Plasmin-mediated Release of the Guidance Molecule F-spondin from the Extracellular Matrix*

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Serine proteases are implicated in a variety of processes during neurogenesis, including cell migration, axon outgrowth, and synapse elimination. Tissue-type plasminogen activator and urokinase-type activator are expressed in the floor plate during embryonic development. F-spondin, a gene also expressed in the floor plate, encodes a secreted, extracellular matrix-attached protein that promotes outgrowth of commissural axons and inhibits outgrowth of motor axons. F-spondin is processed in vivo to yield an amino half protein that contains regions of homology to reelin and mindin, and a carboxyl half protein that contains either six or four thrombospondin type I repeats (TSRs). We have tested F-spondin to see whether it is subjected to processing by plasmin and to determine whether the processing modulates its biological activity. Plasmin cleaves F-spondin at its carboxyl terminus. By using nested deletion proteins and mutating potential plasmin cleavage sites, we have identified two cleavage sites, the first between the fifth and sixth TSRs, and the second at the fifth TSR. Analysis of the extracellular matrix (ECM) attachment properties of the TSRs revealed that the fifth and sixth TSRs bind to the ECM, but repeats 1–4 do not. Structural functional experiments revealed that two basic motives are required to elicit binding of TSR module to the ECM. We demonstrate further that plasmin releases the ECM-bound F-spondin protein.

Development of the nervous system requires neurons to migrate and to extend axons over long distances from their sites of origin to their intended targets in the peripheral and the central nervous system. Cellular cues often provide physical guides for the growing axons, whereas soluble and cell-attached attractant and repulsive molecules influence axonal steering decisions (1). The axonal growth cone has been proposed to participate actively in modulating the tissue/matrix environment, enabling growth through an impeding substrate (2). It was anticipated by Krystosek and Seeds (3) that release of extracellular proteases by the axonal growth cone may facilitate its movement by digesting cell-cell and cell-matrix contacts that block the path of the advancing growth cone. Over the past several years, it became evident that extracellular serine proteases, such as plasminogen, tissue-type plasminogen activator (tPA),1 urokinase-type plasminogen activator (uPA) (for review, see Ref. 4), thrombin (5), and neurotrypsin (6), are expressed in the nervous system. They have been implicated in a variety of processes during neurogenesis, including cell migration, axon outgrowth, and synapse elimination (7, 8). They also play a critical role in the adult nervous system by mediating neuronal plasticity (9, 10), apoptosis (11), and peripheral nerve regeneration (12).

Localization of plasmin activity to neuronal growth cones was initially demonstrated by digestion of a fibrin clot overlay (3). It was further demonstrated that plasmin cleaves the ECM molecules: collagen, fibronectin (13) and laminin (for review, see Ref. 4). In addition to cleaving ECM molecules directly, the extracellular serine proteases may act indirectly by releasing latent proteases and growth factors from the matrix. It was demonstrated that metalloproteases (14), transforming growth factor-β (15), vascular endothelial growth factor (16), fibroblast growth factor (17), platelet-derived growth factor (18), and hepatic growth factor/scatter factor (15) are produced as matrix-attached latent proteins, subjected to cleavage and subsequently to activation by plasmin.

F-spondin, a gene expressed in the floor plate, encodes a secreted, ECM-attached protein (19). It plays a dual role in patterning axonal trajectory in the spinal cord by promoting outgrowth of commissural axons (20) and inhibiting outgrowth of motor axons (21). F-spondin protein is processed in vivo to yield an amino half protein, which contains regions of homology to reelin and mindin, and a carboxyl half protein, which contains either six or four thrombospondin type I repeats (TSRs) (20, 22). F-spondin expression in the nervous system overlaps with expression of several serine proteases. In the floor plate, F-spondin is expressed together with tPA and uPA (19, 22–24), whereas in the hippocampus, F-spondin is coexpressed with tPA and neurotrypsin (6, 25). In addition, several tPA-expressing neurons extend axons toward or through an F-spondin-rich milieu. Embryonic motor neurons are exposed to the floor

