Localization of the Heparin-Binding Site of Glia-Derived Nexin/Protease Nexin-1 by Site-Directed Mutagenesis

Stuart R. Stone,*,‡ Marianne L. Brown-Luedi,§ Giorgio Rovelli,§,‖ Angelo Guidolin,§ Elaine McGlynn,⊥ and Denis Monard§

Department of Haematology, University of Cambridge, MRC Centre, Hills Road, Cambridge CB2 2QH, U.K., Friedrich Miescher-Institut, P.O. Box 2543, CH-4002 Basel, Switzerland, and Research Department, Pharmaceutical Division, Ciba, CH-4002 Basel, Switzerland

Received December 10, 1993; Revised Manuscript Received March 31, 1994®

ABSTRACT: Recombinant rat glia-derived nexin was expressed in insect cells using the baculovirus system. The kinetics for the inhibition of thrombin by this recombinant material were indistinguishable from those observed with natural glia-derived nexin and recombinant nexin expressed in yeast. In addition, the dependence of the rate of inactivation on the concentration of heparin was similar for the three preparations. At the optimal heparin concentration, the association rate constant was 330-fold higher than that observed in the absence of heparin. A putative heparin-binding site is found in glia-derived nexin between residues 71 and 86; heparin-binding sites are found in homologous regions of antithrombin III and heparin cofactor II. Lysines in this region were mutated to glutamates, and the kinetics for the inhibition of thrombin by mutant proteins were determined. Concurrent mutation of all seven lysines in this region (residues 71, 74, 75, 78, 83, 84, and 86) did not affect the rate constant for the association of glia-derived nexin with thrombin in the absence of heparin, but it resulted in complete loss of the heparin acceleration of the rate of association. Mutations of residues 83, 84, and 86 together also caused a marked decrease in the acceleration by heparin of the reaction between glia-derived nexin and thrombin. These results support the hypothesis that the heparin-binding sites of glia-derived nexin, antithrombin III, and heparin cofactor II are found in homologous regions of the molecules. Heparin was also found to potentiate the ability of wild-type glia-derived nexin to inhibit the thrombin-induced retraction of neurites from neuroblastoma NB2a cells. This heparin potentiation was not observed with the GDN mutants lacking a functional heparin-binding site.

Glia-derived nexin (GDN)[1] is a 43-kDa glycoprotein that was originally isolated on the basis of its ability to promote neurite elongation from neuroblastoma cells (Guenther et al., 1985). The amino acid sequence of GDN showed it to be a member of the serpin (serine protease inhibitor) superfamily (Gloor et al., 1986) and identical to protease nexin-1 (McGrogan et al., 1988). GDN inhibits a range of trypsin-like serine proteases (Scott et al., 1985; Stone et al., 1987), and it is thought that GDN plays a role in the control of such enzymes in the extravascular space. In particular, it reacts rapidly with thrombin to form stable complexes, and its rate of reaction with thrombin is markedly stimulated by heparin (Scott et al., 1987; Stone et al., 1987; Wallace et al., 1989; Evans et al., 1991; Rovelli et al., 1992). Heparin is a negatively charged glycosaminoglycan that also stimulates the rate of reaction of other serpins, including antithrombin III and heparin cofactor II, with their target proteases. It has been suggested that heparin-like molecules on the cell surface and in the extracellular matrix could serve to regulate the activity of GDN *in vivo* (Farrell & Cunningham, 1986).

