Glycosaminoglycans Differentially Bind HARP and Modulate Its Biological Activity*

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Francis Vacherot‡§, Jean Delbès, Mélanie Heroult‡, Denis Barritault‡, David G. Fernig¶, and José Courtý‡‡

From the ‡Laboratoire de Recherche sur la Croissance Cellulaire, la Réparation et la Régénération Tissulaires (CRRET), Unité Propre de Recherche de l’Enseignement Supérieur Associées au CNRS (UPRES-A) CNRS 7053, Université Paris XII-Val de Marne, avenue du Général de Gaulle, 94010 Créteil, France and the ¶School of Biological Sciences, University of Liverpool, Liverpool L69 7ZB, United Kingdom

Heparin affin regulatory peptide (HARP) is a polypeptide belonging to a family of heparin binding growth/differentiation factors. The high affinity of HARP for heparin suggests that this secreted polypeptide should also bind to heparan sulfate proteoglycans derived from cell surface and extracellular matrix defined as extracellular compartments. Using Western blot analysis, we detected HARP bound to heparan sulfate proteoglycans in the extracellular compartments of MDA-MB 231 and MC 3T3-E1 as well as NIH3T3 cells overexpressing HARP protein. Heparitinase treatment of BEL cells inhibited HARP-induced cell proliferation, and the biological activity of HARP in this system was restored by the addition of heparin. We report that heparan sulfate, dermatan sulfate, and to a lesser extent, chondroitin sulfate A, displaced HARP bound to the extracellular compartment. Binding analyses with a biosensor showed that HARP bound heparin with fast association and dissociation kinetics ($k_{\text{ass}} = 1.6 \times 10^6 \text{M}^{-1} \text{s}^{-1}$; $k_{\text{diss}} = 0.02 \text{s}^{-1}$), yielding a $K_d$ value of 13 nM; the interaction between HARP and dermatan sulfate was characterized by slower association kinetics ($k_{\text{ass}} = 0.68 \times 10^6 \text{M}^{-1} \text{s}^{-1}$) and a lower affinity ($K_d = 51 \text{nM}$). Exogenous heparin, heparan sulfate, and dermatan sulfate potentiated the growth-stimulatory activity of HARP, suggesting that corresponding proteoglycans could be involved in the regulation of the mitogenic activity of HARP.

Heparin affin regulatory peptide (HARP) belongs to a growing group of heparin binding extracellular regulatory molecules, and it has mitogenic and neurite outgrowth activities. Independently purified from perinatal rat brain as a polypeptide that induces neurite outgrowth of embryonic neurons (1), this protein, also named pleiotrophin (2), has been purified from uterus (3) and adult brain (4) as a growth factor for fibroblastic and endothelial cells. Expression of HARP mRNA is developmentally regulated, and major expression is found during the perinatal growth period in rat brain (5, 6), pointing to the possible function of this molecule in the maturation of nerve cells. Moreover, HARP mRNA is found in several adult tissues, and HARP has been implicated in tumor growth (7).

HARP cDNA has been cloned and sequenced from several species. The predicted amino acid sequence has been determined in humans, mice, and rats (2, 6, 8, 9) and shows 98% homology among the three species. In addition, the HARP amino acid sequence shares 55% homology with the midkine gene product (10). In contrast to their neurite outgrowth activity, initial studies differed in their results about the mitogenic activity of midkine and HARP (3, 9, 11–15), possibly because of differences in the cell type used or to the isolation procedure. More recent studies clearly demonstrate that this family of polypeptides have growth-stimulatory activity (16).

The high affinity of HARP for heparin suggests that HARP may bind to heparan sulfate proteoglycans (HSPGs) present in extracellular compartments defined as cell surface and extracellular matrix (ECM). Recent studies have demonstrated that syndecan-1, syndecan-3 (N-syndecan), and syndecan-4 (rudpocan) bind HARP with high affinity (13, 17, 18). In addition, biochemical and cell biological studies have pointed to syndecan-3 as the HARP receptor involved in neurite outgrowth activity (19). Despite the correlation derived from previous studies, there has been no direct biochemical demonstration that HARP is trapped in the extracellular compartment as a mitogenic molecule bound to HSPGs. In this study we investigated whether HARP is present in the extracellular compartment as well as the role of glycosaminoglycans (GAGs) in controlling HARP mitogenic activity.

