A Peptide Sequence of Heparin/Heparan Sulfate (HP/HS)-interacting Protein Supports Selective, High Affinity Binding of HP/HS and Cell Attachment†∗

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We previously have identified a novel cell surface heparan sulfate/heparin (HS/HP)-interacting protein (HIP) found in 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). The amino acid sequence predicted for HIP revealed a potential HS/HP-binding motif. In the present studies, a synthetic peptide corresponding to this putative HS/HP-binding motif, HIP peptide, was synthesized and examined with regard to its HS/HP binding and cell attachment promoting activity. Results using solid phase binding assays demonstrate that HIP peptide binds HS/HP with high selectivity and has high affinity for bulk HP (50% saturation ≈ 300 nM) and even higher affinity for a subset of polysaccharides found in commercial [3H]HP (half-saturation ≈ 10 nM). Moreover, HIP peptide binds subsets of cell and extracellular matrix-associated HS and dermatan sulfate expressed by RL95 cells, a human uterine adenocarcinoma cell line. HIP peptide also binds a similar fraction of HS as well as dermatan sulfate expressed by JAR cells, a human choriocarcinoma cell line. In contrast to binding of cell- or extracellular matrix-associated HS, HIP peptide does not bind secreted or released forms of HS or DS from either RL95 or JAR cells to a significant extent. HS species that bind to HIP peptide are generally larger, have a higher negative charge density, and have a larger proportion of di- and trisulfated disaccharide units than HS species that do not bind to HIP peptide, demonstrating structural differences among these polysaccharides. This same peptide supports HS-dependent JAR cell attachment. Collectively, these data demonstrate that a linear peptide sequence found within HIP can account, at least in part, for the HS/HP binding and cell adhesion promoting activities of this protein.

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Heparin/heparan sulfate (HP/HS)† and heparan sulfate proteoglycans (HSPGs) participate in diverse biochemical and physiological processes via interactions with a variety of proteins. HP/HS and HSPGs bind and modulate activities of growth factors (3), maintain extracellular matrix integrity (4, 5), modulate hemostasis (6), participate in cell adhesion, growth, and differentiation (7), and regulate smooth muscle cell proliferation (8, 9). Studies in our laboratory have demonstrated that HP/HS and HSPGs are involved in the initial stages of mouse embryo implantation. HSPGs can be detected as early as the two-cell stage (10), and HSPG expression on cell surfaces of mouse blastocysts increases 4–5-fold at the peri-implantation stage (11–13). Inhibition of HSPG synthesis or enzymatic removal of HS from blastocyst surfaces inhibits embryo attachment (12, 14). Mouse embryo attachment to fibronectin, laminin, and isolated mouse uterine epithelial cells is HP/HS-dependent (11). Complementary specific, high affinity HP/HS sites on the cell surface of mouse uterine epithelial cells also have been characterized (15). Thus, it is suggested that HSPGs and their corresponding binding sites are involved in the initial attachment of mouse embryos to uterine epithelium.

The initial attachment of the human trophoblastic cell line, JAR, to RL95 cells, a human uterine epithelial cell line, is HP/HS-dependent (16). Furthermore, a single class of highly specific, cell surface HP/HS-binding sites has been identified on RL95 cell surfaces (17). Cell surface, HP/HS-binding peptides were isolated from RL95 cells, and partial NH2-terminal amino acid sequence was obtained. A full-length cDNA sequence corresponding to one of these peptides was identified (1). Predicted peptide sequence from this cDNA revealed an antigenic sequence that also has features expected for a HP/HS-binding motif (18). Polyclonal antibodies directed against this synthetic peptide recognize a novel HP/HS-interacting protein (HIP) with a Mr on SDS-polyacrylamide gel electrophoresis of 24,000. HIP is expressed by a variety of epithelial and endothelial cells and cell lines and on RL95 cell surfaces (1, 2). In addition, HIP is highly expressed by human cytотrophoblast at the fetal-maternal interface throughout pregnancy in juxtaposition to a potential HSPG ligand, perlecain (19). Previous studies (20) suggested that a large fraction of the HP/HS binding and cell adhesion promoting activity of intact HIP is attributable to this same peptide sequence.

