The Two Thrombospondin Type I Repeat Domains of the Heparin-binding Growth-associated Molecule Bind to Heparin/Heparan Sulfate and Regulate Neurite Extension and Plasticity in Hippocampal Neurons*

Erkki Raulo1,†, Sarka Tumova1, Ivan Pavlov1, Mari Pekkanen1, Anni Hienola1, Emilia Klangki1, Nisse Kalkkinen1, Tomi Taira1, Ilkka Kilpelainen1,‡, and Heikki Rauvala1,‡

From the 1Department of Chemistry, 2Neuroscience Center, and 3Institute of Biotechnology, University of Helsinki, Helsinki FIN-00014, Finland

HB-GAM (heparin-binding growth-associated molecule, also designated as pleiotrophin) and midkine form a two-member family of extracellular matrix proteins that bind tightly to sulfated carbohydrate structures such as heparan sulfate. These proteins are used by developing neurons as extracellular cues in axonal growth and guidance. HB-GAM was recently reported to enhance differentiation of neural stem cells. Based on the solution structure of HB-GAM, we have recently shown that HB-GAM consists of two \( \beta \)-sheet domains flanked by flexible lysine-rich N- and C-terminal tails with no apparent structure. These domains are homologous to thrombospondin type 1 repeats present in numerous extracellular proteins that interact with the cell surface. Our findings showed that the two \( \beta \)-sheet domains fold independently. We showed that the domains (but not the lysine-rich tails) in HB-GAM are required and sufficient for interaction with hippocampal neurons. The individual domains bind heparan sulfate weakly and fail to produce significant biological effects in neurite outgrowth and long term potentiation assays. The amino acids in the linker region joining the two domains may be replaced with glycines with no effect on protein function. These results suggest a co-operative action of the two \( \beta \)-sheet domains in the biologically relevant interaction with neuron surface heparan sulfate.

The formation of neuronal connections in the developing brain is regulated by interactions of the cell surface with extracellular matrix molecules, soluble growth factors, and cell surface adhesion molecules. Similar mechanisms are implicated in the control of plastic changes in the adult nervous system. Heparan sulfate (HS)-mediated interactions of growing neuronal processes with proteins of the extracellular matrix are involved in axonal path finding during development and plasticity. Understanding the molecular mechanisms of heparin/HS binding by these proteins is therefore of key importance in molecular neurobiology.

HB-GAM3 has been described as a heparin-binding protein expressed at the extracellular matrix of axonal tracts in the developing brain (1–3, 26). In addition, the protein has been implicated in hippocampal synaptic plasticity (4, 5) and bone development (6). Neurite outgrowth and osteoblast migration induced by HB-GAM depend on the neuronal cell surface heparan sulfate proteoglycan N-syndecan, also designated as syndecan-3 (6–8). HB-GAM is also described as a developmentally regulated cytokine pleiotrophin (9).

We have suggested in our previous work (10) that HB-GAM and mid-gestation kidney protein MK are structurally similar heparin-binding proteins homologous to the thrombospondin type 1 repeat (TSR) (Fig. 1). The TSR repeat is found in a larger superfamily of extracellular matrix-associated and cell surface proteins, such as thrombospondins 1 and 2, F-spondin, mindin, semaphorins F and G, and TRAP (thrombospondin-related anonymous protein from malaria parasite \( \text{Plasmodium falciparum} \)) (for review, see Ref. 11). A common feature for proteins in this superfamily is the function in cell surface and matrix binding that is dependent on heparin-type polysaccharides. Indeed, HB-GAM has been shown to bind the cell surface receptor N-syndecan via the heparan sulfate side chains of N-syndecan (7, 8). This interaction leads to signaling by N-syndecan to cortactin/Src kinase pathway and reorganization of the cytoskeletal network resulting in the neuronal growth cone and osteoblast migration (6, 12). Similarly, mid-gestation kidney protein MK has been shown to interact with syndecans 1 and 4 earlier in development (13). In addition to N-syndecan, HB-GAM interacts with the receptor-type protein tyrosine phosphatase RPTP\( \beta \)/\( \xi \) at the cell surface (14–16). We have recently shown that HB-GAM inhibits its proliferation and induces differentiation of neural stem/progenitor cells. These activities of HB-GAM are proposed to occur through competition with fibroblast growth factor-2 for binding to cell surface heparan sulfate (17).