Heparin-binding regions in antithrombin III have been identified by chemical modification studies and from the effects of naturally occurring mutations (Blackburn et al., 1984; Koide et al., 1984; Chang & Tran, 1986; Brennan et al., 1987; Liu & Chang, 1987; Peterson & Blackburn, 1987; Peterson et al., 1987; Owen et al., 1987; Chang, 1989). Comparison of the amino acid sequences of GDN, heparin cofactor II, and antithrombin III indicates that positively charged residues in the region corresponding to Lys^{114}–Lys^{136} of antithrombin III are well conserved in all three molecules (Figure 1). Moreover, a number of the conserved residues are involved in the binding of heparin and/or dermatan sulfate to heparin cofactor II (Figure 1; Blinder et al., 1989, 1990; Ragg et al., 1990; Whinna et al., 1991). In terms of tertiary structure, this region corresponds to α-helix D of α_1-antitrypsin and a loop following the helix (Huber & Carrell, 1989). In a previous study, an 81-residue CNBr fragment of GDN containing this region was shown to inhibit the heparin stimulation of the reaction of GDN with thrombin (Rovelli et al., 1992). However, a shorter peptide based on the sequence of α-helix D and the positively charged loop following the helix was unable to inhibit the heparin stimulation. Thus, it was not possible to prove unequivocally from these data that the region of GDN around α-helix D was involved in the heparin stimulation of the GDN–thrombin reaction. It was suggested that the failure of the shorter peptide to inhibit could be due to its failure to assume a helical structure (Rovelli et al., 1992). In order to investigate

* Address correspondence to this author at Department of Haematology, University of Cambridge, MRC Centre, Hills Road, Cambridge CB2 2QH.

‡ University of Cambridge.

§ Friedrich Miescher-Institut.

‖ Present addresses: Department of Neurobiology, Stanford University School of Medicine, Stanford, CA 94305-5401, and Department of Clinical Immunology, Flinders Medical Centre, Bedford Park, South Australia 5042, Australia.

⊥ Ciba.

® Abstract published in *Advance ACS Abstracts,* May 15, 1994.

[1] Abbreviations: GDN, glia-derived nexin/protease nexin-1; bac-GDN, recombinant GDN produced in insect cells using the baculovirus expression system; bac-GDN-3E, bac-GDN with Lys^{83}, Lys^{84}, and Lys^{86} mutated to glutamate; bac-GDN-7E, bac-GDN with Lys^{71}, Lys^{74}, Lys^{75}, Lys^{78}, Lys^{83}, Lys^{84}, and Lys^{86} mutated to glutamate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium.

0006-2960/94/0433-7731$04.50/0

ATIII	114	**K**	T	S	D	-	Q	I	H	F	F	F	A	**K**	L	N	C	**R**	L	Y	R	**K**	A	N	**K**136
HCII	173	**K**	Y	E	I	T	T	I	H	N	L	F	R	**K**	L	T	H	**R**	L	F	**R**	**R**	-	N	F195
GDN	63	R	Y	N	V	N	G	V	G	K	V	L	K	K	I	N	K	A	I	V	S	K	K	N	K86

FIGURE 1: Comparison of the proposed heparin-binding domains of GDN, antithrombin III (ATIII), and heparin cofactor II (HCII). Residues that have been shown to be involved in the binding of heparin and/or dermatan sulfate to antithrombin III and heparin cofactor II are shown in bold. The region of GDN corresponding to α-helix D in α_1-antitrypsin is underlined (Loebermann et al., 1984).

the location of the heparin-binding site of GDN further, a recombinant expression system for GDN in insect cells has been established and mutations that affect the charge properties in the region of α-helix D in GDN have been made. The results are consistent with the hypothesis that interactions with positively charged residues in α-helix D and the loop following the helix are responsible for the heparin stimulation of the GDN–thrombin reaction.

MATERIALS AND METHODS

Materials. *Spodoptera frugiperda* (Sf9) cells, wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV), also known as baculovirus, and the transfer vector pVL1392 were kindly provided by Dr. M. A. Summers. Restriction and DNA-modifying enzymes were purchased either from Boehringer Mannheim (Mannheim, Germany) or Biofinex (Praroman, Switzerland). Cell culture media and antibiotics were from Gibco-BRL (Basel, Switzerland). Prothrombin was purified from human plasma and activated to thrombin as previously described (Stone & Hofsteenge, 1986). The concentration of thrombin was determined by active site titration (Jameson et al., 1973). The thrombin synthetic substrate D-Ile-Pro-Arg-*p*-nitroanilide (S-2288) was from Chromogenix (Molndal, Sweden). Yeast recombinant GDN was produced and characterized as previously described (Sommer et al., 1989). All other chemicals were of the highest purity available commercially.

