Histidine-Rich Glycoprotein and Platelet Factor 4 Mask Heparan Sulfate Proteoglycans Recognized by Acidic and Basic Fibroblast Growth Factor

Kathryn J. Brown* and Christopher R. Parish

Division of Cell Biology, John Curtin School of Medical Research, Australian National University, Canberra ACT 2601, Australia

Received April 25, 1994; Revised Manuscript Received August 22, 1994[⊗]

ABSTRACT: Recent studies have shown that fibroblast growth factors (FGFs) need to interact with cell-surface heparan sulfate proteoglycans (HSPGs) in order to bind to and activate FGF receptors. In this paper, three major heparin-binding proteins, histidine-rich glycoprotein (HRG) and antithrombin III (ATIII), which are constitutively present at high concentrations in plasma, and platelet factor 4 (PF4), which is released locally at high concentrations by degranulating platelets, were tested for their ability to act as modulators of FGF activity by competing with the FGFs for cell-surface HSPGs. HRGs from both chicken and human, and human PF4, were demonstrated to compete with each other and with acidic FGF (aFGF) and basic FGF (bFGF) for binding to BALB/c 3T3 cell-surface HSPGs, whereas ATIII did not compete. Thus, HRG, PF4, aFGF, and bFGF all interact with the same HS chains on the 3T3 cell surface, either binding to the same or binding to adjacent saccharide sequences on the chains. In terms of their relative binding affinity for cell-surface HSPGs, the hierarchy was shown to be PF4 ≥ bFGF > aFGF = cHRG > hHRG. HRG was also shown to significantly inhibit both FGF-stimulated and endogenous 3T3 cell DNA synthesis. HRG also binds to extracellular matrices (ECM), originating from bovine corneal endothelial cells, in a heparin-inhibitable manner. Indeed, both HRG and PF4, at physiological concentrations, were shown to effectively inhibit the binding of ^{125}I-aFGF and ^{125}I-bFGF to ECM. In addition, HRG was able to displace biologically active bFGF from the ECM. On the basis of these findings, it is proposed that HRG and PF4 may act as positive regulators of FGF activity by displacing FGF from the ECM or basement membrane and making FGF available to responsive cells. Alternatively, they could act as negative regulators by masking HSPGs on responsive cells and preventing FGF receptor activation.

It is becoming increasingly clear that heparan sulfate proteoglycans (HSPGs)[1] play a major role in regulating fibroblast growth factor (FGF) activity. For example, it is now known that the interaction between FGF and cell-surface HSPG is essential for the binding of acidic and basic FGF to their signal-transducing receptors (Yayon et al., 1991; Olwin & Rapraeger, 1992). In addition, HSPGs sequester FGFs and store them in a stable and protected form in the extracellular matrix (ECM) (Jeanny et al., 1987; Folkman et al., 1988). There is also evidence to suggest that the interaction between HSPGs and FGFs may exhibit considerable specificity with acidic FGF (aFGF) and basic FGF (bFGF), possibly interacting with related but subtly different HS sequences (Turnbull et al., 1992; Mach et al., 1993; Nurcombe et al., 1993).

In addition to the FGFs, there are three well-characterized heparin-binding proteins present in plasma which could compete with acidic and basic FGF for binding to HSPG present on the surface of BALB/c 3T3 cells. The three heparin-binding proteins are histidine-rich glycoprotein (HRG), platelet factor 4 (PF4), and antithrombin III (ATIII). If able to compete with FGFs for binding to cell-surface HSPGs, it is possible that these molecules may be able to maintain an equilibrium in which FGFs are only able to access their receptors when their activity is specifically required. Histidine-rich glycoprotein (molecular mass 75 kDa) (Haupt & Heimburger, 1972) is present in mammalian plasma at concentrations as high as 100 μg/mL and, thus, is a major heparin-binding protein in plasma (Lijnen et al., 1983a,b). HRG contains a remarkably high content of histidine and proline residues, these residues comprising approximately 20% of its amino acids (Heimburger et al., 1972). HRG has been shown to exhibit a number of biological effects which include neutralization of the anticoagulant activity of heparin (Lijnen et al., 1983b) and neutralization of the ability of heparin to inhibit smooth muscle cell proliferation (Hajjar et al., 1987).

Platelet factor 4 exists as a high molecular weight complex of a tetramer of PF4 polypeptide and chondroitin sulfate (Barber et al., 1972; Moore et al., 1975) in the α-granules of platelets. It also has a high affinity for heparin (Maione et al., 1990), eluting from heparin−Sepharose with 1.5 M NaCl (Niewiarowsky et al., 1976). Although normally found

* Address correspondence to this author. Telephone: +61 6 249 2604. Fax: +61 6 249 2595.

[⊗] Abstract published in *Advance ACS Abstracts,* October 15, 1994.

[1] Abbreviations: aFGF, acidic fibroblast growth factor; ATIII, antithrombin III; bFGF, basic fibroblast growth factor; BM, basement membrane; BSA, bovine serum albumin; cHRG, chicken histidine-rich glycoprotein; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ECM, extracellular matrix; FACS, fluorescence-activated cell sorter; GAG, glycosaminoglycan; GlcAc, glucuronic acid; GlcN, D-glucosamine; HBSS, Hanks BSS/0.1% BSA, pH 7.0; hHRG, human histidine-rich glycoprotein; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; IdoA, iduronic acid; PBS, phosphate-buffered saline; PF4, platelet factor 4; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

0006-2960/94/0433-13918$04.50/0 © 1994 American Chemical Society

in human plasma at concentrations as low as 10 ng/mL, concentrations as high as 25 μg/mL are thought to be achieved at sites of vascular injury where platelets aggregate and the α-granules are released (Files et al., 1981). The physiological significance of PF4 is uncertain although it has been shown to inhibit angiogenesis (Taylor & Folkman, 1982).

Antithrombin III, present in plasma at concentrations of 100−200 μg/mL (Murano et al., 1980), is the major inhibitor of the activated serine proteases of the blood coagulation cascade. The binding of heparin to ATIII greatly facilitates the inactivation of the enzymes involved in the coagulation cascade (Bjork & Nordenman, 1976).

This paper describes studies in which we examined whether chicken and human HRG, human ATIII, and human PF4 interact with the same or different HSPGs as do acidic and basic FGF, on the 3T3 cell surface and in the ECM. The ability of HRG and PF4 to inhibit FGF-induced 3T3 cell DNA synthesis and of HRG to displace bFGF from the ECM was also investigated.

MATERIALS AND METHODS

Growth Factors and Heparin-Binding Proteins. Human recombinant acidic and basic fibroblast growth factors were obtained from BioSource International (Camarillo, CA) and PeproTech Inc. (Rocky Hill, NJ). Acidic and basic FGFs were reconstituted at 200 μg/mL in PBS/0.1% CHAPS to prevent adherence to the walls of the tubes and aliquots stored in polyethylene tubes (Kartell, Milan, Italy) at −70 °C. Frozen aliquots were not thawed more than once and were not used any longer than 2 weeks after being thawed. ^{125}I-aFGF(1234 Ci/mmol) and ^{125}I-bFGF (920 Ci/mmol) were obtained from Amersham International plc, Amersham, U.K. ^{125}I-aFGF was resuspended in distilled water to a final concentration of 600 ng/mL, and aliquots were frozen. ^{125}I-bFGF was resuspended in distilled water to give a final concentration of 200 ng/mL, and aliquots were frozen.

Human antithrombin III (ATIII) and human platelet factor 4 (PF4) were obtained from Sigma Chemical Co., St. Louis, MO. ATIII was reconstituted in distilled water to give a final concentration of 143 μg/mL. PF4 was desalted and resuspended in PBS to give a final concentration of 100 μg/mL. Human (hHRG) and chicken (cHRG) HRGs were prepared from human and chicken plasma by phosphocellulose ion-exchange chromatography (Rylatt et al., 1981). The purity of the two HRG preparations was demonstrated by SDS−PAGE, in each case a single Coomassie Blue stained band being detected. Human HRG has a molecular mass of approximately 75 kDa whereas chicken HRG is a somewhat larger molecule with a molecular mass of approximately 135 kDa. Chicken HRG was radiolabeled with ^{125}I by the iodogen method as previously described by Glabe et al. (1983).