In this paper, we describe functional studies on the synthetic peptide corresponding to this putative HP/HS-binding motif: CRPKAKAKAKAKDQTK, referred to as HIP peptide. The current studies demonstrate that HIP peptide is a highly selective ligand, binding to cell surface HS/HP and supporting JAR cell attachment.

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HP/HS-binding peptide, recognizes certain forms of HP and cell- and extracellular matrix-associated HS expressed by human cell lines, and can support the HS-dependent attachment of a human trophoblast cell line. Therefore, this peptide motif can account, at least in part, for the HS/HP binding and adhesion promoting activities of HIP.

**EXPERIMENTAL PROCEDURES**

**Materials**—Heparin, bovine kidney heparan sulfate, bovine intestinal mucosa heparan sulfate, chondroitin sulfates A and C, dermatan sulfate, keratan sulfate, hyaluronic acid, heparinase from the cow lung, heparinases I and III, BSA, and CHAPS were purchased from Sigma. [3H]HP (0.44 Ci/mmol) and [35S] sulfate (43 Ci/mg) were purchased from NEN Life Science Products and ICN Biochemicals Inc. (Irvine, CA), respectively. [3H]HP was labeled by reduction with sodium [3H]borohydride. [6-3H]Glucosamine was purchased from ICN. Fetal bovine serum, and Dulbecco's phosphate-buffered saline (PBS) media, and supplements were from Irvine Scientific (Santa Ana, CA).

**Cell Culture**—JAR and RL95 cells were grown in a 1:1 mixture of Dulbecco's modified Eagle medium (DMEM) and Ham's F-12 medium (F-12) containing 10% (v/v) heat-inactivated fetal bovine serum, 15 mM HEPES, pH 7.4, 50 units of penicillin/mL, and 50 μg of streptomycin sulfate/mL.

**Peptide Synthesis, Conjugation to Maleimide-activated BSA, and HIP Peptide Affinity Chromatography**—A synthetic peptide (HIP peptide) derived from HIP, a novel cell surface HS/HP-binding protein (I, 2), CRPKAKAKAKAKAQKTK, and a randomly scrambled peptide, CQKAKRAAKAPQDKK, were synthesized on a Venga 250 peptide synthesizer using Fmoc (N-(9-fluorenlyl)methoxycarbonyl) methodology (21). This synthetic peptide was conjugated to maleimide-activated BSA (Irvine) through the sulfhydryl group of cysteine in the peptide following the coupling protocol provided by the manufacturer. The HIP peptide affinity matrix was formed by cross-linking the BSA-conjugated HIP peptide to cyagenon bromide-activated Sepharose (Sigma) in the presence of N-acetylated HIP. Inclusion of acetylated HIP was adopted to produce a more stable affinity matrix by shielding the HP-binding sites of the HIP peptide complex from cross-linking to the Sepharose beads. Acetylation of HP was performed following the method described previously (22). In a parallel study, the pH affinity classes of HP, peaked at 0.15 μM NaCl, 0.3 μM NaCl, and 2.2 μM NaCl, respectively, were separated by HIP peptide affinity chromatography using gradient elution (23). Therefore, in most cases, stepwise elution of HIP peptide affinity chromatography was employed. The HIP peptide affinity matrix was packed into a 3-mL column and equilibrated with 0.15 μM NaCl-PBS. [3H]HP or [35S] HS resuspended in 0.15 μM NaCl-PBS was loaded into the column. The column was then washed sequentially with 0.15 μM NaCl-PBS (run-through) (RT-HP or HS), 0.45 μM NaCl-PBS (low affinity (LA)-HP or HS), and 3.0 μM NaCl-PBS (high affinity (HA)-HP or HS). Radioactivity of each fraction was determined by liquid scintillation counting.

