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The Anticoagulation Factor Protein S and Its Relative, Gas6, Are Ligands for the Tyro 3/Axl Family of Receptor Tyrosine Kinases

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Summary

We report the identification of ligands for Tyro 3 (alternatively called Sky, rse, brt, or tif) and Axl (alternatively, Ark or UFO), members of a previously orphan family of receptor-like tyrosine kinases. These ligands correspond to protein S, a protease regulator that is a potent anticoagulant, and Gas6, a protein related to protein S but lacking any known function. Our results are reminiscent of recent findings that the procoagulant thrombin, a protease that drives clot formation by cleaving fibrinogen to form fibrin, also binds and activates intracellular signaling via a G protein–coupled cell surface receptor. Proteases and protease regulators that also activate specific cell surface receptors may serve to integrate coagulation with associated cellular responses required for tissue repair and growth, as well as to coordinate protease cascades and associated cellular responses in other systems, such as those involved in growth and remodeling of the nervous system.

Introduction

An important class of cell surface receptors are those with intrinsic protein-tyrosine kinase activity, thus known as receptor tyrosine kinases (RTKs) (Schlessinger and Ullrich, 1992). Binding of the extracellular portion of an RTK to its cognate polypeptide ligand results in receptor dimerization or oligomerization, which in turn activates the intracellular catalytic domain of the receptor, resulting in tyrosine phosphorylation of the receptor as well as of multiple intracellular signaling molecules. These molecules initiate a variety of signaling cascades that determine the wide variety of phenotypic responses that can be mediated by RTKs. Thus, the insulin receptor is critical for maintaining glucose homeostasis and is a major regulator of metabolism in many cell types; receptors for vascular endothelial growth factor appear to be critical for angiogenesis; and the Trk receptors for the neurotrophins mediate survival and differentiation effects upon particular neurons (Schlessinger and Ullrich, 1992).

The extensive sequence similarity shared by tyrosine kinase domains has allowed for homology-based cloning of a large number of proteins that appear to be RTKs, in that they are predicted transmembrane proteins with large ectodomains and a cytoplasmic tyrosine kinase domain (e.g., Lai and Lemke, 1991). These proteins have been designated as orphan RTKs because their presumed ligands have yet to be identified. Recent progress has allowed for the identification of ligands for previously orphan RTKs. For example, a receptor-based affinity purification approach (Bartley et al., 1994), as well as receptor-based detection screens for ligand-expressing cells in pools of cells randomly transfected with cDNA expression libraries (Beckmann et al., 1994; Cheng and Flanagan, 1994; Davis et al., 1994), has yielded several related ligands for what had previously been the largest family of orphan RTKs, those related to Eph and Eck. Here we have focused on the identification of ligands for another family of previously orphan receptor-like tyrosine kinases. This family was first recognized when a search for receptor-like tyrosine kinases in the rat nervous system yielded DNA fragments encoding portions of three candidate receptor-like tyrosine kinases that appeared to be closely related to each other (Lai and Lemke, 1991); these candidate receptor-like tyrosine kinases were designated Tyro 3, Tyro 7, and Tyro 12. Full-length versions of cDNAs encoding these three receptor-like tyrosine kinases have now been characterized: Tyro 3 has also been named Sky, rse, brt, or tif (Lai et al., 1994; Ohashi et al., 1994; Mark et al., 1994; Fujimoto and Yamamoto, 1994; Dai et al., 1994); Tyro 7 has also been designated Axl, UFO, or Ark (O'Bryan et al., 1991; Janssen et al., 1991; Rescigno et al., 1991; Bellosta et al., 1995); what is likely the chicken version of Tyro 12 has been called Eyk (Jia and Hanafusa, 1994), while the human form has been dubbed c-Mer (Graham et al., 1994). These three related receptor-like tyrosine kinases display differential patterns of expression. Tyro 3/Sky/rse/brt/tif (hereafter called Tyro 3) is most prominently expressed in the adult nervous system, although it is notably expressed in some nonneural tissues such as kidney, ovary, and testis, and it is also found in a number of hematopoietic cell lines. Axl/UFO/Ark (hereafter called Axl) and Tyro 12/

Eyk/Mer (hereafter called Mer) are also expressed in the nervous system, but are more widely expressed than Tyro 3 in peripheral tissues. All three of these related receptor-like tyrosine kinases have similar ectodomains, sharing about 35% amino acid identity and consisting of two immunoglobulin-like domains followed by two fibronectin type III repeats, suggesting that they recognize related ligands.

We have utilized receptor-based detection and affinity purification approaches to identify ligands for Tyro 3 and Axl. These ligands correspond, respectively, to a previously identified protein, designated protein S (Dahlback, 1991), known for its potent anticoagulant actions, and a protein S relative known as Gas6 (Manfioletti et al., 1993) because it was cloned as a growth arrest–specific gene. The critical anticoagulant role of protein S is most dramatically revealed by the massive thrombotic complications suffered by infants homozygous for protein S deficiency (Mahasandana et al., 1990; Pegelow et al., 1992). Protein S seemingly acts by indirectly inhibiting proteases involved in the coagulation cascade, although the precise mechanism by which protein S mediates this inhibition remains unclear (Dahlback, 1991); several additional functions for protein S, not directly involving coagulation, have also been proposed (Hessing, 1991; Gasic et al., 1992; Maillard et al., 1992; Phillips et al., 1993). Protein S is a heavily modified ~70 kDa protein that contains several modules, an N-terminal region containing vitamin K–dependent γ-carboxylation sites, a thrombin-sensitive module, a series of epidermal growth factor (EGF)-like repeats that undergo hyroxylation modification, and a module with homology to steroid-binding globulin (Dahlback, 1991). Although nothing is known about the function of Gas6, it shares all but the thrombin-sensitive module with protein S (Manfioletti et al., 1993).

