Heparin/Heparan Sulfate (HP/HS) Interacting Protein (HIP) Supports Cell Attachment and Selective, High Affinity Binding of HP/HS*

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Heparin/heparan sulfate (HP/HS), HS proteoglycans, and their binding proteins play important roles in a variety of biological processes. Previously, we identified a novel cell surface HP/HS interacting protein (HIP) from human uterine epithelia and a variety of other human epithelial and endothelial cells and cell lines (Liu, S., Smith, S. E., Julian, J., Rohde, L. H., Karin, N. J., and Carson, D. D. (1996) J. Biol. Chem. 271, 11817–11823; Rohde, L. H., Julian, J., Babaknia, A., and Carson, D. D. (1996) J. Biol. Chem. 271, 11824–11830). In the current studies, we have purified and characterized HIP from HEC cells, a human uterine epithelial cell line, as well as recombinant HIP from a bacterial expression system. HIP supports attachment of the human trophoblastic cell line, JAR, in a HS-dependent fashion. Predigestion of JAR cells with a mixture of heparitinases, but not chondroitinase ABC, abolished cell attachment to HIP. In addition, JAR cell attachment to HIP is highly sensitive to HP inhibition and much more selective for HP/HS than other glycosaminoglycans. Dermatan sulfate displays partial inhibitory activity as well, consistent with the observation that chondroitinase ABC digestion partially reduces JAR cell attachment to HIP. Solid-phase binding assays indicate HIP binds \(^{3}H\)HP with high affinity (apparent \(K_D\) = 8 nM). Furthermore, HIP bound cell surface/extracellular matrix-associated HS, expressed by RL95 cells, a human uterine epithelial cell line. Anti-HIP antibody generated against a synthetic peptide derived from a putative HP/HS-binding motif resident within HIP inhibited about half of \(^{125}I\)HP binding to HIP, indicating that this domain is a functional HP/HS-binding domain of HIP. Similarly, this same synthetic peptide motif of HIP could block about 50% of \(^{3}H\)HP binding to HIP; however, this peptide almost completely inhibited cell attachment to HIP, suggesting a critical role, in this regard. Collectively, these results suggest that HIP can function as a HP/HS-binding cell-cell/cell-matrix adhesion molecule.

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1 The abbreviations used are: HP, heparin; HS, heparan sulfate; GAG, glycosaminoglycan; Hep3, a mixture of heparinas I, II, and III; HIP, heparin-heparan sulfate interacting protein; PBS, phosphate-buffered saline; PICS, protease inhibitor mixture solution; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis.

** EXPERIMENTAL PROCEDURES

Materials—Heparin, low molecular weight heparin, bovine intestinal mucosa heparan sulfate, bovine kidney heparan sulfate, dermatan sulfate, chondroitin sulfate C, keratan sulfate, hyaluronic acid, heparinas I, II, and III, chondroitinases AC and ABC, BSA, and heparinase agars were purchased from Sigma. \(^{3}H\)HP (0.44 mCi/mg) was purchased from NEN Life Science Products. \(^{125}I\)Sulfate (43 Ci/mg) and \(^{125}I\)-protein A (50 µCi/µg) were purchased from ICN Biochemical Inc. (Irvine, CA). Tissue culture media components were obtained from...
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Irvine Scientific (Santa Ana, CA). Fetal bovine serum and Dulbecco's phosphate-buffered saline (PBS) were from Life Technologies, Inc. (Grand Island, NY). All chemicals used were reagent grade or better.

**Cell Culture—HEC and JAR cells** were grown to 60% confluence in Dulbecco's modified Eagle's medium and Ham's F-12 medium (F-12) containing 10% fetal bovine serum. Activating heparin-agarose was prepared by the manufacturer.

**Construction of Anti-HIP Immunoaffinity Matrix**—Production of polyclonal anti-HIP antibodies and their affinity purification was as described before (11). Anti-HIP immunoaffinity matrix was formed by cross-linking affinity-purified anti-HIP antibody to cyanogen bromide-activated Sepharose (Sigma) following the procedures provided by the manufacturer.

