Biochem. J. (1987) 245, 543–550 (Printed in Great Britain)



# Glycosaminoglycans on fibroblasts accelerate thrombin inhibition by protease nexin-1

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Protease nexin-1 (PN-1) is a proteinase inhibitor that is secreted by human fibroblasts in culture. PN-1 inhibits certain regulatory serine proteinases by forming a covalent complex with the catalytic-site serine residue; the complex then binds to the cell surface and is internalized and degraded. The fibroblast surface was recently shown to accelerate the rate of complex-formation between PN-1 and thrombin. The present paper demonstrates that the accelerative activity is primarily due to cell-surface heparan sulphate, with a much smaller contribution from chondroitin sulphate. This conclusion is supported by the effects of purified glycosaminoglycans on the second-order rate constant for the inhibition of thrombin by PN-1. Also, treatment of $^{35}SO_4^{2-}$-labelled cells with heparitin sulphate lyase or chondroitin sulphate ABC lyase demonstrated two discrete pools of $^{35}S$-labelled glycosaminoglycans; subsequent treatment of plasma membranes with these glycosidases showed that heparitin sulphate lyase treatment abolished about 80% of the accelerative activity and chondroitin sulphate ABC lyase removed the remaining 20%. These results show that two components are responsible for the acceleration of PN-1–thrombin complex-formation by human fibroblasts. Although dermatan sulphate is also present on fibroblasts, it did not accelerate the inhibition of thrombin by PN-1.

## INTRODUCTION

PN-1 is a protein proteinase inhibitor which inhibits certain regulatory serine proteinases such as thrombin, urokinase and plasmin (Baker *et al.*, 1980; Scott *et al.*, 1985). PN-1 is synthesized and secreted by cultured human fibroblasts and forms a stable complex with proteinases in the extracellular environment; this complex then binds to the fibroblast surface and is rapidly internalized and degraded (Low *et al.*, 1981). In this manner, PN-1 regulates proteolytic activity at and near the cell surface (Baker *et al.*, 1986). For example, in cell-culture systems, PN-1 has been shown to inhibit thrombin-stimulated cell division (Low *et al.*, 1982) and to prevent extracellular-matrix destruction by human fibrosarcoma cells (Bergman *et al.*, 1986). Also, an inhibitor from glial cells which resembles PN-1 has been shown to stimulate neurite outgrowth in neuroblastoma cells (Guenther *et al.*, 1985).

PN-1 shares several properties with two plasma proteinase inhibitors, namely ATIII and HCII. All three proteins inhibit thrombin with rapid second-order kinetics. In addition, each of these inhibitors has a heparin-binding site, and the rate of thrombin inhibition by these proteins is markedly accelerated by the addition of heparin (Rosenberg & Damus, 1973; Baker *et al.*, 1980; Tollefsen & Blank, 1981). However, PN-1 does not cross-react immunologically with ATIII or HCII, and CNBr peptide maps of these inhibitors are different (Farrell *et al.*, 1986). In addition, each of these inhibitors has a different *N*-terminal amino acid sequence (Petersen *et al.*, 1979; Witt *et al.*, 1983; Scott *et al.*, 1985). PN-1 is also different in that its site of action appears to be in tissues, as judged by its release from several cultured extravascular cells (Eaton & Baker, 1983) and by the inability to detect significant quantities of it in plasma.

Recent studies indicate that glycosaminoglycans at cell surfaces and in the extracellular matrix may accelerate the reactions between these proteinase inhibitors and certain proteinases in ways that reflect their sites of action. For example, the rate of inhibition of thrombin by ATIII is accelerated by vascular endothelial cells (Marcum *et al.*, 1983), but not by fibroblasts or smooth-muscle cells (Brandt *et al.*, 1985; Marcum & Rosenberg, 1985). This specificity reflects the site of action of ATIII in plasma and appears to be involved in haemostasis by limiting thrombin activity, and thus thrombus formation, near the endothelial-cell surface. This accelerative activity of endothelial cells on the reaction between ATIII and thrombin is due to a subpopulation of heparin-like heparan sulphate molecules (Marcum & Rosenberg, 1984). Heparan sulphate has also been shown to accelerate the rate of thrombin inhibition by HCII, another thrombin inhibitor found in plasma. Interestingly, the inhibition of thrombin by HCII, but not by ATIII, is also accelerated by dermatan sulphate (Tollefsen *et al.*, 1983). This suggests that HCII may also function in extravascular tissues where dermatan sulphate is present. For example, during injury, this mechanism could limit thrombin activity at the surfaces of certain extravascular cells.

Our recent complementary studies have shown that the rate of inhibition of thrombin by PN-1 is accelerated by the surface of human fibroblasts (Farrell & Cunningham, 1986). This finding, coupled with the ability of these cells to secrete PN-1 and degrade PN-1–thrombin complexes,

Abbreviations used: ATIII, antithrombin III; Bz-Arg-[$^3$H]OEt, α-*N*-benzoyl-L-arginine [2-$^3$H]ethyl ester (hydrochloride); HCII, heparin cofactor II; PN-1, protease nexin-1.