Site-Directed Mutagenesis. A *Hin*dIII–*Ssp*I fragment of the cDNA for rat GDN which included the entire coding sequence (Sommer et al., 1987) was cloned into the *Eco*RI/*Hin*dIII sites of M13mp18. Oligonucleotide-directed mutagenesis was performed using the *in vitro* mutagenesis system (version 2.1) supplied by Amersham (Amersham, U.K.). Mutants were selected by isolation of single-stranded DNA followed by sequencing using Sequenase (USB, Cleveland, OH). The *Pst*I/*Eco*RI fragment encoding the mutant was then isolated from the replicative form of the phage.

Cell Culture. Sf9 cells were maintained in spinner flasks and monolayers in TC100 medium containing 10% fetal calf serum (Summers & Smith, 1988). For the expression of GDN, cells were transferred to serum-free Sf900 medium (Summers & Smith, 1988). Mouse neuroblastoma NB2a cells were grown as previously described (Suidan et al., 1992) in glucose-enriched and pyruvate-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin G (100 units/mL), and streptomycin sulfate (100 μg/mL).

Neurite retraction assays were performed as described by Suidan et al. (1992). NB2a mouse neuroblastoma cells were plated out at a density of 50 000 per 35-mm Falcon dish and left to attach and proliferate in the presence of 10% fetal calf serum. After 24 h, cells were washed three times with serum-free DMEM, and the medium was replaced with DMEM supplemented with insulin (5 μg/mL), transferrin (5 μg/mL), sodium selenate (5 ng/mL), putrescine (16 μg/mL), and progesterone (10 ng/mL). After an additional 24 h, the cells had grown neurites, and the assays were initiated. Recombinant GDN (mutant or wild type) was added to the cells at a concentration of 0.2 nM. Heparin was added simultaneously at concentrations of 0, 0.2, 2, and 100 nM. After incubation for 30 min at 37 °C, thrombin was added to a final concentration of 0.06 nM. After an additional 15 min at 37 °C, the cells were fixed with 2.5% glutaraldehyde in PBS and the average neurite length per cell was determined (Suidan et al., 1992). The results are expressed as the neurite length after treatment as a percentage of that observed with control cells to which no thrombin had been added.

Isolation of Recombinant Baculovirus. Procedures used for growing Sf9 cells and for purifying recombinant baculovirus were those described by Summers and Smith (1988). *Pst*I/*Eco*RI fragments encoding wild-type and mutant GDN were subcloned into the baculovirus transfer vector pVL1392. This vector was then cotransfected with wild-type viral DNA into Sf9 cells by using lipofectin (Gibco-BRL, Basel, Switzerland). Pure recombinant virus was isolated by limiting dilution. DNA dot–blot hybridization was used to identify recombinant virus (Fung et al., 1988). Sequencing of the recombinant inserts confirmed their identity.

Expression and Purification of Recombinant GDN. Sf9 monolayers were infected with recombinant virus and grown for 3 days in serum-free medium. Western blotting analysis indicated the presence of GDN in the medium. Media (100 mL) from monolayer cultures were harvested, centrifuged, and filtered (0.22 μm). This material was diluted with an equal volume of 50 mM Tris-HCl buffer, pH 8.0, containing 100 mM NaCl, and loaded onto a 5-mL Econo-Pac Blue-Agarose cartridge equilibrated with the same buffer. The column was washed extensively with the equilibration buffer until the absorbance of the effluent was zero. GDN activity was eluted with 1.2 M NaCl in 50 mM Tris-HCl, pH 8.0. Fractions of 1.0 mL were collected and tested for their thrombin inhibitory activity (see below). The fractions were also analyzed by SDS–PAGE (Laemmli, 1970) and were pooled on the basis of their activity and purity. The pooled fractions were concentrated and dialyzed against 50 mM Tris-HCl, pH 8.0, containing 400 mM NaCl. This sample was applied to a 1.6 × 93-cm column of Sephacryl S-200 (Pharmacia) equilibrated with the same buffer. The column was developed at a flow rate of 15 mL/h, and fractions of 3.0 mL were collected. The fractions were analyzed by SDS–PAGE and tested for their thrombin inhibitory activity. Active fractions containing predominantly a band of the correct molecular mass for GDN (43 kDa) were pooled. Western blotting analysis of the pooled fractions confirmed that the band of 43 kDa was GDN.

