Biochem J. (1989) 257, 191–196 (Printed in Great Britain)

Effect of heparin on the glia-derived-nexin–thrombin interaction

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In order to determine the specificity of the interaction between thrombin and glia-derived nexin (GdN), the inactivation of proteolytically modified human thrombin species by GdN has been studied. The second-order rate constants for the inactivation of α-, β_T-, γ_T- and ϵ-thrombin by GdN were 1.41, 0.63, 0.33 and 1.91 $\mu M^{-1} \cdot s^{-1}$ respectively. The kinetic properties of gdN were also investigated in the presence of different types of heparin, fractionated according to antithrombin III-binding affinity. Association rate constants of both gdN and antithrombin III with α-thrombin were obtained using unfractionated, low- and high-affinity heparin types. The different heparin types gave optimal rates of inhibition at similar heparin concentrations for both inhibitors. At optimal heparin concentrations, the rate of inactivation of α-thrombin by GdN was 0.5–1.2 $nM^{-1} \cdot s^{-1}$, which suggests that, under these conditions, the interaction is diffusion-controlled.

INTRODUCTION

Antithrombin III (AT-III) is the major inhibitor of thrombin found in the blood (Travis & Salvesen, 1983). However, two other inhibitors of thrombin found in blood have been characterized: heparin cofactor II (Tollefsen *et al.*, 1982) and platelet protease nexin (Gronke *et al.*, 1987). Glia-derived nexin (GdN), a proteinase inhibitor that promotes neurite outgrowth *in vitro* (Monard *et al.*, 1973), appears to be highly homologous, or even identical, with protease nexin (Sommer *et al.*, 1987; Scott *et al.*, 1985). The mechanism of inhibition of thrombin by GdN and AT-III appears to be similar in that they both form SDS-resistant complexes with thrombin, and the rate of inhibition of thrombin by the proteins is stimulated by heparin (Travis & Salvesen, 1983; Scott *et al.*, 1985; Stone *et al.*, 1987*a*). In the absence of heparin, however, GdN inactivates thrombin about 200-fold faster than antithrombin III (Stone *et al.*, 1987*a*; Olson & Shore, 1982). The reason for this increased rate in GdN is not clear, but it seemed possible that GdN interacted with one of the secondary binding sites of thrombin which give thrombin its unique specificity (Fenton, 1981). Three proteolysed forms of α-thrombin are available that allow this hypothesis to be tested. γ_T-Thrombin can be obtained by either extensive trypsin digestion of thrombin or autolytic digestion in high salt (Berliner, 1984). This form contains proteolytic cleavages in two surface loops of thrombin corresponding to residues 65–83 and 146–150 in chymotrypsin (Birktoft & Blow, 1972). After brief trypsin treatment of α-thrombin, a form of thrombin termed 'β_T-thrombin', containing a single cleavage in the loop 65–83, can be isolated (Braun *et al.*, 1988). Treatment of α-thrombin with pancreatic elastase (Kawabata *et al.*, 1985) results in ϵ-thrombin, which has a single cleavage in the loop 146–150. β_T- and γ_T-thrombin show a markedly diminished ability to cleave fibrinogen (Hofsteenge *et al.*, 1988) as well as a decreased affinity for the thrombin receptor thrombomodulin (Hofsteenge *et al.*, 1988) and the inhibitor hirudin (Stone *et al.*, 1987*b*). In contrast, the interaction of all three ligands with ϵ-thrombin was not greatly affected. Thus it was concluded that a region in the loop 65–83 or nearby forms a secondary binding site which gives thrombin its unique specificity for fibrinogen, hirudin and thrombomodulin.

The rates of inactivation of β_T-, γ_T- and ϵ-thrombin by AT-III were not markedly different from that observed with α-thrombin (Hofsteenge *et al.*, 1988), suggesting that AT-III probably did not interact with the above secondary binding site. Moreover, AT-III and thrombomodulin do not compete for a common binding site on thrombin (Hofsteenge *et al.*, 1986; Jakubowski *et al.*, 1986). In the present study, β_T-, γ_T- and ϵ-thrombin have been used to examine whether the GdN interacts with the same secondary binding site as thrombomodulin, hirudin and fibrinogen.