**Metabolic Labeling and Isolation of Different Fractions of Cell-expressed HS**—Near confluent cultures of RL95 and JAR cells were rinsed several times with serum-free medium consisting of RPMI 1640 (minus sulfate) supplemented with 3.3 mM MgCl2, 1.2 g/liter NaHCO3, 15 mM HEPES, pH 7.2, 2.5 units/ml penicillin, and 2.5 μg/ml streptomycin sulfate. Streptomycin sulfate served as the sole source of nonradioactive sulfate in this medium. The cells were incubated overnight in the same low sulfate medium described above containing 0.5 μCi/ml H3[35S] SO4. For generation of [3H]-labeled HS, RL95 cells were labeled overnight in media containing 25 μCi/ml [3H]glucosamine. The following day, the medium was collected and the cell layers were rinsed several times with ice-cold PBS. The cell layers were then incubated for 30 min on ice with PBS containing 50 μg/ml trypsin to release cell surface proteoglycans. Cells were not released from the tissue culture surfaces under these conditions, nor was cell viability compromised as indexed by trypan blue exclusion. At the end of the labeling period, the trypsinated cell layers were suspended in fresh warm medium and incubated at 37°C for 2 h. Afterward, the cell layers were rinsed with 300 μl of PBS three times, and 100 μl of ice-cold PBS containing 0.1% (v/v) heat-denatured BSA (50°C for 30 min) was added and incubated at 37°C for 1 h. Afterward, the cells were rinsed with 200 μl of PBS three times, and then 50 μl of [3H]HP (or [35S]HS)-containing reaction mixture ([3H] HP typically was added at a concentration of 1.5 × 105 dpm/well (approximately 5000 nmol) with 0.1% (w/v) BSA and 0.1% (w/v) CHAPS in PBS) was added, and incubated at 37°C for 1 h.

**HIP binding to HIP peptide** was performed in 96-well microassay plates (Corning, Corning, NY). Fifty μl of BSA-conjugated HIP peptide in PBS (100 μg/ml), as well as the same amount of concentration of other controls, was added to each well and dried at 37°C overnight. The next day, each well was rinsed with 200 μl of PBS three times, and 100 μl of ice-cold PBS containing 0.1% (w/v) heat-denatured BSA (50°C for 30 min) was added and incubated in a 37°C incubator for at least 1 h. Afterward, the wells were rinsed with 200 μl of PBS three times, and then 50 μl of [3H]HP (or [35S]HS)-containing reaction mixture ([3H]HP typically was added at a concentration of 1.5 × 105 dpm/well (approximately 300 nmol) along with 0.1% (w/v) BSA and 0.1% (w/v) CHAPS in PBS) was added, and incubated in a 37°C incubator overnight. There were no differences in...
the amount of [3H]HP bound after two to four washes under these conditions. Therefore, the amount of bound radioactivity detected after three washes was considered to represent a functional equilibrium and was used to calculate K_{eq,app}. Where indicated, unlabeled HP, bovine kidney HS, bovine intestinal mucousa HS, chondroitin sulfates A and C, dermatan sulfate, keratan sulfate, or hyaluronic acid were included as competitors. At the end of each experiment, unbound [3H]HP (or [35S]HS) was removed with 200 μl of PBS three times. Bound [3H]HP (or [35S]HS) was extracted from each well by overnight incubation at 37 °C with 100 μl of extraction buffer: 4 μl guanidine HCl, 25 μM Tris-HCl, pH 8.0, 2.5 mM EDTA, and 0.02% (w/v) sodium azide. Half of the extract was counted in a Beckman scintillation counter.

**Cell Attachment Assay of HIP Peptide**—Cell attachment assays employed 24-well tissue culture plates (Costar). Each well was coated either with 200 μl of BSA-conjugated HP peptide (100 μg/ml) or with the same amount of other controls. The plates were incubated in a 37 °C incubator. The next day, each well was rinsed with 0.5 ml of PBS three times. Subsequently, 500 μl of 0.1% (w/v) heat-denatured BSA in PBS was added to each well and incubated at 37 °C for at least 1 h. Each well was rinsed again with 0.5 ml of PBS three times prior to the addition of cells. JAR cells were grown to approximately 90% confluence. Cells were detached with trypsin/EDTA solution for 5 to 10 min at 37 °C, washed with DMEM/F-12 (1:1) plus 1% (w/v) BSA three times, and resuspended in DMEM/F-12 (1:1) plus 1% (w/v) BSA. This cell suspension (100 μl/well) was then added to the wells described above, and incubated for 24 h at 37 °C in a 3% CO2 incubator. At the end of each experiment, unattached cells were gently rinsed away with 0.5 ml of PBS three times, and the relative cell attachment was determined using the hemocytometer assay as described (28).