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suggests a localized mechanism in non-vascular tissues to control certain regulatory proteinases. In the present studies we have identified glycosaminoglycans that can accelerate the inactivation of thrombin by PN-1 and have shown that the acceleration by the fibroblast surface can be accounted for primarily by heparan sulphate and, to a lesser extent, by chondroitin sulphate. Furthermore, we show that, unlike that by HCII, the inactivation of thrombin by PN-1 is not accelerated by dermatan sulphate.

## MATERIALS AND METHODS

### Proteins and glycosaminoglycans

PN-1 was purified as previously described (Farrell *et al.*, 1986). Highly purified human $\alpha$-thrombin was generously given by Dr. John W. Fenton, II, New York State Department of Health, Albany, NY, U.S.A. (Fenton *et al.*, 1977). Active-site titrations showed that the thrombin was 99% active (Chase & Shaw, 1969). Thrombin was iodinated with $Na^{125}I$ (New England Nuclear Corp., Boston, MA, U.S.A.) to $(5–10) \times 10^5$ c.p.m./pmol, using the chloroglycouril (Pierce) method (Fraker & Speck, 1978; Baker *et al.*, 1980).

Heparin lyase (heparinase), heparitin sulphate lyase (heparitinase) and chondroitin sulphate ABC lyase (chondroitinase ABC) were obtained from Seikagaku Kogyo Co. through Miles Scientific (Elkhart, IN, U.S.A.). Each of these glycosidases had 1% or less of the other activities as contaminants.

The glycosaminoglycans which were tested for their effect on PN-1-mediated thrombin inhibition included heparin (Calbiochem, San Diego, CA, U.S.A.), chondroitin sulphate A and C, dermatan sulphate (chondroitin sulphate B), keratan sulphate and hyaluronic acid (Sigma). Heparan sulphate (heparitin sulphate), referred to as type I (bovine liver) and type II (human aorta), were generously given by Dr. Magnus Höök, University of Alabama in Birmingham, Birmingham, AL, U.S.A. (Kjellén *et al.*, 1980). Dextran sulphate was obtained from Pharmacia (Piscataway, NJ, U.S.A.). Our studies indicated that the only glycosaminoglycan preparation which contained a contaminant that was significant for these studies was dermatan sulphate (see the Results section).

### Cell culture, preparation of plasma membranes and metabolic labelling

Human foreskin fibroblasts were cultured as previously described (Farrell *et al.*, 1986). Cells were judged free of mycoplasmal contamination (Barile & McGarrity, 1983). Fractions enriched for plasma membranes were prepared by the method of Thom *et al.* (1977). Tissue-culture dishes (35 mm diameter) were plated with $1 \times 10^5$ cells in 2 ml of Dulbecco's modified Eagle's medium containing 10% calf serum (GIBCO, Grand Island, NY, U.S.A.). After 2 days the cells were rinsed once with minimum essential medium (GIBCO) containing 0.08 mM-$SO_4^{2-}$, and labelled for 2 days in 1 ml of this medium containing dialysed 10% calf serum and 25 $\mu$Ci of $Na_2{}^{35}SO_4$/ml (New England Nuclear, Boston, MA, U.S.A.) (Gill *et al.*, 1981). The cells were rinsed five times with Dulbecco's phosphate-buffered saline (137 mM-NaCl/2.7 mM-KCl/8.1 mM-$Na_2HPO_4$/1.5 mM-$KH_2PO_4$/0.9 mM-$CaCl_2$/0.5 mM-$MgCl_2$, pH 7.4) at 4 °C before digestion with trypsin or glycosidases.

### Kinetics of PN-1-mediated thrombin inhibition

The substrate Bz-Arg-[$^3$H]OEt (New England Nuclear, Boston, MA, U.S.A.) was used to measure thrombin activity after inhibition with PN-1 as described by Tollefsen *et al.* (1983) for HCII and ATIII. PN-1 (30 nM) was substituted for HCII and ATIII in the assay, and the concentration of thrombin was 4.4 nM in 0.15 mM-NaCl/0.1% poly(ethylene glycol)/20 mM-Tris, pH 7.4. The glycosaminoglycans were added at the indicated concentrations, which varied from 0 to 1000 $\mu$g/ml, in a total volume of 200 $\mu$l at 37 °C. The reaction with Bz-Arg-[$^3$H]OEt was quenched with 2 mM-phenylmethanesulphonyl fluoride rather than with hirudin. Second-order rate constants were calculated by using the equation (where Th is thrombin):

$$k_{assoc.} = \frac{\ln\left[\frac{[Th]([PN\text{-}1]-[complex])}{[PN\text{-}1]([Th]-[complex])}\right]}{([PN\text{-}1]-[Th])(0.33\ min)}$$

where [Th] is the initial concentration of thrombin, [PN-1] is the initial concentration of PN-1, and [complex] is the final concentration of PN-1–thrombin complexes in the reaction mixture.

In control experiments, 140 $\mu$l of a 143 $\mu$g/ml dermatan sulphate solution was incubated in the assay buffer with heparinase, heparitinase or chondroitinase ABC (1 unit/ml) for 1 h at 37 °C. The digested glycosaminoglycans were then used in the Bz-Arg-[$^3$H]OEt assay described above by adding 50 $\mu$l of 120 nM-PN-1 and 10 $\mu$l of 88 nM-thrombin.