Polysaccharides. Heparin (bovine lung), heparan sulfate (bovine kidney), heparan sulfate fast-moving fraction (bovine intestinal mucosa), heparan sulfate (bovine intestinal mucosa), hyaluronic acid (human umbilical cord), chondroitin 4-sulfate (whale cartilage), chondroitin 6-sulfate (whale cartilage), dermatan sulfate (porcine skin), and keratan sulfate (bovine cornea) were all obtained from Sigma Chemical Co. Sodium sucrose octasulfate was a generous gift from Bukh meditec A/S, Denmark. Heparan sulfate (porcine mucosal, 32 kDa) was a generous gift from Organon International bv, Oss, The Netherlands.

Cell Culturing. Mouse BALB/c 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; gibco, Gaithersburg, MD) supplemented with $NaHCO_3$, 1% L-glutamine, 10 mM HEPES, pH 7.4, and 10% fetal calf serum (FCS; Commonwealth Serum Laboratories, Melbourne, Australia) at 37 °C (5% CO_2 incubator) in 80 cm^2 tissue culture flasks (Nunc, Roskilde, Denmark). Cell monolayers were released for subculturing with 0.1% trypsin (Cytosystems, Sydney, Australia) and 0.1% EDTA in PBS when cells were subconfluent (every 3 days), and cells were then resuspended at 1.5×10^4 cells/mL in culture medium. Confluent monolayers used in binding and mitogenic assays were prepared by seeding 3×10^3 cells/well in 96-well plates (Nunc) in the same medium as used for subculturing, and incubating at 37 °C for 4−5 days. For mitogenic assays, cells were serum-starved for a minimum of 24 h before the addition of treatments.

Binding of Radiolabeled Acidic and Basic FGF to BALB/c 3T3 Cells. ^{125}I-FGF binding was conducted with confluent BALB/c 3T3 cells. Prior to the initiation of ^{125}I-FGF binding, the monolayers were washed once with 200 μL/well of ice-cold binding buffer (DMEM/1% L-glutamine/0.1% BSA/10 mM HEPES, pH 7.4) and then incubated at 4 °C for 10 min to precool the monolayers.

To determine the ability of cHRG, hHRG, ATIII, PF4, heparin, aFGF, and bFGF to inhibit the binding of ^{125}I-aFGF and ^{125}I-bFGF to the 3T3 cell surface, cells were incubated with 95 μL/well of each inhibitor at the concentration indicated for 1 h on ice before adding 5 μL of either ^{125}I-aFGF or ^{125}I-bFGF to each well to give a final concentration of 10 ng/mL ^{125}I-FGF. The plate was incubated for a further 2 h on ice before aspirating unbound ^{125}I-FGF and washing cells 3 times with ice-cold Hanks BSS/0.1% BSA (HBSS). Cells were lysed by incubation with 100 μL/well of PBS/0.5% Triton X-100 for 30 min, mixed, and transferred to counting tubes for counting by a γ counter.

Binding of Radiolabeled aFGF, bFGF, and cHRG to Extracellular Matrices. Extracellular matrices (ECMs) were prepared as follows: bovine corneal endothelial cells at passages 6−12 were seeded into 96-well plates (Nunclon) at a concentration of 1.25×10^5 cells/mL. Cells were grown to confluence and confluent monolayers denuded of cells by incubating monolayers with 150 μL/well of 0.02 M ammonium hydroxide for 5 min at room temperature, followed by a PBS rinse and incubation of each well with 150 μL of 0.5% Triton X-100 in PBS for 30 min at 37 °C. Lysed cells were removed to expose the subendothelial matrix. ECMs were stored in PBS/0.1% azide at 4 °C until required and washed 3 times with 200 μL/well of PBS just prior to use. To determine the relative affinities of ^{125}I-HRG, ^{125}I-aFGF, and ^{125}I-bFGF for ECM HSPGs, serial dilutions of each radiolabeled molecule (supplemented with unlabeled molecule) were made in HBSS, and 100 μL of each dilution was mixed with 100 μL of medium or medium containing heparin (500 μg/mL). Following incubation for 1 h on ice, the mixtures (50 μL/well) were transferred to ECM-containing wells and incubated for 2 h on ice. The unbound radiolabeled molecules were removed by aspiration, and the wells were washed 3 times with 200 μL/well of ice-cold HBSS. The ECMs and bound radiolabeled molecules were solubilized by incubating each well with 200 μL of 4 M

guanidine hydrochloride/2% Triton X-100, overnight at 4 °C. The amount of radiolabeled aFGF, bFGF, and cHRG associated with the ECM was then determined by a γ counter.

In binding−inhibition experiments, 50 μL of either cHRG, hHRG, PF4, ATIII, heparin, aFGF, or bFGF was added per well to give the final concentration required. The inhibitors were allowed to bind to the ECM for 1 h at 4 °C before 5 μL/well of either ^{125}I-bFGF (22 ng/mL) or ^{125}I-aFGF (440 ng/mL) was added to each well. Incubation was for 2 h at 4 °C before the reaction was terminated with the removal of unbound growth factor by washing and the release of radiolabeled growth factor bound to the ECM, as described above.

Ability of HRG To Displace Biologically Active bFGF from the ECM. Basic FGF (10 μg/mL; 1 mL/well) was incubated with ECM in a 6-well plate for 1 h at 4 °C. Control wells received 1 mL of DMEM per well. After incubation, all wells were washed 3 times with DMEM and incubated with DMEM for a further 1 h at 4 °C. Wells were washed again to remove any released bFGF before the addition of either medium alone or cHRG (100 μg/mL; 1 mL/well) to both control or bFGF-pretreated wells. Wells were then incubated for 2 h at 4 °C. The supernatants were removed and serially diluted in DMEM, 1% L-Gln, and 10 mM HEPES, pH 7.4, and 100 μL aliquots of each dilution were added in triplicate to confluent monolayers of serum-starved BALB/c 3T3 cells in 96-well plates. The plates were incubated for 48 h, with [*methyl*-^{3}H]thymidine (0.5 μCi/well) (60 Ci/mmol) (Amersham) being added for the final 24 h. Incubation was stopped by freezing and thawing the cultures 3 times, the assay harvested with a 1295-004 Betaplate 96-well harvester (Pharmacia, Uppsala, Sweden), and the radioactivity counted with a liquid scintillation counter (Pharmacia).

Ability of HRG To Inhibit FGF-Induced DNA Synthesis in BALB/c 3T3 Cells. Serial dilutions of cHRG (1 mg/mL initial concentration) in DMEM, 1% L-Gln, 20 μM $ZnSO_4$, and 10 mM HEPES, pH 7.4, were made and 50 μL aliquots added in triplicate to confluent monolayers of serum-starved 3T3 cells. Zinc was included in the medium as it enhances the binding of HRG to heparin/HS structures (Kazama & Koide, 1992). The HRG was allowed to bind to the cells for 1 h at 4 °C before 50 μL aliquots of aFGF (20 ng/mL) or bFGF (2 ng/mL) were added to wells containing either HRG or medium. Control wells contained HRG without growth factor or medium alone. Incubation was for 48 h at 37 °C and DNA synthesis measured by [*methyl*-^{3}H]thymidine incorporation as described above.

Rose Bengal Cell Adhesion Assay. A modified version of the cell adhesion assay developed by Ishihara et al. (1992) was used to investigate the interaction of BALB/c 3T3 cell-surface HSPG with FGFs immobilized on plastic. Ninety-six-well round-bottom poly(vinyl chloride) (PVC) microtiter plates (Dynatech Laboratories, Chantilly, VA) were coated with 50 μL/well of either aFGF (2.5 μg/mL), bFGF (0.313 μg/mL), cHRG (10 μg/mL), or hHRG (5 μg/mL), overnight at 4 °C. Wells were aspirated, the plate was dunked twice in a PBS bath, and nonspecific binding sites were blocked by incubation with HBSS at 37 °C for 1 h. BALB/c 3T3 cells were suspended in HBSS at a cell density of 2.5×10^6 cells/mL; 0.1 mL (2.5×10^5 cells) was applied to each coated well and incubated at 37 °C for 1 h. The plate was flicked to remove unbound cells and 100 μL/well of 0.25% Rose Bengal dye (Koch-Light Laboratories Ltd., Colnbrook, Berks, England) in PBS added for 3 min at room temperature. Rose Bengal stains the nuclei and cytoplasm of both live and dead cells (O'Neill & Parish, 1983). The unadsorbed dye was removed by flicking the plate and dunking it twice in two separate PBS baths. The plate was allowed to drain before adding 200 μL/well of 50% ethanol in PBS. Each well received a constant amount of mixing with a multichannel pipet to allow liberation of the dye from the cells. Nonspecific binding of the dye to HRG and FGF-coated and uncoated wells in the absence of cells was also determined and subtracted from experimental points. The relative number of cells in each well was quantified by determination of the optical density of each well (λ_1 = 540 nm, λ_2 = 650 nm) using a Thermomax microplate reader (Molecular Devices, Menlo Park, CA).