Isolation of Heparin with High Affinity for GDN. Heparin with high affinity for GDN was purified as previously described (Rovelli et al., 1992). Heterogeneous heparin was fractionated according to its size on a Sephadex G-100 column. Fractions with a molecular mass of 15 kDa were pooled and applied to a column of yeast recombinant GDN coupled to Sepharose equilibrated with 50 mM Tris-HCl, pH 8.0, containing 50

mM NaCl. The column was washed with 0.35 M NaCl in the equilibration buffer, and high-affinity heparin was eluted with 1.0 M NaCl. The concentration of active heparin was determined from the concentration dependence of its stimulation of the inhibition of thrombin by yeast recombinant GDN (Rovelli et al., 1992).

Thrombin Assays. Amidolytic assays were performed at 37 °C as described previously (Wallace et al., 1989) in 50 mM Tris-HCl, pH 7.8, 0.1 M NaCl, and 0.1% poly(ethylene glycol) (M_r 6000). The concentration of active GDN was determined by incubating thrombin (2.0 nM) for 40 min with varying concentrations of GDN and measuring the residual activity (Stone et al., 1987). The association rate constants for thrombin–GDN reactions were determined by progress curve kinetics as previously described (Wallace et al., 1989; Rovelli et al., 1992). The concentrations of GDN, thrombin, and the substrate D-Ile-Pro-Arg-*p*-nitroanilide were respectively 10 nM, 20–200 pM, and 50–400 μM. The concentrations of enzyme and substrate were adjusted such that the steady-state velocity was achieved before significant (>10%) substrate depletion had occurred.

The progress curve for the thrombin-catalyzed formation of chromogenic product (P) in the presence of GDN can be described by the equation for slow-binding inhibition (eq 1; Stone et al., 1987; Wallace et al., 1989; Rovelli et al., 1992):

$$P = v_s t + \frac{(v_0 - v_s)}{k'}[1 - \exp(-k't)] \quad (1)$$

where k' is an apparent first-order rate constant, and v_0 and v_s are the initial and steady-state velocities, respectively. Nonlinear regression analysis of progress curves according to eq 1 yielded estimates for k', v_s, and v_0. The value of the second-order association rate constant, k_1, can be related to I, k', v_0, v_s, the Michaelis constant (K_m), and the substrate concentration (S) by eq 2 (Rovelli et al., 1992), and this equation was used to calculate estimates for k_1. The K_m value

$$k_1 = k'(1 - v_s/v_0)\frac{1 + S/K_m}{I} \quad (2)$$

for D-Ile-Pro-Arg-*p*-nitroanilide under the assay conditions is 5.1 μM (Stone & Hofsteenge, 1986). Values of k_1 are reported as the weighted means of triplicate determinations.

RESULTS AND DISCUSSION

Recombinant GDN has previously been expressed in yeast (Sommer et al., 1989). However, good levels of expression of GDN were only obtained when the yeast were grown in a fermenter; using laboratory shaker flasks, negligible amounts of GDN were expressed by the yeast. Therefore, in the present study, the baculovirus system has been tested as an alternative means for the expression of recombinant GDN. Although the levels of expression obtained were low, they were sufficient for functional studies. On the basis of a thrombin inhibition assay, 1–5 μg of active GDN per milliliter of medium was obtained when the cells were grown under serum-free conditions. GDN was purified by affinity chromatography on Blue-Agarose followed by gel filtration. This procedure resulted in preparations of GDN that displayed one major band with a molecular mass of about 43 kDa on SDS–PAGE. Minor contaminants (<10%) of higher molecular mass were observed (data not shown). Western blotting confirmed that the major band was GDN. The average yield of the purification procedure was about 10%. The apparent molecular mass of GDN expressed in insect cells was the same as that of recombinant GDN from yeast, but both recombinant proteins exhibited apparent molecular masses about 2 kDa lower than that of the protein isolated from C6 rat glioma cells. The differences in molecular mass between the recombinant proteins and the native protein from C6 glioma cells most probably represent differences in glycosylation patterns. The differences in glycosylation do not, however, appear to affect the activity of GDN. All three proteins had similar kinetic parameters for inhibition of thrombin in the presence and absence of heparin [see below compared with Wallace et al. (1989) and Rovelli et al. (1992)]. It appears that the glycosylation state of GDN does not affect its inhibitory activity with thrombin. In support of this proposal, it was found that treatment of C6 glioma cells with the glycosylation inhibitor tunicamycin resulted in a decrease of about 3 kDa in the apparent molecular mass of GDN isolated from these cells but did not affect the kinetic parameters for the inhibition of thrombin (data not shown).