**Glycosaminoglycans (GAG) Competition and the Effects of GAG Lyases on JAR Cell Attachment to HIP Peptide**—A variety of GAGs, including unlabeled HP, bovine kidney HS, bovine intestinal mucousa HS, chondroitin sulfates A and C, dermatan sulfate, keratan sulfate, and hyaluronic acid, were used as competitors in assays of cell attachment to HIP peptide. Different GAGs (100 or 200 μg/ml) were included in the incubation medium for cell attachment assay in the competition studies. To determine if cell surface HP/HS or other GAGs of JAR cells mediate JAR cell attachment to HIP peptide, some cell attachment assays were performed in the presence of different GAG lyases (500 milliunits/ml), including heparinases I, II, and III, chondroitinas AC and ABC, with the addition of a protease inhibitor solution (11).

## RESULTS

### [3H]HP Binding to HIP Peptide—Solid phase assays were employed to determine the HP/HS binding activity of HIP peptide. Preliminary studies established that a coating concentration at 100 μg/ml HP peptide in PBS gave optimal binding for both [3H]HP and cell attachment. Furthermore, [3H]HP did not bind to BSA or BSA conjugated to an irrelevant peptide (CSSLSYTNPVAATSANL) corresponding to the cytoplasmic tail region of the mucin, MUC1 (29). Therefore, 100 μg/ml HP peptide was used typically in [3H]HP or [35S]HS binding and cell attachment studies. In addition, solid-phase assays also were performed using HIP peptide not conjugated to maleimide-activated BSA as coating material, and similar results were obtained as described above for BSA-conjugated HIP peptide (data not shown). Collectively, these results demonstrate HP binding to HIP peptide and not the BSA carrier. [3H]HP binding also was time-dependent, reaching maximal values within 10–15 min (data not shown).

As shown in Fig. 1A, [3H]HP binding to HIP peptide is concentration-dependent with a 50% saturation at approximately 245 nM when these data are analyzed as a Scatchard plot (Fig. 1A, inset). Four separate experiments yielded an average 50% saturation at a point of 300 nM. A peptide with the same amino acid composition as HIP peptide, but with a scrambled sequence, CQKAKTRAKAAKPDKKK, bound [3H]HP with a 10–20-fold lower affinity than HIP peptide (data not shown). Therefore, the peptide sequence, rather than composition, was crucial to obtain high affinity binding. In a parallel study, HIP peptide affinity chromatography of commercial [3H]HP revealed three affinity classes of HP (RT-HP, LA-HP, and HA-HP) with regard to binding to HIP peptide (23). To determine the affinity of HA-HP binding to HIP peptide, HA-HP was collected and subjected to the solid-phase HIP peptide binding assay. Fig. 1B shows that HIP peptide binds HA-HP with higher affinity (50% saturation at approximately 10 nM) than bulk HP. Thus, these results suggest that HIP peptide can recognize and bind certain forms of HP better than other forms of HP.

### Selectivity of [3H]HP Binding to HIP Peptide—Selectivity of [3H]HP binding to HIP peptide was determined using different GAGs, including unlabeled heparin (HP), bovine kidney HS (HS-BK), bovine intestinal mucosa HS (HS-BIM), chondroitin sulfate A (CS-A), dermatan sulfate (DS), chondroitin sulfate C (CS-C), keratan sulfate (KS), and hyaluronic acid (HA), as competitors. As shown in Fig. 2, HIP most effectively inhibited [3H]HP binding to HIP peptide. Dermatan sulfate at 100 μg/ml inhibited [3H]HP binding to HIP peptide; however, other GAGs were ineffective in this regard (Fig. 2). We noticed that commercially available HS (HS-BIM and HS-BK) did not significantly inhibit HP binding to HIP peptide. As shown below, this agrees with other lines of evidence, indicating that only certain forms of HS bind to HIP peptide. A dose dependence study demonstrated that HIP was approximately 50-fold more effective than dermatan sulfate at inhibiting [3H]HP binding to HIP peptide (Fig. 2, inset). Collectively these studies demonstrated that HIP peptide binds HP much more selectively than other GAGs.