To determine the optimal cell concentration to use in this assay, doubling dilutions of cells in HBSS were prepared, ranging between 1×10^6 cells/well and 3.9×10^3 cells/well, and incubated with HRG-coated (10 μg/mL) wells for 1 h at 37 °C. To determine the optimal concentrations of cHRG and hHRG with which to coat the wells, wells were coated with a range of concentrations of either cHRG or hHRG prior to the addition of cells as described above.

The ability of various glycosaminoglycans (100 μg/mL) to inhibit the binding of cell-surface HSPGs to immobilized cHRG and hHRG was also tested. Glycosaminoglycans tested were heparin, HS from bovine kidney, bovine intestinal mucosa, and porcine mucosa, hyaluronic acid, chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, keratan sulfate, and sodium sucrose octasulfate. Fifty microliters per well of glycosaminoglycan was incubated with HRG-coated wells for 1 h at 4 °C before 50 μL of cells (2.5×10^5 cells/well) was added to each well and incubated at 37 °C for 1 h. To titrate the inhibitory activity of some GAGs or proteins, doubling dilutions of each inhibitor were prepared in HBSS, and 50 μL/well of each concentration was aliquoted in triplicate into HRG-coated wells and the same procedure followed as described above. Control wells received medium without inhibitor.

To determine whether PF4, ATIII, cHRG, hHRG, aFGF, and bFGF cross-react with the same HSPGs on the 3T3 cell surface, each of these factors was tested for their ability to inhibit the binding of 3T3 cells to PVC plates coated with one of the following: aFGF, bFGF, cHRG, or hHRG. Serial dilutions of each of PF4, cHRG, hHRG, aFGF, and bFGF were prepared and equal volumes of each dilution incubated with an equal volume of cells for 2 h at 4 °C before aliquoting 100 μL of each sample to triplicate wells coated with HRG. Control wells received cells which had been preincubated with medium in place of growth factor.

Heparanase Treatment of Cells. Confluent monolayers of BALB/c 3T3 cells were released with 0.4% EDTA in PBS and 1.3×10^6 cells resuspended in approximately 47 units of heparinase 1 (EC 4.2.2.7; Sigma) in 20 mM HEPES, pH 7, 2 mg/mL BSA, and normal saline to give a final volume of 200 μL. Control cells were resuspended in the same buffer without heparinase. The cells were rotated slowly for 1 h at 37 °C, washed twice, and resuspended in HBSS (1×10^6 cells/mL) before 0.1 mL of cells was applied to each cHRG-coated well and the Rose Bengal adhesion assay performed, as described above. Heparinase treatment had no effect on cell viability. The specificity of the heparinase

was demonstrated by the ability of heparin to partially inhibit the effect of the enzyme.

Fluorescence Flow Cytometry. In order to confirm the results of the Rose Bengal plate assays, an assay was developed which utilized the ability of a FACScan to quantify the binding of a fluoresceinated form of cHRG to 3T3 cells. FITC conjugation of chicken HRG was performed as follows: 1 mL of cHRG (2.14 mg/mL) in PBS was dialyzed overnight at 4 °C against 500 mL of 0.05 M boric acid/0.2 M NaCl, pH 9.2. Ten milligrams of fluorescein 5-isothiocyanate (FITC; Molecular Probes, Eugene, OR) was dissolved in 2.0 mL of DMSO and 50 μL of FITC−DMSO immediately added to the cHRG solution. Incubation was for 2 h at room temperature in the dark. The sample was applied to a PD-10 column and fluorescent HRG eluted in PBS to give a final concentration of 1 mg/mL.

To test the ability of cHRG, hHRG, aFGF, bFGF, and PF4 to inhibit the binding of FITC−cHRG to 3T3 cells, cells were grown to confluence in DMEM/20% FCS/1% L-glutamine/10 mM HEPES, detached with PBS/EDTA, and adjusted to 5×10^6 cells/mL in HBSS. To wells of a 96-well V-bottom plate (Serocluster Costar, Cambridge, MA) were added 20 μL of cells and 20 μL of inhibitor, spanning the concentration ranges required. The plate was mixed gently and incubated on ice for 30 min. Two microliters of FITC−cHRG (1 mg/mL) was added to each well, and after further mixing, the plate was incubated on ice for 1 h. Unbound FITC−cHRG was removed by washing the wells 3 times with 150 μL/well of ice-cold HBSS, the cells being pelleted by a GS-6R centrifuge (Beckman, Sydney, Australia) at 200*g* for 1 min at 4 °C. Cell pellets were resuspended in 100 μL of ice-cold HBSS, and the fluorescence intensity of each sample was determined by a FACScan (Becton Dickinson, Mountain View, CA). Control samples contained medium in place of inhibitor, or medium in place of FITC−cHRG.

To test the ability of heparin to inhibit the binding of FITC−cHRG to 3T3 cells, serial dilutions of 20 μL of FITC−cHRG were made in a V-bottom plate, and 2 μL of heparin (2 mg/mL) was added to each well. After the plate was incubated on ice for 30 min, 20 μL of 3T3 cells (5×10^6 cells/mL) was added to each well. Control samples included HRG in the absence of heparin, and cells in the presence and absence of heparin without HRG. The plate was mixed gently and incubated for a further 30 min on ice. Unbound FITC−cHRG were removed by washing the plate 3 times in the same manner as described above, and fluorescence was quantified by a FACScan.

RESULTS

Binding of HRGs to Cell-Surface HSPGs. In order to examine the interaction of chicken and human HRG with BALB/c 3T3 cell-surface HSPGs, an assay based on that of Ishihara et al. (1992) was developed. The assay entailed immobilizing HRG in PVC plastic wells and quantifying cell adhesion to the HRG using the vital stain Rose Bengal. In initial experiments, the optimum concentration of chicken and human HRG for coating PVC plates for use in the adhesion assay was determined (data not shown). It was found that both HRGs could mediate BALB/c 3T3 cell adhesion when immobilized to plastic, although lower coating concentrations of cHRG than hHRG were required for maximum binding. In subsequent assays, cHRG was used at 10 μg/mL and hHRG at 5 μg/mL to coat the plates. An interesting feature of this adhesion assay was that plastic-immobilized HRG is able to act as a spreading factor for BALB/c 3T3 cells in the absence of fetal calf serum. After 2 h incubation at 37 °C, cells added to an HRG-coated dish had attached quite firmly to the HRG substrate and had changed from a rounded to a more spread-out morphology. In contrast, cells seeded on an uncoated dish remained rounded and showed no inclination to attach to the plastic surface (data not shown).

Two lines of evidence were obtained which indicated that the immobilized HRGs were interacting with HS structures on the surface of BALB/c 3T3 cells. First, pretreatment of the 3T3 cells with bacterial heparinase I resulted in a 97% inhibition of adherence of the cells to HRG-coated plates. Second, heparin was a potent inhibitor of the binding of 3T3 cells to immobilized HRG, virtually complete inhibition of cell binding to both cHRG and hHRG occurring at heparin concentrations >0.8 μg/mL (data not shown). Subsequently, various other GAGs were tested for their inhibitory activity (100 μg/mL). Only heparin, fast-moving HS, and bovine intestinal HS were able to inhibit HRG binding (data not shown). However, fast-moving HS and bovine intestinal HS were much less effective inhibitors than heparin, requiring concentrations as high as 100−300 μg/mL to completely inhibit cells from binding to the immobilized HRGs. The lower inhibitory activity of the two HS for human and chicken HRG may be because the particular HS motifs on the HS chain with which the HRGs interact occur less frequently in these HS than they do in heparin.

Inhibition of Binding of Heparin-Binding Proteins to Cell-Surface HSPGs. A series of cross-blocking studies were carried out using the plate-binding assay to determine whether the FGFs and the heparin/HS-binding proteins, HRG, ATIII, and PF4, bind to similar or different HS structures on the surface of BALB/c 3T3 cells. Chicken and human HRG, acidic and basic FGF, and PF4 were found to totally block 3T3 cell binding to each of the HRGs immobilized on the plate (Figure 1). These data suggest that the HRGs, FGFs, and PF4 interact either with the same HS sequence or with closely associated HS sequences on the 3T3 cell surface, although based on the inhibition curves, each factor exhibits a different affinity for 3T3 HSPGs.