**Heparin-Agarose and Anti-HIP Immunoaffinity Chromatography for Purification of Natural HIP**—Confluent HEC cell cultures were rinsed with Dulbecco's PBS three times and cells released from dishes with 10 ml EDTA, 1 mM NaCl, and 10 ml Tris-HCl, pH 7.2. The harvested cells were disrupted, in the presence of a protease inhibitor mixture solution (PICS) (5), by three cycles of freezing and thawing, followed by sonication on ice for 30 s. The mixture then was centrifuged at 25,000 × g for 30 min and the supernatant was collected and diluted in 10 ml Tris-HCl, pH 7.2, to reach the final NaCl concentration of 0.15 M. The diluted cell lysate was then mixed with heparin-agarose and incubated with constant rotary agitation at 4 °C overnight. The following day, the mixture was loaded onto a column and washed sequentially with 0.15, 0.5, 1.0, and 2.0 M NaCl in 10 ml Tris-HCl, pH 7.2. The 1.0 M eluate from the heparin-agarose column was collected and concentrated with Centricon-10 concentrators (Amicon, Inc., Beverly, MA). The concentrated 1.0 M eluate was then diluted with 10 ml Tris-HCl, pH 7.2, to reach a final NaCl concentration of 0.15 M, and incubated with anti-HIP immunoaffinity matrix, in the presence of PICS at 4 °C overnight in a rotary agitator. The mixture then was loaded into a 3-ml syringe column, and the resin washed extensively first with 0.15 M, then 0.5 M NaCl 10 ml Tris-HCl, pH 7.2, and then 0.1 M glycine-HCl, pH 4.0. Finally, the bound protein(s) was eluted with 0.1 M glycine-HCl, pH 2.5, into a tube containing 1.0 ml Tris-HCl, pH 8.0, and immediately dialyzed against 10 ml Tris-HCl, pH 7.2, overnight. The dialyzed eluate then was concentrated with Centricon-10 and stored at −80 °C until use.

**Expression and Isolation of Recombinant HIP**—PCR primers were made with EcoRI adaptors to the human HIP sequence nucleotides 25–508 with an engineered mutation at position 507 G → A to make a stronger stop codon for Escherichia coli. The forward and reverse primers were: 5′-CGGCGGGCTCTCATGGGGTCG-3′ and 5′-GCTGACTGCAGGAAATGCTAGTCTGGGGAGTGGGCAAGTCT-3′

**SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis**—Solubilized protein samples were precipitated with 10% (w/v) trichloroacetic acid at 4 °C for 2 h or overnight. The precipitates were collected by centrifugation at 1,200 × g for 10 min at 4 °C, washed sequentially with 10% (w/v) trichloroacetic acid and 100% acetone, and air-dried. The pellets were dissolved in equal volumes of sample buffer (50 mM Tris-HCl, pH 7.0, 1% (v/v) β-mercaptoethanol, and 0.01% (v/v) phenylmethylsulfonyl fluoride; and sample buffer (13), heated in boiling water for 5 min and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were resolved by SDS-PAGE on a 15% (w/v) acrylamide resolving gel as described previously by (14), and detected with silver staining (15). For Western blot analysis, the gel was rinsed briefly with transfer buffer (100 mM Tris base and 100 mM glycine, pH 9.2) and transferred to a nitrocellulose membrane at 4 °C for 5 h at 40 V in a Transblot apparatus (Bio-Rad). The transferred blot was blocked with 1% (w/v) BSA in PBS, 0.01% (v/v) sodium azide, and 0.05% (v/v) Tween 20 (PAT) overnight at room temperature. The blot then was incubated with primary anti-HIP antibodies incubated in PAT containing 1% (w/v) BSA at room temperature for at least 6 h, rinsed with PAT three times for 5 min each, and incubated with 6 µg of 125I-protein A in 70 ml of PAT plus 0.1% (w/v) BSA at room temperature for more than 6 h. Finally, the blot was rinsed with PAT three times for 5 min each, air-dried, and exposed to Kodak X-AR film at −20 °C.