While our findings that protein S–related proteins are ligands for the Tyro 3/Axl family of RTKs were quite unexpected, they are reminiscent of recent findings with another protein critical for regulating coagulation, the procoagulant thrombin, which is a protease that drives clot formation by cleaving fibrinogen to form fibrin. Thrombin, like protein S, has also been found to bind and activate intracellular signaling via a specific cell surface receptor; in the case of thrombin, however, the receptor is a G protein–coupled receptor (Vu et al., 1991; Coughlin, 1993). These thrombin receptors may be invoived in regulating coagulation pathways or related processes (Coughlin, 1993), but it has also been suggested that they act to regulate thrombin involvement in other protease cascades, such as those involved in remodeling of the nervous sytem (Monard, 1993); thrombin receptors also mediate more traditional growth factor–like actions of thrombin (Coughlin, 1993). It seems quite remarkable (and perhaps not coincidental) that a procoagulant/protease (thrombin) and an anticoagulant/protease regulator (protein S) would both have the ability to activate intracellular signaling cascades via specific cell surface receptors. The realization that the protein S–related proteins are ligands for the Tyro 3/Axl family of RTKs will likely provide new insights into the regulation of coagulation and adjunctive systems necessary for tissue repair and growth, as well as unrelated systems that also exploit both these factors and their receptors.

Results

Identification of an Activity That Binds and Induces Autophosphorylation of Tyro 3

To identify a source of a ligand for Tyro 3, we evaluated concentrated conditioned media from a number of different cell lines for their ability to induce tyrosine phosphorylation of the Tyro 3 receptor expressed in Rat2 fibroblasts. Only a small number of the cell lines conditioned in serum-free media displayed Tyro 3–phosphorylating activity, the most notable being the ABAE bovine endothelial cell line (Figure 1A, left). In contrast, all conditioned media prepared in serum displayed prominent Tyro 3–phosphorylating activity, suggesting that serum itself was a source of this activity. Consistent with this possibility, evaluation of fetal bovine serum (FBS) revealed that it alone contained notable levels of Tyro 3–phosphorylating activity (Figure 1A, left); various batches of 10-fold concentrated ABAE conditioned medium contained activity at a concentration comparable to that found in 1%–3% serum (e.g., Figure 1A; data not shown).

If the Tyro 3–phosphorylating activity present in serum

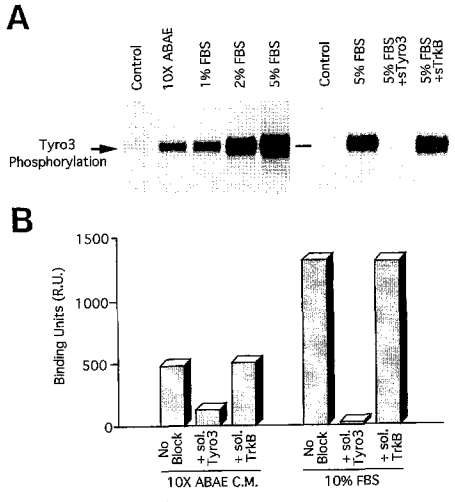

Figure 1. Tyro 3 Phosphorylation-Inducing and Binding Activity Is Identified in Conditioned Medium from ABAE Cells and in FBS

(A) ABAE conditioned medium (10-fold concentrated) and FBS (at indicated concentrations) induce Tyro 3 tyrosine phosphorylation that is blocked in the presence of excess soluble Tyro 3–Fc. Rat2 fibroblasts transfected with an expression vector encoding mouse Tyro 3 were challenged for 5 min with indicated samples, then analyzed for levels of tyrosine-phosphorylated Tyro 3 as described in the Experimental Procedures; in indicated lanes, FBS was premixed with 50 μg/ml of either soluble Tyro 3–Fc or soluble TrkB–Fc prior to being added to the reporter cells.

(B) BIAcore assay of Tyro 3–binding activity. The indicated samples of ABAE conditioned medium (10-fold concentrated) or FBS were passed over a BIAcore sensor chip surface that had Tyro 3–Fc covalently coupled to it; prior to being passed over the sensor chip, samples were in some cases premixed with 10 μg/ml soluble Tyro 3–Fc (sol. Tyro 3) or TrkB–Fc (sol. TrkB), as indicated. Binding to the sensor chip surface is indicated (as RUs).

or in ABAE conditioned medium were a bona fide Tyro 3 ligand that directly bound to the Tyro 3 ectodomain and thus activated catalytic activity of the Tyro 3 endodomain, we reasoned that excess amounts of a soluble Tyro 3 ectodomain would act as a blocker of this activity by binding to it and preventing its interaction with cell surface Tyro 3 receptors. To evaluate this possibility, we constructed a fusion between the ectodomain of Tyro 3 and the Fc portion of human immunoglobulin G1 (designated soluble Tyro 3 or Tyro 3–Fc), as previously described for the Eph-related receptors (Davis et al., 1994); we also constructed as a control a similar Fc fusion for the TrkB receptor (designated soluble TrkB or TrkB–Fc). This soluble form of Tyro 3, but not the soluble form of TrkB, effectively blocked the ability of serum (Figure 1A, right) or ABAE conditioned medium (data not shown) to induce phosphorylation of the full-length Tyro 3 expressed in Rat2 cells. Thus, serum and ABAE contain an activity that appears to behave like a bona fide Tyro 3 ligand in that it specifically binds to the ectodomain of Tyro 3 and induces Tyro 3 phosphorylation.

To measure directly binding of this activity to the ectodomain of Tyro 3, we purified recombinant soluble Tyro 3–Fc and covalently coupled it to the dextran surface of a BIAcore sensor chip and used the BIAcore (Johnsson et al., 1991; Fagerstam, 1991) to quantitate binding to this Tyro 3 surface; specific binding to the Tyro 3 immobilized on the surface was assessed by comparing binding in the presence or absence of excess soluble Tyro 3–Fc or soluble TrkB–Fc. Different batches of 10-fold concentrated ABAE conditioned medium exhibited specific Tyro 3–binding activity approximately equivalent to that found in 1%–3% serum (e.g., Figure 1B; data not shown).

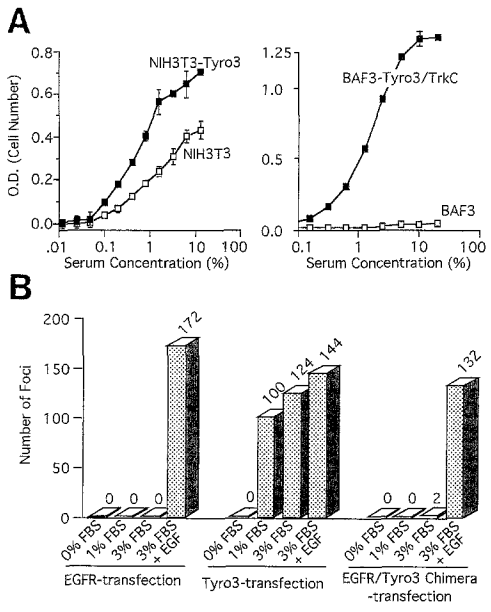

Figure 2. Tyro 3 Ligand Identified in Serum Can Induce Biological Responses Mediated by Tyro 3

(A) NIH 3T3 and BAF cells expressing Tyro 3 display enhanced growth responses to serum. Either parental cells or transfectants expressing introduced vectors encoding mouse Tyro 3 or the chimeric Tyro 3–TrkC receptors were plated in increasing concentrations of FBS, as indicated. After 3 days, cell number was evaluated by use of an MTT assay (see Experimental Procedures).