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1 The abbreviations used are: tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; ECM, extracellular matrix; TSR(s), thrombospondin repeat(s); PCR, polymerase chain reaction; HEK, human embryonic kidney; AP, alkaline phosphatase; mAb, monoclonal antibody; DM, double mutant; Tsp-1, thrombospondin-1.
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plate-derived F-spondin (21). As motor axons emerge from the spinal cord and up-regulate the expression of tPA (26), they encounter the somite-derived F-spondin (27); subsequently, at the peripheral nerve, they are ensheathed by Schwann cell-expressing F-spondin (22). Similarly, embryonic sensory neurons and sympathetic ganglia neurons expressing tPA are also surrounded by F-spondin-expressing cells in the ganglia and along their axonal path (22, 26, 28).

In the current study we provide evidence demonstrating that F-spondin is a substrate for plasmin. Plasmin cleaves F-spondin at two sites, the first located between the fifth and sixth TSRs and the second at the fifth TSR. The cleavage sites are located between the extracellular matrix binding TSRs (repeats 5 and 6) and the nonbinding repeats (repeats 1–4). In accordance, treatment of F-spondin with plasmin yields a diffusible, ECM-free, TSR domain protein containing TSRs 1–4.

EXPERIMENTAL PROCEDURES

DNA Constructs—DNA plasmids were constructed by PCR as indicated in Table I. Forward and backward primers were used for PCR using a “template plasmid.” The PCR products were subcloned into a suitable plasmid (pBluescript II KS, Strategene) or into the restriction sites indicated in the table. The mutant plasmids PL1m, PL2m, and DM were generated as follows. Two PCRs were set up with two sets of primers (the upper and lower row of primers in the table). The PCR products of the two reactions were combined, and an additional PCR was performed with the forward primer of the upper row and the backward primer of the lower row. The PCR products were digested and subcloned as indicated in the table.

Plasmin Cleavage Assay—HEK293 T cells were transfected with the various plasmids using the liposome-mediated transfection reagent DOTAP (Roche, Manheim, Germany), and LipofectAMINE (Life Technologies, Inc.). Conditioned medium was collected after 2–4 days and treated with the appropriate reagents at 37 °C for 1 h. For plasmin cleavage assays, conditioned medium was treated with plasmin (Chromogenix, Sweden) at the indicated concentrations and the chromogenic substrate specific for plasmin, S-2251 (Val-Leu-Lys-p-nitroanilide, Chromogenix) in 100 mM Tris-HCl, pH 7.4, in a final volume of 100 μl, in microtiter plates for 1 h at 37 °C. Plasmin activity was measured by monitoring the increase of absorbance at 405 nm, using a Thermomax thermostat plate reader (Molecular Devices Corp.). In other cases, plasmin cleavage of proteins was analyzed by electrophoresis on 12% SDS-polyacrylamide gels, transferred to nitrocellulose, and the protein products were analyzed by the anti-reelin domain antibody (R8). Except for TSR5a, the size of all the plasmin-treated proteins was unchanged. TSR5a protein, however, was reduced, and thus the treated protein migrated faster than its untreated counterpart. This suggests that the cleavage site is carboxyl to the fourth TSR.

To visualize the two protein cleavage products, an anti-TSR domain antibody (R2) was used. Proteins containing TSRs 1–6 and TSRs 2–6 (Fig. 2B) were subjected to plasmin treatment. The 55-kDa TSR1–6 protein yielded a 40-kDa protein and a 16-kDa protein. The 46-kDa TSR2–6 protein yielded a 28-kDa and a 16-kDa protein (Fig. 2B). The 16-kDa proteolytic protein migrated to the same extent in both proteins, suggesting that the cleavage site in these proteins is identical. It is difficult to ascertain the precise cleavage site by the molecular masses of the plasmin proteolytic fragments because an N-linked glycosylation site is present at amino acids 681–683, within the fifth TSR.

