Neurite Outgrowth in Brain Neurons Induced by Heparin-binding Growth-associated Molecule (HB-GAM) Depends on the Specific Interaction of HB-GAM with Heparan Sulfate at the Cell Surface*

(Received for publication, July 13, 1995, and in revised form, October 31, 1995)

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Heparin-binding growth-associated molecule (HB-GAM) is a cell surface- and extracellular matrix-associated protein that lines developing axons in vivo and promotes neurite outgrowth in vitro. Because N-syndecan (syndecan-3) was found to function as a receptor in HB-GAM-induced neurite outgrowth, we have now studied whether the heparan sulfate side chains of N-syndecan play a role in HB-GAM-neuron interactions. N-Syndecan from postnatal rat brain was found to inhibit HB-GAM-induced but not laminin-induced neurite outgrowth when added to the assay media. The inhibitory activity was abolished by treating N-syndecan with heparitinase, but it was retained in N-syndecan-derived free glycosaminoglycan chains, suggesting that N-syndecan heparan sulfate at the cell surface is involved in HB-GAM-induced neurite outgrowth. Binding to HB-GAM and inhibition of neurite outgrowth was observed with heparin-related polysaccharides only; galactosamino-glycans were inactive. Significant inhibition of neurite outgrowth was induced by heparin and by N-syndecan heparan sulfate but not by heparan sulfates from other sources. A minimum of 10 monosaccharide residues were required for HB-GAM binding, as well as for inhibition of HB-GAM-induced neurite outgrowth. Experiments with selectively desulfated heparins indicated that 2-O-sulfated iduronic acid units, in particular, are of importance to the interaction with HB-GAM, whereas glucosamine N-sulfate and 6-O-sulfate groups were implicated to a lesser extent. Structural analysis of N-syndecan from 6-day-old rat brain indicated that the heparan sulfate chains contain sequences of contiguous, N-sulfated disaccharide units with an unusually high proportion (82%) of 2-O-sulfated iduronic acid residues. We suggest that this property of N-syndecan heparan sulfate is essential for HB-GAM binding and induction of neurite outgrowth.

Cell surface proteoglycans are suggested to function as receptors or co-receptors for molecules that mediate cell-to-extracellular matrix and cell-to-cell interactions and for growth factors of the extracellular milieu (for reviews see Refs. 1–7). Most of these interactions depend on binding of proteins to the negatively charged glycosaminoglycan (GAG)* chains of the proteoglycans. Binding of proteins to the GAG chains can vary with regard to affinity and specificity and generally involves electrostatic interactions with negatively charged GAG structures (3, 8). A growing number of heparin-binding molecules involved in various biological processes have attained interest during the recent years. Among the best characterized members of the heparin-binding growth factors are the acidic and basic fibroblast growth factors. Several reports on the heparin/heparan sulfate involved in fibroblast growth factor action have appeared (9–13).

HB-GAM (p18) was isolated from rat brain as a neurite outgrowth-promoting protein, the expression of which in brain corresponds to the stage of rapid axonal growth (14). The full-length cDNA encoding rat HB-GAM has been cloned and sequenced (15). The same amino acid sequence has been reported for pleiotrophin, a protein isolated from uterus as a mitogen for NIH 3T3 cells (16). The HB-GAM sequence is more than 90% conserved from man to chicken (15–19), and it shares about 50% homology with the mouse midgestation kidney protein (20, 21) and its chicken homolog retinoic acid-inducible heparin-binding protein (22). The midgestation kidney protein is involved in retinoic acid-induced cell differentiation (20, 21).

The neurite outgrowth-promoting property of HB-GAM in vitro has been shown in a number of studies (14, 16, 18, 19, 23). Furthermore, during embryonic and perinatal development, HB-GAM is strongly expressed in axon pathways of the brain (24) and as a component of basement membranes outside the brain (25). These findings have raised the question as to the occurrence of cell surface receptors for HB-GAM.