(B) Tyro 3–transfected NIH 3T3 cells are transformed by low concentrations of serum. NIH 3T3 cells were transfected with expression vectors encoding the indicated receptors and then selected in the indicated concentrations of serum (in some cases supplemented with EGF). Number of morphologically transformed foci that developed in these cultures is indicated.

Tyro 3–Binding Activity Also Mediates Tyro 3–Dependent Growth Responses

To determine whether the Tyro 3–binding and Tyro 3 phosphorylation–inducing activity was also capable of mediating Tyro 3–dependent biological responses, we first generated NIH 3T3 fibroblasts stably expressing full-length Tyro 3 receptors. These cells exhibited enhanced growth responsiveness to serum, as would be expected if the transfected Tyro 3 was mediating a gain of responsiveness to an abundant growth factor present in serum (Figure 2A, left); this assay was, however, complicated by the fact that the parental NIH 3T3 cells responded quite well to factors other than the Tyro 3 ligand, such as platelet-derived growth factor, normally found in serum. To accentuate differences between the serum responsiveness of parental and Tyro 3–expressing NIH 3T3 cells, we utilized a different assay, in which parental NIH 3T3 cells do not normally respond. In low concentrations of serum, NIH 3T3 cells normally undergo growth arrest and do not appear morphologically transformed. The Tyro 3 receptor has previously been shown to have transforming capabilities (Lai et al., 1994), so we reasoned that if serum did indeed contain abundant levels of a functional Tyro 3 ligand, Tyro 3–expressing NIH 3T3 cells might differ from parental NIH 3T3 cells in that they would become morphologically transformed in low concentrations of serum. To evaluate this possibility, NIH 3T3 cells were transfected with a Tyro 3 expression vector and selected in medium containing low (0%–3%) concentrations of serum; foci of morphologically transformed fibroblasts grew out (Figure 2B), indicating that serum does indeed have a ligand that activates the Tyro 3 receptor. In contrast, neither parental NIH 3T3 cells nor NIH 3T3 cells transfected with a vector encoding the EGF receptor (EGFR) yielded transformed foci in low serum (serum does not contain EGF), although foci were formed as expected when the serum was supplemented with EGF (Figure 2B). To rule out the remote possibility that the Tyro 3 receptor catalytic domain was promoting transformation in a serum-dependent yet ligand-independent fashion, we constructed a vector encoding a chimeric receptor in which the ectodomain of the EGFR was fused to transmembrane and cytoplasmic domains of the Tyro 3 receptor (designated EGFR–Tyro 3). Fibroblasts transfected with this chimeric receptor did not yield transformed foci when grown in low serum, but did when grown in the presence of EGF (Figure 2B).

To develop another convenient assay that would be able to detect the presence of a functional Tyro 3 ligand in serum, we utilized the interleukin-3 (IL-3)–dependent BAF-3 cell line, which normally does not respond to serum; transfection of certain, but not all, RTKs into the BAF-3 cells allows them to exhibit growth responses to the ligand spe-

cific for the introduced RTK (Collins et al., 1988). EGFR–Tyro 3 introduced into BAF-3 cells did not allow these cells to grow in the presence of EGF, presumably because the Tyro 3 cytoplasmic domain does not activate signaling pathways that elicit growth in BAF-3 cells; this inability to promote BAF-3 growth responses has previously been seen with some receptors, whereas others, such as the Trk receptors, efficiently mediate growth in BAF-3 cells (data not shown). Thus, we constructed a chimeric receptor in which the ectodomain of the Tyro 3 receptor was fused to the cytoplasmic domain of TrkC, which we presumed would allow the Tyro 3 ligand to promote growth responses in BAF-3 cells. BAF-3 cells expressing the chimeric Tyro 3–TrkC receptor, in contrast with parental BAF-3 cells, did indeed display dose-dependent growth responses to serum (Figure 2A, right). The chimeric Tyro 3–TrkC receptor also proved more potent than the parental Tyro 3 receptor in NIH 3T3 assays (data not shown) and thus was substituted for the parental Tyro 3 receptor in NIH 3T3 assays presented below.

Receptor-Based Affinity Chromatography Identifies Protein S as a Tyro 3 Ligand

All together, the above data indicate that both ABAE conditioned medium and FBS contain a ligand that binds the Tyro 3 ectodomain and induces Tyro 3 tyrosine phosphorylation as well as Tyro 3–mediated biological responses. To isolate this ligand, we took advantage of the fact that it could bind to the ectodomain of Tyro 3. FBS (~450 mg of total protein) was directly loaded onto a column containing immobilized Tyro 3–Fc. After extensive washing (in buffer containing 0.5 M NaCl), material (~7.5 μg) eluted by using 10 mM glycine, 250 mM NaCl at pH 2.5 exhibited highly enriched binding to Tyro 3 (starting material contained 0.657 resonance units [RUs] of Tyro 3 binding per microgram, while purified material contained 13,900 RUs/μg, corresponding to a ~21,000-fold purification) as determined via the BIAcore. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) analysis revealed a single major band with a molecular mass of ~75 kDa (Figure 3A); sequence analysis of peptides resulting from tryptic digestion of electroblotted proteins in this band revealed sequence identity with a previously characterized protein, the anticoagulation factor protein S (Figure 3B).

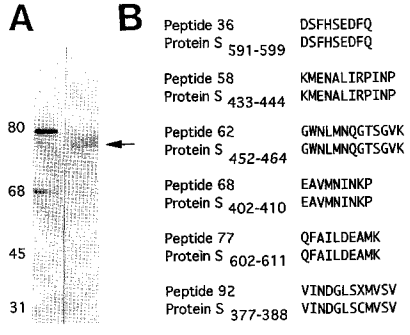

Figure 3. Receptor-Based Affinity Purification of Protein S as the Ligand for Tyro 3

(A) SDS–PAGE analysis depicts eluate from Tyro 3 affinity column, revealing a major species of ~75 kDa.

