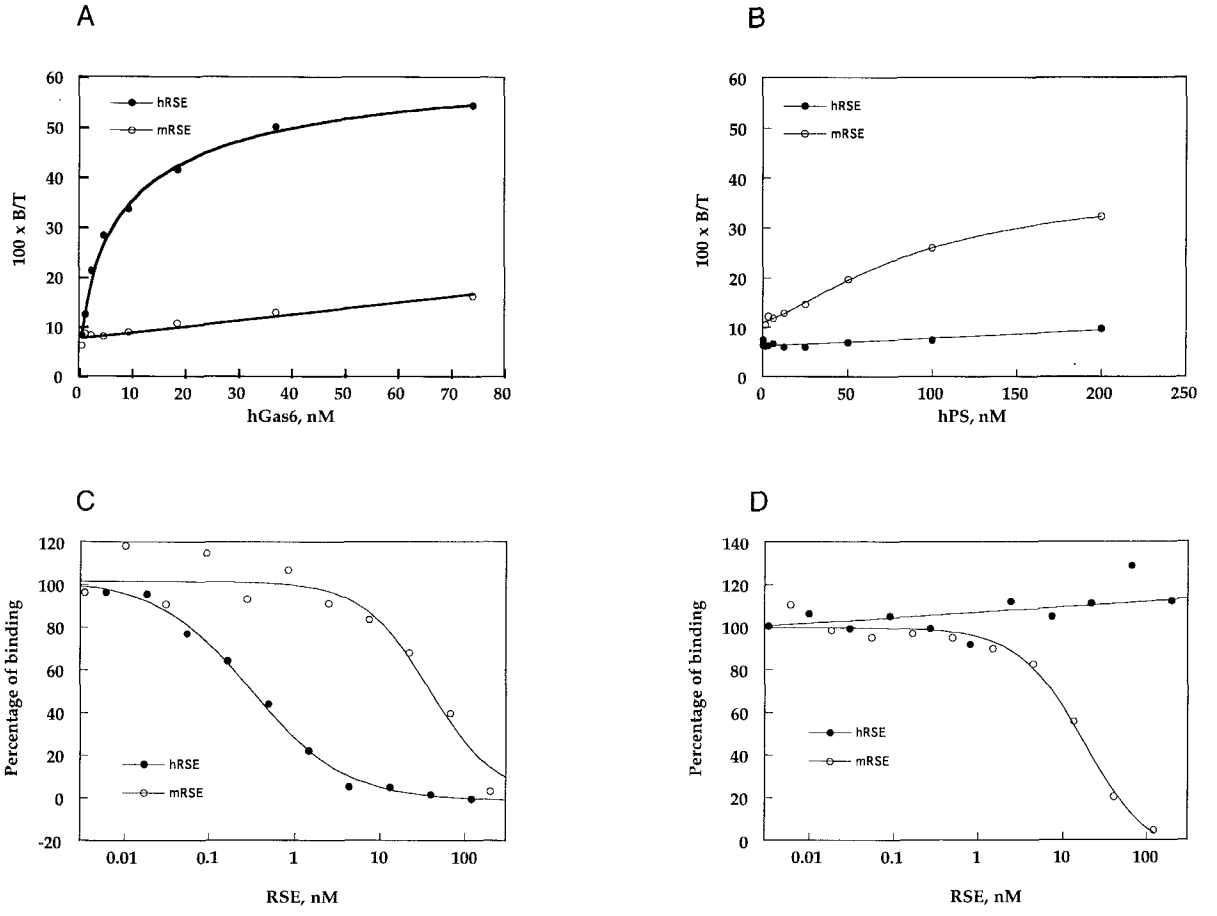

Figure 3. Solution Phase Binding of Recombinant hGas6 and Plasma-Derived hPS to hRse–IgG and mRse–IgG

To measure saturation with ligand, increasing concentrations of hGas6 (A) or hPS (B) were mixed with a fixed amount (50,000 cpm) of ^{125}I-hRse/Tyro 3 or ^{125}I-mRse/Tyro 3, and precipitable radioactivity was measured as described in Experimental Procedures. Binding in (A) and (B) is expressed as 100 × B/T, where T is total radioactivity added and B is radioactivity recovered in the barium pellet. To measure inhibition of binding, concentrations of hGas6 and hPS were chosen to give 100 × B/T of 8–12. A constant amount of hGas6 and ^{125}I-hRse/Tyro 3 (C) or a constant amount of hPS and ^{125}I-hRse/Tyro 3 (D) was mixed with the indicated concentrations of hRse–IgG or mRse–IgG, barium precipitable radioactivity was measured as in Experimental Procedures, and binding was normalized to the percent of that obtained in the absence of added inhibitor with appropriate background subtraction (background of 3%–5% of total counts per minute).