** Experimental Procedures

Materials—Cell culture reagents were from Life Technologies, Inc. Heparin lyase I (Flavobacterium heparinium; EC 4.2.2.7), heparin lyase III (F. heparinium; EC 4.2.2.8), chondroitin ABC lyase (Proteus vulgaris; EC 4.2.2.4), leupeptin, pepstatin, phenylmethylsulfonyl fluoride, heparan sulfate (HS) from bovine intestinal mucosa, keratan sulfate (KS) from bovine cornea, chondroitin sulfate A (CS-A) from bovine trachea, dermatan sulfate (DS) from porcine skin, and chondroitin sulfate C (CS-C) from shark cartilage were purchased from Sigma. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins were obtained from Diagnostics-Pasteur (Marne la Coquette, France). The affinity-purified HARP antibodies were obtained as described previously by Ledoux (20). Heparin-Sepharose was found to bind to Millipore Corporation (Saint Quentin en Yvelines, France).
and Amersham Pharmacia Biotech, respectively. Porcine mucosal heparin was a kind gift from M. Petitiou (Sanofi, France), ABC alkaline phosphatase substrate kit 1 and Levamisol were from Biosys, Vector laboratories (Compiegne, France). FGF2 was a gift from G. Mazue (Amersham Pharmacia Biotech).

Cell Culture—Benign epithelial lens (BEL) cells were isolated by the method previously described (21). The cells were grown in DMEM supplemented with 10% fetal calf serum in the presence of 5 ng/ml FGF2 and were used between passages 10 and 20. NIH3T3 cells line stably transfected with pJK12 that overexpressed HARP (H-NIH3T3) were cultured as described previously (22). MDA-MB 231 cells were a gift from M. Crepin (Bobigny, France) and MC 3T3-E1 cells were a gift from M. Meunier (Lyon, France).

Western Blot Analysis of HARP—The presence of HARP in extracellular compartments was defined as cell surface and extracellular matrix was investigated by washing the cells grown to confluency in a 150-cm² tissue culture dish with 2 × 5 ml of 20 mM Hepes, pH 7.4, containing 0.15 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 1 μg/ml pepstatin A, and 5 mM EDTA. The 2 mM NaCl washes diluted 1:4 and the conditioned medium of cells were incubated for 4 h with 100 μl of 10% (w/v) heparin-Sepharose at 4 °C on a shaker. The heparin-Sepharose was washed four times with 10 ml of 20 mM Hepes, pH 7.4, 0.5 mM NaCl and twice with 10 ml of 20 mM Hepes, pH 7.4.

Western blotting was carried out on 1A family members using human recombinant HARP cDNA were seeded at a density of 4 × 10⁴/cm² in 150-cm² culture dishes. Cells were seeded at 10⁴/well in 48-well culture plates in DMEM supplemented with 10% (v/v) fetal calf serum, and the cells were maintained in serum-free DMEM. Twenty h later the cells were treated with 3 international units/ml heparin and incubated for 4 h at 37 °C. The medium was discarded, and the cells were incubated in serum-free DMEM in the presence of 1.25 ng/ml HARP (ED₅₀ efficiency dose for 40% of maximal stimulation induced by HARP) with or without increasing concentrations of heparin (10 to 10⁴ ng/ml) in triplicate for 18 h, and the incorporation of [methyl-3H]thymidine incorporation was determined as described. Experiments were performed in duplicate.