The present paper shows that the TSR domains in HB-GAM fold independently, and they do not interact with each other in solution conditions. Individual TSR domains derived from HB-GAM bind weakly to heparin/HS and fail to influence neurite outgrowth and plasticity. The domain structure of HB-GAM, in which both TSR domains are present, is required for heparin/HS binding and interaction with hippocampal neurons.

**EXPERIMENTAL PROCEDURES**

**PCR Mutagenesis of TSR Domain Proteins**—The TSR domains of HB-GAM as individual recombinant proteins and as a di-domain polypeptide (TABLE ONE) were produced in \( \text{Escherichia coli} \) using the glutathione \( \delta \)-transferase fusion vector pGEX-2T (Amersham Biosciences) as described previously (10). The domain boundaries were
predicted from previous NMR structural data (10, 18). The lysine-rich tails were excluded from the domain constructs. The single domain spans were as follows: N-terminal domain, amino acids Ser13-Asn58; C-terminal domain, amino acids Ala65-Gly110. The di-domain construct spanned amino acids Ser13–Gly110. Mismatch primers were used to generate differentially truncated forms of rat HB-GAM cDNA coding region as presented in TABLE ONE. To study the contribution of the linker region between the two TSR domains in heparin binding and biological activity constructs, G-linked di-TSR, TSR-N+Linker, and TSR-C+Linker were generated. All DNA constructs were sequenced to exclude PCR-born mutations.

Heparin Affinity Chromatography of HB-GAM Domains—The bacterial lysates were subjected to heparin affinity chromatography. A linear NaCl gradient from 0 to 2 M in 20 mM sodium phosphate buffer, pH 7.5, was used to compare the elution profiles of separate domains with the intact HB-GAM produced similarly in E. coli. All recombinant products described above were purified by this method and verified for >90% homogeneity on SDS-PAGE. The proteins were subjected to matrix-assisted laser desorption ionization mass spectroscopy to verify molecular weight before they were used in the subsequent studies. The $^{15}$N- and $^{13}$C-labeled protein samples for NMR were produced by growing the bacteria in a minimal media as described previously (10), followed by normal workup.

NMR Measurements of HB-GAM-derived TSR Domains—All the spectra were acquired at 30°C on Varian Inova 600- and 800-MHz spectrometers using protein concentrations of 0.2–1 mM. The spectra were processed with Vnmr (Varian Inc., Palo Alto, CA) and analyzed manually with Felix 97 software (Biosym/Molecular Simulations, Inc., San Diego, CA). The backbone assignments for the individual domains were carried out by recording a typical set of triple resonance spectra (CBCA(CO)NH, HNCACB, HNCA and HN(CO)CA, (19)) followed by normal spectral interpretation/analysis. The possible interaction of the individual domains was followed by titrating a $^{15}$N-labeled N-terminal domain sample (1 mM, residues 13–58) to a 5-fold excess of unlabeled C-domain (residues 65–110), and vice versa. The heparin interaction experiments were carried out by titrating the protein samples with a purified 14-meric heparan sulfate preparte (20).