A 200-kDa heparan sulfate proteoglycan isolated from cultured brain neurons and from rat brain has been implicated as a receptor or a co-receptor for HB-GAM (26). Based on partial peptide sequencing and on immunochemical analysis, this component has been identified as N-syndecan (syndecan-3). N-Syndecan cDNA has been previously cloned based on its homology with other syndecans, and N-syndecan has been shown to be strongly expressed in developing nervous tissues, for example in perinatal rat brain (27). A temporal co-expression during brain development has also been recently demonstrated for HB-GAM and N-syndecan (28). Furthermore, both proteins are preferentially localized to developing fiber tracts of the rat brain (28).

Because biochemical and cell biological studies have impli-
cated N-syndecan as an HB-GAM receptor (26), we have carried out further studies on HB-GAM-N-syndecan interactions. The carbohydrate molety of N-syndecan consists mostly or exclusively of heparan sulfate chains (26, 27), and we have therefore focused our studies on heparin-type structures that are likely to function as HB-GAM-binding sites. Because N-syndecan and N-syndecan/heparan sulfate are not commercially available, we have taken the strategy to define the structural requirements of active carbohydrates using heparin and other glycosaminoglycans as model components and attempted to correlate these findings to studies on N-syndecan. We suggest that the binding of HB-GAM to carbohydrates is specific for heparin-type structures and that N-syndecan heparan sulfate fulfills the requirements for such interaction.

EXPERIMENTAL PROCEDURES

Glycosaminoglycans—Polysaccharides, selectively modified saccharides, and oligosaccharides used in the experiments were as described previously (13). In addition, samples of completely desulfated or of selectively N-desulfated heparin were N-acetylated by treatment with acetic anhydride. Bovine lung heparan sulfate was given by K. Yoshida of the Seikagaku Corporation.

Recombinant HB-GAM—Recombinant HB-GAM was produced with the aid of baculovirus expression in SF9 cells and purified from the culture medium as described previously (29). For saccharide binding assays purified recombinant HB-GAM was dialyzed against 130 mM NaCl, and 50 mM Tris-HCl, pH 7.4.

N-Syndecan and N-Syndecan-derived Saccharides—N-Syndecan was isolated as described previously (26). Alcian blue-silver staining that detects both proteins and proteoglycans (30) was used in combination with SDS-polyacrylamide gel electrophoresis to detect fractions that contain N-syndecan.

N-Syndecan glycopeptides were prepared by digestion with sequencing grade trypsin (Boehringer Mannheim). Heparan sulfate-containing fractions were concentrated by HB-GAM affinity chromatography, and the 100-kDa glycopeptides were ethanol precipitated for 30 min at −70 °C and centrifuged at 20,000 × g for 30 min at 4 °C.

Glycosaminoglycan chains were released from intact N-syndecan by treatment with alkaline borohydride (31). The released saccharides were purified by HB-GAM affinity, desalted by passage through Sephadex G-25, and lyophilized. The carbohydrate contents of intact N-syndecan or its released GAG chains were measured by the carbazole reaction for hexuronic acid (32).

Binding of Polysaccharides and Oligosaccharides to HB-GAM—Competitive binding of polysaccharides and 3H-labeled heparin to HB-GAM were carried out as described previously (13). HB-GAM (10 μg, 1.85 μM) was incubated with 334 Bq of 3H-labeled heparin (120 nmol) and various amounts of unlabeled polysaccharide competitors. In oligosaccharide binding assays, HB-GAM (10 μg, 1.85 μM) was incubated with 3H-labeled heparin oligosaccharides (167 Bq, 60 nmol) in the absence of any unlabeled competitor.

Compositional Analysis of N-Syndecan Heparan Sulfates—The N-syndecan-derived glycopeptides (5 μg of saccharide) were cleaved at N-sulfated glucosamine units by treatment with nitrous acid at pH 1.5 (33). The resulting disaccharides and oligosaccharides were reduced with NaBH₄ (1.8–3.7 MBq) as described previously (33). The labeled saccharides were isolated by gel chromatography on a column of Biogel P-100 in 0.5 M NH₄HCO₃. The disaccharides were pooled, desalted by lyophilization, and analyzed further by anion-exchange HPLC on a Whatman Partisil-10 SAX column (34). Samples were eluted with a stepwise gradient of KH₂PO₄. Identification of the disaccharides was based on previously characterized standard disaccharides (35).