(B) Tryptic peptide sequences obtained from major ~75 kDa species indicated in (A) (see Experimental Procedures) correspond to portions of the bovine protein S sequence.

Confirmation That Protein S Is a Functional Ligand for Tyro 3

Because protein S is an abundant serum protein with a previously characterized function as an anticoagulation factor, it seemed imperative to verify independently and definitively that it acted as a bona fide ligand for Tyro 3. Thus, we confirmed the ability of recombinant protein S to activate Tyro 3: conditioned medium from COS cells transfected with a vector encoding human protein S (producing nanomolar levels of protein S), but not conditioned medium from COS cells transfected with a control vector, was very effective at inducing Tyro 3 tyrosine phosphorylation (Figure 4A) and Tyro 3–dependent growth (Figure 4B) in reporter cells.

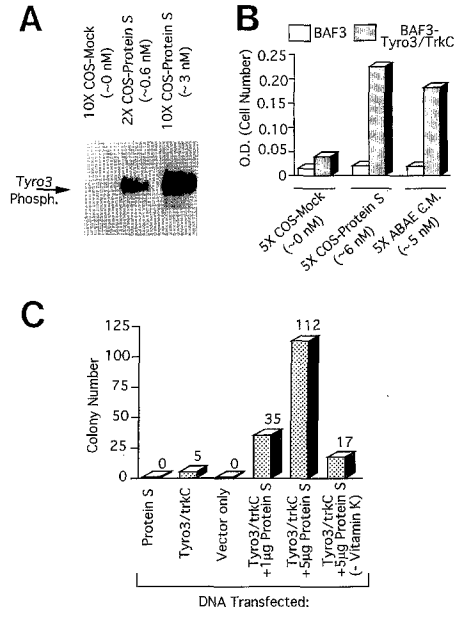

Figure 4. Recombinant Human Protein S Induces Tyro 3–Mediated Responses

(A) Recombinant protein S induces Tyro 3 phosphorylation. Conditioned medium from mock-transfected (10-fold concentrated) COS cells or from COS cells expressing a transfected human protein S expression vector (either 2-fold or 10-fold concentrated, shown by immunoblotting to contain the indicated concentrations, either ~0.6 nM or ~3 nM, of human protein S) induced tyrosine phosphorylation of Tyro 3 in Rat2 reporter cells.

(B) Recombinant protein S specifically supports BAF-3 cells expressing Tyro 3 receptor chimera. Parental BAF-3 cells or the Tyro 3–reporter BAF-3 transfectants described in Figure 2A were cultured in the presence of the indicated concentration of conditioned medium from either mock-trasnfected COS cells, COS cells expressing human protein S, or ABAE cells. The concentrations of protein S in the various samples, as assayed by immunoblotting for protein S, are indicated. After 3 days, cell number was evaluated by use of an MTT assay (see Experimental Procedures).

(C) Cotransfection of vectors encoding human protein S and a mouse Tyro 3 chimera results in vitamin K–dependent autocrine transformation of NIH 3T3 cells; quantitation of foci obtained is depicted.

We also demonstrated that protein S could collaborate with Tyro 3–TrkC to transform NIH 3T3 cells autocrinely: individual transfections of NIH 3T3 cells with either a vector encoding protein S or a vector encoding Tyro 3–TrkC did not yield transformed foci when the cells were plated in the absence of serum, although cells cotransfected with both protein S and Tyro 3–TrkC yielded large numbers of transformed foci (Figure 4C). Since protein S production requires γ-carboxylation mediated by a vitamin K–dependent carboxylase (Dahlback, 1991), we also performed the cotransfections in the absence of vitamin K and found greatly reduced numbers of foci, consistent with the foci formation requiring production of vitamin K–dependent protein S (Figure 4C). All together, the genetic evidence confirms the results of the affinity purification and demonstrates that protein S is a functional ligand for the Tyro 3 receptor.

Identification of Protein S–Related Gas6 as a Ligand for Axl

The surprising finding that protein S is a ligand for the Tyro 3 receptor would suggest that other Tyro 3–related receptors, such as Axl, would similarly bind to protein S or perhaps to protein S–related ligands. Defining such a ligand-binding specificity for Axl would in turn further verify the assignment of protein S as a functionally relevant ligand for Tyro 3. To attempt identification of a ligand for Axl, we constructed a vector encoding a chimeric protein in which the ectodomain of Axl was fused to the Fc portion of human immunoglobulin G1, as we had previously done for Tyro 3. The chimeric Axl–Fc protein produced from this vector was purified and covalently coupled to the dextran surface of a BIAcore sensor chip and used to screen for binding activities in serum and a panel of conditioned media. In contrast with the situation with Tyro 3, little or no binding activity specific for Axl was detected in serum. However, large amounts of a specific binding activity, as judged by its competition by excess soluble Axl–Fc, was detected in the conditioned media of several cell lines. Interestingly, ABAE cells, which had displayed prominent Tyro 3–binding activity, also displayed notable specific binding to Axl. The Tyro 3–binding activity in ABAE cells appeared distinct from the Axl-binding activity in the same cells, since excess soluble Tyro 3–Fc could almost completely compete the binding activity specific for the Tyro 3 surface but only marginally compete the binding activity specific for the Axl surface, while excess soluble Axl–Fc had essentially the reciprocal competition profile (Figure 5A).