Table 1 compares the concentration of each heparin-binding factor required to inhibit 3T3 cell binding by 50% and may only be compared within each data column due to different concentrations of factor used to coat the plates. ATIII was completely unable to inhibit 3T3 cells from binding to immobilized FGFs, or HRGs. This result indicates that the FGFs and HRGs interact with HS structures on 3T3 cells which differ from the pentasaccharide sequence in heparin and HS with which ATIII is known to bind. On the basis of the inhibition data presented in Table 1, the overall hierarchy of inhibition for the five inhibitory factors was PF4 ≥ bFGF > aFGF = cHRG > hHRG. This hierarchy was generally maintained irrespective of the heparin-binding protein immobilized on the plate. Thus, the general trend suggests that PF4 and bFGF bind cell-surface HSPGs with the highest affinity while cHRG generally exhibits a lower affinity but always an affinity greater than that exhibited by hHRG. Direct comparisons of aFGF and cHRG showed some variation as to which was the more potent inhibitor,

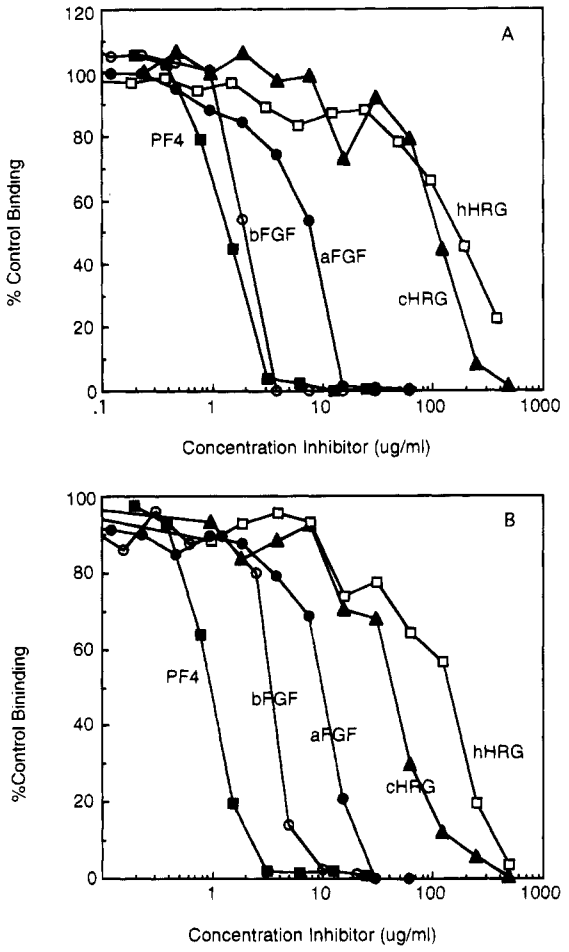

FIGURE 1: Comparison of the ability of soluble PF4 (■), bFGF (○), aFGF (●), cHRG (▲), and hHRG (□) to inhibit the binding of BALB/c 3T3 cells to immobilized (A) cHRG (10 μg/mL) and to (B) hHRG (5 μg/mL). Cells and inhibitors were incubated together for 2 h on ice before being added to HRG-coated wells for a further 1 h incubation at 37 °C, 100% binding representing that occurring in the absence of inhibitor. Values represent the mean of triplicate treatments. Standard errors of means were <20%.

Table 1: Ability of Different Heparin-Binding Molecules To Inhibit Binding of FGFs and HRGs to 3T3 Cells

	molecule bound to plate			
inhibitor	bFGF	aFGF	cHRG	hHRG
PF4	64[a]	42	33	21
bFGF	34	200	114	200
aFGF	125	1875	500	625
cHRG	148	815	940	407
hHRG	2667	5333	2400	2000
ATIII	>16000	>16000	>16000	>16000

[a] Values represent the concentration (nanomolar) required to inhibit BALB/c 3T3 cell binding by 50%, based on the molecular masses of inhibitors being 31.2 kDa for PF4, 17.5 kDa for bFGF, 16 kDa for aFGF, 135 kDa for cHRG, 75 kDa for hHRG, and 62.3 kDa for ATIII.

but aFGF always bound HSPGs with lower affinity than did bFGF and PF4.

Since all of the inhibition assays described above were performed at 37 °C with plastic-immobilized heparin-binding molecules, it was important to determine whether similar results could be obtained at 4 °C with heparin-binding molecules in solution. Such studies were performed with soluble, FITC-conjugated, cHRG, and cell binding was detected by fluorescence cytometry. Figure 2 shows that

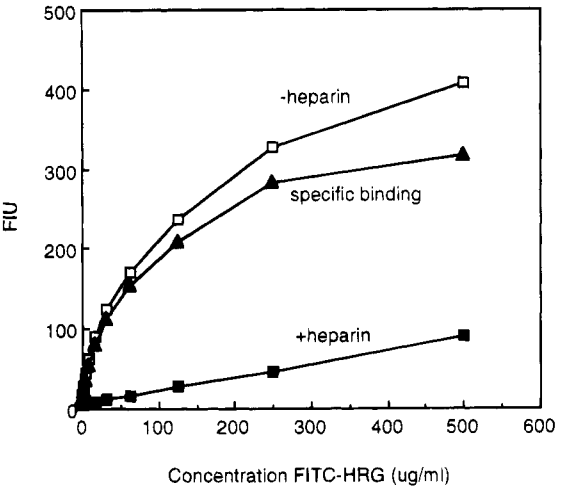

FIGURE 2: Ability of FITC−cHRG to bind BALB/c 3T3 cells in the presence and absence of heparin (200 μg/mL). FITC−cHRG and heparin were incubated together for 30 min on ice before being added to cells and incubated for a further 30 min on ice. After washing, the FITC−cHRG remaining bound to the cells in the presence and absence of heparin was determined by a FACScan. The amount of FITC−cHRG bound to cells is given in fluorescence intensity units (FIU).

the binding of soluble FITC−cHRG to 3T3 cells at 4 °C is both saturable and heparin-inhibitable. This confirms the result seen in the plate assay where heparin was a potent inhibitor of 3T3 cell binding to immobilized cHRG at 37 °C. Binding−inhibition experiments with soluble FITC−cHRG (Figure 3) showed that FGFs and HRGs totally block FITC−cHRG binding to 3T3 cells with a hierarchy of inhibition (i.e., bFGF > aFGF = cHRG > hHRG) similar to that observed with the plate-binding assay. An additional experiment also demonstrated that PF4 totally inhibited FITC−cHRG binding (data not shown).

The inhibition of binding of soluble radiolabeled acidic and basic FGF to 3T3 cell-surface HSPGs was also examined. This assay had the advantage that physiological concentrations (10 ng/mL) of the FGFs were used with physiologically relevant concentrations (i.e., micromolar) of the HRGs, ATIII and PF4, which were added as inhibitors. Figure 4B compares the ability of bFGF, aFGF, cHRG, hHRG, and ATIII to inhibit the binding of 10 ng/mL ^{125}I-bFGF to the 3T3 cell surface. The results are in agreement with those obtained with the Rose Bengal plate assays (Table 1). Basic FGF was the most potent inhibitor of ^{125}I-bFGF binding, reducing binding to <25% of that observed in the absence of inhibitor. Acidic FGF, cHRG, and hHRG were somewhat less effective inhibitors, reducing binding to approximately 40−60% of the control. In agreement with the Rose Bengal plate assay, ATIII was unable to significantly inhibit the binding of ^{125}I-bFGF to the 3T3 cell surface. The ability of heparin to inhibit ^{125}I-bFGF binding by >80% indicates the heparin/HS dependence of the binding being observed. Similar inhibition results were obtained with ^{125}I-aFGF (Figure 4A). Heparin, the FGFs, and HRGs as well as PF4 significantly inhibited the binding of ^{125}I-aFGF to 3T3 cells, whereas ATIII was again inactive. These data confirm that all of the heparin-binding molecules, except ATIII, interact with the same species of HSPG on the 3T3 cell surface and very likely with the same or adjacent HS sequences.