Cells and Neurite Outgrowth Assays—Cells from cerebral hemispheres of 16-day-old rat embryos were prepared as described previously (14). The cells used in the assays have been previously characterized and shown to be mainly (80–90%) neurons (36). Freshly prepared neurons in a serum-free Dulbecco’s modified Eagle’s medium containing 10 mg/ml of bovine serum albumin were used in all assays. Neurite outgrowth on substrates coated with 4 μg/ml of baculovirus-derived recombinant HB-GAM (29) was estimated in 20-h assays as described previously (14). To induce a similar neurite outgrowth response, the substrates were coated with laminin (Sigma; 50 μg/ml) in control cultures.

RESULTS

Inhibition of HB-GAM-induced Neurite Outgrowth by N-syndecan—N-Syndecan is expressed in cultured brain neurons based on metabolic labeling experiments and on immunohistochemistry (26), and it is also found in the cultured neurons by Western blotting using anti-peptide antibodies (28) against N-syndecan (data not shown). Because it has been suggested that HB-GAM induces neurite outgrowth by binding to N-syndecan at the cell surface (26), the effect of exogenously added brain N-syndecan on HB-GAM-mediated neurite outgrowth was studied. N-Syndecan isolated from perinatal rat brain was found to clearly inhibit HB-GAM-induced neurite outgrowth; the concentration required for 50% inhibition (IC₅₀) was −0.7 μg/ml (Figs. 1, A and C, and 2B). Monitoring of the affinity isolation (26) by neurite outgrowth assays and by SDS-polyacrylamide gel electrophoresis showed that the inhibitory activity co-purified with N-syndecan. Thus, a control fraction eluting immediately after N-syndecan did not display any inhibitory activity (44 ± 9% of cells with neurites as compared with the control value 48 ± 8.2%; calculated as in Fig. 2) when analyzed in parallel with the N-syndecan-containing fraction. The inhibitory activity was resistant to heating (100 °C, 5 min; data not shown) but was abolished by heparitinase digestion of N-syndecan (Fig. 1, C and D). Even rather high concentrations of heparitinase-treated N-syndecan (17 μg/ml) did not inhibit neurite outgrowth (47.8 ± 8.3% of cells with neurites). Glycosaminoglycan chains released from N-syndecan by alkaline borohydride treatment inhibited neurite outgrowth with an IC₅₀ of −2 μg/ml (Fig. 2B).

To study whether the inhibitory effect of N-syndecan is specific with respect to the substrate used, the assays were also carried out on laminin-coated culture wells. No effect on neurite outgrowth or other aspects of cell morphology could be observed on laminin-coated substrates at the concentrations of N-syndecan that inhibited HB-GAM-induced neurite outgrowth (Fig. 1, E and F). An N-syndecan fraction that completely inhibited HB-GAM-induced neurite outgrowth at 5 μg/ml was tested up to 41 μg/ml on laminin-coated substrate, but no inhibition was observed (33 ± 3.3% of cells with neurites as compared with 33 ± 5.7% in the control).

Inhibition of HB-GAM-induced Neurite Outgrowth by Heparin and Its Modified Forms, Other Glycosaminoglycans, and Heparin-derived Oligosaccharides—The specificity of the inhibitory effect of heparin-type carbohydrates on HB-GAM-induced neurite outgrowth was studied using several glycosaminoglycans and their fragments in the assay media. The IC₅₀ of heparin was found to be 25 ng/ml (Fig. 2A). In contrast, laminin-induced neurite outgrowth was insensitive to heparin at the concentrations found to perturb HB-GAM-neuron interaction (30 ± 5% of cells with neurites in the absence or the presence of 0.5 μg/ml heparin).