### Glycosidase digestion of fibroblasts

After the $^{35}SO_4^{2-}$ labelling described above, the cells were incubated in 0.5 ml of Dulbecco's phosphate-buffered saline containing 0–4 units of heparitinase or chondroitinase ABC/ml for 30 min at 37 °C. This supernatant was removed and saved. The cells were then rinsed with 0.5 ml of $Ca^{2+}$/$Mg^{2+}$-free phosphate-buffered saline and the rinse pooled with the previous supernatant. A 10 ml portion of Hydrofluor (National Diagnostics, Sommerville, NJ, U.S.A.) was added to the pooled supernatants and the radioactivity was measured by using a scintillation counter. The attached cells were trypsin-treated by using 1 ml of 0.05% trypsin/0.02% EDTA in $Ca^{2+}$/$Mg^{2+}$-free phosphate-buffered saline for 20 min at 37 °C. The trypsin-treated material was centrifuged for 5 min at 4 °C in a Microfuge (Beckman). A 10 ml portion of Hydrofluor was added to the resulting supernatant and the radioactivity was measured as described above.

### PN-1–$^{125}$I-thrombin complex formation assay

The SDS/polyacrylamide-gel electrophoretic assay for PN-1–$^{125}$I-thrombin complex-formation was used as previously described (Low *et al.*, 1981). Briefly, 1.25 $\mu$g of membranes in 10 $\mu$l of 0.15 M-NaCl/20 mM-imidazole, pH 7.4, was incubated with 5 $\mu$l of Dulbecco's phosphate-buffered saline containing 0–0.06 unit of heparitinase or chondroitinase ABC for 30 min at 37 °C. A 5 $\mu$l portion of 68.3 nM-PN-1/bovine serum albumin (0.25 mg/ml) [RIA (radioimmunoassay) grade; Sigma, St. Louis, MO, U.S.A.] in 0.15 M-NaCl/20 mM-imidazole, pH 7.4, was

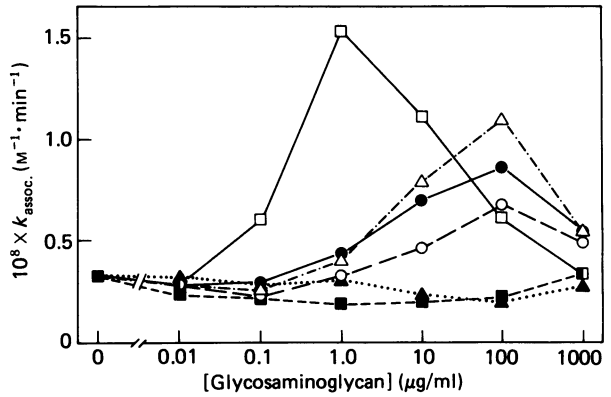


**Fig. 1. Effect of purified glycosaminoglycans on the second-order rate constant for PN-1-mediated thrombin inhibition**

PN-1 (30 nM) was incubated with 4.4 nM-thrombin in 200 μl of Tris/saline/0.1% poly(ethylene glycol) for 20 s at 37 °C in the presence of the following glycosaminoglycans at the indicated concentrations: chondroitin sulphate A (○), chondroitin sulphate C (△), heparan sulphate type I (□), heparan sulphate type II (●), hyaluronic acid (▲) and keratan sulphate (■). The glycosaminoglycans were precipitated by adding 2 mg of Polybrene/ml, and the remaining thrombin activity was quantified by measuring the amount of Bz-Arg-[$^3$H]OEt hydrolysis (0.66 μCi/ml) over a 5 min period. The reaction was quenched with 2 mM-phenylmethanesulphonyl fluoride and the released [$^3$H]ethanol was extracted and counted for radioactivity. The values for $k_{assoc.}$ were calculated as described in the text. This experiment was conducted twice with similar results.

added to the reaction mixture, followed immediately by 5 μl of 6.83 nM-$^{125}$I-thrombin/bovine serum albumin (0.25 mg/ml) in the same buffer. The reaction mixture was incubated for 8 min at 37 °C and then quenched with 25 μl of SDS/polyacrylamide-gel-electrophoresis sample buffer. It was then subjected to electrophoresis by the method of Ornstein (1964) and Davis (1964) as modified by Laemmli (1970). The samples were not boiled, in order to minimize hydrolysis of PN-1–$^{125}$I-thrombin complexes. Sample preparation and electrophoresis resulted in hydrolysis of less than 8% of the PN-1–$^{125}$I-thrombin complexes. (8% was the amount of free $^{125}$I-thrombin remaining uncomplexed after a 16 min incubation at 37 °C with a 10-fold excess of PN-1 and 500 μg membrane/ml. This 8% represents a maximum value of dissociation, since the 8% may also have come from inactive $^{125}$I-thrombin.) The bands corresponding to PN-1–$^{125}$I-thrombin complexes were excised and the radioactivity was measured in a counter by a method similar to that used to quantify C1 inhibitor–C1r–C1s (complement component) complexes (Sim *et al.*, 1980).