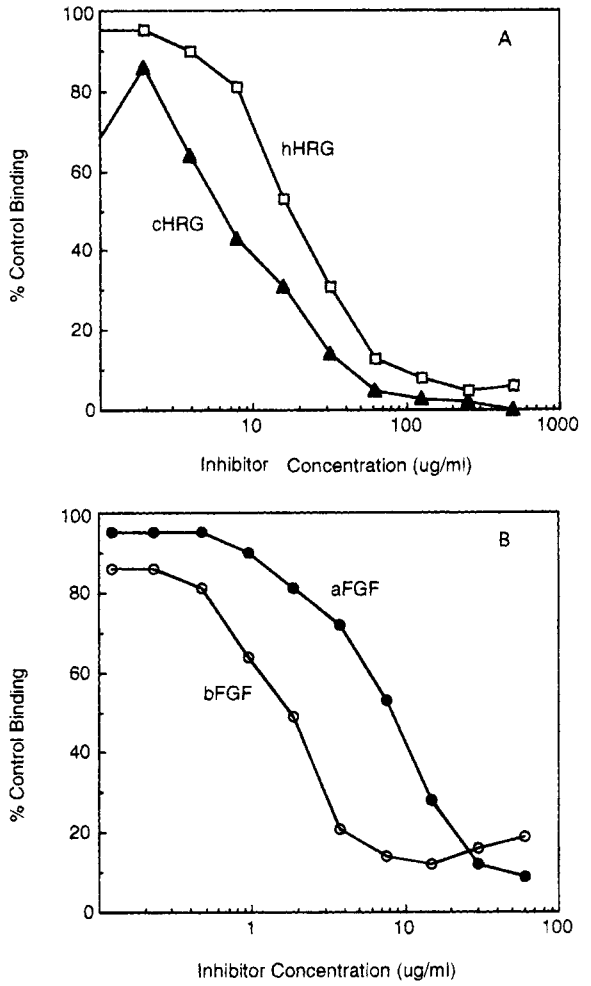

FIGURE 3: Ability of different concentrations of (A) cHRG (▲) and hHRG (□) and (B) aFGF (●) and bFGF (○) to inhibit the binding of FITC−cHRG (50 μg/mL) to BALB/c 3T3 cells. cHRG, hHRG, aFGF, or bFGF were incubated with BALB/c 3T3 cells for 30 min on ice before FITC−cHRG was added to each sample and incubated for a further 1 h on ice. After washing, the FITC−cHRG remaining bound to the cells in the presence and absence of inhibitor was determined by a FACScan. The amount of FITC−cHRG bound to cells is expressed as a percent of control binding, i.e., that occurring in the absence of inhibitor.

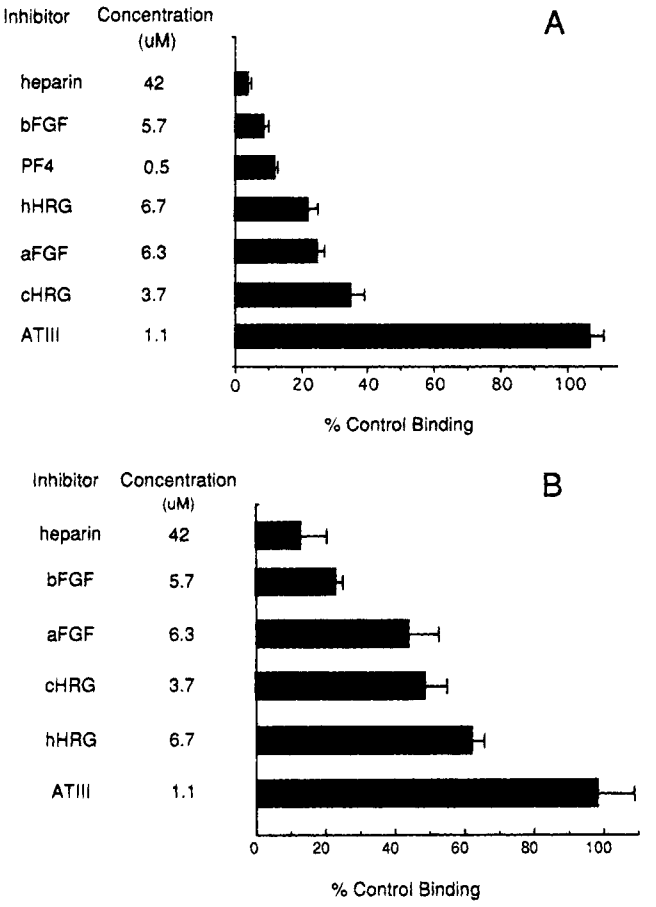

FIGURE 4: Ability of heparin, bFGF, aFGF, cHRG, hHRG, ATIII, and PF4 to inhibit the binding of (A) ^{125}I-aFGF (10 ng/mL; 0.63 nM), and of heparin, bFGF, aFGF, cHRG, hHRG, and ATIII to inhibit the binding of (B) ^{125}I-bFGF (10 ng/mL; 0.57 nM), to BALB/c 3T3 cells. Cells were incubated with inhibitors for 1 h on ice before the addition of ^{125}I-aFGF or ^{125}I-bFGF, and a further 2 h incubation on ice. After washing away unbound ^{125}I-FGF, the amount of cell-associated radioactivity was determined by a γ counter. ^{125}I-FGF binding to the cell surface is presented as percent control binding, i.e., ^{125}I-FGF binding occurring in the absence of inhibitor. Each value represents the mean of triplicate treatments ± SEM. The protein inhibitors, PF4, HRG, and ATIII were used at physiological concentrations.

Inhibition of Binding of Heparin-Binding Proteins to HSPGs in the ECM. Since FGFs have been shown to be associated with ECM HSPGs *in vivo* (Jeanny et al., 1987; Folkman et al., 1988), it was of interest to assess whether the different heparin-binding proteins could inhibit the binding of radiolabeled FGFs to the ECM. Initial experiments examined the binding of radiolabeled FGFs and HRG to bovine corneal endothelial cell ECM in the presence and absence of a high concentration of heparin. Scatchard analysis of the experimental data found heparin-inhibitable binding of ^{125}I-aFGF, ^{125}I-bFGF, and ^{125}I-cHRG to the ECM to have dissociation constants of 2.7 ± 0.5, 1.7 ± 0.2, and 146 ± 37 nM, respectively. In most cases, heparin inhibited binding of radiolabeled aFGF, bFGF, and cHRG by >80%. However, at concentrations of ^{125}I-cHRG >30 μg/mL, binding could not be inhibited by heparin, which may indicate that at high concentrations cHRG binds to low-affinity, non-HSPG, sites in the ECM. Thus, the reason for HRG's relatively high binding affinity, compared with the FGFs, may be due to it binding cooperatively to HSPG and non-HSPG sites in the ECM. Such a dual interaction may not occur at the cell surface.

In order to determine whether physiological concentrations of molecules such as PF4 and HRG could act as *in vivo* regulators of FGF activity by modulating FGF uptake and release from the ECM, their ability to inhibit the binding of physiologically relevant concentrations of ^{125}I-aFGF and ^{125}I-bFGF to the ECM was determined. Figure 5A,B shows that bFGF, aFGF, PF4, cHRG, and hHRG are all effective inhibitors of ^{125}I-aFGF (Figure 5A) and ^{125}I-bFGF (Figure 5B) binding to the ECM, usually reducing binding by 60−90%. As observed in other assays, ATIII showed no inhibitory activity. The high degree of inhibition by heparin (70% with bFGF and 95% with aFGF) demonstrated the heparin/HS dependence of the binding assay. These results show that HRG and PF4, at concentrations approaching the physiological level, are able to substantially reduce the binding of ^{125}I-aFGF (40 ng/mL) and ^{125}I-bFGF (2 ng/mL) to the ECM.