The inhibitory activity of heparin was essentially lost upon selective 2-O-desulfation (Fig. 2A), which resulted in an IC₅₀ 10³-fold higher than that of heparin. Also selective 6-O-desulfation and N-desulfation followed by N-acetylation resulted in an appreciable, albeit less dramatic, loss of inhibitory capacity, shifting the dose-response curves to about 10²-fold higher concentrations. The inhibition of HB-GAM-induced neurite outgrowth by heparin thus appears to depend on the concerted effect of different sulfate substituents, the 2-O-sulfate groups on iduronic acid units being of particular importance.

In contrast to heparin, chondroitin sulfate, aorta heparan sulfate, kidney heparan sulfate, lung heparan sulfate, and dermatan sulfate (data not shown) were all ineffective or displayed little inhibitory activity. Notably, detergent-free N-syndecan and its heparan sulfate chains were clearly more
active than any of the other heparan sulfate preparations tested (Fig. 2B).

Dose-response curves similar to those shown in Fig. 2 were also generated for different heparin-derived oligosaccharides. The IC50 concentrations, compiled in Table I, show that heparin oligosaccharides from disaccharide to octasaccharide were not inhibitory, whereas some inhibition was observed for the decasaccharide. For larger oligosaccharides the inhibitory activity was clearly increased with increasing polymer length.

Binding of Polysaccharides and Oligosaccharides to HB-GAM in Solution—Unlabeled intact heparin induced a 50% inhibition of [3H]heparin binding to HB-GAM at 1 mg/ml (Fig. 3A). Approximately 101-102-fold higher concentrations of selectively 2-O-desulfated, 6-O-desulfated, and N-desulfated heparin were required for a 50% inhibition as compared with intact heparin. Totally de-O-sulfated and de-N-sulfated re-N-acetylated heparin did not bind to HB-GAM (Fig. 3A). Iduronic acid 2-O-, glucosamine N-, and 6-O-sulfate groups thus all play a role in HB-GAM binding.

Of other polysaccharides tested, only aorta heparan sulfate displayed significant binding activity, although 102-103-fold higher concentrations were required as compared with heparin (Fig. 3B). Chondroitin sulfate, dermatan sulfate, lung heparan sulfate, or kidney heparan sulfate did not bind to HB-GAM (Fig. 3B).

The requirement for polymer size in HB-GAM binding was studied using [3H]-labeled heparin oligosaccharides. The smallest oligosaccharide showing appreciable binding (approximately 10% of added oligosaccharide) was the decasaccharide (Fig. 4).

Characterization of N-Syndecan Heparan Sulfate—N-Syndecan isolated from 6-day-old rat brain was digested with trypsin, and the resulting glycopeptides were subjected to compositional analysis. The glycosaminoglycans were degraded with HNO2 at pH 1.5, which cleaves heparin/heparan sulfate at GlcNSO3-HexA linkages, and the products were reduced with NaB3H4, resulting in the conversion of the deaminated GlcN to [1-3H]aManR units (33). Gel chromatography of the resultant labeled oligosaccharides afforded mainly di- and tetrasaccharides (Fig. 5A). Assessment of N-substituents from the elution pattern (37) indicated that 58% of the glucosamine units were N-sulfated and further suggested that the N-sulfated disaccharide units occurred predominantly in contiguous blocks and in alternating sequence with N-acetylated units. Only 8.4%, on a molar basis, of the deamination products accounted for saccharides larger than tetrasaccharides.

Separation of the disaccharides by anion-exchange HPLC showed a predominant peak of the mono-O-sulfated species, IdoA(2-OSO3)-aManR, along with smaller amounts of nonsulfated, other mono-O-sulfated, and di-O-sulfated components (Fig. 5B; Table II). As much as 82% of the disaccharides contained a 2-O-sulfated IdoA unit, whereas only 30% were 6-O-sulfated. The total (N- and O-) sulfate content of the heparan sulfate chain was calculated to 0.92 residue/disaccharide unit. Interestingly, only about one-third of the tetrasaccharides carried an O-sulfate substituent, as indicated by high voltage paper electrophoresis (data not shown).
DISCUSSION