## RESULTS

### Acceleration of PN-1-mediated thrombin inhibition by glycosaminoglycans

Previous studies showed that the fibroblast surface accelerates the formation of complexes between PN-1 and $^{125}$I-thrombin (Farrell & Cunningham, 1986). In view of the likelihood that the active component in fibroblasts was a glycosaminoglycan, we examined the ability of several purified gycosaminoglycans to accelerate the reaction between PN-1 and thrombin. This was done by measuring the effect of a broad concentration range of each glycosaminoglycan on the second-order rate constant for the PN-1-mediated thrombin-inhibition reaction. Of the glycosaminoglycans likely to be present on the fibroblast surface, heparan sulphate type I was the most active in this assay (Fig. 1). However, chondroitin sulphate C also increased the second-order rate constant, suggesting that both of these glycosaminoglycans could be responsible for the fibroblast activity. Heparan sulphate type II and chondroitin sulphate A were less active, whereas keratan sulphate and hyaluronic acid had no detectable activity in the assay. [Keratan sulphate is also absent from the surface of dermal fibroblasts (Klintworth & Smith, 1981).] Dermatan sulphate displayed a different concentration-dependence and will be discussed further below. It can be seen from Fig. 1 that the rate constants reached a maximum and then decreased at higher glycosaminoglycan concentrations. A similar phenomenon has been described for acceleration by heparin of ATIII inhibition of thrombin (Jordan *et al.*, 1980*a*,*b*). These investigators found that the decrease in the rate constant at high heparin concentrations corresponded to increasing amounts of heparin bound to thrombin.

Interestingly, heparin and synthetic dextran sulphate, a highly sulphated glucose polymer with approx. 2.3 sulphate residues per glucose unit, were the most active compounds tested. Dextran sulphate increased the $k_{assoc.}$ from $3.3 \times 10^7$ $M^{-1} \cdot min^{-1}$ to $5.2 \times 10^8$ $M^{-1} \cdot min^{-1}$. Heparin raised the $k_{assoc.}$ to $4.5 \times 10^8$ $M^{-1} \cdot min^{-1}$. The fact that heparin accelerates the reaction more effectively than either type of heparan sulphate does not imply that heparin is the relevant physiological mediator of the accelerative effect on PN-1. In fact, heparin is not generally present in the extracellular environment of tissue cells.

Although the data in Fig. 1 show that the saccharide subunits of glycosaminoglycans are critical for determining their ability to accelerate the inactivation of thrombin by PN-1, it was interesting to evaluate also the effect of the degree of sulphation. The importance of sulphation within a class of glycosaminoglycans is demonstrated by the relationship between the activities of the heparin/heparan sulphates and their degree of sulphation. Heparin was most active, followed by heparan sulphate type I, which was more active than heparan sulphate type II; their sulphur-to-hexosamine ratios are respectively 2.33, 0.97 and 0.66 (Kjellén *et al.*, 1980). This series shows a marked correlation between sulphation of the glycosaminoglycan and its ability to accelerate the reaction between PN-1 and thrombin (Fig. 2).

In order to ensure that the accelerative activities of the most potent glycosaminoglycans were not due to other contaminating activities, specific glycosidases were used to digest the glycosaminoglycans before use in the Bz-Arg-[$^3$H]OEt assay. In each case, the glycosidase removed virtually all of the activity of its corresponding glycosaminoglycan (results not shown). These results confirmed that the stimulatory activities of the heparin, heparan sulphate type I and chondroitin sulphate C preparations were due primarily to these components, and not due to contaminating activities. In contrast it

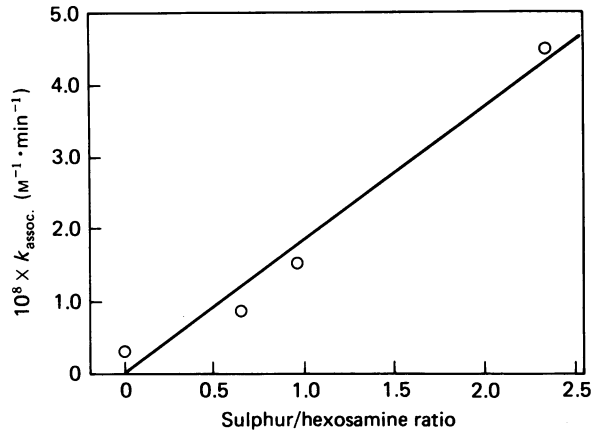


**Fig. 2. Effect of sulphation on the accelerative activity of heparin/heparan sulphates**

The maximum value of $k_{assoc.}$ for heparin, heparan sulphate type I and heparan sulphate type II from Fig. 1 are plotted as a function of the sulphur/hexosamine ratio for each species (Kjellén *et al.*, 1980).