Binding Assays—Porcine intestinal mucoxa heparin was purified and biotinylated as described (26). DS was biotinylated in a similar fashion. Five hundred μg of DS in 100 μl of distilled water was incubated with 30 μl of a 50 mM solution of N-oxidozyxycumidine amino caproate-LC biotin (Pierce-Warriner, Chester, UK) in dimethyl sulfoxide for 72 h. Unreacted biotin was removed by fractionation on a Sephadex G-25 column (1 × 25 cm) equilibrated in distilled water, and DS was lyophilized. Biotinylated DS was immobilized on streptavidin-derivatized planar surfaces as described for biotinylated heparin (26).

Binding reactions were carried out in an IAexs resonant mirror biosensor at 20 °C using planar biotinylated surfaces derivatized with streptavidin according to the manufacturer’s instructions (Affinity Sensors, Bedford, MA). The heparin-Sepharose was added for binding, washed at least twice. The distribution of the immobilized DS, and of the bound HARP on the surface of the biosensor cuvette was inspected by examination of the resonance scan, which showed that at all times these molecules were distributed uniformly on the sensor surface and therefore were not microaggregated. Binding assays were as described (27), and the amount of bound HARP was reported in arc s (1 arc s = 1/360°). Briefly, the ligate, HARP, was added at a known concentration in 100 μl of PBS-T (PBS supplemented with 0.02% (v/v) Tween 20), and then the association reaction was followed over a set time, usually 150 s. The cuvette was then washed twice with 200 μl of PBS-T, and the dissociation of bound ligate into the bulk PBS-T was followed over time. To remove residual bound ligate and thus regenerate the immobilized ligate, the biosensor was washed twice with 200 μl of 2 M NaCl, 10 mM Na₂HPO₄, pH 7.2. Binding parameters were calculated from the association and dissociation rate constants, kₐ and kₐₜₐₜ, respectively, using the nonlinear curve-fitting FastFit software (Affinity Sensors) provided with the instrument, as described (27). HARP did not itself bind to streptavidin-derivatized surfaces.

RESULTS—

Presence of HARP in the Extracellular Compartments—The presence of HARP in the culture medium conditioned by MDA-MB 231 (7) and MC 3T3-E1 (28) cells prompted us to determine whether HARP was also present in the 2 mM NaCl washes of the surface of these cells, which corresponded to most of the material bound to the ECM and cell surface. In MDA-MB 231 (Fig. 1A) and MC 3T3-E1 cells (Fig. 1B), HARP immunoreactivity was observed both in the conditioned medium (lanes 1) and in the 2 mM NaCl washes (lanes 2). Thus, HARP is secreted by cells into the culture medium as well as sequestered in their extracellular compartments.

To determine whether HARP was associated with the ECM, the latter was prepared from MC 3T3-E1 cells that produced a substantial ECM and tested for the presence of HARP by immunocytochemistry. Immunocytochemistry of ECM preparations displayed intense staining against anti-HARP (Fig. 2A). In contrast, no immunostaining was observed when nonspecific antibodies were used (Fig. 2B) or after a 2 mM NaCl wash (result not shown). Furthermore, as shown in Fig. 2C, Western blot analysis of 2 mM NaCl washes of ECM preparations showed HARP immunoreactivity.

Effect of Heparin, Heparitinase, and Chondroitinase ABC on HARP Release—The finding that HARP was bound to the extracellular compartments was reminiscent of other heparin binding growth factors like FGF2 (25, 29, 30), vascular endothelium growth factor (31), and exin 6 platelet-derived growth factor (32). The high affinity of HARP for heparin is consistent with the possibility that this molecule binds to HSPGs. This
was investigated by studying the effect of heparinase, heparitinase, and chondroitinase ABC on the release of HARP from H-NIH3T3 cells. Enzyme concentrations optimal for degradation of the relevant GAGs were determined by using 35S-metabolically labeled GAGs derived from H-NIH3T3 (result not shown). Treatment with 1.2 international milliunit/ml heparinase or heparitinase induced the release of HARP from the extracellular compartments of NIH3T3 cells as compared with the buffer alone (Fig. 3). In contrast, treatment with chondroitinase ABC did not cause the release of HARP beyond that observed in buffer alone (Fig. 3).