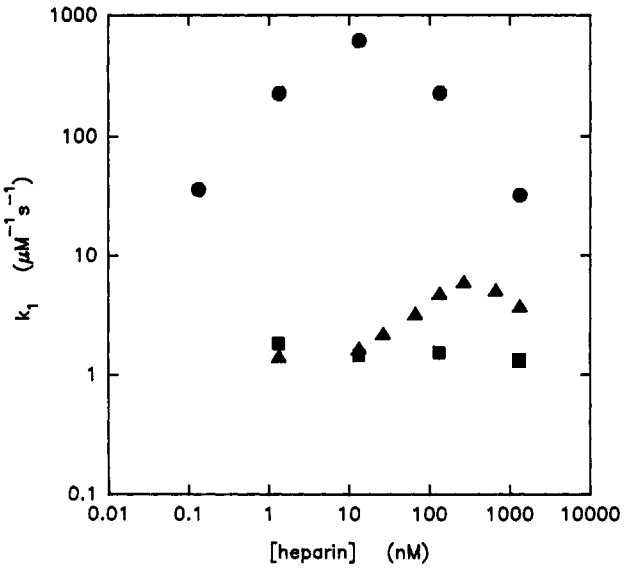

FIGURE 2: Effect of heparin on the association rate constant for the reaction of thrombin with wild-type and mutant GDN. The logarithm of the second-order rate constant (k_1) is plotted against the logarithm of the concentration of heparin. The points represent weighted means of triplicates for bac-GDN (●), bac-GDN-3E (▲), and bac-GDN-7E (■). Kinetic data were obtained and analyzed as described in Materials and Methods.

Wild-type recombinant GDN expressed in insect cells (bac-GDN) had the same kinetic properties as natural (Stone et al., 1987; Wallace et al., 1989) and recombinant GDN expressed in yeast (Rovelli et al., 1992). In the absence of heparin, the association rate constant (k_1) for the reaction of bac-GDN with thrombin was (1.84 ± 0.08) × 10^6 $M^{-1}s^{-1}$, which compares well with previously published values for GDN (Stone et al., 1987; Wallace et al., 1989; Rovelli et al., 1992) and protease nexin-1 (Scott et al., 1985; Evans et al., 1991). Moreover, the profile obtained for the heparin dependence of k_1 was also similar to those previously reported (Wallace et al., 1989; Rovelli et al., 1992): heparin stimulated at low concentrations and inhibited at higher concentrations (Figure 2). As was observed for yeast recombinant GDN under identical conditions (Rovelli et al., 1992), optimal stimulation was seen with about 10 nM heparin. The value k_1 at the optimal concentration of heparin was 6 × 10^8 M^{-1} s^{-1}. This heparin-stimulated rate is also similar to that obtained with yeast recombinant GDN (Rovelli et al., 1992) and represents a 330-fold increase in k_1. Thus, the data indicate that recombinant GDN produced in the baculovirus system responds normally to heparin.