The ability of HRG to release biologically active bFGF from the ECM was also tested. In these experiments, bovine

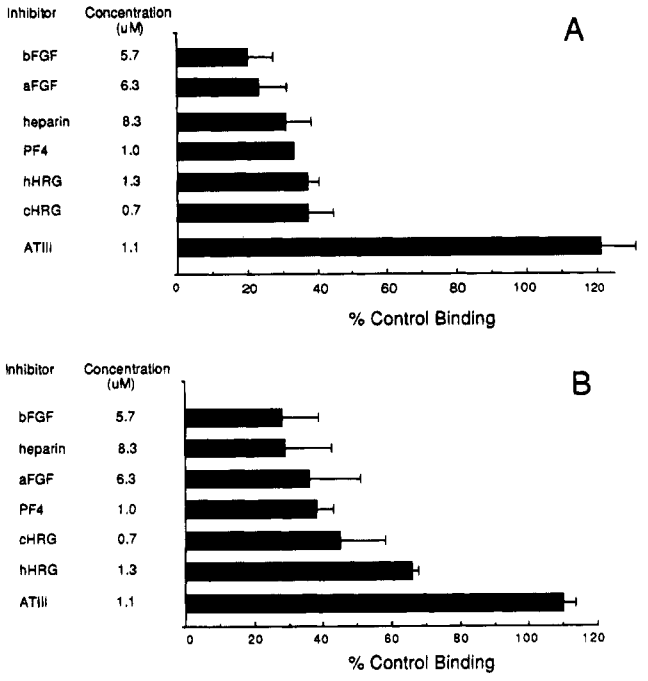

FIGURE 5: Ability of heparin, bFGF, aFGF, cHRG, hHRG, ATIII, and PF4 to inhibit the binding of (A) ^{125}I-aFGF (40 ng/mL; 2.5 nM) and (B) ^{125}I-bFGF (2 ng/mL; 0.11 nM), to bovine corneal endothelial cell ECM. ECMs were incubated with inhibitors for 1 h on ice before the addition of ^{125}I-FGF which was followed by 2 h incubation on ice. After washing away unbound ^{125}I-FGF, bound growth factor was released from the ECM by incubation with 4 M guanidine hydrochloride/2% Triton X-100 overnight at 4 °C. The amount of ^{125}I-FGF released was determined and is presented as percent control binding, i.e., ^{125}I-FGF binding to ECM in the absence of inhibitor. Each value represents the mean of triplicate treatments ± SEM. The protein inhibitors, PF4, HRG, and ATIII were used at physiological concentrations.

corneal endothelial ECMs were exposed to bFGF and washed, and the ability of cHRG at a physiological concentration (100 μg/mL) to displace bFGF from the ECM determined. The bFGF content of supernatants was assessed by measuring the induction of BALB/c 3T3 cell DNA synthesis (Figure 6). There was some spontaneous release of bFGF from the ECMs preincubated with bFGF (Figure 6), whereas there was no mitogenic activity in the medium harvested from control ECMs not preincubated with bFGF (data not shown). The presence of cHRG resulted in considerable displacement of bFGF from the ECM, there being approximately an 8-fold increase in the mitogenic activity of supernatants from cHRG-treated ECMs (Figure 6). In contrast, cHRG released no mitogenic activity from control ECMs.

Effects of HRG and PF4 on the Mitogenic Activity of FGFs. Figure 7 shows the effect of a range of HRG concentrations on both FGF-stimulated and endogenous DNA synthesis of serum-starved BALB/c 3T3 cells. HRG significantly inhibited both aFGF- and bFGF-induced DNA synthesis in 3T3 cells. Interestingly, HRG was particularly effective at inhibiting proliferation in the absence of exogenously added FGFs, concentrations of 80 μg/mL and above, inhibiting endogenous DNA synthesis significantly below background levels. This suggests that HRG may be inhibiting the activity of heparin-binding growth factors produced endogenously by the 3T3 cells. Preliminary studies with PF4 indicated that it was unable to inhibit FGF-induced 3T3 cell

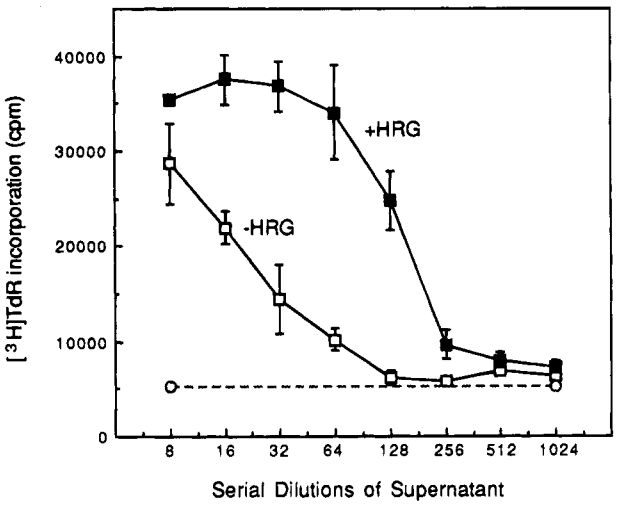

FIGURE 6: Ability of cHRG (100 μg/mL; 0.7 μM) to displace bFGF from bovine corneal endothelial cell ECM. ECMs were incubated with bFGF (10 μg/mL; 0.6 μM) for 1 h at 4 °C, unbound bFGF was washed away, and the ECMs were incubated with DMEM for 1 h at 4 °C to allow for any further spontaneous dissociation of bFGF. Unbound bFGF was removed, and the ECMs were incubated with (■) and without (□) cHRG for 2 h at 4 °C. Two-fold serial dilutions of the culture supernatants were prepared, starting with a 1/8 dilution, and applied to confluent monolayers of serum-starved BALB/c 3T3 cells. Control wells did not receive bFGF but did receive HRG, and the dashed line represents DNA synthesis resulting after ECMs were incubated with medium alone. Incubation was for 48 h, [^{3}H]TdR was added for the second 24 h, and its incorporation is expressed in cpm. Values represent mean ± SEM of triplicate values.

DNA synthesis (data not shown). This confirms the work of Maione et al. (1990), who found that rhPF4 inhibited the proliferation of human umbilical vein endothelial cells *in vitro* but had no effect on the proliferation of human dermal fibroblasts or keratinocytes.

DISCUSSION

The major finding to result from this study is that aFGF, bFGF, cHRG, hHRG, and PF4 all interact with the same HSPG species on the 3T3 cell surface. It appears likely that they either bind to the same or bind to adjacent saccharide sequences on the cell-surface HS chains. In contrast, ATIII fails to bind to the HS structure recognized by these molecules. This conclusion was based on data obtained from three separate binding assays, namely, inhibition of the interaction of immobilized HRG and FGFs, soluble fluoresceinated cHRG, and soluble radiolabeled FGFs, with 3T3 HSPGs.

It has been proposed for many years that clusters of basic residues are important in protein−heparin interactions (Deuel et al., 1977). X-ray crystallogrpahic studies of acidic and basic FGF implicate Lys-112, Lys-118, and Arg-122 (in human aFGF) in the heparin-binding site of both FGFs (Zhu et al., 1991; Eriksson et al., 1991; Zhang et al., 1991). Similarly, lysine residues have also been implicated in the interaction between heparin and PF4 (Deuel et al., 1977) and between heparin and ATIII (Rosenberg & Damus, 1973). The PF4 tetramer contains two extended, six-stranded β sheets, each formed by two subunits and arranged back-to-back (St. Charles et al., 1989). The carboxyl-terminal α helices contain the lysine residues (Lys-76, -77, -80, -81) (Handin & Cohen, 1976) thought to be involved in heparin

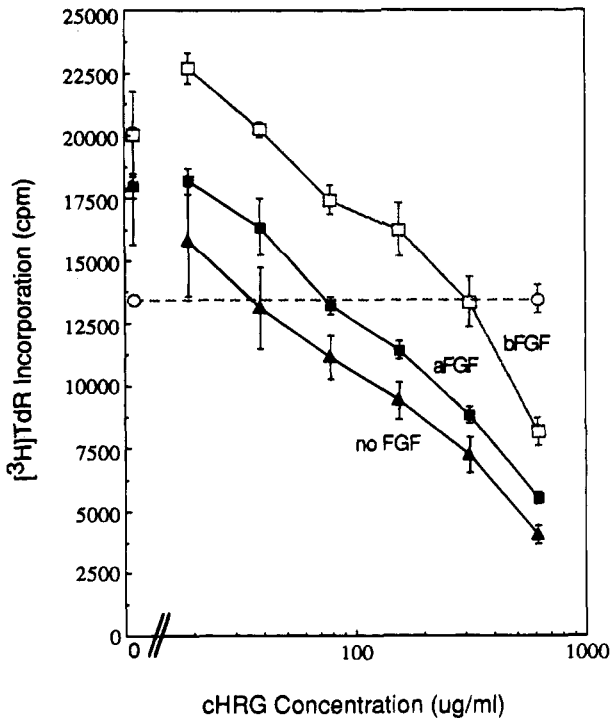

FIGURE 7: Effect of different concentrations of cHRG on FGF-stimulated and endogenous DNA synthesis of serum-starved BALB/c 3T3 cells. Confluent monolayers of 3T3 cells were initially incubated with different concentrations of cHRG for 1 h on ice before the addition of either aFGF (20 ng/mL; 1.25 nM) (■), bFGF (2 ng/mL; 0.11 nM) (□), or medium without FGFs (▲). The dashed line shows background DNA synthesis occurring in the absence of FGFs and cHRG. DNA synthesis was measured by [^{3}H]TdR, with data being expressed in cpm. Values represent the mean ± SEM of triplicate values.

binding and are arranged as antiparallel pairs on the surface of each extended β sheet (St. Charles et al., 1989). It is predicted that in ATIII, a helix containing Lys-282, -286, and -299 would have three positive charges on the same side which would interact with three critical sulfates on the octasaccharide-binding unit in heparin, preferred by ATIII (Villanueva, 1984). To date, little is known about the heparin-binding site on HRG except that the region is rich in histidine residues (Burch et al., 1987).