**Effect of Glycosaminoglycans on HARP Release**—To confirm that HARP binds to extracellular compartments through interactions with HSPGs, we determined whether the exogenous addition of HSPGs could release HARP from cellular binding sites. Cell monolayers of H-NIH3T3 cells were incubated at 4 °C with various GAGs, including HS, KS, heparin, CS-A, DS, or CS-C at concentrations ranging from 0.1 to 100 μg/ml. Incubation with HS or heparin at 10 μg/ml resulted in the efficient release of HARP (Fig. 4A). The same result was observed with 100 μg/ml heparin or HS (result not shown). Studies performed with other GAGs indicated that treatment with DS at 10 μg/ml or 100 μg/ml (result not shown) released a similar amount of HARP as HS (Fig. 4A). In contrast, CS-A (10 μg/ml) released considerably less HARP than CS-C (10 μg/ml), even less (Fig. 4A). KS (10 μg/ml) was without effect. NaCl (2 mM) was able to release residual HARP (Fig. 4B). Quantitative studies using indirect enzyme-linked immunosorbent assay revealed that DS released up to 84% bound HARP, and HS released 78%. In contrast, treatment with 100 μg/ml CS-A resulted in 40% displacement of bound HARP, and no significant effect was observed with CS-C or KS used at the same concentration (data not shown).

**Characterization of the HARP-GAGs Interaction**—The HARP-GAGs interaction was characterized by fast association kinetics, as exemplified by HARP binding to immobilized DS (Fig. 5A). The dissociation of HARP from immobilized DS was also fast (Fig. 5B). The binding of HARP to heparin and DS was always homogenous; there was no evidence for the presence of more than one binding site for HARP in either of the GAGs (Fig. 5B and Table I). The association rate constant for the HARP-heparin interaction (k association = 1.6 ± 0.3 × 10^6 M^-1 s^-1) was twice as fast as that for the HARP-DS interaction (k association = 0.68 ± 0.07 × 10^6 M^-1 s^-1) (Table I). The dissociation rate constant of HARP from heparin was slightly slower than that of HARP from DS (Table I). Thus, when the kinetic parameters were used to calculate the affinity of the HARP-GAG interactions, the interaction between HARP and heparin had a considerably higher affinity (K_d 13 ± 3 nM) than the interaction between HARP and DS (K_d 51 ± 14 nM). The K_d values calculated from the extent of binding observed at equilibrium were very similar to those calculated from the kinetic binding parameters (Table I).

Competition binding assays were used to further characterize the interactions between HARP and the GAGs. In the first set of experiments, we examined whether HARP recognized a structural motif in the GAGs that was also recognized by the archetypal heparin binding growth factor, FGF2. The extent of binding of 111 nM HARP to DS was 97 ± 4 arc s (33, 34). The cuvette surface was then saturated with FGF2 by repeatedly adding 167 nM FGF2 until no further binding was observed. The extent of binding of 111 nM HARP to the FGF2-saturated DS surface was similar to that observed in the absence of FGF2, 92 ± 5 arc s. This result suggests that HARP recognizes a structural motif distinct from that recognized by FGF2. The same experiment was performed on the heparin surface. The extent of binding of 55 nM HARP to the heparin surface was 99 ± 3 arc s, whereas only 65 ± 10 arc s HARP bound to the heparin surface saturated with FGF2, suggesting that the structural motifs recognized by HARP and FGF2 in heparin may overlap to a certain extent. In the second set of experiments, the ability of different GAGs to inhibit the binding of 111 nM HARP to immobilized heparin or DS was determined. On immobilized heparin (Fig. 5C), 140 arc s HARP was bound at equilibrium, and soluble heparin was the most effective inhibitor of HARP binding, followed by DS and CS-A. Thus 500 ng/ml heparin inhibited HARP binding by 87%, whereas 50 μg/ml DS and 500 μg/ml CS-A reduced HARP binding by 79%. On immobilized DS (Fig. 5D), 53 ± 6 arc s HARP was bound at equilibrium, and the rank order of the inhibitory ability of the GAGs on HARP binding was the same as on immobilized heparin. Thus 500 ng/ml heparin inhibited HARP binding by 82%, whereas 5 μg/ml DS was required for 85% inhibition, and 50 μg/ml CS-A for 88% inhibition.