**Ligand-binding Assays**—HB-GAM domain binding to heparin was analyzed by surface plasmon resonance using the IAsys instrument (Thermo Electron Corporation). Heparin-derivatized surfaces were prepared by immobilizing heparin-BSA (Sigma) to one cell of the double-well planar aminosilane cuvette according to the manufacturer’s instructions. Briefly, 0.2 mg/ml heparin-BSA in 15 mM Na$_2$HPO$_4$, pH 7.4, was coupled via amino groups to the aminosilane surface activated with polymerized glutaraldehyde, resulting in an immobilization level of ~0.9 ng of heparin-BSA/mm$^2$ planar surface. The second cell was coated with BSA alone to generate a control surface. Unbound molecules were removed by washing the cuvettes with the immobilizing buffer and 2 M NaCl in phosphate-buffered saline (PBS). Remaining activated sites were blocked with $\beta$-casein. The binding data for each ligand were obtained by successive additions of increasing ligand concentrations in PBS and measuring the equilibrium response after each addition. Between the measurements for different ligands, the cuvette was regenerated by washing with 2 M NaCl in PBS. All ligands displayed low or negligible binding to the control BSA surface, as was monitored simultaneously in the control cell. The response in the control cell was subtracted from the value measured in the heparinized cell to obtain the specific response $R$, which was used to calculate the dissociation constant $K_d$ by fitting in the equation $r = R_{\text{max}} [L]/(K_d + [L])$, where [L] is

### TABLE ONE

| Mutants produced by PCR mutagenesis | K-rich | TSR | Linker | TSR | K-rich |
|-----------------------------------|--------|-----|--------|-----|--------|
| HB-GAM                           | 1-12   | 13-58 | Linker | 59-64 | 111-138 |
| DI-TSR                           |        |      |        |      |        |
| G-Linked                         |        |      |        |      |        |
| TSR-N                            |        |      |        |      |        |
| TSR-C                            |        |      |        |      |        |
| TSR-N+Linker                     |        |      |        |      |        |
| TSR-C+Linker                     |        |      |        |      |        |

**FIGURE 1. Secondary structure of HB-GAM (see Ref. 10).** The protein folds into two $\beta$-sheet domains (blue) connected by a flexible linker sequence (red). The N- and C-terminal highly cationic tails (red) appear as random coils having no specific structure. Amino acids at the $\beta$-sheet boundaries are labeled.
the ligand concentration and $R_{max}$ corresponds to the $R$ value at saturation.

**Binding to Heparan Sulfate**—Recombinant rat N-syndecan ectodomain was expressed as an Fc-fusion protein in 293T cells using the pIgG expression vector (21). The construct was transfected to semiconfluent 293T cells using FuGENE® transfection reagent (Roche Diagnostics, Mannheim, Germany). Three days post-transfection the medium was harvested. The medium was centrifuged to remove debris and used as a reagent in binding assays. As a control, a mock medium transfected with FuGENE® alone was prepared. Wells of Nunc maxisorb plates were coated with 1 μg/ml protein A (Amersham Biosciences) and blocked with 1% BSA in PBS, 0.05% Tween 20. Conditioned medium from transfected 293T cells was added to the wells. After washing three times with 0.1% BSA, 0.05% Tween 20 in PBS, biotinylated di-TSR was added with or without competing unlabeled ligands. The level of biotin bound to the wells was monitored with biotinylated di-TSR was added with or without competing unlabeled ligands. The level of biotin bound to the wells was monitored with horseradish peroxidase-conjugated streptavidin using the ortho-phenylenediamine chromogenicsubstrate according to the manufacturer’s instructions (Sigma). To control the binding through heparan sulfate glycosaminoglycans, heparinase II (Sigma) was used. 0–2 IU/ml heparinase II was added in the assay medium to cleave heparan sulfate glycosaminoglycans, heparinase II was added in the assay medium to cleave heparan sulfate glycosaminoglycan side chains of the recombinant N-syndecan-Fc-fusion protein.

**Heparin-binding Assay**—96-well Nunc maxisorb plates were coated with 100 μl of 1 μg/ml HB-GAM for an hour. The wells were blocked with 1% BSA in PBS, 0.05% Tween 20 for 1 h. After washing the wells three times with the blocking buffer, 0.1 μg/ml biotin-BSA-heparinase II (Sigma) with 20 μg/ml competing unlabeled ligands in 0.1% BSA in PBS, 0.05% Tween 20 was incubated in the wells for 1 h at +4 °C. The level of biotin bound to the wells after three washing steps was monitored with horseradish peroxidase-conjugated streptavidin using the ortho-phenylenediamine chromogenic substrate according to the manufacturer’s instructions (Sigma).