The nature of the heparin/HS motif which interacts with these proteins has also been investigated. Contiguous sequences of IdoA(2-OSO_3)α1,4-GlcNSO_3 are thought to be important in mediating high-affinity binding between HS and bFGF. In particular, the 2-*O*-sulfate of IdoA(2-OSO_3) and the *N*-sulfate of GlcNSO_3 appear to be essential for strong binding to bFGF (Turnbull et al., 1992; Habuchi et al., 1992). There is some data to suggest that aFGF interacts with a slightly different sequence on HS, IdoA(2-OSO_3)α1,4-GlcNSO_3(6-OSO_3) (Mach et al., 1993; Barzu et al., 1989). It is currently thought that five contiguous disaccharides of IdoA(2S)-GlcNSO_3 are sufficient to bind and activate bFGF (Walker et al., 1994). There are four heparin-binding sites on the PF4 tetramer, and for strong binding between heparin and PF4 to occur, heparin molecules larger than 10 000 Da in size or containing at least eight disaccharide units are required (Luscombe & Holbrook, 1983; Denton et al., 1983). A 2-fold helical structure of heparin with a linear polydisaccharide consisting mainly of alternating IdoA(2-OSO_3)α1,4-GlcNSO_3(6-OSO_3) residues has been proposed and would complement the 2-fold symmetry of the PF4 tetramer (Atkins & Nieduszynski, 1976). The smallest fragment of heparin that will bind to ATIII is an octasaccharide (Thunberg et al., 1980) containing a unique pentasaccharide sequence, GlcNSO_3(6-OSO_3)-GlcAc-GlcNSO_3(3-OSO_3)(6S)-IdoA(2-OSO_3)-GlcNSO_3(6-OSO_3), as the binding region (Lindahl et al., 1980, 1983, 1991) of which the 3-*O*-sulfate group of the internal glucosamine residue is essential for high-affinity binding (Petitou et al., 1988). It is not yet known which regions on heparin/HS interact with HRG, but it is anticipated that like the FGFs it would interact with the more highly sulfated regions of HSPGs. Collectively, these data are consistent with the FGFs and PF4 binding to similar or adjacent motifs on HS whereas ATIII binds to a more restricted saccharide sequence.

Cell-surface HSPGs have been previously shown to be of critical importance in FGFR activation by FGFs (Yayon et al., 1991; Olwin & Rapraeger, 1992), and the ability of molecules such as HRG and PF4 to compete with FGFs for binding to the same HSPGs suggests that HRG and PF4 may play a fundamental role in regulating FGF activity. Support for this idea comes from the observation that HRG and PF4 are very effective inhibitors of the binding of physiological concentrations of radiolabeled acidic and basic FGF to cell-surface HSPGs.

The continuous presence of HRG in plasma at concentrations as high as 100 μg/mL and its ability to substantially inhibit the binding of aFGF and bFGF to HSPGs on the 3T3 cell surface make it a prime candidate as a regulator of FGF activity. It is envisioned that by binding to HSPGs on the cell surface, HRG would prevent acidic and basic FGF from constitutively stimulating FGFRs. Indeed, HRG was shown in this study to significantly inhibit acidic and basic FGF-induced 3T3 cell DNA synthesis.

PF4's distribution is highly localized, being released from blood platelets at sites of vascular injury, and is therefore less likely than HRG to act as a general modulator of FGF activity. In addition, PF4 has a very low plasma concentration (Lane et al., 1984), although FGFs do play an important role in wound repair and, like PF4, are released at sites of vascular injury. At such sites, PF4 may, on the one hand, aid the displacement of FGFs from the ECM, thus enabling the FGFs to bind to the cell surface and activate FGFRs and, on the other hand, inhibit FGF-induced proliferation by masking cell-surface HSPGs required for FGF action. PF4 has already been reported to have antiangiogenic properties, possibly as a result of its ability to inhibit endothelial cell proliferation (Taylor & Folkman, 1982; Sharpe et al., 1990). The ability of PF4 to potently inhibit the binding of acidic and basic FGF to HSPGs on the fibroblast cell surface suggests that when added exogenously to endothelial cells, PF4 inhibits the FGF-induced proliferation by successfully competing with the FGFs for cell-surface HSPGs.

Although the FGFs, HRGs, and PF4 all interact with the same HSPG species on BALB/c 3T3 cells, they differ in their affinities for them. Basic FGF and PF4 have the highest binding affinity, followed by cHRG and aFGF which appear to exhibit approximately equal affinity, and hHRG has the lowest affinity. This may be due to PF4's tetrameric structure and its ability to form a complex with up to four heparin-like molecules simultaneously (Marshall et al., 1984) while human HRG probably has only one heparin-binding site per molecule (Burch et al., 1987). The inhibition data presented in this paper, using three different binding assays,

demonstrated that aFGF has a substantially lower affinity (approximately 5-fold) for 3T3 cell-surface HSPG than bFGF. Cross-blocking studies also indicated that ATIII did not interact with the HS sequences on 3T3 cells recognized by FGFs and HRGs.

Acidic and basic FGF's are known to be localized to basement membranes (BMs), both growth factors having been identified in ECMs deposited by cultured myoblasts (Weiner & Swain, 1989) and endothelial cells (Baird & Ling, 1987; Vlodavsky et al., 1987). Basic FGF is also present in BMs of rat fetus (Gonzalez et al., 1990), bovine cornea (Folkman et al., 1988), and human blood vessels (Cordon-Cardo et al., 1990). Scatchard analysis of heparin-inhibitable binding of acidic and basic FGF to the ECM suggests that both FGFs bind to HSPG in the ECM with considerably lower affinity (K_ds of 2.7 ± 0.5 and 1.7 ± 0.2 μM, respectively) than they are thought to bind to HSPGs on the cell surface (K_d of 2−10 nM) (Moscatelli, 1987). Thus, FGFs may be able to pass from low-affinity receptors in the ECM to relatively higher affinity HSPGs on the cell surface according to kinetic equilibrium. The ability of HRG to also bind to the ECM in a heparin-inhibitable manner suggests that HRG may also be able to modulate FGF activity by regulating its release from the matrix.

Although matrix-bound FGF is known to be biologically active, the presence of FGF in BMs *in vivo* does not appear to stimulate the overlying endothelial and epithelial cells to proliferate (D'Amore, 1990). It has therefore been suggested that while bound to HS in the ECM/BM, FGFs are not accessible to their cell-surface receptors. Indeed, the ECM/BM has been proposed to act as a physiological reservoir for growth factors, holding them in a stable and protected form until they are required to bind to and activate FGFRs on the cell surface. This would also explain how FGFs are prevented from acting on and stimulating vascular endothelium continuously. It is not clear, though, how FGFs are released from the ECM when they are required to stimulate cell proliferation, although it has been proposed that BM degradation by hydrolytic enzymes may render FGFs accessible to cells (Flaumenhaft et al., 1989). The data presented in this paper suggest that an alternative means by which FGFs could be released from the ECM would be if HRG (or PF4) were to displace them from their HSPG-binding sites in the ECM, making FGF available for binding to HSPGs on the cell surface. Our data with bFGF and HRG show that this is certainly a possibility.

In conclusion, studies described in this paper have demonstrated that PF4 and HRG are potentially important regulators of FGF action by competing for the same or closely associated HS motifs on HSPGs. These molecules could regulate FGF action in both a positive and a negative sense, on the one hand by displacing FGF from ECM/BM and making these FGFs available to responsive cells, and on the other hand by masking HSPGs on responsive cells and preventing FGFR activation.