**Effect of Glycosaminoglycans on the Mitogenic Activity of HARP**—We then examined whether HSPGs played a role in the mitogenic response to HARP as demonstrated for the neurite outgrowth activity by studying whether the response of heparitinase-treated BEL cells to HARP was dependent on the presence of heparin. As shown in Fig. 6A, stimulation induced by 1.25 ng/ml HARP was reduced by 50% in cells pretreated with heparitinase. The addition of heparin in the concentration range from 10 to 10^4 ng/ml clearly restored and potentiated the mitogenic activity of HARP. Maximum activity with heparin was seen at 100 ng/ml, corresponding to a stimulation of 4.2 times over the control, i.e. cells treated with heparitinase and without the addition of heparin. In contrast, no effect was observed when heparin was added alone to untreated cells at concentrations ranging from 10 to 10^4 ng/ml (result not shown). In our hands, treatment of BEL cells with chondroitinase ABC resulted in the loss of cell adhesion followed by cell death; these cells were thus unsuitable for testing the role of chondroitin sulfate in the mitogenic response (data not shown).

![Fig. 1. Presence of HARP in the extracellular compartments of MDA-MB 231 and MC 3T3-E1 cells.](image-url)
Fig. 2. HARP immunoreactivity staining of isolated ECM. MC 3T3-E1 cells were grown to confluence, and ECM was prepared by NH$_4$OH incubation as described under "Experimental Procedures." Immunoreactivity was detected by using affinity-purified anti-HARP immunoglobulin fractions (1 μg/ml) as negative control (B). C, a 1:4 dilution of 2 M NaCl wash of the extracellular matrix of MC 3T3-E1 was incubated with 100 μl of 10% (w/v) suspension of heparin-Sepharose. Bound proteins were eluted with Laemmli sample buffer and were analyzed by Western blot as described under "Experimental Procedures."

Fig. 3. Western blot analysis of the effect of enzymatic treatment on HARP release from extracellular compartments. H-NIH3T3 cells were seeded at 6 × 10$^5$ cells in 150-cm$^2$ tissue culture dishes. Conditioned medium was removed 72 h later. Cells were washed with PBS and incubated for 2 h at 4 °C with DMEM (lane 2), DMEM containing 1.2 international milliunits/ml heparitinase (lane 3), DMEM containing 1.2 international milliunits/ml chondroitinase ABC (lane 4), or DMEM containing 1.2 international milliunits/ml chondroitinase ABC and 20 μg/ml heparin (24). Maximal potentiation was observed at 1 μg/ml, whereas higher concentrations (10 to 100 μg/ml) inhibited the mitogenic activity of HARP. In control experiments, HS used alone (10 ng/ml to 100 μg/ml) had no effect on DNA synthesis. We also tested the effect of KS, CS-A, DS, and CS-C for their ability to modulate the mitogenic activity of HARP. CS-A and DS modulated the mitogenic activity of HARP. Potentiation occurred at concentrations of CS-A and DS between 1 and 100 μg/ml; the maximum effect was obtained at a concentration of 10 μg/ml, leading to a 2-fold increase in thymidine incorporation over that induced by HARP alone. In contrast, KS (Fig. 6B) and CS-C (Fig. 6C) had no effect in the same range of concentrations. The effect of DS on DNA synthesis induced by HARP or FGF2 on BEL cells was also compared (Fig. 6D). At 1 μg/ml, HS leads to a 2- and 1.7-fold stimulation of DNA synthesis by FGF2 and HARP, respectively. As illustrated, CS-A and DS potentiate only the HARP stimulation, and no effect of chondroitin sulfate was observed on FGF2 stimulation of DNA synthesis in the range of concentrations tested (1–10 μg/ml).