A Highly Plasmin-sensitive Site Is Located between the Fifth and Sixth TSRs—To pinpoint the plasmin cleavage site, nested deletions of 5 amino acids each, covering the 30-amino acid region that interspaced repeats 5 and 6, were generated. All of the proteins were equipped with the Myc epitope at the carboxyl terminus. The proteins were subjected to plasmin digestion and analyzed with anti-Myc mAb, to visualize the cleaved product, and anti-TSR antibody to detect the unprocessed and processed protein. TSR1–5–5 was resistant to 10 μg/ml plasmin, whereas TSR1–5–10, TSR1–5–15, and TSR1–5–20 were sensitive to plasmin (Fig. 3, A and B). Thus, a sensitive site (designated PL1) to plasmin is located carboxyl to the fifth TSR. It is plausible that another site is located amino to the PL1 site, within the fifth TSR. To test this hypothesis we incubated the TSR1–5–5 and TSR1–5–20 with increasing con-
### TABLE I

**Construction of DNA plasmids used in this paper**

DNA constructs were generated by PCR using template plasmids, forward and backward primers, and cloning vectors as indicated in the table. The F-spondin template plasmid is the cDNA of the rat F-spondin (19).

| Name       | Template plasmid | Forward primer | Backward primer | Cloning vector | Cloning sites |
|------------|------------------|----------------|-----------------|----------------|---------------|
| TS5a       | F-spondin        | GCAAGCTTGCTGGCTGCTTTCTCGAGTTG | CGTCTAGAGAAGCTGCTCTCCAT | pSecTagB | HindIII-XbaI |
| TS0        | F-spondin        | GCAAGCTTGCTGGCTGCTTTCTCGAGTTG | GAGATCTAGAGAAGCTGCTCTCCAT | pSecTagB | HindIII-XbaI |
| TS1        | F-spondin        | GCAAGCTTGCTGGCTGCTTTCTCGAGTTG | GTCTAGAGAAGCTGCTCTCCAT | pSecTagB | HindIII-XbaI |
| TS2        | F-spondin        | GCAAGCTTGCTGGCTGCTTTCTCGAGTTG | GTCTAGAGAAGCTGCTCTCCAT | pSecTagB | HindIII-XbaI |
| TS3        | F-spondin        | GCAAGCTTGCTGGCTGCTTTCTCGAGTTG | GTCTAGAGAAGCTGCTCTCCAT | pSecTagB | HindIII-XbaI |
| TS4        | F-spondin        | GCAAGCTTGCTGGCTGCTTTCTCGAGTTG | GTCTAGAGAAGCTGCTCTCCAT | pSecTagB | BamH-XbaI    |
| TSRI–6     | F-spondin        | GAGAGATCTTTGAGCTCTCTCCCTCTGGCTT | CGTCTAGAGAAGCTGCTCTCCAT | pSecTagB | BamH-XbaI    |
| TSRI–5 + 5 | F-spondin        | GAGAGATCTTTGAGCTCTCTCCCTCTGGCTT | GTCTAGAGAAGCTGCTCTCCAT | pSecTagB | BamH-XbaI    |
| TSRI–5 + 10| F-spondin        | GAGAGATCTTTGAGCTCTCTCCCTCTGGCTT | GTCTAGAGAAGCTGCTCTCCAT | pSecTagB | BamH-XbaI    |