As indicated above, heparin accelerates the inactivation of thrombin by both AT-III and GdN. The mechanism of this acceleration with AT-III appears to be due to heparin acting as a template to which both AT-III and thrombin bind (Pomeranz & Owen, 1978; Griffith, 1982; Nesheim, 1983). Heparin also induces a conformational change in AT-III which may also contribute to the rate acceleration (Rosenberg & Damus, 1973; Li *et al.*, 1976; Villanueva & Danishefsky, 1977; Nordenman *et al.*, 1977). The region of heparin that specifically binds to AT-III has been shown to consist of a pentasaccharide with a unique sulphation pattern (Riesenfeld *et al.*, 1981; Beetz & van Boeckel, 1986; Atha *et al.*, 1987). Only heparin molecules containing this pentasaccharide show a high affinity for AT-III and are efficient catalysts of the inactivation of coagulation proteinases (Choay *et al.*, 1981; Thunberg *et al.*, 1982). Moreover, heparin with a high affinity for AT-III is a more efficient catalyst of the inactivation of thrombin (Höök *et al.*, 1976; Lam *et al.*, 1976). The template mechanism does not apply to the heparin acceleration of the factor Xa–AT-III interaction, where only heparin binding to AT-III appears to be important (Danielsson

Abbreviations used: GdN, glia-derived nexin [previously known as GdNPF (Monard *et al.*, 1973)]; AT-III, antithrombin III; Pip, pipecolyl; *p*-NA, *p*-nitroanilide.

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et al., 1986). The mechanism by which heparin accelerates the α-thrombin–GdN interaction is, however, not known. Therefore we have initiated studies with heparins fractionated on the basis of their affinity for AT-III to examine the mechanism of acceleration of the thrombin–GdN interaction.

EXPERIMENTAL

Materials

The thrombin substrate D-Phe-Pip-Arg *p*-NA (S-2238) was purchased from Kabi AB (Molndal, Sweden) and the substrate Tos-Gly-Pro-Arg *p*-NA acetate (Chromozym TH) was purchased from Boehringer-Mannheim G.m.b.H. (Mannheim, Germany). Heparin (Sigma, St. Louis, MO, U.S.A.) was fractionated according to molecular size and antithrombin affinity (Nordenman & Björk, 1978) as described by Stone & Hofsteenge (1987). GdN was prepared as previously described (Guenther *et al.*, 1985), with an additional ion-exchange purification step using fast protein liquid chromatography with a Mono-Q column (Pharmacia, Uppsala, Sweden). The purified GdN gave a single band with a molecular mass of 43 kDa on SDS/polyacrylamide-gel electrophoresis using silver staining (Eschenbruch & Buerk, 1982). The concentration of GdN was determined by titration with α-thrombin as previously described (Stone *et al.*, 1987*a*). Human AT-III was isolated as described by Miller-Andersson *et al.* (1974). Human α-thrombin was prepared as previously described (Hofsteenge *et al.*, 1986; Stone & Hofsteenge, 1986) and was found to be 97% active by active-site titration with 4-methylumbelliferyl *p*-guanidinobenzoate (Jameson *et al.*, 1973). Proteolysed forms of α-thrombin were prepared as described previously (Stone *et al.*, 1987*b*; Braun *et al.*, 1988). A schematic representation of the various forms of thrombin is shown in Fig. 1. All other reagents were of the highest purity available commercially.