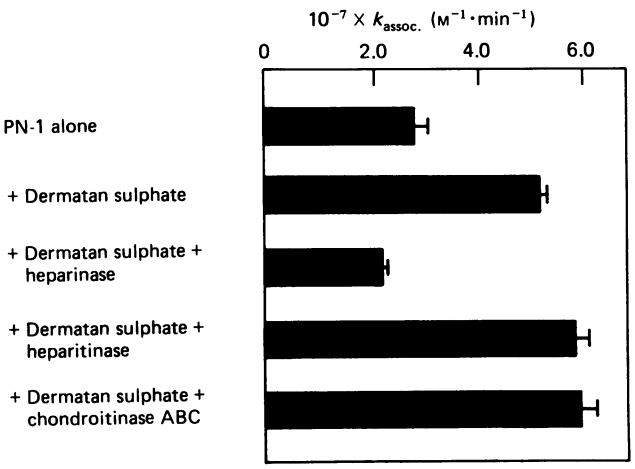


**Fig. 3. Glycosidase digestion of dermatan sulphate**

Dermatan sulphate (143 $\mu$g/ml) was digested with heparinase, heparitinase or chondroitinase ABC (1 unit/ml) in 140 $\mu$l of Tris/saline/0.1% poly(ethylene glycol) for 1 h at 37 °C. The digested dermatan sulphate was then assayed for its stimulatory activity in the PN-1-mediated thrombin-inhibition assay described in Fig. 1. The error bars represent S.E.M. values for duplicate determinations.

appeared that the accelerative effect of dermatan sulphate was likely to be due to a contaminant, since its effect was not saturable over the concentration range used in this assay, namely up to 1 mg/ml. To test this, dermatan sulphate was digested with heparinase, heparitinase and chondroitinase ABC (Fig. 3). If the activity in the preparation had been due to dermatan sulphate, then digestion with chondroitinase ABC should have destroyed the activity. However, only heparinase treatment decreased the ability of the preparation to accelerate the reaction between PN-1 and thrombin. This demonstrates that the activity of the dermatan sulphate preparation was due to a contaminating heparin-like species. It is noteworthy that the functionally related

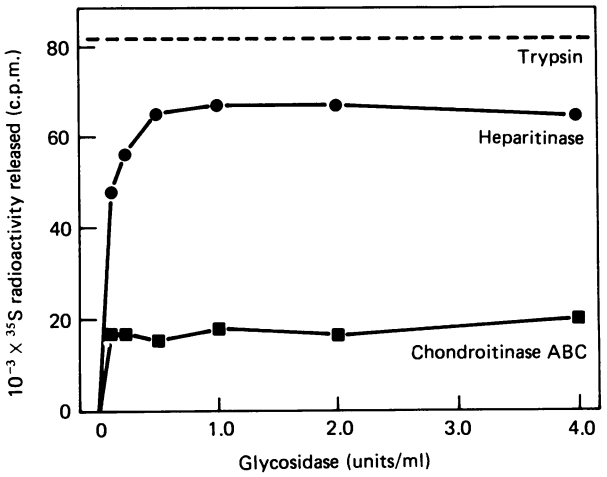


**Fig. 4. Release of $^{35}$S-labelled glycosaminoglycans by glycosidases**

Fibroblasts were labelled for 2 days with 25 $\mu$Ci of $^{35}SO_4^{2-}$/ml. The cells were washed five times with Dulbecco's phosphate-buffered saline and digested with the indicated concentrations of heparitinase (●) or chondroitinase ABC (■) for 30 min at 37 °C. The released $^{35}$S-labelled components were counted. Control plates were incubated for 30 min at 37 °C in Dulbecco's phosphate-buffered saline, and then digested with trypsin (----) for 20 min at 37 °C. The cells were centrifuged and the trypsin-treated $^{35}$S-labelled components were counted for radioactivity. (About $2 \times 10^4$ c.p.m. of radioactivity was released into control supernatants containing only Dulbecco's phosphate-buffered saline; this amount was subtracted from each value.)

plasma inhibitor HCII is markedly accelerated by dermatan sulphate (Tollefsen *et al.*, 1983).

### Release of $^{35}$S-labelled glycosaminoglycans from fibroblasts by glycosidases

The stimulatory activities of heparan sulphate and chondroitin sulphate in the Bz-Arg-[$^3$H]OEt assay suggested that cell-surface heparan sulphate and chondroitin sulphate on human fibroblasts could both be responsible for the accelerative effect of fibroblast plasma membranes on the formation of PN-1–$^{125}$I-thrombin complexes. Both of these glycosaminoglycans are present on the surface of human dermal fibroblasts (Gill *et al.*, 1981). In order to test the ability of purified glycosidases to remove glycosaminoglycans from foreskin fibroblasts, the cells were metabolically labelled with $^{35}SO_4^{2-}$; sulphated proteoglycans labelled by this procedure account for the vast majority of $^{35}$S-labelled cell-surface components (Kleinman *et al.*, 1975). Treatment of the fibroblasts with heparitinase released about 80% of the trypsin-sensitive $^{35}$S-labelled components in a dose-dependent manner (Fig. 4). Virtually identical results were obtained with heparinase (results not shown). However, heparitinase was used in all subsequent experiments, since heparinase has a broader specificity and will degrade both heparin and heparan sulphate (Linker & Hovingh, 1972). In contrast, treatment with chondroitinase ABC removed only about 20% of the trypsin-sensitive components, even at concentrations as high as 4 units/ml for 30 min. It should be emphasized