In order to assess the role of positively charged residues in α-helix D and in the loop following this helix, all seven lysines between residues 71 and 86 were mutated to glutamates (Figure 1). The choice of glutamate for the replacement of the lysine residues was made to eliminate nonspecific as well as specific binding to this region of the molecule. Heparin is a negatively charged glycosaminoglycan, and thus, nonspecific binding to a negatively charged region around α-helix D is unlikely to occur. In the absence of heparin, the mutant GDN with the seven lysine → glutamate replacements (bac-GDN-7E) displayed kinetics that were indistinguishable from those of the wild-type protein. A value of k_1 of $(1.79 \pm 0.07) \times 10^6$ M^{-1} s^{-1} was obtained. This result indicates that the mutations have not affected the structure of the reactive site loop of GDN. In contrast to its effect on the wild-type protein, however, heparin (0.13–1330 nM) did not accelerate the rate of inhibition of thrombin by bac-GDN-7E (Figure 2). Similar results were obtained when only the three lysines in the loop following α-helix D were mutated to glutamate. This mutant (bac-GDN-3E) also exhibited kinetics indistinguishable from wild type in the absence of heparin [$k_1 = (1.61 \pm 0.13) \times 10^6$ M^{-1} s^{-1}]. The ability of heparin to accelerate the reaction of bac-GDN-3E with thrombin was also greatly impaired (Figure 2). A slight increase in the value of k_1 (4-fold) was seen at higher heparin concentrations (100–1000 nM).

The results obtained with the mutants bac-GDN-7E and bac-GDN-3E indicate that interactions of heparin with positively charged residues in the region of GDN between residues 71 and 86 are required for the heparin stimulation of the GDN–thrombin reaction. This conclusion is in agreement with the results of studies on antithrombin III and heparin cofactor II which indicate that the heparin-binding sites of these molecules are found in homologous positions. The GDN region between residues 71 and 86 contains seven lysine residues, which are organized in two groups (Lys^{71}/Lys^{74}/Lys^{75}/Lys^{78} and Lys^{83}/Lys^{84}/Lys^{86}; Figure 1). The first four residues are found in a region corresponding to α-helix D of α_1-antitrypsin (Huber & Carrell, 1989), while the other three are in a surface loop following the helix. As shown in Figure 1, residues in homologous positions in both of these regions have been shown to be involved in heparin binding to antithrombin III (Peterson & Blackburn, 1987; Peterson et al., 1987; Liu & Chang, 1987; Chang, 1989) and heparin and/or dermatan sulfate binding to heparin cofactor II (Blinder et al., 1989; Blinder & Tollefsen, 1990; Ragg et al., 1990; Whinna et al., 1991). Molecular modeling of GDN based on the three-dimensional structure of α_1-antitrypsin (Loebermann et al., 1984) indicates that the side chains of Lys^{71}, Lys^{74}, Lys^{75}, and Lys^{78} would be gathered on the solvent-exposed face of α-helix D. Moreover, one or more of the side chains of Lys^{83}, Lys^{84}, and Lys^{86} could be well positioned with respect to α-helix D to form a positively charged cluster. In a previous study, a CNBr fragment of GDN containing this region was shown to inhibit the heparin stimulation of the reaction of GDN with thrombin, whereas a shorter peptide consisting of residues 71–86 was ineffective (Rovelli et al., 1992). Molecular modeling indicates that the helical structure of residues 68–82 would be essential for the clustering of the lysine residues, and thus, the poor heparin-binding activity of the peptide 71–86 would be explained by the failure of this peptide to form a helical structure.