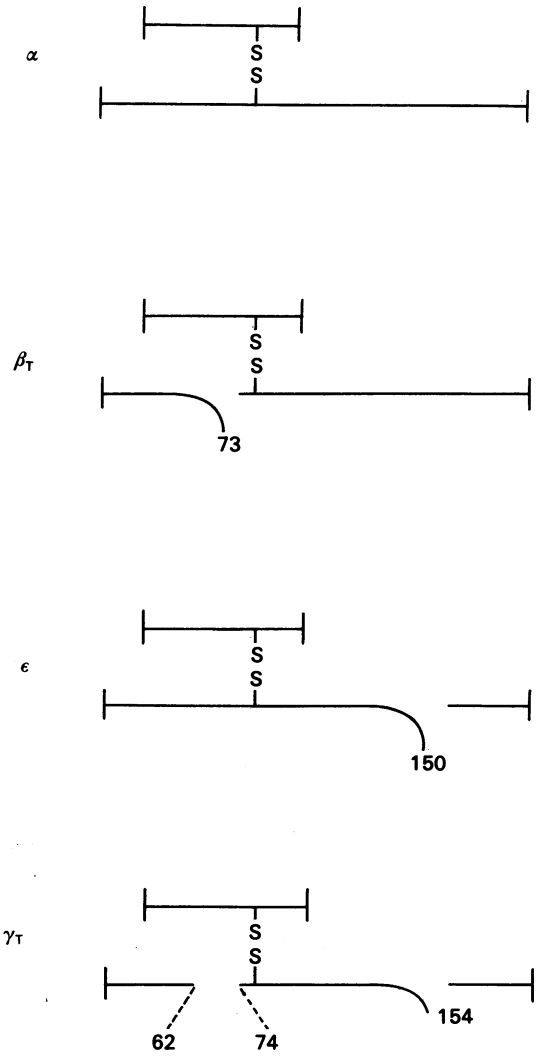

Fig. 1. Schematic representation of the proteolysed forms of α-thrombin

The sites of proteolytic cleavage of the forms of thrombin are shown schematically in relation to the native form of α-thrombin. The numbers given show the sites (residue numbers) of proteolytic cleavage.

Enzyme assays

The assays were performed at 37 °C in 0.05 M-Tris/HCl buffer, pH 7.8, which contained 0.1% poly(ethylene glycol) (M_r 6000) and 0.1 M-NaCl, with the addition of bovine serum albumin (0.02 mg/ml) in experiments with GdN (Hofsteenge *et al.*, 1986; Stone *et al.*, 1987*a*). For progress-curve experiments the concentration of the substrate (D-Phe-Pip-Arg *p*-NA) was varied between 50 and 600 μM in order to allow the steady-state velocity of the reaction to develop such that an accurate determination of the apparent first-order rate constant of inhibition could be made. The concentration of the substrate was determined spectrophotometrically at 342 nm by using a molar absorption coefficient of 8270 $M^{-1} \cdot cm^{-1}$ (Lottenberg & Jackson, 1983).

THEORY AND DATA ANALYSIS

Slow-binding inhibition by GdN and AT-III

In the presence of heparin, both GdN and AT-III were slow-binding inhibitors of α-thrombin. The inhibition of thrombin by GdN has previously been shown to conform to the mechanism shown in Scheme 1 both in the absence and in the presence of high concentrations of heparin (Stone *et al.*, 1987*a*). To show that the mechanism also applies at optimal and low concentrations of heparin, the apparent first-order rate constant was determined (as described below) at various concentrations of GdN in the presence of constant total concentrations of heparin. The results for optimal (66.6 nM) and low (0.67 nM) heparin concentrations showed that the apparent first-order rate constant increases linearly with increasing concentrations of GdN (results not shown), showing that the mechanism of inhibition corresponds to slow-binding inhibition type A (Morrison, 1982). The data of Olson & Shore (1982) indicate that the inhibition by AT-III would also follow this mechanism at the concentration of AT-III used in the present study.

$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} ES \xrightarrow{k_{cat.}} E + P$$
$$+$$
$$I$$
$$k_2 \uparrow\downarrow k_1$$
$$EI$$

Scheme 1

For this mechanism, the amount of product at time t is given by eqn. (1) (Morrison, 1982):

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

where P is the amount of product at time t, k' is an apparent first-order rate constant, and v_o and v_s are the initial and steady-state velocities respectively. In all experiments, pseudo-first-order conditions were maintained by using a concentration of inhibitor at least one order of magnitude greater than the concentration of enzyme, i.e. [I] ≫ [E].

The value of the second-order rate constant (k_1) can be related to the inhibitor concentration, [I], k', the initial and steady-state velocities (v_o and v_s respectively), the Michaelis–Menten constant (K_m) and the substrate concentration, [S], by eqn. (2) (derived from equations given by Morrison & Stone, 1985):

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

This relationship was used to calculate the value of the k_1 by using the values of k', v_o and v_s determined from the analyses of the data according to eqn. (1) together with the known concentrations of the substrate and inhibitor, and the previously determined K_m value of 3.6 μM for the substrate S-2238 (Hofsteenge *et al.*, 1986). In experiments with Chromozym TH, the previously determined K_m value of 13 μM was used (Hofsteenge *et al.*, 1988).