| TSRI–5 + 15| F-spondin        | GAGAGATCTTTGAGCTCTCTCCCTCTGGCTT | GTCTAGAGAAGCTGCTCTCCAT | pSecTagB | BamH-XbaI    |
| PL1m       | F-spondin        | GAGAGATCTTTGAGCTCTCTCCCTCTGGCTT | GTCTAGAGAAGCTGCTCTCCAT | pSecTagB | Xba-I-SacII  |
| PL2m       | F-spondin        | GAGAGATCTTTGAGCTCTCTCCCTCTGGCTT | GTCTAGAGAAGCTGCTCTCCAT | pSecTagB | Xba-I-SacII  |
| DM         | PL1m             | TGGTCTGAGATAGAAGCTGCTGTTGCTGGGA | CAGGATCCGCTTACCCCACTACCTACCA | pSecTagB | Xba-I-SacII  |
| AP-TSR1    | TS1              | GAGAGATCTTTGAGCTCTCTCCCTCTGGCTT | GTCTAGAGAAGCTGCTCTCCAT | pSecTagB | Xba-I-SacII  |
| AP-TSR1–2  | TS2              | GAGAGATCTTTGAGCTCTCTCCCTCTGGCTT | GTCTAGAGAAGCTGCTCTCCAT | pSecTagB | Xba-I-SacII  |
| AP-TSR1–4  | TS4              | GAGAGATCTTTGAGCTCTCTCCCTCTGGCTT | GTCTAGAGAAGCTGCTCTCCAT | pSecTagB | Xba-I-SacII  |
| AP-TSR1–5  | TS5              | GAGAGATCTTTGAGCTCTCTCCCTCTGGCTT | GTCTAGAGAAGCTGCTCTCCAT | pSecTagB | Xba-I-SacII  |
| AP-TSR1–6  | TS5              | GAGAGATCTTTGAGCTCTCTCCCTCTGGCTT | GTCTAGAGAAGCTGCTCTCCAT | pSecTagB | Xba-I-SacII  |
| AP-TSR5    | TS5              | GAGAGATCTTTGAGCTCTCTCCCTCTGGCTT | GTCTAGAGAAGCTGCTCTCCAT | pSecTagB | Xba-I-SacII  |
| AP-TSR6    | TS2–6            | GAGAGATCTTTGAGCTCTCTCCCTCTGGCTT | GTCTAGAGAAGCTGCTCTCCAT | pSecTagB | Xba-I-SacII  |
| AP-TSR5–6  | TS2–6            | GAGAGATCTTTGAGCTCTCTCCCTCTGGCTT | GTCTAGAGAAGCTGCTCTCCAT | pSecTagB | Xba-I-SacII  |
| GST-TSR4   | TS4              | GAGAGATCTTTGAGCTCTCTCCCTCTGGCTT | GTCTAGAGAAGCTGCTCTCCAT | pSecTagB | Xba-I-SacII  |
| GST-TSR5   | TS5              | GAGAGATCTTTGAGCTCTCTCCCTCTGGCTT | GTCTAGAGAAGCTGCTCTCCAT | pSecTagB | Xba-I-SacII  |
| AP-TSR4 & 5| GST-TSR4         | GAGAGATCTTTGAGCTCTCTCCCTCTGGCTT | GTCTAGAGAAGCTGCTCTCCAT | pSecTagB | Xba-I-SacII  |
| AP-TSR5 & 4| GST-TSR5         | GAGAGATCTTTGAGCTCTCTCCCTCTGGCTT | GTCTAGAGAAGCTGCTCTCCAT | pSecTagB | Xba-I-SacII  |
| TSSR2–6    | F-spondin        | GAGAGATCTTTGAGCTCTCTCCCTCTGGCTT | GTCTAGAGAAGCTGCTCTCCAT | pSecTagB | Xba-I-SacII  |
| pSec4X4    | F-spondin        | GAGAGATCTTTGAGCTCTCTCCCTCTGGCTT | GTCTAGAGAAGCTGCTCTCCAT | pSecTagB | Xba-I-SacII  |
| TSSR1–6Δ5  | F-spondin        | GAGAGATCTTTGAGCTCTCTCCCTCTGGCTT | GTCTAGAGAAGCTGCTCTCCAT | pSecTagB | Xba-I-SacII  |
conditioned media were subjected to proteolysis with 10 mM HEK293 T
Myc epitope.