**Cells and Neurite Outgrowth Assays**—Hippocampal primary cultures were prepared essentially as described previously (22). For neurite outgrowth assays, neurons were plated at 12,500/well density in microwells coated with recombinant HB-GAM and different TSR domains. For the counting of neurite outgrowth, images were taken from living cells using randomly selected microscopic fields, and the extensions exceeding 10 μm in length were considered as neurites. The induction of neurite outgrowth was calculated as percentage of cells growing neurites in the 48-h assay.

The ability of different TSR domains to inhibit neurite outgrowth on HB-GAM-coated wells when presented as soluble factors was monitored as described previously (23). Briefly, neurons were plated at 12,500/well density in microwells coated with HB-GAM (1 μg/ml). The cells were grown for 48 h in assay medium containing different concentrations of soluble domains and analyzed for neurite outgrowth.

**Transfilter Migration Assays**—Migration assays were performed with NMRI mice strain embryonic day 14–15 forebrain neurons prepared as described previously (24). The cells were plated at the density of 150,000 cells/well on 12-mm COSTAR transfilter plates with a 12-μm pore size. The outer surfaces of the filters were precoated with 1 mM HB-GAM. Because the di-TSR polypeptide was apparently unable to coat the wells, we performed the assay in the presence of 1 mM HB-GAM or di-TSR as soluble competitors in the assay medium (BSA at 10 mg/ml, Dulbecco’s modified Eagle’s medium). The inhibitory effect on migration after 16 h was followed.

**In Vitro Electrophysiology**—Transverse hippocampal slices (400 μm thick) from adult Wistar rats (1.5–2 months old) were cut using Vibratome 3000 Plus for *in vitro* electrophysiological experiments. Slices were allowed to recover at room temperature for at least 60 min before the experiments. Recordings were made in the interface-type chamber (volume 1 ml; perfusion rate 1 ml/min) at +32 °C. Artificial cerebrospinal fluid containing (in mM) NaCl (124), KCl (3), CaCl₂ (2), NaHCO₃ (25), NaH₂PO₄ (1.1), MgSO₄ (1.3), glucose (10), was equilibrated with the mixture of 5% CO₂ and 95% O₂ to yield a pH of 7.4.

Extracellular recordings from stratum radiatum of the CA1 area of the hippocampi were obtained using glass capillary microelectrodes filled with 150 mM NaCl. Field excitatory postsynaptic potentials (fEPSPs) were elicited by stimulating Schaffer collaterals with a bipolar stimulation electrode. The stimulus intensity was adjusted to gain a half-maximal fEPSP amplitude. Base-line synaptic transmission was monitored at 0.05 Hz, pulse length 0.1 ms. The slope of fEPSP was used as an indicator of synaptic efficacy and was calculated between 20 and 80% of the maximal amplitude. Long term potentiation (LTP) was induced by high frequency stimulation (100 Hz/1 s), during which the pulse length was doubled. TSR domains were dissolved in PBS (0.1 mM NaHPO₄, 150 mM NaCl, pH 7.4) at a concentration of 200 μg/ml. Because of the limited amounts of TSR domains, they were pressure-injected (~0.2 μl) into the dendritic area of the hippocampal CA1 region close to the recording site 10 min before LTP induction (as described in Ref. 4). Control experiments with PBS injections of the same volume were carried out to ensure that the procedure did not interfere with the base-line synaptic response or induction of LTP.

LTP program, version 230d (25), was used for data acquisition and analysis. Student’s $t$ test was used for statistical analysis of the data. Changes were considered to be significant at $p$ values $<0.05$.

**RESULTS AND DISCUSSION**

**TSR Domains of HB-GAM Fold Independently**—The $^1H$-$^15N$ heteronuclear single quantum coherence (HSQC) spectra of the isolated domains coincide with the corresponding spectrum of full-length HB-GAM (Fig. 2). Only very minor changes are observed in the chemical shifts, indicating that the structures of the isolated domains are similar to those in the native protein. Also the di-TSR $^1H$-$^15N$ HSQC spectrum coincides fully with the spectrum of the whole protein (data not shown), indicating a proper fold.
Domain-domain titration experiments were carried out to gain information on possible domain-domain interactions. In these titrations, a sample of the \(^{15}\text{N}\)-labeled domain was titrated with an unlabeled second domain. The titrations were carried out in both ways, i.e. the N-terminal domain was titrated with the C-domain, and vice versa. However, no changes in chemical shifts were observed in either case. Therefore, it is evident that the two domains fold independently and they do not have domain-domain interactions in solution state.