### Cell Surface and Extracellular Matrix-associated HS Binds to HIP Peptide—Fig. 3 shows that, in solid-phase assays, [35S]HS isolated from RL95 cell surfaces binds to HIP peptide in a concentration-dependent and saturable fashion. Previous studies established the median molecular weight of cell surface HS chains of RL95 cells (26). Assuming the specific activity of the [35S]sulfate in these HS preparations was similar to that of the sulfate precursor used for metabolic labeling, a 50% saturation point of approximately 0.2 nM was estimated. Inclusion of 100 μg/ml unlabeled HP inhibited almost all of this binding (>90%). Furthermore, HS isolated from cell surfaces of various breast cancer cell lines also specifically binds to HIP peptide (30). Collectively, these data illustrate that certain forms of HS expressed at the cell surface or the extracellular matrix by human cells binds to HIP peptide.

Given the ability of dermatan sulfate to partially compete for [3H]HP binding in the solid-phase assays as well as in JAR cell adhesion assays (see Ref. 16, and studies below), [35S]O4-labeled HS and dermatan sulfate were isolated from either RL95 or JAR cells and tested for HIP peptide binding by HIP peptide affinity chromatography. Representative HIP peptide affinity column elution profiles of [35S]DS and [35S]HS are shown in Fig. 4 (A and B, respectively). These experiments demonstrate that a much higher percentage of cell-associated glycosaminoglycans bound to HIP peptide than secreted forms. In most fractions, bound glycosaminoglycans eluted between 0.2 and 0.3 M NaCl; however, cell surface HS contained higher affinity species that required more than 0.4 M NaCl for elution. Table I summarizes the results of HIP peptide affinity chromatography of [3H]HP and different fractions of [35S]HS or [35S]DS isolated from RL95 or JAR cells. Some variability (46–63%) was observed in binding with different [3H]HP preparations. Most (78–93%) extracellular matrix HS from RL95 cells bound to HIP peptide affinity matrix at physiological salt concentrations. Cell-associated [35S]HS of JAR cells, either accessible or nonaccessible to trypsin release, also bound to HIP peptide, albeit a lower percentage than the corresponding fractions from RL95 cells. In contrast, secreted [35S]HS from both cell lines
displayed very little binding to HIP peptide. A similar pattern was observed for JAR cell DS, i.e., a fraction of cell-associated forms, either accessible or inaccessible to trypsin release, bound to HIP peptide whereas secreted DS bound relatively poorly. It was concluded that cell- or extracellular matrix-associated forms of HS or DS were capable of binding to HIP peptide under physiological conditions; secreted HS or DS bound poorly.

**Characterization of HIP Peptide-binding HS Fractions**—

**[3H]glucosamine- or [35S]O4-labeled HS were prepared from**

**FIG. 1.** Concentration dependence of [3H]HP binding to HIP peptide. A, the indicated concentrations of bulk commercially available [3H]HP were added to wells coated with HIP peptide in the presence (○) or absence (●) of 1 mg/ml unlabeled HP, and incubated at 37 °C for 2 h. At the end of the experiment, unbound [3H]HP was removed and the bound [3H]HP was determined. Results are means ± S.D. of triplicate determinations. Inset, kinetic analyses using assumptions for Scatchard analysis of the data indicating an apparent $K_D = 245$ nM. B, bulk [3H]HP was fractionated by HIP peptide affinity chromatography as described under “Experimental Procedures.” The HIP peptide affinity-purified [3H]HP also was subjected to solid phase assays as described in A. The inset shows that Scatchard analyses of the data and demonstrates an apparent $K_D = 10$ nM.