In the absence of heparin, higher concentrations of GdN were used and the inhibition appeared irreversible, i.e. the steady-state velocity was negligible. Under these conditions, eqns. (1) and (2) reduce to eqns. (3) and (4) respectively, and these equations were used to evaluate the value of k_1 in the absence of heparin (Morrison & Stone, 1985).

$$P = \frac{v_o[1 - \exp(-k't)]}{k'} \quad (3)$$

$$k_1 = \frac{k'}{1 + [S]/K_m} \quad (4)$$

When the rate constant for the inhibition of thrombin by AT-III is plotted against the concentration of heparin, a bell-shaped curve is obtained (for example, see Nesheim, 1983). Such a curve is empirically described by eqn. (5), which can be derived for the template model (Nesheim, 1983):

$$k_1(\text{obs.}) = \frac{k_1(\text{opt.})}{1 + \frac{[H]}{K_1} + \frac{K_2}{[H]}} \quad (5)$$

where [H] is the total heparin concentration, k_1(obs.) is the observed second-order rate constant, k_1(opt.) is the maximum value of k_1 obtained at an optimal heparin concentration, and K_1 and K_2 are composite constants whose actual meaning will depend on the mechanism of heparin stimulation. Values of k_1 obtained at different concentrations of heparin were weighted according to the inverse square of their standard error and fitted to eqn. (5).

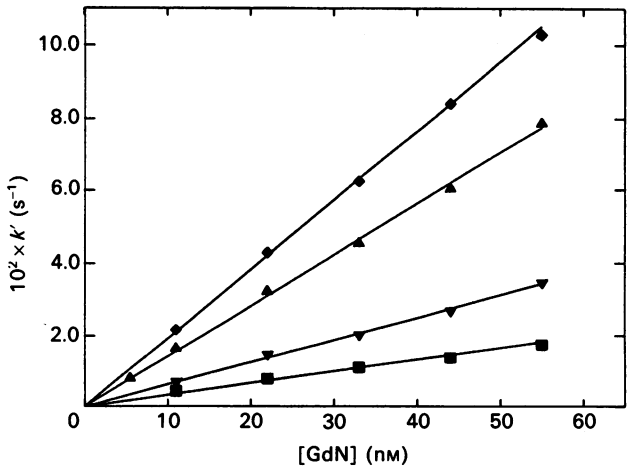

Fig. 2. Inhibition rate constants of proteolysed thrombins by GdN

First-order rate constants of inhibition of proteolysed thrombins by GdN were determined as described in the Experimental section. The plot shows the relation of these rate constants to the concentration of GdN with each form of thrombin as follows: ▲, 50 pM-α-; ▼, 120 pM-β_T-; ■, 58 pM-γ_T-; and ◆, 67 pM-ε-thrombin.

RESULTS

Inhibition of proteolysed forms of thrombin by GdN

Fig. 2 shows the dependence of the apparent first-order rate constant for the inhibition of various forms of thrombin on the concentration of GdN (5–55 nM; for concentrations of thrombins, see the legend to Fig. 2). Analysis of these data yielded the values for the second-order rate constants which are given in Table 1. These values can be compared with those previously obtained for AT-III (Table 1). The rate constants of inactivation of the proteolysed forms of thrombin relative to α-thrombin are similar for both GdN and AT-III.

Effect of heparin on the rate of inhibition of α-thrombin by GdN

Fig. 3 shows a plot of the observed rate constant, k_1, against heparin concentration in the presence of constant total concentrations of GdN (2.0 nM) and thrombin (0.2 nM) for high- and low-affinity types of heparin. It can be seen from comparison with Fig. 4 (which shows the corresponding results for 26.7 mM-AT-III and 0.2 nM-α-

Table 1. Comparison of the inhibition of various forms of thrombin by GdN and AT-III

	GdN		AT-III*	
Form of thrombin	$10^{-6} \times k_1$ ($M^{-1} \cdot s^{-1}$)	Relative rate†	$10^{-4} \times k_1$ ($M^{-1} \cdot s^{-1}$)	Relative rate†
α	1.41 ± 0.02	1.0	1.08	1.0
β_T	0.63 ± 0.01	0.45	0.47	0.44
γ_T	0.33 ± 0.01	0.23	0.17	0.16
ε	1.91 ± 0.02	1.35	0.95	0.88

* From Hofsteenge *et al.*, (1986).
† Value of k_1 relative to that obtained with α-thrombin.