**Binding of Heparin to HB-GAM as Followed by NMR Spectroscopy**—We have previously shown (10) that during the titration of full-length HB-GAM with heparin, the \(^{1}\text{H}-^{15}\text{N}\) HSQC resonances of the structural areas of the protein (i.e. N- and C-terminal domain) gradually disappear from the spectrum, but the resonances of the non-structural, lysine-rich tails (residues 1–12 and 111–136) remain in the spectrum with high intensity and show no significant changes in their chemical shifts. In this respect, the di-TSR (Gly\(^{13}\)-Ser\(^{110}\)) protein behaves in an exactly similar way as the full-length protein. During the titration with heparin, the \(^{1}\text{H}-^{15}\text{N}\) resonances of the protein gradually broaden under the detection limit (data not shown).

The individual N- and C-terminal domains interact with heparin in fast exchange in the NMR timescale, as shown in Fig. 3A for the N-terminal domain (spectral data for C-terminal not shown). The titration data, as a function of amino acid sequence for the isolated domains, are presented in Fig. 3, B and C. The binding data show that individual domains bind the 14-meric heparin fragment with a stoichiometry of 1:1. The \(K_d\) values for the separate N- and C-terminal domains were 53 and 20 \(\mu\text{M}\), respectively, as calculated from the chemical shift changes during the titration. In the heparin titration experiments, residues 14–18, 39–43, and 52–54 in the N-terminal domain and residues 66–69 and 89–93 in the C-terminal domain show significant changes in their chemical shifts, indicating that these residues are playing a role in the interaction with heparin. However, these findings should be confirmed with mutation experiments.

A titration of a sample containing the N- and C-terminal domains in a 1:1 ratio with heparin shows a similar effect (data not shown), as seen with the whole protein and the di-TSR sample. However, the interaction of heparin with the two domains appears in intermediate exchange in the NMR timescale (not slow, as in the case of the intact protein). The titration was carried out by first titrating a sample of the \(^{15}\text{N}\)-enriched N-terminal domain with \(^{15}\text{N}\)-enriched C-terminal domain to a ratio of 1:1, followed by titration with heparin 14-mer and observing changes in the \(^{1}\text{H}-^{15}\text{N}\) HSQC spectrum. During the course of titration with heparin, the original signals from the domains in solution gradually disappear from the spectrum. With an excess of heparin, a part of the signals becomes visible, but a large number of signals remains below detection limit, even in the presence of a large excess of heparin. Further, a good number of signals are just weakly visible at the end of the titration. Thus, it was not possible to obtain reliable assignments for the (TSR-N-TSR-C-heparin) complex. Similar results were obtained with commercial low molecular weight heparin. The disappearance of the signals of the structural domains of the protein when both domains are present in the solution cannot solely be explained by the molecular weight of the complex. It seems that HB-GAM undergoes a conformational change in the complex form, which causes the broadening of the signals. This is in agreement with the circular dichroism spectroscopy data described previously (10).

The NMR titration data shows that the N- and C-terminal domains interact with heparin in a co-operative manner. The domains do not...
interact with each other in the absence of heparin, but the timescale of the heparin interaction changes when both domains are present, even when as separate domains.