Fig. 4. Effect of glycosaminoglycans on HARP release. Cell monolayers of NIH3T3 cells were incubated with various GAGs (10 μg/ml) including heparin (lane 2), HS (lane 3), KS (lane 4), CS-A (lane 5), DS (lane 6), CS-C (lane 7), or with incubation buffer alone (lane 1). Cells were treated as described under "Experimental Procedures," and the presence of HARP in GAG-treated cells (A) or treated with GAGs as described in A following treatment with 2 M NaCl washing buffer (B) was performed by Western blot.

Since GAGs derived from the extracellular compartments are involved in the binding of HARP, we investigated whether the addition of exogenous GAGs modulated the mitogenic activity of HARP. As shown in Fig. 6B, a dual effect (potentiation/inhibition) was observed when increasing concentrations of HS (10 ng/ml to 100 μg/ml) were added, as described previously for heparin (24). Maximal potentiation was observed at 1 μg/ml, whereas higher concentrations (10 to 100 μg/ml) inhibited the mitogenic activity of HARP. In control experiments, HS used alone (10 ng/ml to 100 μg/ml) had no effect on DNA synthesis. We also tested the effect of KS, CS-A, DS, and CS-C for their ability to modulate the mitogenic activity of HARP. CS-A and DS modulated the mitogenic activity of HARP. Potentiation occurred at concentrations of CS-A and DS between 1 and 100 μg/ml; the maximum effect was obtained at a concentration of 10 μg/ml, leading to a 2-fold increase in thymidine incorporation over that induced by HARP alone. In contrast, KS (Fig. 6B) and CS-C (Fig. 6C) had no effect in the same range of concentrations. The effect of DS on DNA synthesis induced by HARP or FGF2 on BEL cells was also compared (Fig. 6D). At 1 μg/ml, HS leads to a 2- and 1.7-fold stimulation of DNA synthesis by FGF2 and HARP, respectively. As illustrated, CS-A and DS potentiate only the HARP stimulation, and no effect of chondroitin sulfate was observed on FGF2 stimulation of DNA synthesis in the range of concentrations tested (1–10 μg/ml).

DISCUSSION

Many growth factors bind to heparin, and the interaction of these growth factors with HSPGs at the cell surface and in the extracellular matrix is a central event in regulating the transport and effector functions of the growth factors. The HS-FGF2 interaction is one of the most extensively investigated and has at least two distinct functions. The first function relates to HS acting as a local storage site for FGF2. Several possible mechanisms has been demonstrated in the release of biologically active FGF2 from HSPGs. FGF2 can be released from the HS by an excess of soluble heparin or HS or by enzymatic digestion of HSPGs. Enzymatic digestion by heparitinase (35, 36), plasmin (37), and phospholipases (38, 39) generates soluble HS-FGF2 complexes that can induce a mitogenic response. Secondly, FGF2 only stimulates a mitogenic response through its high affinity tyrosine kinase receptors if it also interacts with HS (40). Other heparin binding growth factors like transforming growth factor-β1 and -2 (41), exon 6 platelet-derived growth factor (32), vascular endothelium growth factor (31), hepatocyte growth factor/scatter factor (42), and heparin binding -EGF (43) are associated with HSPGs present in the ECM, and the bioavailability and activity of these growth factors is also regulated by HSPGs.

Cells overexpressing HARP were used to study the specific properties of HARP bound to the extracellular compartments. The presence of HARP in the extracellular compartment was confirmed by Western blotting as an 18,000-Da polypeptide...
and by N-terminal sequencing (not shown). Interestingly, 90% of the HARP from the extracellular compartment preparation was inactive in our mitogenic assay. Sequence analysis of this HARP isolated by Mono S chromatography showed that it had the N-terminal sequence NH$_2$-Gly-Lys-Lys-Glu-Lys-Pro. Comparison with the N-terminal sequence of the mitogenic form supports the data that the mitogenicity of HARP is directly or indirectly related to the presence of three N-terminal amino acids (22).