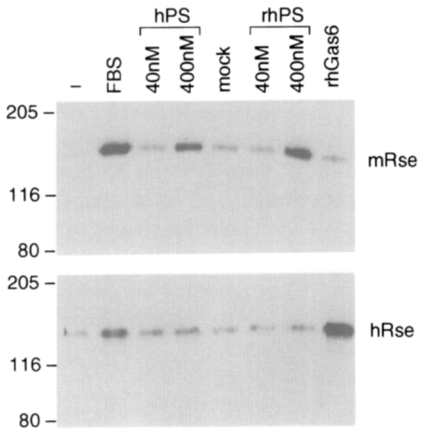

Figure 4. Conditioned Medium from Cells Expressing hGas6 Induces Phosphorylation of hRse/Tyro 3

Phosphorylation of mRse/Tyro 3 is induced by purified hPS or by recombinant hPS (rhPS). NIH 3T3 cells expressing either mRse/Tyro 3 or hRse/Tyro 3 were serum starved and then left untreated (minus) or treated with 20% FBS or the indicated concentrations of hPS purified from human serum or treated with conditioned medium from 293 cells either mock transfected (M) or expressing recombinant hPS or recombinant hGas6 (rhGas6). The amounts of PS and Gas6 in the conditioned medium were estimated by Western blotting and by comparison to purified material of known concentration.

with the relatively modest affinity of hPS for mRse/Tyro 3 that we observe (see Figure 3).

Rse/Tyro 3, along with the related receptors Axl/Ufo and c-Mer, comprises a family of receptor tyrosine kinases whose extracellular domains are reminiscent of neural cell adhesion molecules. Our data demonstrate that hGas6 is a bona fide ligand for hRse/Tyro 3. Interestingly, hGas6 has also been shown to activate human Axl/Ufo (Varnum et al., 1995; our unpublished data), and bovine Gas6 binds to the extracellular domain of murine Axl/Ufo (Stitt et al., 1995). Thus, Gas6 appears to activate multiple receptor signaling pathways.

We also demonstrate a pattern of reciprocal preferences between hRse/Tyro 3 and mRse/Tyro 3 and hPS and hGas6; hRse/Tyro 3 prefers hGas6 over hPS while mRse/Tyro 3 prefers hPS over hGas6. One explanation for the inability of bovine Gas6 to bind to mRse/Tyro 3 (Stitt et al., 1995) is that this interaction may be governed by species differences in receptor–ligand recognition. While our data that hPS binds to and activates mRse/Tyro 3 are in agreement with that of Stitt et al. (1995), the inability of hPS to serve as a ligand for hRse/Tyro 3 brings into question the conclusion that PS is a potent ligand for Rse/Tyro 3. Our results do not rule out the possibility that hPS might activate hRse/Tyro 3 under certain circumstances. For example, modified forms of either the ligand or the receptor might behave differently than the versions we have tested. However, we have tested several sources of hPS (plasma-derived hPS from Enzyme Research Laboratories, Calbiochem, and Celsus Laboratories, as well as recombinant hPS expressed in human 293 and CHO cells) and do not detect significant activation of hRse/Tyro 3. Given its homology to Gas6, a potent ligand for Rse/Tyro 3, and the related receptor Axl (Varnum et al., 1995), PS could yet prove to have a role as a physiologically relevant ligand for a member of the Rse/Axl family.

Paul J. Godowski,* Melanie R. Mark,* Jian Chen,*
Michael D. Sadick,† Helga Raab,‡
and R. Glenn Hammonds‡
*Department of Cell Genetics
†Department of Bioanalytical Technology
‡Department of Protein Chemistry
Genentech
South San Francisco, California 94080

Experimental Procedures

Construction and Expression of Recombinant Proteins

hRse–IgG and mRse–IgG were constructed by fusing the sequence encoding amino acids 1–428 of hRse/Tyro 3 or amino acids 1–418 of mRse/Tyro 3 (Mark et al., 1994) to amino acids 216–443 of human IgGγ1. Epitope-tagged gD.hPS or gD.Gas6 were constructed by linking the coding sequences for the gD signal sequence and epitope tag (Mark et al., 1994) by polymerase chain reaction to coding sequences immediately before the first amino acid of mature hGas6 or hPS. Recombinant proteins were expressed in stable 293 cells grown in serum-free medium. For purification, conditioned medium containing receptor–IgG proteins was mixed 1:1 with Pierce ImmunoPure gentle Ag/Ab binding buffer and passed through a 1 ml HiTrap protein A–Sepharose column. Bound proteins were eluted with Pierce ImmunoPure eluting buffer and desalted on a Pharmacia PD10 column into phosphate-buffered saline (PBS). Protein concentration was determined by an anti-Fc enzyme-linked immunosorbent assay (ELISA).