HB-GAM was initially isolated as a neurite outgrowth-promoting protein that was eluted from heparin-Sepharose by \(1\) M NaCl (14). The salt concentration needed to reverse the HB-GAM-heparin interaction thus approaches that required to impede high affinity binding to heparin of other proteins such as antithrombin and fibroblast growth factors (38, 39). The strong binding of HB-GAM to heparin suggests that heparin-type carbohydrates might be involved in the biological function(s) of HB-GAM. This assumption was strengthened by the recent findings that N-syndecan serves as a receptor or a co-receptor for HB-GAM (26) and that addition of exogenous heparin, as well as heparitinase treatment of neurons, both inhibit HB-GAM-induced neurite outgrowth (24).

The purpose of the present study was to define the glycosaminoglycan structure(s) responsible for HB-GAM binding and implicated in the functional properties of the protein. To this end we employed a neurite outgrowth bioassay as well as a protein binding assay, comparing the effects on these assays of various exogenous carbohydrates. In the bioassay we used rat forebrain neurons that were freshly prepared for each assay, thus attempting to retain the normal cell surface carbohydrate structures that tend to be rapidly changed in vitro.
Interaction of HB-GAM with Cell Surface Heparan Sulfate

Disaccharides generated by deamination of N-syndecan

N-Syndecan-derived heparan sulfate was degraded with nitrous acid, and the resultant products were reduced with NaB\(^{3}\)H\(_{4}\). The disaccharide fraction was recovered (see Fig. 5A) and separated by anion exchange HPLC on a Partisil-10 SAX column as described under "Experimental Procedures." The percentages of disaccharide structures were estimated from the radioactivity of the corresponding peaks (see Fig. 5B).

| Structure | Total |
|-----------|-------|
| HexA-aMan\(_{n}\) | 7.3 |
| GlcA(2-OSO\(_{3}\))-aMan\(_{n}\) | <2 |
| GlcA-aMan\(_{n}\)(6-OSO\(_{3}\)) | 5.1 |
| Idoa-aMan\(_{n}\)(6-OSO\(_{3}\)) | 6.6 |
| Idoa(2-OSO\(_{3}\))-aMan\(_{n}\) | 63 |
| Idoa(2-OSO\(_{3}\))-aMan\(_{n}\)(6-OSO\(_{3}\)) | 19 |

Heparin at very low concentrations (10–30 ng/ml) was found to clearly inhibit neurite outgrowth induced by substrate-bound HB-GAM. By contrast, heparin did not affect neurite outgrowth on laminin-coated substrates at concentrations clearly exceeding those active with HB-GAM. Interestingly, preparations of detergent-free N-syndecan also inhibited HB-GAM-induced but not laminin-induced neurite outgrowth. This finding is in agreement with observations that brain N-syndecan does not bind to commonly occurring matrix proteins, such as laminin or fibronectin (40).

Although the inhibitory effect of N-syndecan was clearly attributed to the heparan sulfate side chains, the potency was significantly lower than that displayed by heparin, expressed on a carbohydrate basis (Fig. 2B). Heparan sulfate is generally more heterogeneous in structure than is heparin, and it thus seems reasonable to assume that a particular saccharide sequence implicated in the biological activity would be less abundant in heparan sulfate. Release of the polysaccharide chains from the core protein resulted in a further modest decrease in inhibitory capacity (Fig. 2B), suggesting that the biological activity is facilitated by the intact proteoglycan assembly.

Compared with N-syndecan and N-syndecan-derived glycosaminoglycans, other glycosaminoglycan preparations were clearly less active (Fig. 2B). A preparation of heparan sulfate from human aorta thus was required in ~10-fold higher concentration than the N-syndecan polysaccharide to similarly inhibit HB-GAM-induced neurite outgrowth; other polysaccharides tested, including lung and kidney heparan sulfates, chondroitin sulfate, and dermanan sulfate, showed even lower activity.