**Cell Attachment to HIP, Glycosaminoglycan (GAG) Competition, and GAG Lyase Digestion**—JAR cell attachment to HIP was performed on 96-well tissue culture plates (Corning, Corning, NY). Each well was coated with approximately 3 µg of purified HIP, fibronectin, or heat-denatured BSA and the plates were dried overnight. Each well was then rinsed with 100 µl of PBS three times and blocked with 100 µl of heat-denatured BSA (0.1% (w/v) for 1–2 h. After blocking, the wells were rinsed with PBS three times again prior to the addition of cells. JAR cells at 70–90% confluence were detached with trypsin/EDTA solution for 5–10 min at 37 °C. The detached cells were rinsed three times with Dulbecco's modified Eagle's medium/F-12 (1:1) plus 0.1% (w/v) BSA and resuspended in the same medium at a concentration of 5 × 10⁵ cells/ml. Then 100 µl of this cell suspension was added to each well, and the wells were incubated with glycosaminoglycan competitors, and incubated in a 37 °C incubator for 5–6 h. At the end of each experiment, unattached cells were gently rinsed with PBS, and the relative cell attachment was determined using the hexazonidase assay (16).

For glycosaminoglycan competition assays, 10 or 100 µg/ml HIP, low molecular weight HIP, bovine intestinal mucosa HS, bovine kidney HS, dermatan sulfate, chondroitin sulfate C, hyaluronic acid, or keratan sulfate were included in the incubation medium and used as competitors in the assays of JAR cell attachment to HIP. To determine if HIP cell surface/HIP or other GAGs mediate JAR cell attachment to HIP, JAR cells were digested with 500 milliunits/ml of Hep3 (a mixture of heparinases I, II, and III), chondroitinase AC, or chondroitinase ABC, in the presence of PICS, at 37 °C for 30 min prior to addition to HIP coated wells.

**[3H]HIP or [35S]HS Binding to HIP—[3H]HIP or [35S]HS, obtained following metabolic labeling of RL95 cells with H[3]SO₄O₃ as described (11), were used in solid-phase HIP-binding assays performed in 96-well microassay plates. Cell surface [35S]HS was prepared as described (11). Extracellular matrix from [35S]-labeled RL95 cells was prepared as described previously (17). Briefly, cell layers were gently rinsed with PBS twice, treated with 0.5% (w/v) heat-denatured BSA and the plates were dried overnight. Afterward, the dish was rinsed four times with PBS, and the residue, defined as “extracellular matrix,” was removed with a rubber policeman. Glycosaminoglycans were released by mild alkaline hydrolysis, digested with chondroitinase ABC, and the identity of the remaining high molecular weight (> 10,000) material as HS was confirmed by nitric acid digestion as described previously (9). Approximately 3 µg of HIP or heat-denatured BSA as a control was added to each well and dried at 37 °C overnight. The next day, each well was rinsed with PBS three times, and blocked with 100 µl of 0.1% (w/v) heat-denatured BSA in a 37 °C incubator for at least 1 h. Each well was then rinsed with PBS three times, and then [3H]HIP or [35S]HS (typically 1.0 × 10⁶ dpm) were added in a final volume of 50 ml in PBS containing 0.1% (w/v) BSA, and incubated in a 37 °C incubator for 2 h. In some assays, the [3H]HIP concentration was varied. In other assays, different amounts of HIP were used to coat wells. For glycosaminoglycan competition assays, different concentrations of glycosaminoglycans, including unlabeled HIP, low molecular weight HIP, bovine intestinal mucosa HS, bovine kidney HS, dermatan sulfate, chondroitin sulfate C, keratan sulfate, and hyaluronic acid were added to the binding assays. At the end of each experiment, unbound [3H]HIP (or [35S]HS) was removed by rinsing three times with PBS. Bound [3H]HIP (or [35S]HS) was extracted with 100 ml of extraction buffer (4 ml guanidine HCl, 25 ml Tris-HCl, pH 8.0, 2.5 ml EDTA, and 0.02% (w/v) sodium azide) overnight at 37 °C. Forty ml of the extract was counted in a Beckman scintillation counter.