Panel B: sixth TSRs (designated “a” region). The gray boxes
represent the 30 amino acids that are interspaced between the fifth and
sixth TSRs (designated “a” region). The black triangle represents the
Myec epitope. Panel B, plasmid cleaves the recombinant F-spondin. HEK293 T cells were transfected with the TS5a construct (panel A). The
conditioned media were subjected to proteolysis with 10 µg/ml plasmin (Plm), plasminogen (Plg), tPA, and the cofactor fibrin (Fb) in various
combinations as indicated. The protein products were analyzed by
Western blotting with the 9E10 mAb. Cleavage is evident with plasmin
(lane 2), plasmin + fibrin (lane 3), and with plasminogen + tPA + fibrin
(lane 8). Partial cleavage is also obtained by plasminogen + fibrin (lane 4). Panel C, the plasmid-mediated cleavage of F-spondin is inhibited by
plasmin-specific inhibitor. Inhibitors to either plasmin (aprotinin) or to
metalloproteinase (EGTA) were added to the plasmin system. The protein
products were analyzed by Western blotting with the 9E10 mAb.
Aprotinin fully blocks F-spondin cleavage (lanes 3 and 9), whereas
EGTA does not inhibit its cleavage (lanes 4 and 10).

The size products of the fully cleaved proteins were identical.
Hence, it appears that a second site (PL2) is located amino to
PL1. In the absence of PL1, the PL2 site is less sensitive to
plasmin. We assume that an intermediate protein product ap-
plies in the absence of PL1, the PL2 site is less sensitive to
plasmin. The mutated protein was resistant to low concentrations of
plasmin (Fig. 4B). At higher concentrations, 50 µg/ml, a
cleaved product was apparent (Fig. 4, C and D). The size of the
cleaved product was identical to the size of the cleaved control
protein TS5a. Thus, mutating the arginines at positions 730–732 created a PL1-resistant protein.

A Second Plasmid Cleavage Site Is Located within the Fifth
TSR—To locate the PL2 site, a similar approach was taken.
Nested deletion proteins, of 5-amino acid intervals, in the fifth
TSR, between TS5a and TS4 were generated. TS5a + 5 (Fig. 5A)
TS4 + 10 and TS4 + 15 (data not shown) were resistant to plasmin at concentrations ranging from 10 to
100 µg/ml, as assayed by the anti-TSR antibody R2, and the
anti-carboxyl end 9E10. Thus, it can be determined that the cleavage site is carboxyl to the fourth TSR. Panel
B, TS1–6 and TSR2–6 conditioned media were analyzed before and
after plasmin incubation. The reaction products were separated on 15%
SDS-polyacrylamide gel electrophoresis and blotted with the anti-TSR
antibody R2. The 55-kDa TRS1–6 protein yielded a 40-kDa protein
and a 16-kDa protein. The 46-kDa TSR2–6 protein yielded a 28-kDa and
a 16-kDa protein.

The mutated protein was resistant to low concentrations of plasmin (Fig. 4B). At higher concentrations, 50 µg/ml, a
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and a 16-kDa protein. The 46-kDa TSR2–6 protein yielded a 28-kDa and
a 16-kDa protein.

The mutated protein was resistant to low concentrations of plasmin (Fig. 4B). At higher concentrations, 50 µg/ml, a
cleaved product was apparent (Fig. 4, C and D). The size of the
cleaved product was identical to the size of the cleaved control
protein TS5a. Thus, mutating the arginines at positions 730–732 created a PL1-resistant protein.
PL2m (Fig. 6A). A double mutant, DM, containing the PL1m and PL2m was also constructed. Analysis of the sensitivity of the proteins to plasmin with the 9E10 antibody, revealed that the carboxyl end of the wild type, TSR1–5a protein and the mutant PL2m protein were cleaved at low concentrations of plasmin. The PL1m and the DM were both resistant to low plasmin, with the DM protein being more resistant to higher concentrations of plasmin than PL1m (Fig. 6A). The high sensitivity of PL2m, as judged by the elimination of the carboxyl end of the wild type, TSR1–5a protein and the mutant PL2m protein was plotted in a logarithmic scale ($y$) as a function of plasmin concentrations ($x$).

those two sites did not change the plasmin sensitivity of F-spondin (data not shown). It is conceivable that mutating the 682 and 686 sites changed the conformation of the protein and exposed arginines 693 and 695 to plasmin.