Heparinase or heparitinase treatment of the cells, but not chondroitinase treatment, released the extracellularly stored HARP of H-NIH3T3 cells, indicating that such HARP is associated almost exclusively with HS. In keeping with this conclusion, heparin and HS were able to displace the extracellularly stored HARP of H-NIH3T3 cells. However, DS was also able to displace the extracellularly stored HARP of H-NIH3T3 cells, although other GAGs that were tested were much less effective. This result suggested that HARP may interact with DS, although it is not associated with this molecule in the extracellular compartment of H-NIH3T3 cells.

To further characterize the interactions between HARP and GAGs, in vitro analyses were used to determine the binding parameters of HARP for heparin and DS and to examine the relative ranking of HARP-GAG interactions. The HARP-hepa-

![Image](FIG.5 Interaction of HARP with GAGs. Biotinylated DS (A and D) or heparin (C) was immobilized on a streptavidin-derivatized aminosilane surface as described under “Experimental Procedures.” A, the binding of different concentrations of HARP (14–555 nM) to immobilized DS was followed in real time for 80–110 s, and then, after two quick washes with 200 μl of PBS-T, the dissociation of the bound HARP into 200 μl of PBS-T was followed for the next min. Two independent sets of binding reactions were performed, of which one is presented. B, a plot of $k_{on}$ against ligand concentration yields a straight line ($r = 0.973$), the slope of which corresponds to the association rate constant, $k_{on} = 0.68 (\pm 0.07) \times 10^6$ M$^{-1}$ s$^{-1}$. The $k_{on}$ of HARP for DS at each concentration of HARP was determined using the FastFit software. Competition for HARP bound to immobilized heparin (C) or DS (D) by various GAGs. The binding of HARP (111 nM) was followed in the biosensor in presence of various concentrations of GAGs, as indicated in the figure. [] control; [■] heparin; [□] CS-A; [■] CS-B.

### Table I

| GAGs (μg/ml) | Extent of binding (arc s) |
|-------------|--------------------------|
| 0 0.005 0.5 5 50 500 |                             |

![Image](FIG.5)

**A** Interaction of HARP with GAGs. Biotinylated DS (A and D) or heparin (C) was immobilized on a streptavidin-derivatized aminosilane surface as described under “Experimental Procedures.” A, the binding of different concentrations of HARP (14–555 nM) to immobilized DS was followed in real time for 80–110 s, and then, after two quick washes with 200 μl of PBS-T, the dissociation of the bound HARP into 200 μl of PBS-T was followed for the next min. Two independent sets of binding reactions were performed, of which one is presented. B, a plot of $k_{on}$ against ligand concentration yields a straight line ($r = 0.973$), the slope of which corresponds to the association rate constant, $k_{on} = 0.68 (\pm 0.07) \times 10^6$ M$^{-1}$ s$^{-1}$. The $k_{on}$ of HARP for DS at each concentration of HARP was determined using the FastFit software. Competition for HARP bound to immobilized heparin (C) or DS (D) by various GAGs. The binding of HARP (111 nM) was followed in the biosensor in presence of various concentrations of GAGs, as indicated in the figure. [] control; [■] heparin; [□] CS-A; [■] CS-B.

**B** Extent of binding (arc s) for various concentrations of HARP (14–555 nM) to immobilized DS. Two independent sets of binding reactions were performed, of which one is presented.

**C** Extent of binding (arc s) for various concentrations of GAGs (0–500 μg/ml) to immobilized heparin. **D** Extent of binding (arc s) for various concentrations of GAGs (0–500 μg/ml) to immobilized DS.

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**C** Extent of binding (arc s) for various concentrations of GAGs (0–500 μg/ml) to immobilized heparin. **D** Extent of binding (arc s) for various concentrations of GAGs (0–500 μg/ml) to immobilized DS.