Having defined a source of a specific Axl-binding activity, we once again took advantage of a receptor-based affinity purification approach. We loaded 10-fold concentrated ABAE conditioned medium (~2250 mg of total protein) directly onto a column containing immobilized Axl–Fc. After extensive washing (in buffer containing 0.5 M NaCl), material (~28 μg) eluted by using 10 mM glycine, 250 mM NaCl at pH 2.5 exhibited highly enriched binding to Axl (starting material contained 0.95 RUs/μg Axl binding, while purified material contained 9723 RUs/μg, corresponding to a ~10,234-fold purification) as determined

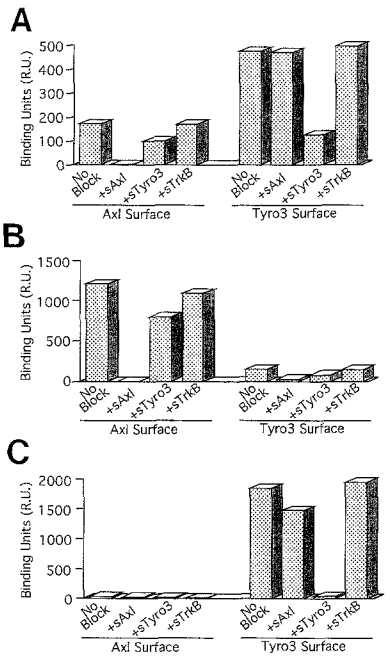

Figure 5. Distinct Ligands for Both Axl and Tyro 3 Can Be Found in ABAE Conditioned Medium

(A) Distinct Axl- and Tyro 3–binding activities in ABAE conditioned medium (concentrated 10-fold). The conditioned medium was passed over a BIAcore sensor chip coupled to either Axl–Fc or Tyro 3–Fc; prior to being passed over the sensor chip, samples were in some cases premixed with 10 μg/ml soluble Axl–Fc (sAxl), Tyro 3–Fc (sTyro3), or TrkB–Fc (sTrkB), as indicated. Binding to the sensor chip surface is indicated (as RUs). Note that the activity binding to the Axl surface was only marginally competed with soluble Tyro 3–Fc, while the activity binding to the Tyro 3 surface was not competed by soluble Axl–Fc.

(B) The affinity-purified Axl ligand (as described in Figure 6) binds rather specifically to Axl and not to Tyro 3; binding to Axl and Tyro 3 surfaces and competitions with soluble Axl (sAxl) and Tyro 3 (sTyro3) were performed as in (A).

(C) The affinity-purified Tyro 3 ligand (as described in Figure 3) binds rather specifically to Tyro 3 and not Axl; binding to Tyro 3 and Axl surfaces and competitions with soluble Tyro 3 (sTyro3) and Axl (sAxl) were performed as in (A).

via the BIAcore. SDS–PAGE analysis revealed a single major band with a molecular mass of ~75 kDa (Figure 6A); sequence analysis of peptides resulting from tryptic digestion of electroblotted proteins in this band revealed marked sequence homology with a recently characterized relative of protein S, known as Gas6 (Figure 6B). Gas6 has been described in mouse and human (Manfioletti et al., 1993). The peptide sequences we obtained from a bovine source (the ABAE cells) displayed 76% and 78% sequence identity with the corresponding regions of the mouse and human Gas6 proteins, respectively, while the mouse and human sequences were 74% identical to each other in these regions (Figure 6B; data not shown); in contrast, the peptide sequence we obtained for the Axl ligand displayed only 47% identity to the corresponding regions of bovine protein S. Thus, the affinity-purified ligand for the Axl receptor appears to correspond to the bovine version of Gas6.

The affinity-purified Gas6 was largely specific to Axl as

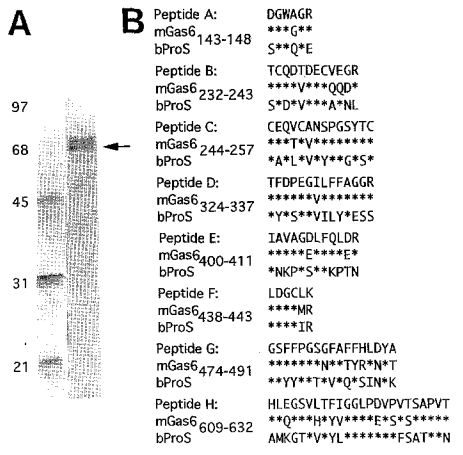

Figure 6. Receptor-Based Affinity Purification of Gas6 as the Ligand for Axl

(A) SDS–PAGE analysis depicting eluate from Axl affinity column, revealing a major species of ~75 kDa.

(B) Tryptic peptide sequences obtained from major ~75 kDa species indicated in (A) (see Experimental Procedures) compared with corresponding regions of mouse Gas6 (mGas6) as well as bovine protein S (bProS). Note that the purified sequence is from a bovine source (ABAE cells) and thus does not exactly match mouse Gas6.

compared with Tyro 3; much more ligand bound to an Axl surface than to a Tyro 3 surface, and the binding was efficiently competed by soluble Axl–Fc but not Tyro 3–Fc (see Figure 5B). The affinity-purified Tyro 3 ligand (i.e., protein S) displayed reciprocal specificity for Tyro 3 in the same assays (see Figure 5C). These findings demonstrate that protein S and its relative Gas6 are rather specific ligands for Tyro 3 and Axl, respectively.

Northern Blot Analysis Reveals That Protein S mRNA Is Expressed by Cultured Schwann Cells and Astrocytes and Is Up-Regulated Following Nerve Injury

To begin to identify physiologically relevant situations in which Tyro 3 might be activated by protein S, we compared expression of transcripts for this receptor and its ligand in an assortment of tissue-derived RNA samples from both neural and nonneural sources (Figure 7). Both Tyro 3 and protein S mRNAs were quite widespread in their expression, although, as noted previously, Tyro 3 mRNA was more prominently expressed in the nervous system (Figure 7A). Protein S mRNA was detected in all brain regions examined, but was more prominently expressed in nonneural tissues (Figure 7B). However, protein S was highly expressed in cultured Schwann cells derived from sciatic nerve, as well as in cultured astrocytes derived from the embryonic brain. In vitro cultured Schwann cells and astrocytes share many of the features that their in vivo counterparts display in response to an injury (Rudge et al., 1994), suggesting that protein S expression in vivo might be up-regulated in response to injury in either the peripheral or the central nervous system. Such expression would certainly support the notion that protein S could provide neurotrophic support to neurons expressing Tyro 3 following injury or otherwise play an important role in