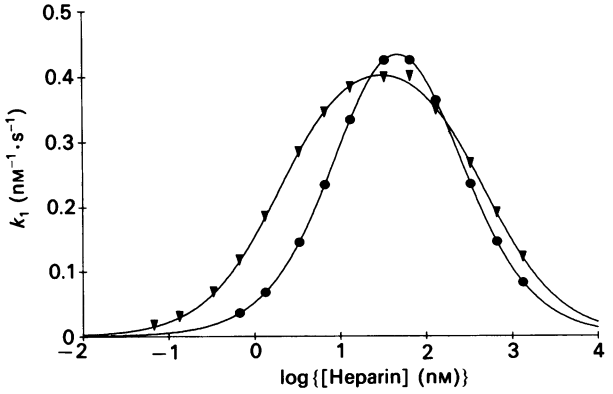

Fig. 3. Inhibition of α-thrombin by GdN in the presence of high- and low-affinity types of heparin

Kinetic data were obtained as described in the Experimental section using heparin concentrations in the range 0.067–1333.24 nM and the following constant total concentrations: GdN, 2.0 nM; α-thrombin, 0.2 nM. These data were analysed as described in the Theory and data analysis section. The plot shows the variation in k_1(obs.) with the logarithm of the heparin concentration. The curves shown are the averages for two experiments, where ● represents low-affinity and ▼ represents high-affinity heparin respectively.

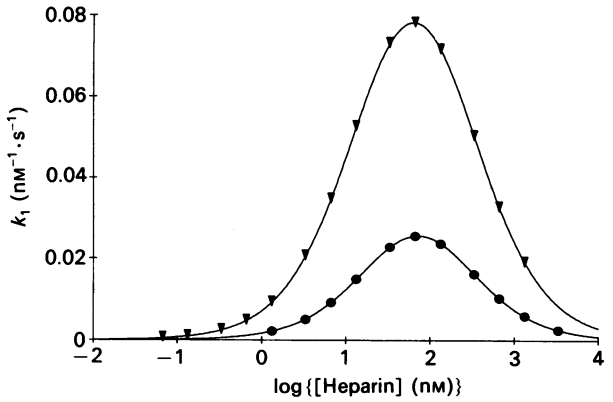

Fig. 4. Inhibition of α-thrombin by AT-III in the presence of high- and low-affinity types of heparin

Kinetic data were obtained as described in the Experimental section, using heparin concentrations in the range 0.067–3333.56 nM and the following constant total concentrations: AT-III, 26.7 nM; α-thrombin, 0.2 nM. These data were analysed as shown in the Theory and data analysis section. The plot shows the variation in k_1(obs.) with the logarithm of the heparin concentration. The curves shown are averages for two experiments, where ● represents low-affinity and ▼ represents high-affinity heparin respectively.

thrombin) that heparin accelerates inhibition by both inhibitors in a similar fashion. Similar results were also obtained with unfractionated heparin (results not shown). For the heparin types used, the optimal rate of inhibition was obtained at approximately the same concentration of heparin with both inhibitors. Analysis of these data according to eqn. (5) yielded estimates for

Table 2. Optimal rate and association constants for thrombin IIa

The units for k_1(opt.) are nM·s^{-1}; those for K_1 and K_2 are nM.

Heparin type	Parameter	GdN	AT-III
Low-affinity	k_1(opt.)	0.64±0.08	0.04±0.01
	K_1	196.2±58.8	211.0±125.2
	K_2	11.35±1.89	24.31±9.68
High-affinity	k_1(opt.)	0.46±0.04	0.12±0.01
	K_1	458.3±159.6	265.5±2.12
	K_2	2.03±0.26	15.23±0.08