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**C** Extent of binding (arc s) for various concentrations of GAGs (0–500 μg/ml) to immobilized heparin. **D** Extent of binding (arc s) for various concentrations of GAGs (0–500 μg/ml) to immobilized DS.

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**C** Extent of binding (arc s) for various concentrations of GAGs (0–500 μg/ml) to immobilized heparin. **D** Extent of binding (arc s) for various concentrations of GAGs (0–500 μg/ml) to immobilized DS.
GAGs bind to HARP

FIG. 6. Effects of GAGs on the mitogenic activity of HARP. A, HARP activation assays by various concentrations of heparin were carried out using BEL cells treated with 3 international milliunits/ml heparitinase as described under “Experimental Procedures.” [3H]thymidine incorporation is expressed as compared with positive control values. The bars indicate the S.E. of triplicate measurements. B, concentration-response curves of DNA synthesis by cells stimulated (filled symbols) or unstimulated (open symbols) by HARP in the absence of serum and the presence of HS (●, ○) or KS (■, □). C, concentration-response curves of DNA synthesis by cells stimulated (filled symbols) or unstimulated (open symbols) by HARP in the absence of serum and in the presence of CS-A (●, ○), DS (■, □), or CS-C (▲, ●). DNA synthesis was determined by measuring [3H]thymidine uptake from 18 to 24 h after addition of GAGs. D, potentialization by GAGs of DNA synthesis stimulations induced by HARP and FGF2 in BEL cells. Stimulations induced by HARP (filled bars) and FGF2 (open bars) in the presence of various concentrations of HS, CS-A, and DS were compared with the stimulations induced by the growth factors alone and referred as 100% relative DNA synthesis. The bars indicate the S.E. of triplicate measurements.

There is good evidence that extracellular HS-like molecules are required for the mitogenic signal induced by HARP. 1) Like heparin, soluble HS potentiates DNA synthesis induced by HARP; 2) binding to extracellular HS plays an important role in the growth-stimulatory activity of HARP, because treatment with heparitinase prevents this activity. However, the precise mechanism of action of HARP is unknown. Although HSPGs molecules, including members of the syndecan family, bind HARP with relatively high affinity, there is no evidence that these cell surface proteins can act as receptors capable of transducing the HARP mitogenic signal. Intriguingly, CS-A and DS also potentiated HARP-induced stimulation of DNA synthesis in BEL cells, and the equal potency of these GAGs (Fig. 6, C and D) is in contrast to the greater affinity of HARP for DS (Figs. 3 and 5).

It has also been reported that N-syndecan binds HARP with high affinity ($K_d = 600$ nM) (13) and plays an important role in the neurite outgrowth activity induced by HARP. Indeed, heparitinase treatment of cells abolishes the neurite outgrowth induced by HARP, clearly demonstrating the key role of N-syndecan (19) or similar HSPGs in HARP-stimulated neurite outgrowth. Small amounts of exogenous heparin and HS both inhibit HARP-induced neurite outgrowth, with ID$_{50}$ (dose that induced 50% inhibition) values of 25 ng/ml and 700 ng/ml, respectively (47). In this study we found that a low concentration of exogenous GAGs potentiated rather than inhibited the mitogenic effect of HARP, suggesting that HARP mitogenic and neurite outgrowth activities might occur via independent mechanisms.
In addition to modulating the mitogenic activity of HARP, DS and HS are likely to regulate the diffusion of HARP within and between cellular compartments. Heparin and DS bind HARP with different affinities and kinetics. Therefore, the relative distribution of HS and DS in mesenchyme, basement membrane, and on epithelial cells at particular stages of tissue development could either be permissive or nonpermissive for the diffusion of HARP from the mesenchyme to the epithelium. Studies would result in an accumulation of HARP in the basement membrane coupled to predominantly DS binding sites on target cells and between cellular compartments. Heparin and DS bind GAGs bind to HARP 7747

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