**Inhibition of [3H]HIP Binding by Anti-HIP**—In one type of experiment, purified HIP was preincubated with anti-HIP antibodies or non-immune rabbit IgG. Briefly, approximately 5 µg of HIP was incubated in a final volume of 180 ml in PBS in the presence of 60 µg/ml affinity purified rabbit anti-HIP IgG or nonimmune rabbit IgG. Anti-HIP is directed against a synthetic peptide, as described in detail previously (11). Five µl of a 50% slurry of protein A-Sepharose was added to this solution and the mixture incubated with constant rotary agitation.
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RESULTS

Purification of HIP—Previous studies have shown that HIP binds to HP-agarose with relatively high affinity. In addition, it was observed that HEC cells, a human uterine cell line, express relatively higher amounts of HIP than other cell lines (11). Therefore, HEC cells were used as a source of HIP, and HP-agarose affinity chromatography was employed as a first step in HIP purification. Stepwise elution (0.15, 0.5, 1.0, and 2.0 M NaCl, respectively) was employed to elute HIP from HP-agarose affinity column. HIP was not eluted with either 0.15 or 0.5 M NaCl. HIP was totally released from the HP-agarose column at 1.0 M NaCl elution as detected by Western blot analysis using anti-HIP antibodies and silver staining (Fig. 1A, lanes 2 and 5). Thus, the 1.0 M NaCl eluate was subjected to further purification. The 1.0 M NaCl eluate from the HP-agarose affinity column was diluted to a final concentration of 0.15 M NaCl, applied to an anti-HIP immunoaffinity resin, and eluted sequentially with 0.15 M NaCl, 0.5 M NaCl in Tris-HCl, pH 7.2, and glycine-HCl, pH 4.0. None of these wash steps released HIP from the anti-HIP immunoaffinity resin (data not shown). As shown in Fig. 1 (lanes 3 and 6) HIP bound to the anti-HIP immunoaffinity column and was released with glycine-HCl at pH 2.5. Silver staining of glycine-HCl, pH 2.5, eluate revealed two major bands, one with the $M_r$ 24,000, which is identical to the size of intact HIP (10, 11) and was verified by Western blotting using anti-HIP antibody (Fig. 1A, lane 6) and another with $M_r$ 14,000, which was repeatedly observed in purified HIP preparations and appears to be a degradation product of HIP. A histidine-tagged version of recombinant HIP was expressed in E. coli and isolated by nickel affinity chromatography (Fig. 1B). Both sources of HIP were used for further studies. HIP isolated from HEC cells was used in most of the experiments shown; however, recombinant HIP displayed similar $[^3H]$HIP binding and cell attachment activities in all assays.

HIP Supports JAR Cell Attachment in a HS-dependent Fashion—To determine if HIP can support cell attachment, JAR cells, a human trophoblastic cell line derived from a placental choriocarcinoma (18), were used for cell attachment assays. JAR cell attachment to HIP-coated wells was HIP concentration-dependent, and time-dependent with maximum attachment at 2–4 h (data not shown). In contrast, heat-denatured BSA-coated wells did not support JAR cell attachment (Fig. 2). Similarly, cell attachment activity was observed with either HIP isolated from HEC cells or expressed in E. coli. To test HIP sensitivity of JAR cell attachment to HIP, different concentrations of HIP were included in the cell attachment incubation media. As shown in Fig. 2, inclusion of HIP at concentrations of 10 ng/ml or more maximally inhibited JAR cell attachment to HIP. Even in the presence of 0.1 μg/ml (approximately 10 nM) HIP, cell attachment was inhibited by more than 60% (Fig. 2B). Therefore, JAR cell attachment to HIP is highly sensitive to HIP. To determine the GAG selectivity of JAR cell attachment to HIP, different GAGs at concentrations of 10 or 100 μg/ml were included in the incubation media for cell attachment assays. As shown in Fig. 2A, among the GAGs tested, dermatan sulfate, bovine intestinal mucosa heparan sulfate, and low molecular weight HP displayed a similar degree of inhibition of JAR cell attachment to HIP as HP at a concentration of 100 μg/ml; however, at a concentration of 10 μg/ml only HP maximally inhibited cell attachment (>80%). Therefore, JAR cell attachment to HIP is more selective for HP than other GAGs.