Binding Assays

Immunoprecipitation with IgG fusion proteins was performed by mixing 1 μg of purified mRse–IgG or hRse–IgG with conditioned medium containing 150 ng of hGas6.gD or hPS.gD and 10 μl of protein A–Sepharose CL4B (Pharmacia) in a final volume of 300 μl. Binding was performed at 4°C on a shaker for 12 hr. Complexes were collected by centrifugation and washed three times with 0.5 ml of PBS with 0.1% NP-40. Proteins were separated by reducing SDS–polyacrylamide gel electrophoresis (SDS–PAGE) on Novex 4%–12% minigels, transferred to nitrocellulose, and blotted with monoclonal anti-gD antibody as described previously (Mark et al., 1994).

Iodination of Rse–IgG was performed using Iodobead (Pierce) oxidation of ^{125}I-NaI; specific activity was 250–500 Ci/mmol. Binding studies were performed by incubating samples containing hGas6 or hPS in 25 mM HEPES (pH 7.2), 0.1% BSA, and 0.05% Tween 20 with 50,000 cpm of ^{125}I-mRse–IgG or ^{125}I-hRse–IgG and cold competitor (if any) in a total volume of 100–120 μl. After 45 min at room temperature, 1 ml of a freshly prepared ice-cold suspension of $BaCl_2$ (10 mM) in PBS was added and precipitable radioactivity collected by centrifugation for 4 min at 14,000 × g.

Phosphorylation Assays

CHO cell lines expressing a Rse.gD (which contains a C-terminal epitope tag recognized by antibody 5B6) were established and screened by Western blotting as described previously (Mark et al., 1994). An ELISA-based kinase receptor activation (KIRA) assay (Sadick et al., submitted) was established to measure quantitatively the ligand-induced phosphorylation of Rse/Tyro 3. CHO-Rse.gD cells were treated with potential ligand sources for 15 min, lysed with detergent, and transferred to surfaces coated with antibody 5B6. The degree of phosphorylation was quantitated using a biotinylated anti-phosphotyrosine antibody (4G10; UBI), streptavidin conjugated to horseradish peroxidase (Zymed Laboratories), and a colorimetric peroxidase assay (two-component substrate kit; Kirkegard & Perry). Purified native hPS was obtained from Enzyme Research Laboratories. NIH 3T3 cells expressing hRse/Tyro 3 or mRse/Tyro 3 were established by infection of NIH 3T3 cells with a recombinant ecotropic retrovirus LXSN.hRse or LXSN.mRse encoding hRse/Tyro 3 or mRse/Tyro 3, respectively. Retroviral vectors were used to produce populations consisting of hun-

dreds of thousands of stable clones to avoid the possibility that an individually selected clone might exhibit an aberrant phenotype. Retroviral stocks (titer of $\sim 1 \times 10^6$ cfu/ml) were prepared, and 1 ml was used to infect 1×10^6 NIH 3T3 cells, which were selected as described previously (Pear et al., 1993). NIH 3T3.hRse or NIH 3T3.mRse cells were washed once in PBS and then cultured in high glucose DMEM without serum for 3 hr prior to treatment. Cells were treated with potential ligand source for 5 min at 37°C. Conditioned medium containing hPS and hGas6 was concentrated 15-fold and then diluted 3-fold and 30-fold to achieve the final concentrations, estimated to be 400 nM or 40 nM, respectively. Control-conditioned medium concentrated 15-fold was used at a 3-fold dilution for the studies. Cells were washed once with cold PBS, lysates were prepared, and receptors were immunoprecipitated with a polyclonal antibody that recognizes both hRse and mRse and were immunoblotted with an anti-phosphotyrosine antibody (4G10; UBI). After incubation with an horseradish peroxidase–conjugated anti-mouse IgG secondary antibody (Amersham), the phosphorylated proteins were revealed using a chemiluminescent detection method (ECL; Amersham).

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