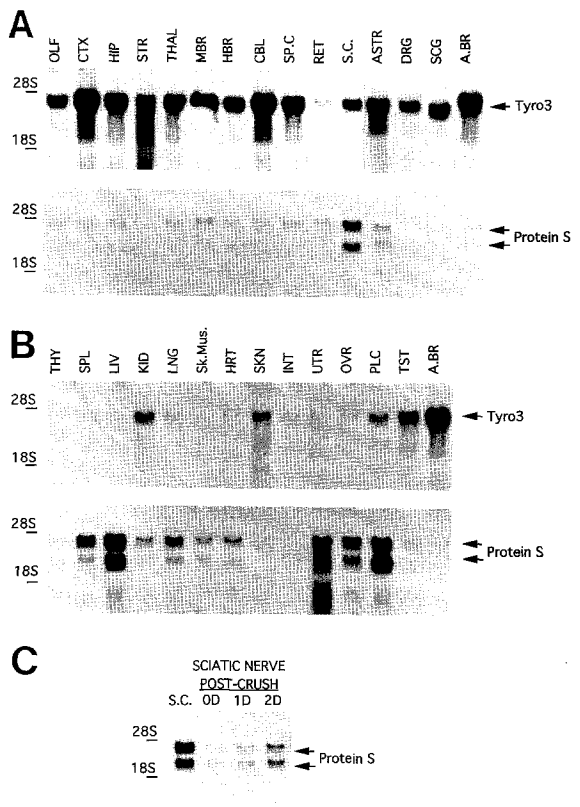

Figure 7. Northern Blot Analysis of Tyro 3 and Protein S Transcript Levels

Northern blot analysis of Tyro 3 and protein S in transcript levels in RNA samples derived from a variety of rat adult neural (A) and nonneural (B) tissues, as well as in RNA samples derived from sciatic nerve (C) (distal to the lesion) at various days following a crush lesion to the nerve, compared with cultured Schwann cells. Abbreviations: OLF, olfactory bulb; CTX, cortex; HIP, hippocampus; STR, striatum; THAL, thalamus; MBR, midbrain; HBR, hindbrain; CBL, cerebellum; SP. C, spinal cord; RET, retina; S. C., cultured schwann cells; ASTR, cultured astrocytes; DRG, isolated dorsal root ganglia; SCG, isolated sympathetic ganglia; A. BR., whole adult brain; THY, thymus; SPL, spleen; LIV, liver; KID, kidney; LNG, lung; Sk. Mus., skeletal muscle from soleus muscle; HRT, heart; SKN, skin; INT, intestine; UTR, uterus; OVR, ovary; PLC, placenta; TST, testes.

injury responses of Tyro 3–expressing central and peripheral neurons. However, Tyro 3 mRNA was also found to be expressed in Schwann cells and astrocytes themselves (Figure 7A), raising the possibility that following injury, protein S acts not only on neurons but on glia as well.

To pursue the possibility that protein S was up-regulated in sciatic nerve in response to injury, protein S mRNA expression was evaluated in samples prepared from normal sciatic nerve, as well as from sciatic nerve distal to a nerve lesion at 1, 2, or 3 days postlesioning. This analysis revealed that protein S expression is clearly up-regulated following nerve injury (Figure 7C).

Discussion

The search for ligands of the Tyro 3/Axl family of RTKs unexpectedly revealed that protein S, a potent anticoagu-

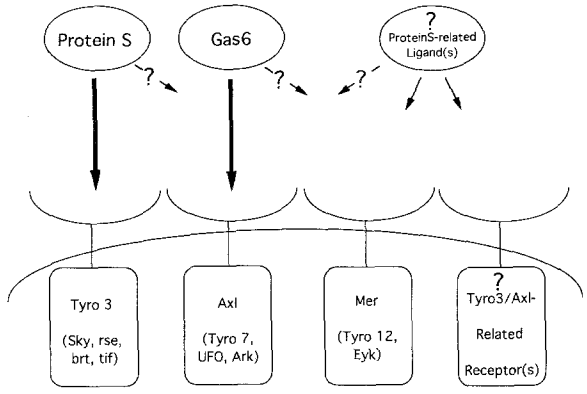

Figure 8. Schematization of the Characterized and Potential Interactions between the Protein S Family of Ligands and the Tyro 3/Axl Family of RTKs

lation factor, binds and activates Tyro 3, while the protein S–related Gas6 binds Axl. Additional Tyro 3–related receptors, such as Mer, presumably bind either these or other as yet unknown members of this ligand family; similarly, these yet to be described protein S relatives could also bind to either Tyro 3 or Axl (Figure 8). Precise comparison of receptor–ligand distributions coupled with gene disruption studies may be required to determine which receptor–ligand pairs are relevant physiologically.

Identification of the physiologic processes triggered by interactions between the Tyro 3–related receptors and their newly defined protein S–related ligands is an important next challenge. The massive thrombotic complications suffered by infants homozygous for protein S deficiency provide dramatic evidence of the critical role of protein S in anticoagulation. Protein S acts as a cofactor for activated protein C, promoting degradation or otherwise inhibiting the coagulation factors Va and VIIIa, thus helping blunt further generation of thrombin (Dahlback, 1991). However, these actions do not seem to account for all of the anticoagulatory activity of protein S (Dahlback, 1991). In addition to its characterized and uncharacterized roles in anticoagulation, protein S has also been suggested to act in a variety of settings outside of the clotting system. For example, protein S interacts with a negative regulator of the complement cascade, C4b-binding protein, and it has been suggested that this interaction underlies potential antiinflammatory actions of protein S (Dahlback, 1991; Hessing, 1991). Based on observations that protein S is produced by bone cells and that osteopenia occurs in protein S–deficient patients, it has also been suggested that protein S may play a role in bone turnover and metabolism (Maillard et al., 1992). Other studies indicate that protein S is also a mitogen for smooth muscle cells (Gasic et al., 1992), and production of protein S by neural tumor cell lines has led to the proposal that protein S might have some function in the brain (Phillips et al., 1993).

Binding and activation of Tyro 3 could clearly be involved in mediating or regulating any of the poorly understood bioactivities of protein S. It is perhaps worthwhile to consider these potential actions of protein S in the context of recent findings with the procoagulant thrombin, which is the protease that drives blood clot formation by cleaving fibrinogen to form fibrin. Remarkably, thrombin, like its indirect regulator, protein S, has also recently been found to bind and activate intracellular signaling via a specific cell surface receptor (Vu et al., 1991; Coughlin, 1993). This G protein–coupled receptor may in some way gauge levels of active thrombin, mediate the activation by thrombin of platelets, or otherwise help in the regulation of thrombin activity during coagulation (Coughlin, 1993). This receptor also apparently mediates the mitogenic effects of thrombin on vascular smooth muscle cells (Coughlin, 1993); such effects may occur in vivo upon exposure of these cells to plasma thrombin after breakdown of the endothelial cell barrier following physical insult or infection. Thrombin receptors have also unexpectedly been found on neurons and glia in the brain (Monard, 1993), and these receptors may be similarly poised to detect breakdown of the blood–brain barrier. However, thrombin is also made within neural tissue, as are inhibitors of thrombin protease activity (Dihanich et al., 1991; Monard, 1993). Furthermore, thrombin, acting via its specific G protein–coupled receptor, has been found to have profound morphologic effects on neurons, astrocytes, and their processes (Monard, 1993). These findings suggest that thrombin may function in brain in a manner totally independent of its actions during clot formation. Axon and synaptic growth and remodeling require the coordinated action of enzymes that digest the extracellular matrix together with factors that regulate neuronal process retraction and outgrowth. Thrombin is an interesting candidate to be involved in this coordination, since it could be part of protease cascades involved in remodeling of the extracellular matrix, while simultaneously activating a G-coupled receptor that would modulate and coordinate changes in process outgrowth or morphology.