**ECM Binding Properties of the TSRs—** We have shown previously that the TSR domain of F-spondin binds to the ECM (19, 25). To examine whether the processing of F-spondin by plasmin modulates its interaction with the ECM, we tested the binding properties of the TSRs. Fusion proteins containing an alkaline phosphatase (AP) fused to various combinations of TSRs were generated (Fig. 7A). The conditioned media of HEK293 T transfected cells were incubated with tissue culture plates coated with bovine corneal endothelial cell ECM. The amount of bound protein was measured by an AP colorimetric reaction. All of the fusion proteins that contained TSR 5 or 6 or
both (AP-TSR1–6, AP-TSR1–5, AP-TSR5, AP-TSR6, and AP-TSR5–6) bound to the ECM (Fig. 7B). Proteins restricted to repeats 1–4 (AP-TSR1, AP-TSR1–2, AP-TSR1–4) did not bind to the ECM (Fig. 7B). The fifth TSR is more adhesive than the sixth. The binding levels of AP-TSR1–5 and AP-TSR1–6 are lower than the isolated fifth and sixth TSRs. This suggests that repeats 1–4 might reduce the affinity of repeats 5 and 6 in the context of the nonprocessed protein. Nevertheless, even binding the entire TSR domain is significantly greater than the processed 1–4 TSR protein. Thus, repeats 5 and 6 are required and sufficient for binding to the ECM.

What are the distinctive properties of repeats 5 and 6 which enable ECM binding? We have shown that F-spondin ECM binding is blocked by heparin sulfate and chondroitin sulfate (19, 25). This suggests that the binding of F-spondin is mediated by proteoglycans. Potential proteoglycan binding sites, BBXB (32), are present in all six TSRs (Fig. 7C). It was shown that each of the two TSRs of the heparin-binding protein HB-GAM has a β-sheet structure composed of three antiparallel β-strands (33). In F-spondin, repeats 1–4 contain one stretch of basic amino acids, a potential binding site in the second antiparallel β-strand, whereas the fifth and sixth repeats have two potential proteoglycan binding sites in the second and third antiparallel β-strands (Fig. 7C). A hypothetical model that accounts for the different binding properties of the TSRs is that there is a requirement for two basic domains in the second and third antiparallel β-strands to facilitate binding to the ECM. To test this hypothesis we replaced the third antiparallel β-strand of repeats 4 and 5 generating the (4-4-5) chimeric repeat 4 (AP-TSR5&4) and the reciprocal (5-5-4) chimeric repeat 5 (AP-TSR5&4) (Fig. 7A). The chimeric repeat 4 bound to the ECM, but the chimeric repeat 5 did not (Fig. 7D). This demonstrates that the third basic antiparallel β-strand of repeat 5 is required to elicit ECM binding of the fifth TSR and sufficient to confer binding properties to the fourth TSR. This supports the hypothesis that two basic motifs are required to enhance the binding of the F-spondin TSRs to the proteoglycans.

Plasmin Releases F-spondin from the ECM—The cleavage of F-spondin at the PL1 site and subsequently at the PL2 site should generate an ECM-free TSR protein composed of repeats 1–4 and the first antiparallel β-strand of repeat 5. To assess this theory, we tested whether plasmin generates an ECM-free protein. Because alkaline phosphatase was found to be degraded by plasmin (data not shown), we generated an amino Myc-tagged TSR protein. Four copies of the Myc epitope were cloned upstream of TSRs 2–6, to generate TSR2–6 protein (Fig. 4A). The protein was preincubated with plasmin and subsequently plated on ECM. The protein was detected by an anti-Myc conjugated antibody and colorimetric horseradish peroxidase reagent. Pretreatment of the TSR2–6 protein with plasmin significantly reduced the binding to ECM compared with the uncleaved untreated protein (Fig. 4B).