k_1(opt.), K_1 and K_2, which are given in Table 2. The term $k_{opt.}$ is the inhibition rate constant at the optimal heparin concentration, such that k_1 is maximized. The range of heparin concentrations between the values of K_1 and K_2 can be considered to be the optimal range for acceleration of the inhibition. The values of k_1(opt.) for low- and high-affinity heparin did not differ greatly with GdN, whereas the value of k_1(opt.) for AT-III was 3-fold higher with the high-affinity heparin. The value of k_1(opt.) for GdN, (0.5–1.2) × 10^9 M^{-1}·s^{-1}, suggests that the rate of association of α-thrombin and GdN in the presence of heparin is diffusion-controlled. The rate for AT-III is an order of magnitude lower at (0.4–1.2) × 10^8 M^{-1}·s^{-1}. For AT-III, at optimal concentrations of heparin, the degree of acceleration by heparin is about 10^4-fold (Table 2; Hofsteenge *et al.*, 1988). For GdN, the degree of acceleration is less (200–500-fold), but, since the rate is probably diffusion-controlled, a greater increase in rate could not be expected.

DISCUSSION

In the present study we have compared the interaction of proteolysed forms of thrombin with both GdN and AT-III to determine whether interactions between GdN and one of the secondary sites of thrombin (Fenton, 1981) can account for the increased affinity of GdN for thrombin over that of AT-III (Stone *et al.*, 1987*a*). It has been shown previously that, in the absence of heparin, the rates of inactivation of β_T-, γ_T- and ε-thrombin by AT-III were not affected to the same extent as the interactions of thrombomodulin, fibrinogen (Hofsteenge *et al.*, 1988) and hirudin (Stone *et al.*, 1987*b*). Thus it was concluded that AT-III probably did not interact with the proposed secondary binding site in the region 65–83 of thrombin (Hofsteenge *et al.*, 1988). The data of Fig. 2 and Table 1 indicate that the effects of proteolytic cleavage on the GdN–thrombin interaction are similar to those observed with AT-III. Thus neither inhibitor appears to interact with region 65–83 of thrombin. However, it can be seen from the values of k_1 (Table 1) that GdN inhibited the various forms of thrombin between 200- and 500-fold faster than AT-III. The increased activity of GdN must arise from some property of the interaction between GdN and thrombin other than interaction with the region described above.

The mechanism of heparin stimulation of both the thrombin–GdN and the thrombin–AT-III interaction

appears to be similar. The only significant difference between the two inhibitors was that high-affinity heparin appeared to be effective at a somewhat lower concentration with GdN; the value of K_2 was 8-fold lower. Thus the mechanism of heparin stimulation of the GdN–thrombin interaction follows the template mechanism proposed for the AT-III–thrombin interaction (Nesheim, 1983) and differs from that proposed for the stimulation of the factor Xa–AT-III interaction (Danielsson *et al.*, 1986; Atha *et al.*, 1987). Previous studies of the heparin-binding site of AT-III have revealed the existence of two essential areas, one in the region of residues 40–60, which is known to contain an important arginine at position 47 (Koide *et al.*, 1984), and an essential tryptophan at position 49 (Blackburn *et al.*, 1984; Peterson & Blackburn, 1987), the other in the vicinity of residues 110–140, which is predominantly basic in character and contains a number of essential lysine residues (Pecon & Blackburn, 1984; Villanueva, 1984; Rosenfeld & Danishefsky, 1986; Liu & Chang, 1987; Peterson *et al.*, 1987). Comparison of the protein sequence similarity by the method of Dayhoff (1979) between GdN and a number of other serpins, including AT-III (Sommer *et al.*, 1987) showed that the latter region of AT-III has significant similarity to the *C*-terminal area of GdN. This region of GdN also shares the predominantly basic character of that AT-III sequence. The *N*-terminal region of GdN, however, shows no overall similarity to the first heparin-binding area of AT-III and is not basic. Thus it seems likely that the region in GdN corresponding to residues 110–140 of AT-III is involved in the binding of heparin, which contributes to the heparin stimulation of the inactivation of thrombin. Other regions of GdN may also be involved in heparin binding, but further protein-chemical studies will be required to locate the exact sites of heparin binding on GdN.

We are grateful to Ms. Elisabeth Fries and Ms. Magda Rentsch for their assistance with the purification of GdN and to Ms. Monique Pedrocca, who fractionated the heparin. We also thank Dr. Dennis Keefe, Dr. Peter Künzler, Dr. Christoph Nager and Dr. Elliott Shaw for their careful pre-submission review of the manuscript.

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Received 22 March 1988/28 June 1988; accepted 21 July 1988