**FIG. 2.** Selectivity of glycosaminoglycan binding to HIP peptide. Equal amounts of [3H]HP (150,000 dpm/well) were added to wells coated with HIP peptide in the presence of 100 μg/ml unlabeled glycosaminoglycans (C, no competitor control; HP, unlabeled heparin; HS-BK, bovine kidney heparan sulfate; HS-BIM, bovine intestinal mucosa heparan sulfate; CS-A, chondroitin sulfate A; DS, dermatan sulfate; CS-C, chondroitin sulfate C; KS, keratan sulfate; HA, hyaluronic acid) and incubated at 37 °C for 2 h. At the end of the experiment, unbound [3H]HP was removed with PBS and the bound radioactivity was determined. The inset is a comparison of [3H]HP binding to HIP peptide in the presence of the indicated concentrations of HP (●) and DS (▼) and demonstrates that HP is at least 50-fold more effective than DS in inhibiting [3H]HP binding. Results are means ± S.D. of triplicate determinations in all cases.
FIG. 3. Concentration dependence of $[^{35}S]$HS binding to HIP peptide. The indicated amounts of $[^{35}S]$HS derived from cell surfaces of RL95 cells, were added to wells coated with HIP peptide in the presence or absence (•) of 100 µg/ml unlabeled HP, and incubated at 37°C for 2 h. At the end of the experiment, unbound $[^{35}S]$HS was removed and bound $[^{35}S]$HS was determined. The data shown are the averages ± range of duplicate determinations in each case.

FIG. 4. HIP peptide affinity chromatography of JAR cell dermatan sulfate and HS fractions. Glycosaminoglycan fractions were prepared from JAR cells and HIP peptide affinity chromatography performed as described under “Experimental Procedures.” Typically, 1–6 × 10$^5$ dpm of $[^{35}S]$-labeled glycosaminoglycan was loaded. For simplicity, the elution profiles of the run-through, i.e. not bound at 0.15 M NaCl, have been omitted and only the elution profiles of the bound material is shown. Profiles of cell surface, i.e. trypsin-releasable (△), cell-associated but not trypsin-releasable (△), and secreted (□) are shown for both dermatan sulfate (A) and HS (B). The salt gradient (0.15–2.0 M NaCl) is indicated by the dashed line. Fractions of 0.45 ml were collected in each case.

RL95 cells representing secreted or released, cell surface (cell-associated and trypsin-releasable), intracellular (cell-associated, but not trypsin-releasable) and extracellular matrix forms. Each fraction was further separated by affinity on a HIP-peptide affinity matrix into three additional fractions. These fractions were: 1) material that did not bind at physiological salt concentrations (RT-HP); 2) material that bound at physiological salt concentrations, but could be eluted with 0.45 M NaCl (LA-HP); and 3) material that remained bound at 0.45 M NaCl, but could be eluted with 3 M NaCl (HA-HP). Each fraction was characterized further by several methods. Anion exchange liquid chromatography (Fig. 5A) demonstrated that cell surface-derived LA- and HA-HS had a slightly higher negative charge density than RT-HS; however, there were no detectable differences in this regard between LA- and HA-HS. These differences between RT-HS and the other HS fractions were found not only in cell surface HS fractions (shown in figure), but also in all other HS fractions examined (data not shown). Molecular exclusion chromatography (Fig. 5B) demonstrated that LA- or HA-HS fractions that bound to HIP peptide were generally larger (median mass > 70 kDa relative to polysaccharide standards) than RT-HS (median mass < 70 kDa). HA-HS was skewed toward lower molecular mass forms relative to LA-HA. These observations were consistently observed in HS derived from the cell surface (shown in figure) as well as secreted, extracellular matrix-associated, and intracellular forms of HS (data not shown).

HS disaccharides generated by exhaustive digestion with a heparinase mixture revealed additional structural differences in HS species that interacted with HIP peptide. A representative disaccharide profile is shown in Fig. 6. The data obtained from all HS fractions are summarized in Table II. In general, RT-HS fractions from all cellular compartments were poorly sulfated with a very high fraction of nonsulfated disaccharides (83–97%). Nonsulfated disaccharides also represented a higher proportion of the total in LA-HS (43–53%) than HA-HS (24–38%). In contrast, HA-HS species were greatly enriched for di- and polysulfated disaccharide species (35–55%). LA-HS species had a larger proportion of di- and trisulfated disaccharides (22–30%) than RT-HS and displayed intermediate proportions of di- and trisulfated disaccharides (18–22%) relative to HA-HS. LA-HS fractions also were generally enriched for monosulfated species relative to RT-HS (10–22% versus 9–15%, respectively). Collectively, these studies indicated that there were structural differences not only between the HS species that bound or did not bind to HIP peptide, but also between the

<table>
<thead>
<tr>
<th>Glycosaminoglycan</th>
<th>Bound at 0.15 M NaCl</th>
<th>%</th>
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<tr>
<td>[3H]HP</td>
<td>46–63</td>
<td></td>
</tr>
<tr>
<td>RL95 secreted [35S]HS</td>
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<td></td>
</tr>
<tr>
<td>RL95 ECM [35S]HS</td>
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<td></td>
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<tr>
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species that bound with low versus high affinity.