To study whether plasmin releases F-spondin from the ECM we performed the converse experiment. To circumvent the non-specific digest of ECM component (which might anchor F-spondin to the ECM) by plasmin (34), we have sensitized the assay by using limited amounts of plasmin and a higher plasmin-sensitive form of F-spondin. Because the PL2 site at the fifth TSR domain is less sensitive to plasmin than the PL1 site, we generated a TSR domain protein, TSR1–6A5, with a deletion of the fifth TSR (and subsequently deletion of PL2 site).
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FIG. 7. Binding properties of the different TSRs to ECM. Panel A, schematic drawing of the constructs used in these experiments. The gray boxes represent the alkaline phosphatase protein, the red boxes are TSRs 1–4, the purple boxes indicate the fifth and sixth repeats, and the white box represents region “a.” The black arrows demarcate the plasmin cleavage sites. Panel B, ECM of bovine corneal endothelial cells was generated in a 96-well plate. All of the fusion proteins that contained TSR 5 or 6 or both of them (AP-TSR1–6, AP-TSR1–5a, AP-TSR5, AP-TSR6, and AP-TSR5–6) bound to the ECM. Proteins restricted to repeats 1–4 (AP-TSR1, AP-TSR1–2, and AP-TSR1–4) did not bind to the ECM (n = 8 for each protein). Panel C, sequence of the TSRs of F-spondin. The blue letters are basic amino acids, and the red letters are acidic amino acids. The gray arrows represent the suggested antiparallel β-strand structure of the TSRs of HB-GAM (33). The underlined amino acids are the cleavage sites of plasmin. Panel D, binding of fourth and fifth TSR replacement proteins to the ECM. The TS4&5 that contains the first and second antiparallel β-strand of the fourth TSR and the third antiparallel β-strand of the fifth TSR binds to the ECM. The TS5&6 that contains the first and second antiparallel β-strand of the fifth TSR and the third antiparallel β-strand of the fourth TSR does not bind to the ECM. SeAP is the conditioned medium of HEK293 T cells transfected with the secreted alkaline phosphatase (n = 8 for each protein).

Treatments with increasing concentrations of plasmin yield a gradual decrease of the ECM-bound TSR1–6A5 protein (Fig. 8C). At 40 μg/ml about 70% of the bound protein was released. Western analysis of the released fraction demonstrates that only the cleaved protein, but not the uncleaved, is released from the ECM after plasmin cleavage (Fig. 8D). Thus, plasmin is generating a diffusible, ECM-free TSR-domain protein.

DISCUSSION

In this work we have identified plasmin as the protease that cleaves F-spondin at the carboxyl terminus. Two cleavage sites have been identified, each with a different sensitivity to plasmin. The cleavage at the more sensitive PL1 site renders high plasmin activity mediates release of a protein domain, containing TSRs 1–4, from the ECM.

Which Protease Cleaves F-spondin in Vivo?—Serine protease expression overlaps F-spondin expression in the nervous system. The protease that is mostly coexpressed with F-spondin is tPA. In addition, tPA is also expressed in neurons that encounter F-spondin at their place of origin as well as along their axonal path (23, 26). The partially cleaved F-spondin in the conditioned medium of transfected HEK293 T cells (Figs. 3B and 6C) suggests that the HEK293 T cell-derived TPA contains a moderate proteolytic activity for F-spondin. Yet, the fully cleaved F-spondin is evident, in vitro, only after activation of plasminogen by tPA, or by plasmin. In vivo, growth cone and floor plate cells may utilize tPA, uPA, or activated plasminogen to cleave F-spondin. The activity of serine proteases is tightly regulated during development by serine protease inhibitors. Neurserpin, a serine protease inhibitor, is expressed in a dynamic fashion in the floor plate and on motor neurons (35). Neurserpin may account for the partial resistance of the floor plate-derived F-spondin because Western analysis of protein extracts from the spinal cord revealed that both the short -4 TSRs, and the long -6 TSRs, proteins are detected (20, 21).

Proteases belonging to other families are also expressed in the floor plate. Metallonpeptinase MMP-11 is expressed in the floor plate (36), and MT5-MMP is expressed ubiquitously in the spinal cord (37). The furin protease SPC4 is also expressed in the floor plate (38). These proteases may cleave F-spondin between the reelin/spondin domain and the TSR domain. Furin and plasmin may be indirectly involved in this activity by activating latent metalloproteinase.

The colocalization of F-spondin, serine proteases, and growth factors in the ECM suggests that they all may interact to facilitate their activities. It was shown that thrombospondin-1 (Tsp-1) binds plasminogen and its activators (39–41). Tsp-1 also binds and activates latent transforming growth factor-β (42). Of special interest is the fact that the recognition signal in Tsp-1 required for activation of transforming growth factor-β, the KRFK motif (42), is also present in the sixth TSR of F-spondin. Hence, specific binding among F-spondin, plasminogen, uPA, tPA, and growth factors might enhance their biological activities.