Our results now indicate that both a procoagulant/protease (thrombin) and an anticoagulant/protease regulator (protein S) have the ability to activate intracellular signaling cascades via specific cell surface receptors. The many roles proposed for thrombin suggest related possibilities for protein S. Just as thrombin receptors are not activated by its inactive precursor (prothrombin), Tyro 3 receptors found in vivo could detect or respond only to a particularly modified form of "active" protein S and in this way act to gauge or to regulate (or both) some aspect of protein S function during coagulation. In addition, activation of cell surface receptors by protein S could serve to integrate coagulation with associated cellular responses required for tissue repair and growth. For example, Tyro 3 could be responsible for mediating the previously described ability of protein S to induce smooth muscle cell proliferation (Gasic et al., 1992). If this is the case, smooth muscle cells in blood vessel walls might only be exposed to circulating protein S (as well as thrombin) after breakdown of the endothelial cell barrier, helping to provide an explanation for why endothelial injury induces the migration and proliferation of underlying smooth muscle cells that can contribute to intimal thickening and subsequent atherosclerosis. Similarly, the Tyro 3 expressed by neurons in the brain

may gauge exposure to plasma-derived protein S following breakdown of the blood–brain barrier and thereby regulate the injury response. Since neural and glial cells express protein S, however, it is likely that the many Tyro 3–expressing cells in the brain encounter this ligand from sources other than plasma and that the Tyro 3–mediated response of these cells to protein S is unrelated to its anticoagulant role in the plasma. Thus, protein S or a related ligand could act as a classical neurotrophic factor for Tyro 3–expressing neurons or as a traditional growth factor for certain Tyro 3–expressing nonneuronal cells. At the same time, protein S could be involved in regulating protease cascades in the brain, such as those proposed for thrombin, that may play key roles in synaptic remodeling and plasticity. In this regard, it is worth noting that Tyro 3 expression in the adult brain is neuronal and is found in structures, such as the hippocampus and olfactory bulb, notable for their activity-dependent plasticity (Lai and Lemke, 1991; Lai et al., 1994). Of particular interest is the preliminary observation that Tyro 3 is primarily localized to the processes of central nervous system neurons (C. L., unpublished data). Finally, the up-regulated expression of protein S in reactive Schwann cells and astrocytes is consistent with the possibility that protein S is involved in growth and remodeling responses to injury, as well as with a role for protein S as a classical neurotrophic factor.

The function of Gas6 is currently unknown, although its homology to protein S and its ability to bind Axl suggest roles both in clotting and in other systems. It is worth noting that Gas6 was cloned on the basis of its dramatic up-regulation following growth arrest in a number of cell lines, including IMR90 fibroblasts, which also express endogenous Axl receptors (Manfioletti et al., 1993), indicating that autocrine Gas6–Axl interactions are not sufficient to elicit a growth response (at least in some cells) and may instead be an intrinsic part of the growth arrest process. It is also of interest that the Axl/Ark receptor has recently been shown to display homophilic interactions (Bellosta et al., 1995); the respective roles of Gas6–Axl as opposed to Axl–Axl interactions in regulating receptor activation and signal transduction remain to be elucidated.

There is an intriguing similarity between our findings and the earlier realization that hepatocyte growth factor, which is similar in structure to plasminogen (Tashiro et al., 1990), also utilizes and activates an RTK, c-Met (Bottaro et al., 1991). In fact, recently described angiostatic activities of a plasminogen fragment may also be attributable to its binding to a specific receptor on endothelial cells (O'Reilly et al., 1994). The structural similarities between hepatocyte growth factor and plasminogen, the previous findings that thrombin can bind and activate a specific G protein–coupled cell surface receptor, and our findings that the protein S–related anticoagulant factors bind and activate members of a family of RTKs demonstrate together that we are far from a complete understanding of the long-studied proteins involved in regulating coagulation cascades and open a wide variety of unexpected areas for investigation. Factors critical for the regulation of coagulation may also be exploited in unrelated pathways, in systems such as the nervous system.

Experimental Procedures

Preparation of Conditioned Medium

ABAE cells were conditioned in defined medium (Zhan et al., 1987), which was subsequently clarified by centrifugation, brought to 1 mM PMSF and 0.14 U/ml aprotinin (both from Sigma Chemical), concentrated 10-fold with 3000 MWCO membranes (Amicon), and stored at −80°C.

Tyrosine Phosphorylation Assays

Rat2 fibroblasts stably transfected with a Tyro 3–expressing plasmid (Lai et al., 1994) were plated 4–5 days before use and serum-starved for 3 hr in DMEM prior to the indicated treatments for 5 min at 37°C. The cells were then lysed in 1% NP-40 in PBS containing 1 mM PMSF, 0.14 U/ml aprotinin, 1 mM EDTA, and 1 mM sodium orthovanadate. The lysates were immunoprecipitated with a phosphotyrosine-specific monoclonal antibody conjugated to agarose (4G10; Upstate Biotechnologies) and immunoblotted with a polyclonal antiserum (1:4000) specific for Tyro 3 (Lai et al., 1994). Following incubation of the blots with HRP-conjugated goat anti-rabbit antibodies (Caltag), bands were visualized by ECL (Amersham). Blocking assays were performed by preincubating with soluble receptor–Fc at indicated concentrations for 30 min at 37°C prior to addition to cells.