To determine if HS or other JAR cell surface GAGs mediated JAR cell attachment to HIP, JAR cells were preincubated with GAG lyases before performing the cell attachment assay. Previous studies have shown that JAR cells express both HS and dermatan sulfate as their major cell surface glycosaminoglycans and that the conditions used remove all detectable HS or dermatan sulfate from JAR cell surfaces (9). Fig. 3 shows that predigestion of JAR cells with heparitinases destroyed (>95%) the ability of JAR cells to attach to HIP. Predigestion with chondroitinase ABC, which degrades dermatan sulfate in addition to chondroitin sulfates A and C, partially (65%) inhibited JAR cell attachment to HIP; however, predigestion with chondroitinase AC, which degrades only chondroitin sulfates A and C, only reduced JAR cell attachment by 20%. Collectively, these results indicate that cell surface HS and, to a lesser extent, dermatan sulfate mediate JAR cell attachment to HIP.

HIP Binds $[^3H]$HIP with High Affinity and Selectivity—Initially, $[^3H]$HIP binding was examined as a function of the amount of HIP used to coat surfaces. A solid-phase binding assay was employed similar to that used by others to approximate various aspects of binding kinetics and specificity (19, 20). As shown in Fig. 4A, $[^3H]$HIP binding to HIP was concentration-dependent and saturable. Kinetic analysis of these data revealed a class of binding sites with an apparent $K_D$ of 8 nM. Lower affinity binding ($K_D = 85$ nM) also was observed that may result from heterogeneity of the $[^3H]$HIP preparations or
more than one class of binding sites in HIP. Both HEC-derived and bacterially-expressed HIP displayed similar [3H]HP binding characteristics.

To further demonstrate that HIP, as opposed to contaminants of HIP preparations, were responsible for the HP binding activity, polyclonal anti-HIP antibody generated against a putative HP/HS-binding domain (11) was employed in the assay. Results are the mean ± S.D. of duplicate determinations. B, JAR cells (5.0 × 10^4 cells/well) were added to 96-well plates coated with 3 μg/well of the purified HIP in the presence of the indicated concentrations of different glycosaminoglycans (HP; DS, dermatan sulfate; CS-C, chondroitin sulfate C; HS-BIM, bovine intestinal mucosa heparan sulfate; HS-BK, bovine kidney heparan sulfate; HP-LMW, low molecular weight heparin; HA, hyaluronic acid; KS, keratan sulfate), and incubated in a 37 °C incubator for 4 h. At the end of the experiment, unattached cells were rinsed with PBS, and cell attachment was determined by hexosaminidase assay. Data are expressed as percentages relative to controls in the absence of competitors. Results are the mean ± S.D. of duplicate determinations.

FIG. 2. JAR cell attachment to HIP is highly sensitive to heparin. A, JAR cells (5.0 × 10^4 cells/well) were added to 96-well plates coated with 3 μg/well of the purified HIP in the presence of the indicated concentrations of different glycosaminoglycans (HP; DS, dermatan sulfate; CS-C, chondroitin sulfate C; HS-BIM, bovine intestinal mucosa heparan sulfate; HS-BK, bovine kidney heparan sulfate; HP-LMW, low molecular weight heparin; HA, hyaluronic acid; KS, keratan sulfate), and incubated in a 37 °C incubator for 4 h. At the end of the experiment, unattached cells were rinsed with PBS, and cell attachment was determined by hexosaminidase assay. Data are expressed as percentages relative to controls in the absence of competitors. Results are the mean ± S.D. of duplicate determinations.