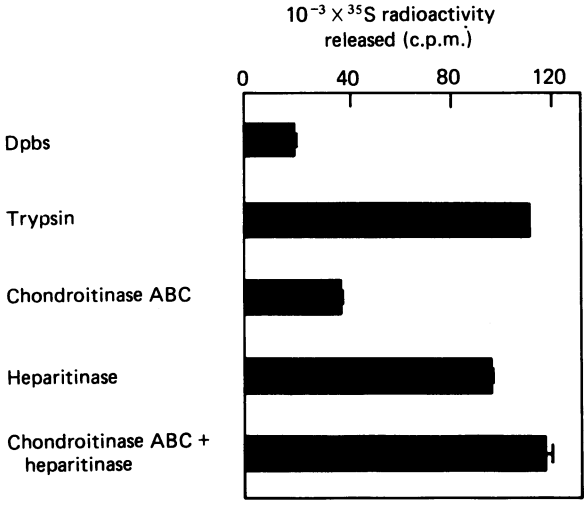


**Fig. 5. Additive release of heparitinase- and chondroitinase ABC-sensitive glycosaminoglycans**

Fibroblasts were labelled with $^{35}SO_4^{2-}$ and then washed as described in Fig. 4. Heparitinase or chondroitinase ABC (1 unit/ml) was then added either separately or together and incubated for 30 min at 37 °C. The released $^{35}$S-labelled components were counted for radioactivity. Control plates were incubated for 30 min at 37 °C with Dulbecco's phosphate-buffered saline (Dpbs), and the released $^{35}$S-labelled components were counted for radioactivity. The cells were then digested with trypsin for 20 min at 37 °C and centrifuged. The trypsin-treated $^{35}$S-labelled components in the supernatant were counted for radioactivity. The error bars represent the S.E.M. values for duplicate determinations.

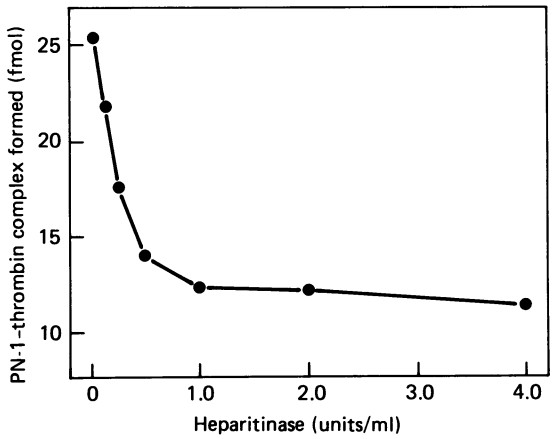


**Fig. 6. Heparitinase digestion of membrane accelerative activity**

Plasma membranes (1.25 $\mu$g) in 15 $\mu$l of 0.15 M-NaCl/20 mM-imidazole, pH 7.4, were incubated for 30 min at 37 °C with the indicated concentrations of heparitinase. A 5 $\mu$l portion of 68.3 nM-PN-1 and one of 6.83 nM-$^{125}$I-thrombin was added and the 25 $\mu$l reaction mixture was incubated for 8 min at 37 °C. The reaction was quenched with 25 $\mu$l of SDS sample buffer and the amounts of PN-1-$^{125}$I-thrombin complexes were quantified by using the electrophoretic assay described in the text. The membrane-accelerated reactions formed 25.4 fmol of complex, and the reactions with PN-1 and $^{125}$I-thrombin alone formed 7.9 fmol of complex.

that these results do not imply that heparan sulphate is more abundant than chondroitin sulphate on the cell surface, since differences in synthetic rate or degree of sulphation could account for the greater amount of $^{35}$S in heparan sulphate. These results do, however, confirm that our preparations of heparitinase and chondroitinase ABC removed discrete classes of glycosaminoglycans from the cell surface, a finding consistent with previous reports (Gill *et al.*, 1981). It should be noted that chondroitinase ABC rather than chondroitinase AC was used in these experiments, since the latter enzyme is inhibited by chondroitin sulphate B, a known component of the fibroblast surface (Yamagata *et al.*, 1968).

If the heparitinase and chondroitinase ABC were removing two different classes of glycosaminoglycans from the cells, then their effects should be additive. To test this, heparitinase and/or chondroitinase ABC were incubated at their optimal concentrations (1 unit/ml) with labelled cells (Fig. 5). As seen above, chondroitinase ABC removed only a small subset of the $^{35}$S-labelled components. Importantly, heparitinase removed most, but not all, of the trypsin-sensitive components. When the two glycosidases were mixed together, however, essentially all of the trypsin-sensitive $^{35}$S-labelled glycosaminoglycans were removed, demonstrating an additive effect of the two glycosidases. This result confirmed that heparitinase and chondroitinase ABC would be useful probes for selectively removing populations of glycosaminoglycans from the fibroblast plasma membranes in subsequent experiments.

### Removal of plasma-membrane accelerative activity by glycosidases

Heparitinase and chondroitinase ABC were used to determine the relative contribution of heparan sulphate and chondroitin sulphate respectively to the accelerative activity of fibroblast plasma membranes on the rate of PN-1-$^{125}$I-thrombin complex-formation. Membranes were used in this assay rather than intact cells, since a larger stimulation could be achieved with membranes, making subsequent measurements of the glycosidase effects more sensitive. Heparitinase-treated membranes were compared with untreated membranes for their ability to accelerate the formation of PN-1-$^{125}$I-thrombin complexes relative to control reaction mixtures, which contained PN-1 and $^{125}$I-thrombin without membranes. The control reactions lacking membranes formed 7.9 pmol of complex compared with 25.4 pmol formed in the presence of membranes (Fig. 6). Heparitinase treatment decreased the amount of membrane-accelerated complex-formation by about 80%, as did heparinase treatment, and reached a maximum effect by about 1 unit/ml. (In control experiments, heparitinase and chondroitinase ABC did not cause a decrease in the basal rate of PN-1-$^{125}$I-thrombin complex-formation in the absence of membranes.) This concentration is consistent with the concentration of heparitinase required to remove the maximum amount of heparitinase-sensitive $^{35}$S-labelled components (Fig. 4), further suggesting that the heparitinase, and not a contaminating enzyme, was responsible for removing the activity.