Binding Properties of F-Spondin TSRs to the ECM—The thrombospondins are a family of proteins widely found in the
malaria circumporozoite protein (50, 54) do bind heparin. Structural studies of the recombinant HB-GAM, using heteronuclear NMR (33), revealed that the native HB-GAM structure is essential for heparin binding. Reduction of the disulfide bonds dramatically reduced heparin binding. The HB-GAM-heparin complex revealed that heparin binds to the β-sheet structure of the TSR, but not to the lysine-rich amino- and carboxyl-terminal tails. This implies that heparin binding requires a specific tertiary structure (33). Basic amino acids are probably essential for binding, but only in the context of the tertiary structure.

Our results obtained with native proteins support the requirement of a specific tertiary structure to elicit binding to the ECM. Repeats 1–4 contain all of the adhesive motifs (WSXW, CSVTCG, and RXR) that were identified in other thrombospondin proteins. Nevertheless, these modules do not bind to the ECM or to cells (data not shown). The second antiparallel β-strand of repeats 1–4 is rich in basic amino acids. The inability of repeats 1–4 to bind to the ECM might be the result of a different tertiary structure imposed by the acidic amino acids in the third antiparallel β-strand. The results of the reciprocal replacement of the third antiparallel β-strand of repeats 4 and 5 supports the hypothesis that basic residues in the third antiparallel β-strand are required for generating an ECM binding module. In support of this, the TSRs HB-GAM and ADAMTS-1, which mediate cell binding, are basic in their third antiparallel β-strand.

F-spondin TSRs represent a unique combination of ECM binding and ECM nonbinding modules. The nonbinding 1–4 TSRs are likely to interact with other receptors because they retain their biological activity (21). The ECM-bound TSRs (two-thirds of the fifth and the sixth) might be involved in activation of latent transforming growth factor-β via the KRFK motive in the sixth TSR, as it was demonstrated for Tsp-1 (42).

Biological Significance of Modulation of F-spondin Binding to the ECM by Plasmin—F-spondin protein was shown to accumulate in the ECM that underlies the floor plate, the endoneurial ECM of the embryonic peripheral nerve, and of the regenerating sciatic nerve (20, 22). An antibody raised against the spondin domain was used in these studies. The anti-TSR domain antibodies that we raised failed to detect the protein in immunohistochemistry studies. We assume that the TSR domain colocalizes with the reelin/spondin domain to the ECM. This assumption is supported by the binding properties of the unprocessed TSR domain protein to the ECM. F-spondin expression in the central nervous system overlaps the expression of other ECM proteins that are targets for plasmin cleavage. Both F-spondin and laminin are expressed in the hippocampus. Thus, F-spondin cleavage by plasmin may also account for the cell death in the hippocampus after seizure and the subsequent elevation of tPA levels, as was demonstrated for laminin (11). In addition, F-spondin may be a target for tPA during activity-dependent forms of synaptic plasticity in the hippocampus and thus mediates the late phase, long term potentiation in both Schaffer collateral and mossy fiber pathways, which is interfered with by tPA inhibitors and in the tPA null mouse (9, 10).

F-spondin processing by the neuronally derived tPA may account for the retarded migration of granule cells in the cerebellum of the tPA null mouse (55). F-spondin is expressed in the internal granule layer of the cerebellum during early postnatal days.2 F-spondin may serve as a repulsive protein for granule neurons, as was demonstrated for the migrating neural crest cells (27). Granule neurons expressing tPA would then be able to clear a path through the F-spondin-rich milieu as

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2 Y. Feinstein, A. Klar, and E. Soriano, unpublished data.
Plasmin Releases F-spondin from the ECM

they migrate from the external granule layer to the internal granule layer. F-spondin plays a dual role in patterning neuronal connectivity in the embryonic nervous system. It promotes outgrowth of commissural neurons (20) and inhibits outgrowth of motor neurons (21). Motor neurons, in rodents, are exposed to F-spondin from different sources during their exogenesis. As they start to extend axons away from the ventral midline of the spinal cord, they might be repelled by a plasminogen-rich milieu? One possibility is that they might change their responsiveness to F-spondin from different sources during their exogenesis. As they migrate from the external granule layer to the internal granule layer, and Heikki Rauvala for comments on the manuscript.

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Plasmin-mediated Release of the Guidance Molecule F-spondin from the Extracellular Matrix
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