FIG. 3. Removal of cell surface heparan sulfate or dermatan sulfate inhibits JAR cell attachment to HIP. JAR cells (5.0 × 10^4 cells/well) were predigested with different glycosaminoglycan lyases as indicated (hep3, ABCase, chondroitinase ABC; A Case, chondroitinase AC) in the presence of a PICS, or 10 μg/ml HP or PICS alone in the absence of enzymes for 30 min at 37 °C, and then added to 96-well plates coated with 3 μg/well of the purified HIP. After incubation at 37 °C for 4 h, unattached cells were rinsed with PBS, and cell attachment was determined by hexosaminidase assay. Data are expressed as percentages relative to controls without enzyme treatment or addition of competitors. Results are the mean ± S.D. of duplicate determinations.

HIP binding activity in HIP. The same peptide to which the antibody was generated was used to compete for JAR cell attachment and [3H]HP binding to HIP. As shown in Fig. 6, HIP peptide inhibited JAR cell attachment to HIP in a dose-dependent fashion with approximately 90% inhibition at 100 μg/ml. In contrast, HIP peptide less effectively competed for [3H]HP binding to HIP reducing this value by approximately 50 and 65% at 100 and 1,000 μg/ml, respectively. Thus, while this HIP peptide sequence did not account for all of the HP binding activity of HIP, it appears to correspond to a motif critical for cell attachment.

HIP Binds HP More Selectively than Other Glycosaminoglycans—Unlabeled HP, dermatan sulfate, chondroitin sulfate C, keratan sulfate, hyaluronic acid, bovine intestinal mucosa HS, bovine kidney HS, and low molecular weight HP were used to test the GAG selectivity of [3H]HP binding to HIP. Fig. 7 shows that HP most effectively inhibited [3H]HP binding to HIP at a concentration of 10 μg/ml (90% of inhibition). Low molecular weight HP and dermatan sulfate showed a partial inhibitory effect (approximately 20%) while other GAGs did not show any significant inhibition on [3H]HP binding to HIP. Therefore, consistent with the cell adhesion assay results, HIP appeared to bind HP preferentially over other GAGs. Finally, HIP-coated wells bound HS isolated from RL95 cell surfaces or extracellular matrix-enriched fractions (Fig. 8). Binding was reduced to background levels by the inclusion of excess unlabeled HP in the assay. The ability of HIP to bind surface or extracellular matrix HS further supported the notion that HIP promotes HS-dependent cell adhesion.

DISCUSSION

In the present studies, HIP purified from a human cell line or expressed in E. coli has been used to study cell adhesion and HP/HS binding activities. Previously, it was suggested that HIP might bind HP/HS and be involved in HS-dependent cell-cell/cell-matrix interactions. This suggestion was based on the observations that HIP is expressed on cell surfaces and binds
HIP either in solution or in solid-phase assays (10, 11). The current studies demonstrate that purified HIP can support JAR cell attachment in a HS-dependent fashion and selectively bind HP/HS with high affinity. JAR cell attachment to HIP is highly sensitive to HP inhibition. Concentrations of HP as low as 0.1 \( \mu \text{g/ml} \) (approximately 10 nM assuming an average molecular mass of HP of 10,000 Da) inhibited cell attachment to HIP greater than 50%. JAR cell attachment to HIP also was more selective for HP than other GAGs tested. At a concentration of 10 \( \mu \text{g/ml} \), HP inhibited JAR cell attachment by more than 80% while dermatan sulfate or low molecular HP showed less than 30% inhibition in this regard. Other GAGs did not show significant inhibition at this concentration. Further, predigestion of JAR cells with heparitinases completely abolished attachment to HIP, demonstrating that HS is the major cell surface molecule mediating JAR cell attachment to HIP. Chondroitinase ABC, but not chondroitinase AC, predigestion of JAR cells also partially inhibited cell attachment, indicating that dermatan sulfate displayed on JAR cell surfaces may also be involved in attachment. Thus, these data suggest that, in addition to HS, dermatan sulfate expressed on JAR cell surfaces also participates in attachment to HIP. These results are consistent not only with the GAG competition studies, but also previous studies showing the presence of both HS and derma-