In contrast, chondroitinase ABC treatment of the membranes caused only a minor decrease in the membrane accelerative activity (Fig. 7). However, this decrease in activity was additive with the decrease seen with heparitinase, as shown by experiments in which

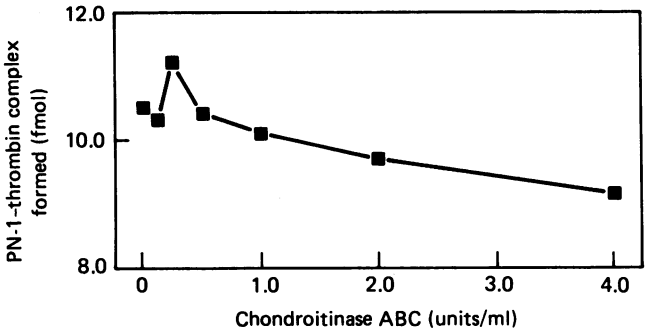


**Fig. 7. Chondroitinase ABC digestion of membrane accelerative activity**

Plasma membranes (1.25 $\mu$g) in 15 $\mu$l of 0.15 M-NaCl/20 mM-imidazole, pH 7.4, were incubated for 30 min at 37 °C with the indicated concentrations of chondroitinase ABC. A 5 $\mu$l portion of 68.3 nM PN-1 and one of 6.83 nM-$^{125}$I-thrombin was added and the 25 $\mu$l reaction mixture was incubated for 8 min at 37 °C. The reaction was quenched with 25 $\mu$l of SDS sample buffer and the amounts of PN-1-$^{125}$I-thrombin complexes were quantified by using the electrophoretic assay described in the text. The membrane-accelerated reactions formed 11.2 fmol of complex, and the reactions with PN-1 and $^{125}$I-thrombin alone formed 5.5 fmol of complex.

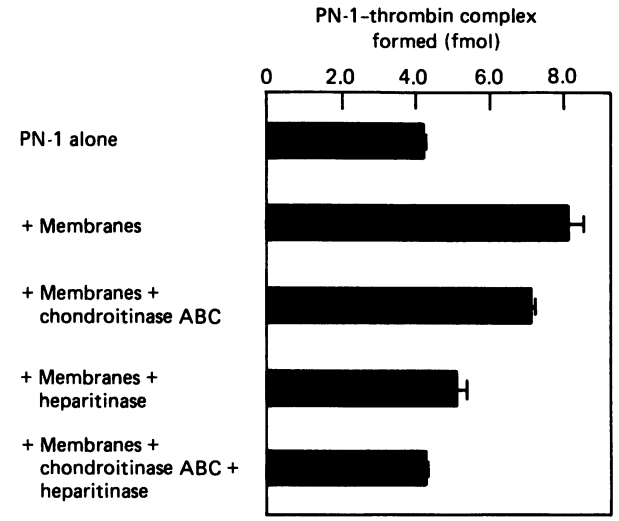


**Fig. 8. Additive removal of membrane accelerative activity by heparitinase and chondroitinase ABC**

Plasma membranes (1.25 $\mu$g) in 15 $\mu$l of 0.15 M-NaCl/20 mM-imidazole, pH 7.4, were incubated for 30 min at 37 °C with 1 unit of heparitinase or chondroitinase ABC/ml, either separately or together. A 5 $\mu$l portion of 68.3 nM-PN-1 and one of 6.83 nM-$^{125}$I-thrombin was added and the 25 $\mu$l reaction mixture was incubated for 8 min at 37 °C. The reaction was quenched with 25 $\mu$l of SDS sample buffer and the amounts of PN-1-$^{125}$I-thrombin complexes were quantified by using the electrophoretic assay described in the text. The error bars represent the S.E.M. values for duplicate determinations.

heparitinase and/or chondroitinase ABC were incubated at their optimal concentrations (1 unit/ml) with membranes. Consistent with the concentration curves seen above (Fig. 6), heparitinase treatment removed most, but not all, of the accelerative activity of the membranes, whereas chondroitinase ABC had much less effect. When the two glycosidases were mixed together, however, essentially all of the accelerative activity was removed (Fig. 8). Thus the effects of heparan sulphate and chondroitin sulphate were additive, and this suggested that heparan sulphate accounts for about 80% of the accelerative activity and chondroitin sulphate accounts for the remaining 20%.