The results of competitive binding of unlabeled polysaccharides, along with [\(^{3}\)H]heparin, to HB-GAM in solution essentially reflected those obtained in the neurite outgrowth assay. Native heparin thus showed the most efficient binding, followed by aorta heparan sulfate, which was required at 10-2-10-3-fold higher concentration to achieve similar displacement of the labeled heparin. Again, other polysaccharides, including heparan sulfate preparations, were essentially inactive. Interestingly, the aorta heparan sulfate is relatively low sulfated, with an overall sulfate content of ~0.6 sulfate groups/disaccharide unit (13), suggesting the occurrence of a specific HB-GAM-binding region. Unfortunately, the amounts of N-syndecan-derived polysaccharide were insufficient for this assay.

Taken together, the results suggest that HB-GAM binds to "heparin-like" (i.e. highly sulfated) regions of heparan sulfate chains and that this interaction is essential to the neurite outgrowth-promoting effect of the protein. This assumption was supported by experiments involving selective chemical desulfation of heparin. In particular, elimination of Idoa 2-O-sulfate groups yielded a product with dramatically impaired ability to bind HB-GAM and to abolish HB-GAM-induced neurite outgrowth. N-Sulfate groups were similarly implicated, although the effects of N-desulfation on neurite outgrowth were somewhat less marked (Fig. 2A). The results of GlcN 6-O-desulfation are less straightforward, because this modification was accomplished by a loss of ~30% of the 2-O-sulfate groups (13). However, it seems justified to also implicate 6-O-sulfate groups in HB-GAM binding, although they appear to be less essential than the 2-O-sulfate substituents. All three sulfate residues of the major disaccharide unit of heparin, Idoa(2-O-SO\(_{3}\))GlcNSO\(_{3}\) that is frequently 6-O-sulfated in the glucosamine residue, thus contribute to the interaction with HB-GAM. The binding epitope apparently differs from those involved in interactions with basic fibroblast growth factor, which requires 2-O- but no 6-O-sulfate groups (11, 13), and with hepatocyte growth factor, which seems to depend more on 6-O- than on 2-O-sulfate groups (41). The occurrence of a dis-
tinct binding site for HB-GAM is emphasized by the demonstration of a minimal molecular size, corresponding to a disaccharide, for a functionally active heparin oligosaccharide (Fig. 4).

The finding that HB-GAM binds to heparin-type saccharide sequences raises the question as to the occurrence of such structures in N-syndecan. In particular, it was anticipated that analysis of the heparan sulfate constituents of N-syndecan, isolated from postnatal rat brain, would elucidate the structural basis for the more efficient interaction of this polysaccharide with HB-GAM, as compared with other heparan sulfates. Indeed, such analysis revealed characteristics of relevance to HB-GAM binding. The degree of N-sulfation, corresponding to ~60% of the total glucosamine units, is intermediary to the values reported for typical heparan sulfates (40–50%) and for heparin (>80%) (42). A similarly high N-sulfate content was recently described for a heparan sulfate from rat liver (43).

However, a more conspicuous feature was noted in the high proportion of 2-O-sulfated IdoA units within the N-sulfated block regions, which amounted to 82% of the total disaccharide units. This value should be compared with the corresponding narrow range 54–58%, which was recently found in a survey of five heparan sulfate preparations from different bovine or swine tissues (13). A minimalist N-syndecan contains regions with unusually high contents of the constituent, 2-O-sulfated IdoA units, which is primarily implicated in HB-GAM binding. It seems likely that this property is reflected by the relatively high activity of N-syndecan in the neurite outgrowth bioassay. Further work is needed to define the minimal binding structure and the distribution of critical block regions, which amounted to 82% of the total disaccharide units.

HB-GAM was also recently shown to co-localize with heparan sulfates in the developing neuromuscular synapse (44); however, the synaptic protein carrying the heparan sulfate chains has not yet been identified.

Acknowledgments—We thank Seija Lehto and Eevi Saarikallio for excellent technical assistance.

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J. Biol. Chem. 1996, 271:2243-2248.
doi: 10.1074/jbc.271.4.2243

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