**FIG. 4.** [\(^{3}H\)HP binds to HIP with high affinity.** A**, the indicated concentrations of [\(^{3}H\)HP were added to 96-well plates coated with 3 \( \mu \text{g/well} \) of purified HIP with or without addition of 100 \( \mu \text{g/ml} \) unlabeled HP. After incubation at 37 °C for 2 h, unbound [\(^{3}H\)HP was rinsed with PBS, and bound [\(^{3}H\) was extracted and determined by liquid scintillation counting. Results are expressed as mean ± S.D. of duplicate determinations. **B**, kinetic analyses using assumptions for Scatchard analyses of the specific [\(^{3}H\)HP binding data from panel A. The dashed lines indicated the calculated classes of binding sites. The calculated \( K_{d,app} \) are also presented for each class of binding site.**

**FIG. 5.** Anti-HIP antibody specifically inhibits [\(^{3}H\)HP binding to HIP. Antibody preabsorption and blocking experiments were performed as described under “Experimental Procedures.” Equivalent portions (approximately 1 \( \mu \text{g/well} \)) were used in each case. [\(^{3}H\)HP binding to HIP-coated surfaces in the absence (HIP) or presence of 100 \( \mu \text{g/ml} \) unlabeled HP (HIP + HP) are shown. Some samples were preabsorbed with rabbit anti-HIP IgG or non-immune IgG as indicated. In other cases, HIP was used to coat surfaces and either non-immune IgG or anti-HIP IgG was included during the [\(^{3}H\)HP binding assay. The results indicate that anti-HIP almost completely removes the components involved in [\(^{3}H\)HP binding; however, anti-HIP only partially blocks [\(^{3}H\)HP binding to HIP. The results shown are the averages ± range of values obtained for duplicate determinations in each case.**

**FIG. 6.** HIP peptide inhibits JAR cell attachment and [\(^{3}H\)HP binding to purified HIP. JAR cells (5 \( \times \) 10\(^{3} \) cells/well) were added to 96-well plates coated with 3 \( \mu \text{g/ml} \) of the purified HIP in the presence of different concentrations of HIP peptide as indicated and incubated in a 37 °C incubator for 4 h (M). At the end of the experiment, unattached cells were rinsed with PBS and cell attachment was determined by hexosaminidase assay. Data are expressed as percentages relative to controls performed in the absence of competitors. Results represent the mean ± S.D. of duplicate determinations. In parallel, [\(^{3}H\)HP (1.0 \( \times \) 10\(^{5} \) dpm/well) was added to 96-well plates coated with 3 \( \mu \text{g/well} \) of purified HIP in the absence or presence of different concentrations of HIP peptide-1 as indicated, and incubated at 37 °C for 2 h (E). At the end of experiment, unbound [\(^{3}H\)HP was rinsed with PBS and bound [\(^{3}H\)HP was extracted and determined by liquid scintillation counting. Data are expressed as percentages relative to controls performed in the absence of peptide. Results represent the mean ± S.D. of duplicate determinations in each case.
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experiment, unbound \(^{35}\)S\(^{-}\)HS was rinsed with PBS, and bound \(^{35}\)S\(^{-}\)HS

plates coated with 3\(^{-}\)H\(^{-}\)HP (1.0 \(\times 10^{6}\) dpm/well) were added to 96-well plates coated with 3 \(\mu\)g/well purified HIP with addition of 10 \(\mu\)g/ml different glycosaminoglycans (HF, DS, dermatan sulfate; CS-C, chondroitin sulfate C; KS, keratan sulfate; HA, hyaluronic acid; HS-BIM, bovine intestinal mucosa heparan sulfate; HS-BK, bovine kidney heparan sulfate; HP-LMW, low molecular weight heparin), respectively. After incubation at 37 °C for 2 h, unbound \(^{3}H\)HP was rinsed with PBS and bound \(^{3}H\)HP was extracted and determined by liquid scintillation counting. Results represent the mean ± S.D. of duplicate determinations.