**Both TSR Domains Are Required for High Affinity Interaction with Heparin/HS**—The affinity of different HB-GAM domains for heparin was analyzed by surface plasmon resonance using a heparin-BSA-coated cuvette. The dissociation constants were determined by equilibrium titration (TABLE TWO). Intact HB-GAM displayed high affinity for heparin ($K_d$ of 176 ± 37 nM). Removal of the lysine-rich tails had no effect on heparin binding of HB-GAM, as the $K_d$ for the di-TSR remained nearly identical (153 ± 40 nM). However, a dramatic change was observed for the individual TSR domains. The C-terminal domain of HB-GAM displayed affinity for heparin ($K_d$ of 13.0 ± 2.5 μM) that was two orders of magnitude lower than that of the intact protein. The N-terminal domain of HB-GAM bound heparin even more weakly. At the concentrations used in our assay, the saturation could not be reached, indicating a $K_d$ value >70 μM. This would indicate that, although the individual domains of HB-GAM can interact with heparin, cooperation between the N-terminal and C-terminal domain results in the optimal heparin binding. The lysine-rich tails do not appear to be directly involved in the HB-GAM interaction with heparin.

Consistent with the surface plasmon resonance measurements, binding of biotinylated heparin-BSA to immobilized HB-GAM in an enzyme-linked immunosorbent assay-type competition assay was reversed by excess soluble concentration of intact HB-GAM and di-TSR. The single domains failed to compete for HB-GAM–heparin interaction when presented in the assay medium. The presence of a 1:1 mixture of TSR-N and TSR-C had no effect on the binding of biotinylated heparin-BSA to immobilized HB-GAM (Fig. 4).

The interaction of HB-GAM with the neuronal cell surface is mediated by N-syndecan heparan sulfate side chains (8). To study the binding characteristics of HB-GAM domains to N-syndecan HS chains, we expressed N-syndecan ectodomain as an Fc-fusion protein in 293T cells. This approach produces N-syndecan with heparan sulfate chains comparable in size and sensitivity to heparinas of those naturally occurring in the brain during development. An enzyme-linked immunosorbent assay-type competition assay using the N-syndecan-Fc-fusion protein was performed for different domain constructs. Binding of biotinylated di-TSR polypeptide to immobilized N-syndecan was effectively decreased in the presence of HB-GAM or di-TSR, whereas the individual TSR domains failed to compete (Fig. 5). Adding heparinase II, which cleaves the heparan sulfate chains, decreased di-TSR binding to individual TSR domains failed to compete for HB-GAM–heparin interaction (Fig. 5). Consistent with the data from coating experiments of the individual TSR domains, we followed neurite outgrowth in hippocampal embryonic neurons plated on different TSR domain polypeptides (Fig. 6A). Consistent with the heparin-binding data, native HB-GAM as well as the di-TSR induced neurites with effective coating concentrations (EC50) of 30 nM and 180 nM, respectively. Both N- and C-terminal single TSR domains failed to induce neurites at the coating concentrations tested (up to 10 μM).

Effects of TSR Domains on Neurite Outgrowth in Hippocampal Neurons—HB-GAM binding to N-syndecan HS chains promotes neurite outgrowth in cultured neurons (7, 23, 24). To monitor the biological activities of the individual TSR domains, we followed neurite outgrowth in hippocampal embryonic neurons plated on different TSR domain polypeptides (Fig. 6A). Consistent with the heparin-binding data, native HB-GAM as well as the di-TSR induced neurites with effective coating concentrations (EC50) of 30 nM and 180 nM, respectively. Both N- and C-terminal single TSR domains failed to induce neurites at the coating concentrations tested (up to 10 μM).

To rule out the possibility that differences in coating efficiency of different domain constructs may affect results, we performed a series of inhibition assays (Fig. 6B). Consistent with the data from coating experiments, the di-TSR polypeptide in the culture medium inhibited neurite outgrowth on HB-GAM-coated substrates in a similar fashion as the intact HB-GAM (Fig. 6B). The single TSR domains failed to inhibit neurite outgrowth induced by HB-GAM when applied to the assay medium in excess to the substrate coating concentration. Likewise, an equimolar mixture of the N- and C-terminal domain polypeptides did not produce any inhibition (not shown), indicating that the two domains must be physically linked to cooperate.