ACKNOWLEDGMENT

We are grateful to Dr. Michael Crouch for supplying us with BALB/c 3T3 cells, Dr. John Morrison for doing the Scatchard analyses, Ms. Karen Jakobsen for preparing the chicken and human HRG, and Ms. Susan Maynes for preparing the ECMs.

REFERENCES

Atkins, E. D. T., & Nieduszynski, I. A. (1976) in *Heparin Chemistry and Clinical Usages* (Kakkar, V. V., & Thomas, D. P., Eds.) pp 21−35, Academic Press, London.

Baird, A., & Ling, N. (1987) *Biochem. Biophys. Res. Commun. 142*, 428−435.

Barber, A. J., Kaser-Glanzmann, R., Jakabova, M., & Luscher, E. F. (1972) *Biochim. Biophys. Acta 286*, 312−329.

Barzu, T., Lormeau, J.-C., Petitou, M., Michelson, S., & Choay, J. (1989) *J. Cell. Physiol. 140*, 538−548.

Bjork, I., & Nordenman, B. (1976) *Eur. J. Biochem. 68*, 507−511.

Burch, M. K., Blackburn, M. N., & Morgan, W. T. (1987) *Biochemistry 26*, 7477−7482.

Cordon-Cardo, C., Vlodavsky, I., Haimovitz-Friedman, A., Hicklin, D., & Fuks, Z. (1990) *Lab. Invest. 63*, 832−840.

D'Amore, P. A. (1990) *Cancer Metastasis Rev. 9*, 227−238.

Denton, J., Lane, D. A., Thunberg, L., Slater, A. M., & Lindahl, U. (1983) *Biochem. J. 209*, 455−460.

Deuel, T. F., Keim, P. S., Farmer, M., & Heinrikson, R. L. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2256−2258.

Eriksson, A. E., Cousens, L. S., Weaver, L. H., & Matthews, B. W. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 3441−3445.

Files, J. C., Malpass, T. W., Yee, E. K., Ritchie, J. L., & Harker, L. A. (1981) *Blood 58*, 607−618.

Flaumenhaft, R., Moscatelli, D., Saksela, O., & Rifkin, D. B. (1989) *J. Cell. Physiol. 140*, 75−81.

Folkman, J., Klagsbrun, M., Sasse, J., Wadzinski, M., Ingber, D., & Vlodavsky, I. (1988) *Am. J. Pathol. 130*, 393−400.

Glabe, C. G., Harty, P. K., & Rosen, S. D. (1983) *Anal. Biochem. 130*, 287−294.

Gonzalez, A. M., Buscaglia, M., Ong, M., & Baird, A. (1990) *J. Cell Biol. 110*, 753−765.

Habuchi, H., Suzuki, S., Saito, T., Tamura, T., Harada, T., Yoshida, K., & Kimata, K. (1992) *Biochem. J. 285*, 805−813.

Hajjar, D. P., Boyd, D. B., Harpel, P. C., & Nachman, R. L. (1987) *J. Exp. Med. 165*, 908−913.

Handin, R. I., & Cohen, H. J. (1976) *J. Biol. Chem. 251*, 4273−4282.

Haupt, H., & Heimburger, N. (1972) Hoppe Seyler's *Z. Physiol. Chem. 353*, 1125−1132.

Heimburger, N., Haupt, H., Kranz, T., & Baudner, S. (1972) Hoppe Seyler's *Z. Physiol. Chem. 353*, 1133−1140.

Ishihara, M., Tyrrell, D. J., Kiefer, M. C., Barr, P. J., & Swiedler, S. J. (1992) *Anal. Biochem. 202*, 310−315.

Jeanny, J. C., Fayein, N., Moenner, M., Chevallier, B., Barritault, D., & Courtois, Y. (1987) *Exp. Cell Res. 171*, 63−75.

Kazama, Y., & Koide, T. (1992) *Thromb. Haemostasis 67*, 50−55.

Lane, D. A., Ireland, H., Wolff, S., Ranasinghe, E., & Dawes, J. (1984) *Thromb. Haemostasis 52*, 183−187.

Lijnen, H. R., Rylatt, D. B., & Collen, D. (1983a) *Biochim. Biophys. Acta 742*, 109−115.

Lijnen, H. R., Hoylaerts, M., & Collen, D. (1983b) *J. Biol. Chem. 258*, 3803−3808.

Lindahl, U., & Kjellen, L. (1991) *Thromb. Haemostasis 66*(1), 44−48.

Lindahl, U., Backstrom, G., Thunberg, L., & Leder, I. G. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6551−6555.

Lindahl, U., Backstrom, G., & Thunberg, L. (1983) *J. Biol. Chem. 258*, 9826−9830.

Luscombe, M., & Holbrook, J. J. (1983) in *Glycoconjugates* (Chester, A. M., Heinegard, D., Lundblad, A., & Svensson, S., Eds.) pp 818−819, Secretariat, Lund.

Mach, H., Volkin, D., Burke, C. J., Middaugh, C. R., Linhardt, R. J., Fromm, J. R., & Loganathan, D. (1993) *Biochemistry 32*, 5480−5489.

Maione, T. E., Gray, G. S., Petro, J., Hunt, A. J., Donner, A. L., Bauer, S. I., Carson, H. F., & Sharpe, R. J. (1990) *Science 247*, 77−79.
Marshall, S. E., Luscombe, M., Pepper, D. S., & Holbrook, J. J. (1984) *Biochim. Biophys. Acta 797*, 34−39.
Moore, S., Pepper, D. S., & Cash, J. D. (1975) *Biochim. Biophys. Acta 379*, 370−384.
Moscatelli, D. (1987) *J. Cell. Physiol. 131*, 123−130.
Murano, G., Williams, L., Miller-Andersson, M., Aronson, D. L., & King, C. (1980) *Thromb. Res. 18*, 259−262.
Niewiarowski, S., Lowery, C. T., Hawiger, J., Millman, M., & Timmons, S. (1976) *J. Lab. Clin. Med. 87*, 720−733.
Nurcombe, V., Ford, M. D., Wildschut, J. A., & Bartlett, P. F. (1993) *Science 260*, 103−106.
Olwin, B. B., & Rapraeger, A. (1992) *J. Cell Biol. 118*, 631−639.
O'Neill, H. C., & Parish, C. R. (1983) *J. Immunol. Methods 64*, 257−268.
Petitou, M., Lormeau, J. C., & Choay, J. (1988) *Eur. J. Biochem. 176*, 637−640.
Rosenberg, R. D., & Damus, P. S. (1973) *J. Biol. Chem. 248*, 6490−6505.
Rylatt, D. B., Sia, D. Y., Mundy, J. P., & Parish, C. R. (1981) *Eur. J. Biochem. 119*, 641−646.
Sharpe, R. J., Byers, H. R., Scott, C. F., Bauer, S. I., & Maione, T. E. (1990) *J. Natl. Cancer Inst. 82*, 848−853.
St. Charles, R., Walz, D. A., & Edwards, B. F. P. (1989) *J. Biol. Chem. 264*, 2092−2099.
Taylor, S., & Folkman, J. (1982) *Nature 297*, 307−312.
Thunberg, L., Backstrom, G., Grundberg, H., Riesenfeld, J., & Lindahl, U. (1980) *FEBS Lett. 177*, 203−206.
Turnbull, J. E., Fernig, D. G., Ke, Y., Wilkinson, M. C., & Gallagher, J. T. (1992) *J. Biol. Chem. 267*, 10337−10341.
Villanueva, G. B. (1984) *J. Biol. Chem. 259*, 2531−2535.
Vlodavsky, I., Folkman, J., Sullivan, R., Fridman, R., Ishai-Michaeli, R., Sasse, J., & Klagsbrun, M. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 2292−2296.
Walker, A., Turnbull, J. E., & Gallagher, J. T. (1994) *J. Biol. Chem. 269*, 931−935.
Weiner, H. L., & Swain, J. L. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 2683−2687.
Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P., & Ornitz, D. M. (1991) *Cell 64*, 841−848.
Zhang, J. D., Cousens, L. S., Barr, P. J., & Sprang, S. R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 3446−3450.
Zhu, X., Komiya, H., Chirino, A., Faham, S., Fox, G. M., Arakawa, T., Hsu, B. T., & Rees, D. C. (1991) *Science 251*, 90−93.