HIP binds \(^{3}H\)HP more selectively than other glycosaminoglycans. \(^{3}H\)HP (1.0 \(\times 10^{6}\) dpm/well) was added to 96-well plates coated with 3 \(\mu\)g/well purified HIP with addition of 10 \(\mu\)g/ml different glycosaminoglycans (HF, DS, dermatan sulfate; CS-C, chondroitin sulfate C; KS, keratan sulfate; HA, hyaluronic acid; HS-BIM, bovine intestinal mucosa heparan sulfate; HS-BK, bovine kidney heparan sulfate; HP-LMW, low molecular weight heparin), respectively. After incubation at 37 °C for 2 h, unbound \(^{3}H\)HP was rinsed with PBS and bound \(^{3}H\)HP was extracted and determined by liquid scintillation counting. Results represent the mean ± S.D. of duplicate determinations.

HIP specifically and selectively binds HP/HS with high affinity. Scatchard analysis revealed at least one class of high affinity binding sites with an apparent \(K_D\) of 8 ns. These affinities are comparable to those reported for other HP/HS-binding proteins (19, 21, 22). The strong binding displayed in these solid-phase assays as well as the tenacity of the HIP interaction in solution with HIP-agarose indicate that HIP is likely to form stable complexes with HS at cell surfaces. In this regard, HS isolated from RL95 cell surfaces or extracellular matrices bound to HIP in solid-phase assays. Thus, HIP is capable of binding HS displayed at sites where cell adhesion normally occurs. Heparanase is an enzyme that partially degrades HS and is found both intracellularly as well as secreted by various normal and tumor cells and cell lines (23, 24). We have found that heparanase selectively acts on HS fractions recognized by HIP and destroys their HIP binding activities (25). This action of enzymes like heparanase may modulate HIP-dependent interactions with cell surface and extracellular matrix forms of HS.

An antibody generated against an HP-binding synthetic peptide sequence of HIP (26) specifically preabsorbed the \(^{3}H\)HP binding activity from HIP preparations. This further demonstrated that HIP, and not a contaminant, contributed this activity. Interestingly, this same antibody also specifically inhibited \(^{3}H\)HP binding to HIP by 55%, indicating that this peptide sequence corresponds to one of the major HP/HS-binding sites of HIP. Solid-phase binding assays also indicated that HIP contains more than one HP/HS-binding site. Competition assays indicated that this peptide motif competed for 50–65% of the total \(^{3}H\)HP binding to HIP, consistent with the antibody inhibition results. However, this same peptide almost entirely inhibited cell attachment. Thus, this peptide motif, in particular, appears to be critical for cell attachment activity. Collectively, the current studies demonstrate that HIP directly participates in HP/HS-binding events and supports cell attachment.

HS proteoglycans have been suggested to function as attachment-promoting factors at embryonic surfaces during the critical event of embryo implantation both \textit{in vitro} and \textit{in vivo} (5, 7). A corollary of this hypothesis is that uterine epithelial cells display complementary HS-binding sites at their cell surfaces. Previous studies have demonstrated that both mouse and human uterine epithelia display such specific, high affinity cell surface HS-binding sites (6, 8). The activity of HIP coupled with its disposition at the cell surface of uterine epithelial cells and cell lines (11) strongly suggest a role in HS-dependent aspects of embryo attachment. Studies of HIP function in embryo implantation in mice should directly address this possibility.

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