GDN was originally isolated on the basis of its ability to promote neurite outgrowth with NB2a neuroblastoma cells (Monard et al., 1973; Guenther et al., 1985), and this cellular effect of GDN was subsequently shown to depend on its ability to inhibit thrombin (Gurwitz & Cunningham, 1988). The data of Figure 3 demonstrate that heparin was to able to potentiate the inhibition by GDN of the thrombin-induced neurite retraction. Thrombin at a concentration of 0.06 nM was able to reduce the average length of the neurites by 90%; the average length of the neurites in the absence of thrombin was between 15 and 20 μm per cell. In the absence of heparin, GDN (0.2 nM) did not markedly inhibit the effect of thrombin (Figure 3). This concentration of GDN was chosen such that no inhibition of thrombin-induced neurite retraction was observed. At a 100-fold higher concentration of GDN, inhibition of neurite retraction was observed (data not shown). Addition of heparin to the cells potentiated the effect of GDN: 100 nM heparin together with 0.2 nM GDN (yeast recombinant or bac-GDN) completely inhibited thrombin-induced neurite retraction. The effect of heparin was dependent on the presence of GDN; in the absence of GDN, no effects were observed with any of the heparin concentrations used. The effect of heparin in the presence of GDN displayed a concentration dependence; the half-maximal effect was observed at about 1–2 nM for bac-GDN and 10 nM for yeast recombinant GDN (Figure 3). No potentiation by heparin was observed with the mutants bac-GDN-3E and bac-GDN-7E (Figure 3). These results confirm in a biological assay that the heparin-binding site on GDN involved in the acceleration of thrombin inhibition is located between Lys^{71} and Lys^{86}. The fact that heparin was able to potentiate the effect of GDN suggests that the glycosaminoglycans on the surface of NB2a cells were not present at a sufficient concentration to stimulate fully the inhibition of thrombin by GDN. Alternatively, the type of glycosaminoglycans produced by NB2a cells may be less efficient than heparin in accelerating the GDN–thrombin interaction. Glycosaminoglycans, like heparin, are synthesized as the carbohydrate portion of proteoglycans. Different types of proteoglycans are found in different regions of the brain, and the expression of these various kinds of proteoglycans is developmentally regulated

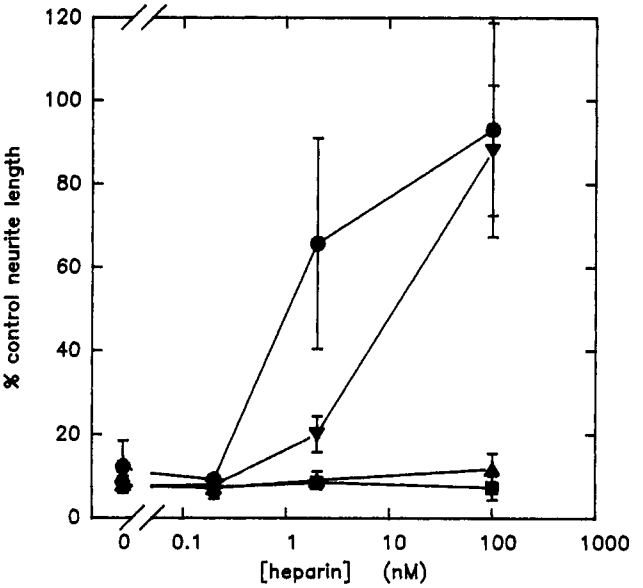

FIGURE 3: Effect of GDN mutants on thrombin-induced neurite retraction in the presence of heparin. The assays were performed as described in Materials and Methods. Average neurite length for each point was determined by measuring the neurite length of about 500 cells. The points shown represent the mean values (± SD) of estimates obtained in two independent experiments, each of which was carried out in duplicate. Four different types of GDN were used: yeast recombinant GDN (▼), bac-GDN (●), bac-GDN-3E (▲), and bac-GDN-7E (■). Heparin alone had no effect on neurite length at any of the concentrations used (data not shown).

(Lander, 1993). Thus, given the possible roles for both GDN and thrombin in the brain (Gurwitz & Cunningham, 1988; Suidan et al., 1992), it would be interesting to determine whether any of these proteoglycans is more efficient in accelerating the interaction of GDN with thrombin or other proteases.

ACKNOWLEDGMENT

We thank E. Fries and S. Reutener for technical assistance, Drs P. C. R. Hopkins, M. R. Wardell and H. S. Suidan for their critical reading of the manuscript and W. Zurcher and F. Fischer for the synthesis of oligonucleotides.