**Effect of TSR Domains on Synaptic Plasticity**—In the adult brain, HB-GAM is demonstrated to act as an inducible inhibitor of hippocampal LTP (4, 5, 27). This effect also seems to be mediated through N-syndecan HS chains. Thus, we further tested the effects of the purified TSR domains on LTP induced by high frequency stim-

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**TABLE TWO**

| Dissociation constants for ligand binding to immobilized heparin-BSA determined by equilibrium titration |
|--------------------------------------------------------------------------------------------------|
| Intact HB-GAM | Di-TSR | TSR-N | TSR-C |
|----------------|--------|-------|-------|
| 176 ± 37 nM | 153 ± 40 nM | >70 μM | 13.0 ± 2.5 μM |

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**FIGURE 4.** Binding of biotinylated heparin-BSA to HB-GAM-coated (1 μg/ml) microtiter wells in the presence of 20 μg/ml different domain constructs. Di-TSR as well as the G-linked form of it effectively compete with HB-GAM for heparin binding. The individual domains fail to compete for binding to heparin when present in the assay medium individually (TSR-N, TSR-C) or as a mixture of both domains (TSR-N + C) = 20 μg/ml TSR-N and 20 μg/ml TSR-C. Error bars indicate the S.D. values from four wells.

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**FIGURE 6A.** Binding of biotinylated heparin-BSA to HB-GAM-coated (1 μg/ml) microtiter wells in the presence of 20 μg/ml different domain constructs. Di-TSR as well as the G-linked form of it effectively compete with HB-GAM for heparin binding. The individual domains fail to compete for binding to heparin when present in the assay medium individually (TSR-N, TSR-C) or as a mixture of both domains (TSR-N + C) = 20 μg/ml TSR-N and 20 μg/ml TSR-C. Error bars indicate the S.D. values from four wells.
ulation of Schaffer collaterals in the hippocampus. In line with previous reports (4), pressure injection of recombinant HB-GAM into the stratum radiatum of the CA1 region of hippocampus suppressed the induction of tetanus-induced LTP so that the slope of fEPSP was 103.5 ± 10.5% of the base-line level 1 h after high frequency stimulation (Fig. 7B). Control injections with PBS did not prevent LTP (fEPSP slope was 153.9 ± 5.6% of the base line). Similar to the effect of native HB-GAM, the di-TSR polypeptide injection as well as the injection of G-linked di-TSR 10 min before high frequency stimulation effectively inhibited LTP. One h after tetanization, the normalized fEPSP slope for di-TSR and G-linked di-TSR was 108.4 ± 8.9% and 114 ± 3.6% of the base line, respectively (Fig. 7, C and E). Single N- and C-terminal β-sheet domains (TSR-N and TSR-C) injections did not abolish LTP. Although the levels of potentiation for TSR-N- and TSR-C-injected slices were lower than those of control PBS-injected slices (TSR-N, 132.7 ± 8.2%; TSR-C, 130 ± 7.8% of the base line), these changes were not statistically significant. These data support the results of neurite outgrowth experiments suggesting that the di-domain structure of HB-GAM is important for heparin/HS-mediated physiological effects of the native protein.

Transfilter Migration Assays—Murine embryonic forebrain neurons were used to test the influence of the TSR domains on neuronal migration. A transfilter migration assay was performed as described under “Experimental Procedures.” Because the di-TSR polypeptide failed to coat the transfilters, we decided to follow the inhibitory effect in a soluble inhibition assay. HB-GAM is a haptotactic guidance molecule in a Boyden chamber assay, displaying at 1 mM coating concentration, a 2-fold increase in the number of migrating cells when compared with poly-L-lysine control. Adding an equimolar concentration of HB-GAM, its di-TSR-fragment or the TSR-C fragment to the medium inhibited the migration. The TSR-N fragment (n = 3) did not have any effect on cell migration in this assay (Fig. 8).

Concluding Remarks—The TSR domains of HB-GAM fold independently and do not interact with each other in the protein structure. These domains are joined by a short linker region and flanked by flexible lysine-rich tails in the N and C termini. Studies using NMR, surface plasmon resonance, and enzyme-linked immunosorbent assay-type binding assays show that HB-GAM binds tightly to heparin/HS via the two TSR domains, whereas the linker region between the domains or the poly-lysine-type tails are not important for the high affinity binding.