## DISCUSSION

PN-1 is secreted by human fibroblasts (Baker *et al.*, 1980) as well as by several other non-vascular cells (Eaton & Baker, 1983). It has been shown to inhibit certain serine proteinases by forming a complex with the proteinase, which blocks its catalytic site. These complexes are then bound, internalized and degraded by the cells which secrete PN-1 (Low *et al.*, 1981). In this series of events, PN-1 can control the activity of several serine proteinases such as thrombin and urokinase (Scott *et al.*, 1985) and thus modulates the events which these proteinases initiate (Low *et al.*, 1982; Scott *et al.*, 1983; Bergman *et al.*, 1986). Recently it was shown that human fibroblasts play another role in this regulation by catalysing the formation of complexes between PN-1 and thrombin (Farrell & Cunningham, 1986). This accelerative activity was found to be associated with the plasma membrane and the extracellular matrix. Preliminary experiments indicated that the activity may be a heparin-like molecule. These findings suggest that fibroblasts may play an active role in regulating thrombin inhibition by PN-1, and that this regulation might be modulated by changes in the cell surface. Thus it was important to identify the cell-surface molecules responsible for the acceleration.

The present studies show that purified heparan sulphate and chondroitin sulphate accelerate the rate of PN-1-mediated thrombin inhibition. This indicated that both of these glycosaminoglycans could be responsible for the fibroblast accelerative activity. Purified hyaluronic acid and keratan sulphate did not accelerate the reaction between PN-1 and thrombin. Although dermatan sulphate is present on fibroblasts, the present studies indicated that it does not accelerate the reaction between PN-1 and thrombin. Thus the commercial preparation of dermatan sulphate accelerated the inactivation of thrombin by PN-1, but further experiments showed that this was attributable not to dermatan sulphate but rather to a heparin-like contaminant. An important aspect of this finding is that HCII is activated by dermatan sulphate, suggesting that HCII and PN-1 may be activated at different sites *in vivo* during wounding.

On the basis of these studies we employed specific glycosidases for heparan sulphate and chondroitin sulphate (heparitinase and chondroitinase ABC respectively) to determine the relative contribution of these glycosaminoglycans to the total fibroblast accelerative activity. Experiments on $^{35}$S-labelled cells showed that heparitinase and chondroitinase ABC recognized two discrete pools of sulphated glycosaminoglycans on the fibroblasts. This indicated that these glycosidases would be useful in distinguishing between cellular heparan sulphate and chondroitin sulphate, as previously shown for dermal fibroblasts (Gill *et al.*, 1981). About 80% of the $^{35}$S-labelled glycosaminoglycans was removed by

treatment with heparitinase. The remaining 20% was removed by treatment with chondroitinase ABC. These results should not be interpreted to mean that 80% of the glycosaminoglycans on the fibroblasts are heparan sulphate, since differences in the rate of synthesis or degree of sulphation could account for the greater incorporation into heparan sulphate. However, the data do show that the two glycosidases removed two discrete pools of $^{35}S$-labelled components. These pools account for all the trypsin-sensitive surface $^{35}S$-labelled glycosaminoglycans, as shown by the additive effects of heparitinase and chondroitinase ABC. Neither glycosidase alone was able to remove all the $^{35}S$-labelled components; however, all the labelled components were removed when the two glycosidases were added together. The fact that neither glycosidase affected the activity of the heparin-like contaminant in dermatan sulphate also suggests that their effect on heparan sulphate and chondroitin sulphate was not due to a non-specific hydrolase. When fibroblast plasma membranes were then digested with heparitinase, an 80% decrease in the amount of stimulated PN-1–$^{125}I$-thrombin complex-formation was seen relative to the rate in the presence of untreated membranes. The remaining 20% of the fibroblast accelerative activity was removed by digestion with chondroitinase ABC, indicating that heparan sulphate and chondroitin sulphate account for virtually all of the fibroblast accelerative activity.

The involvement of cell-surface glycosaminoglycans in the regulation of extracellular proteinases raises the intriguing possibility that cells might actively control extracellular proteinases by modulating the amount and composition of cell-surface glycosaminoglycans. This could provide an additional level of control for proteinase-regulated cellular processes, including cell division (Chen & Buchanan, 1975; Low *et al.*, 1982) and cell migration (Reich, 1978; Danø *et al.*, 1985). Indeed, aberrant glycosaminoglycan metabolism is seen in certain disease states (Wight, 1985), particularly in malignancy (Chiarugi *et al.*, 1980), and may contribute to the pathology of the disease (Kramer, 1979).

The presence of heparan sulphate and chondroitin sulphate on fibroblasts also raises the possibility that secreted PN-1 may bind to these extracellular-matrix glycosaminoglycans. Previous studies have shown that PN-1 has a heparin-binding site (Baker *et al.*, 1980). The bound PN-1 would be ideally situated topologically to protect the cell against proteolysis while simultaneously being present in an activated form. This would not preclude the interaction of free PN-1 in solution with proteinases, but would serve to localize better the inhibitory activity of PN-1 at sites in the extracellular matrix that are particularly sensitive to proteolysis.

We thank Dr. Magnus Höök for providing the heparan sulphate preparations, Dr. John W. Fenton, II, for generously providing human thrombin, and Dr. Terrence Giugni for preparing plasma membranes from human fibroblasts. The excellent technical assistance of Ms. Laura Cho and Ms. Alice Lau is gratefully acknowledged. This work was supported by Grant GM 31609 from the National Institutes of Health.

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Received 1 September 1986/9 December 1986; accepted 6 April 1987