REFERENCES

Blackburn, M. N., Smith, R. N., Carson, J., & Sibley, C. C. (1984) *J. Biol. Chem. 259*, 939–941.
Blinder, M. A., & Tollefsen, D. M. (1990) *J. Biol. Chem. 265*, 286–291.
Blinder, M. A., Andersson, T. R., Abildgaard, U., & Tollefsen, D. M. (1989) *J. Biol. Chem. 264*, 5128–5133.
Brennan, S. O., George, P. M., & Jordan, R. E. (1987) *FEBS Lett. 219*, 431–436.
Chang, J.-Y. (1989) *J. Biol. Chem. 264*, 3111–3115.
Chang, J.-Y., & Tran, T. H. (1986) *J. Biol. Chem. 261*, 1174–1176.
Evans, D. Ll., McGrogan, M., Scott, R. W., & Carrell, R. W. (1991) *J. Biol. Chem. 266*, 22307–22312.
Farrell, D. H., & Cunningham, D. D. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6858–6862.
Fung, M.-C., Chiu, K. Y. M., Weber, T., Chang, T.-W., & Chang, N. T. (1988) *J. Virol. Methods 19*, 33–42.
Gloor, S., Odink, K., Guenther, J., Nick, H., & Monard, D. (1986) *Cell 47*, 687–693.
Guenther, J., Nick, H., & Monard, D. (1985) *EMBO J. 4*, 1963–1966.
Gurwitz, D., & Cunningham, D. D. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 3440–3444.
Huber, R., & Carrell, R. W. (1989) *Biochemistry 28*, 8951–8966.
Jameson, G. W., Roberts, D. V., Adams, R. W., Kyle, W. S. A., & Elmore, D. T. (1973) *Biochem. J. 131*, 101–117.
Koide, T., Odani, S., Takahashi, K., Ono, T., & Sakuragawa, N. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 289–293.
Laemmli, U. (1971) *Nature 227*, 680–685.
Lander, A. D. (1993) *Curr. Opin. Neurobiol. 3*, 716–723.
Liu, C.-S., & Chang, J.-Y. (1987) *J. Biol. Chem. 262*, 17356–17361.
Loebermann, H., Tokuoka, R., Deisenhofer, J., & Huber, R. (1984) *J. Mol. Biol. 177*, 531–556.
McGrogan, M., Kennedy, J., Li, M. P., Hsu, C., Scott, R. W., Simonsen, C. C., & Baker, J. B. (1988) *Biotechnology 6*, 172–177.
Monard, D., Solomon, F., Rentsch, M., & Gysin, R. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1894–1897.
Owen, M. C., Borg, J. Y., Soria, C., Soria, J., Caen, J., & Carrell, R. W. (1987) *Blood 69*, 1275–1279.
Peterson, C. B., & Blackburn, M. N. (1987) *J. Biol. Chem. 262*, 7559–7566.
Peterson, C. B., Noyes, C. M., Pecon, J. M., Church, F. C., & Blackburn, M. N. (1987) *J. Biol. Chem. 262*, 8061–8065.
Ragg, H., Ulshöfer, T., & Gerewitz, J. (1990) *J. Biol. Chem. 265*, 5211–5218.
Rovelli, G., Stone, S. R., Guidolin, A., Sommer, J., & Monard, D. (1992) *Biochemistry 31*, 3542–3549.
Scott, R. W., Bergman, B. L., Bajpai, A., Hersh, R. T., Rodriguez, H., Jones, B. N., Barreda, C., Watts, S., & Baker, J. B. (1985) *J. Biol. Chem. 260*, 7029–7034.
Sommer, J., Gloor, S. M., Rovelli, G., Hofsteenge, J., Nick, H., Meier, R., & Monard, D. (1987) *Biochemistry 26*, 6407–6410.
Sommer, J., Meyhack, B., Rovelli, G., Buergi, R., & Monard, D. (1989) *Gene 85*, 453–459.
Stone, S. R., & Hofsteenge, J. (1986) *Biochemistry 25*, 4622–4628.
Stone, S. R., Nick, H., Hofsteenge, J., & Monard, D. (1987) *Arch. Biochem. Biophys. 252*, 237–244.
Suidan, H. S., Stone, S. R., Hemmings, B. A., & Monard, D. (1992) *Neuron 8*, 363–375.
Summers, M. D., & Smith, G. E. (1988) *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*, Texas Agricultural Experiment Station, College Station, TX.
Wallace, A., Rovelli, G., Hofsteenge, J., & Stone, S. R. (1989) *Biochem. J. 257*, 191–196.
Whinna, H. C., Blinder, M. A., Szewczyk, M., Tollefsen, D. M., & Church, F. C. (1991) *J. Biol. Chem. 266*, 8129–8135.