The HB-GAM sequence contains multiple matches to the XBBXBX or XBBBXXBX consensus for heparin binding, where B is a basic residue and X is a hydrophobic amino acid (28). These consensus sequences are found in the lysine-rich tails but not in the TSR domains. Yet, the lysine-rich tails of HB-GAM are not required for the high affinity binding to

![FIGURE 5. Binding of biotinylated di-TSR protein to recombinant N-syndecan-Fc fusion protein immobilized in protein A-coated microtiter wells. Different domain constructs were used to inhibit the binding. Also, the effect of heparinase II (Hase II) was assayed to control binding through heparan sulfate side chains. Error bars indicate the S.D. values from four wells.](image1)

![FIGURE 6. Role of different HB-GAM recombinant domain fragments in neurite outgrowth in hippocampal neurons. A, for neurite outgrowth assays, neurons were plated at 12,500/well density in microwells coated with recombinant HB-GAM and different TSR domains, as indicated in the chart. B, neurite outgrowth inhibition from primary cultured rat hippocampal neurons grown on HB-GAM-coated microwells. Intact HB-GAM and different domain constructs were applied as neurite outgrowth inhibitors in the assay medium. Induction of neurite outgrowth was calculated as percentage of cells growing processes exceeding 10 μm in a 48-h assay. The error bars indicate the S.D. values from six wells. C, morphology of hippocampal neurons in the presence of different soluble factors, as indicated in the picture.](image2)
heparin/HS. These results agree with a recent notice that heparin-binding domains show no absolute dependence on specific sequences, making them difficult to predict from genomic data (29). In the case of HB-GAM, co-operative action of the two TSR domains is essential for strong binding, whereas single domains only bind weakly. Upon binding, the TSR domains appear to undergo a conformational change.

Previous studies have suggested that HB-GAM regulates neurite outgrowth and synaptic plasticity through binding to the heparan sulfate chains of N-syndecan that communicate with the neuronal cytoskeleton via the cortactin/Src kinase pathway (4, 12, 27, 30). In studies of synaptic plasticity, HB-GAM has been found to act as an inhibitor of hippocampal LTP that is induced by electrical activity (4, 5, 27). The involvement of N-syndecan in the regulation of LTP by HB-GAM is suggested by the finding that HB-GAM is unable to affect LTP in N-syndecan knock-out neurons (31).

If the heparan sulfate chains of N-syndecan are involved in the regulation of neurite outgrowth and plasticity by HB-GAM, it is to be expected that the heparin/HS binding regions play a role in the regulation. According to the current results, this indeed is the case; the di-TSR domain retains the activity of HB-GAM in neurite outgrowth and hippocampal LTP, whereas the single TSR domains, the linker region, or the lysine-rich tails display little, if any, activity.

In addition to neurite outgrowth and synaptic plasticity, HB-GAM has been found to induce migration of embryonic brain neurons in a manner that depends on receptor-type tyrosine phosphatase β/ζ and possibly on syndecan (14, 32). In the case of the transmembrane phosphatase receptor, HB-GAM binds to the chondroitin sulfate chains and possibly to the core protein (15). The present study suggests that regulation of neuron migration by HB-GAM differs from the regulation of neurite outgrowth and LTP in that the C-terminal TSR domain is approximately as active as the di-TSR domain. This might be due to a difference in the receptor mechanism. However, binding of the different HB-GAM regions to the phosphatase receptor has to be studied to resolve this issue.

TSR domains occur in clusters in a wide variety of proteins that bind to the cell surface and matrix (11). This raises the possibility that, in these proteins also, multiple TSR domains may be required for binding to heparin/HS, the cell surface, and the matrix. However, not much can be predicted about this possibility, because the heparin/HS binding properties of other TSR domain proteins have been mostly studied using competing peptides. A similar design as in HB-GAM, for confor-
mational heparin binding requiring two adjacent domains, has been described for the fibronectin type III domains 10 and 11 in tenascin-X (33).

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