Production and Purification of Soluble Tyro 3–Fc, TrkB–Fc, and Axl–Fc

The TrkB–Fc, Tyro 3–Fc, and Axl–Fc proteins resulted from the fusion of the ectodomains of rat TrkB, mouse Tyro 3, and mouse Axl to a spacer with the sequence Gly-Pro-Gly, followed by the hinge, CH2, and CH3 regions of human IgG1, beginning with the residues Glu-Pro-Lys, as described (Davis et al., 1994). Tyro 3–Fc and Axl–Fc proteins were produced as serum-free supernatants from transiently transfected COS cells. TrkB–Fc and Tyro 3–Fc were also produced in Spodoptera frugiperda SF-21AE cells via recombinant baculovirus infection performed by standard methods (O'Reilly et al., 1992). Soluble receptors from either COS- or baculovirus-derived material were purified by protein A–Sepharose (Pharmacia) chromatography.

BIAcore Analysis

The binding activity was measured by use of BIAcore biosensor technology (Pharmacia Biosensor) (Fagerstam, 1991; Johnsson et al., 1991). Purified soluble receptors were covalently coupled through primary amines to the carboxymethyl dextran layer of a CM5 research grade sensor chip (Pharmacia Biosensor). Samples to be assayed for binding to the chip were brought to a final concentration of 2 μg/ml dextran (ICN Biochemicals; MWav, 67,000 g/mol) and 0.005% P20 surfactant. Aliquots of 40 μl were injected across the immobilized surfaces at a flow rate of 5 μl/min, and the receptor binding was monitored for 8 min. The binding activity (in RUs) was measured as the difference between a baseline value determined 30 s prior to the sample injection and a measurement taken at 30 s postinjection. Regeneration of the surface was accomplished with one 12 μl pulse of 3 M $MgCl_2$ (Tyro 3) or Actisep elution medium. Specific binding was evaluated by incubating the samples with 10 or 25 μg/ml of Tyro 3–Fc, Axl–Fc, or TrkB–Fc prior to assaying the binding activity.

Growth and Transformation Assays in NIH 3T3 and BAF-3 Cells

Stable transfections of NIH 3T3 cells were performed as previously described (Glass et al., 1991). The chimeric receptor EGFR–Tyro 3 was derived from the ectodomain of human EGFR fused to the transmembrane and cytoplasmic domain of Tyro 3. The Tyro 3–TrkC chimeric receptor was derived from fusion of the first 455 amino acids of Tyro 3 to the transmembrane and cytoplasmic domains of rat TrkC. The expression plasmid encoding human protein S, pD5-hPS, has been described previously (Nelson and Long, 1992). Transformation assays involving cotransfection of Tyro 3–TrkC constructs utilized 5 μg of receptor expression plasmid, 1 μg or 5 μg of protein S expression plasmid, 500 ng of LTR–*neo* plasmid to confer G418 resistance, and sufficient human placental genomic DNA to bring the total DNA concentration to 30 μg per transfection. Transformation and survival assays in NIH 3T3 cells were carried out as previously described (Ip et al., 1993b), with the exception that survival assays of cells cotrans-

fected with Tyro 3–TrkC and protein S vectors were cultured in defined medium in the presence or absence of 1 mM menadione sodium bisulfite (Sigma Chemical), a water-soluble vitamin K analog. BAF-3 cells, grown in RPMI containing 1 ng/ml IL-3, 10% FBS, and antibiotics were transfected with linearized plasmid by electroporation (200 V, 960 μF) and selected in growth medium plus 400 μg/ml G418 (GIBCO BRL). For growth assays in BAF-3 cells, cells were washed three times with RPMI and plated in 96-well dishes at a density of 10,000 cells per well in 50 μl of RPMI, together with 50 μl of the indicated additions at a 2× concentration in RPMI. The number of viable cells was assessed after 3 days by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described (Ip et al., 1992).

Receptor-Based Affinity Purification of Tyro 3 and Axl Ligands

For receptor-based affinity purification of the Tyro 3 ligand, 1.5 mg of Tyro 3–Fc was cross-linked to a 1 ml HiTrap column (Pharmacia) according to the instructions of the manufacturer, with >90% efficiency. The column was equilibrated in PBS, and 15 ml of FBS then loaded at 0.5 ml/min. The column was washed with PBS until OD_{280} <0.01 was seen in the eluate and then was washed with approximately 6 column vol of 20 mM HEPES (pH 7.4) containing 0.5 M NaCl. Purified ligand was then eluted from the column with 10 mM glycine (pH 2.5) containing 250 mM NaCl. Affinity purification of the Axl ligand was performed as above, except that only 150 μg of Axl–Fc was cross-linked to the HiTrap column and that the PBS-equilibrated column was loaded with 500 ml of 10× concentrated serum-free ABAE conditioned medium at 0.5 ml/min.

Peptide Sequencing of Affinity-Purified Tyro 3 and Axl Ligands

Protein (3 μg) resulting from either the Tyro 3 or Axl affinity purification was concentrated to 100 μl, separated by SDS–PAGE, transferred to a PVDF (Bio-Rad) membrane, and visualized by Ponceau S staining. Membrane fragments containing the protein bands were destained, placed in 0.6 ml of 0.1 mM Tris–HCl (pH 8.0), 50% 2-propanol to which 10 μl of 4-vinylpyridine and 5 μl of tributylphosphine (Aldrich) were added to reduce and pyridylethylate cysteine residues. After 1 hr of incubation, the membrane pieces were washed in 50% propanol and digested for 15 hr with 1:10 ratio of trypsin in 20 μl of 0.1 M Tris–HCl (pH 8.0) containing 1% Triton X-100 (Calbiochem) and 10% acetonitrile. The supernatant was diluted three times with 2% TFA, and the peptides released from the membrane were separated by reverse phase chromatography on a 0.8 mm × 250 mm C18 column (LC Packings), eluted directly onto Beckman sequencing membranes, and sequenced on an ABI Procise protein sequencer.

Production of Recombinant Human Protein S

COS cells, transiently transfected with pD5-hPS (encoding human protein S) or with empty vector, were cultured for 3 days in serum-free defined medium supplemented with 1 mM sodium menadione bisulfite, and the clarified culture supernatants were then concentrated 10-fold. Protein S concentration was estimated by comparison to purified human protein S (Haematologic Technologies) on nonreducing immunoblots probed with a monoclonal antibody to protein S (AHPS 5091; Haematologic Technologies).

Northern Blot Analysis

Northern blot analysis was done as previously described (Ip et al., 1993a), utilizing a randomly labeled PCR probe corresponding to amino acids 267–370 of mouse protein S or a DNA fragment corresponding to amino acids 132–694 of mouse Tyro 3.

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