**JAR Cell Attachment to HIP Peptide**—JAR cells, a human trophoblastic cell line derived from a placental choriocarcinoma (31), was used in a detailed examination of cell attachment to HIP peptide. As described above, preliminary studies determined that 100 µg/ml peptide was an optimal coating concentration and was used for routine JAR cell attachment assays. Kinetic studies showed that JAR cell attachment to HIP peptide was time-dependent with near maximal attachment observed between 4 and 8 h of incubation at 37 °C (data not shown). Thus, the routine JAR cell attachment assays were performed using 4–6-h incubations. Table III shows that JAR cells attached to HIP peptide, and more than 90% of this attachment was inhibited in the presence of 1 mg/ml HP. Heat-denatured BSA, used to block nonspecific binding sites after coating, did not support JAR cell attachment. JAR cells attached well to fibronectin (FN); however, attachment to FN was not inhibited by 1 mg/ml HP. CT-1 peptide, a peptide sequence corresponding to a cytoplasmic region of the human epithelial mucin, MUC1 (29), conjugated to maleimide-activated BSA also was used as a nonspecific peptide control as well as a control for the activated BSA used in HIP peptide conjugation, and did not support JAR cell attachment. Thus, JAR cells specifically attached to HIP peptide in a HP-inhibitable fashion. The calculated pI of HIP peptide is relatively high (9.9). Therefore, it was considered that JAR cell attachment to HIP peptide was due to a nonspecific charge effect; however, lysosome (pI = 10.5–11.0), failed to support JAR cell attachment. Furthermore, JAR cells also failed to attach to a peptide containing a randomly scrambled sequence of the same amino acid composition and therefore, identical pI, as the HIP peptide. As an additional test of the specificity of cell attachment to HIP peptide, surfaces were coated with HIP peptide and soluble HIP peptides or a scrambled peptide were used as competitors. As shown in Table IV, soluble HIP peptide at 100 µg/ml effectively inhibited cell attachment; however, the scrambled sequence, even at 500 µg/ml, had little effect in this regard. Consequently, cell recognition of HIP peptide appeared to be sequence-specific.

**Fig. 6.** Anion exchange liquid chromatography of HS disaccharides. [3H]Glucosamine-labeled HS derived from RL95 cell surfaces was prepared and fractionated by stepwise HIP-peptide affinity chromatography as described under "Experimental Procedures." These fractions then were digested to completion with a heparinase mixture and analyzed by strong anion exchange liquid chromatography on a Hydro-pore-5-AX column as described under "Experimental Procedures." Fraction numbers were collected every 0.5 min, and the profile of the radioactivity eluting in each fraction as determined by liquid scintillation counting is shown. The numbers above the peaks refer to the elution positions of the following disaccharides used as internal controls and monitored by measuring A232 using a flow-through spectrophotometer (where DUA represents 4-deoxy-α-threo-hex-4-enopyranosyluronic acid, GlcNAc represents 2-acetamido-2-deoxy-D-glucose, and GlcNS represents 2-deoxy-2-sulfamido-D-glucose); 1, DUA—GlcNAc; 2, DUA—GlcNS; 3, DUA—GlcNAc-6-O-sulfate; 4, DUA—GlcNAc; 5, DUA—GlcNS-6-O-sulfate; 6, DUA-2-O-sulfate—GlcNS; 7, DUA-2-O-sulfate—GlcNAc-6-O-sulfate; 8, DUA-2-O-sulfate—GlcNS-6-O-sulfate.
In fact, very low concentrations (1 μM) of dermatan sulfate, and keratan sulfate demonstrated that both of the latter polysaccharides failed to inhibit JAR cell attachment to HIP peptide. Further studies on the HP selectivity of JAR cell attachment to HIP peptide, and the peptide affinity column with 0.45M NaCl; HA, material that bound and that remained bound to the peptide affinity column at 0.45 M NaCl and eluted with 3 M NaCl; disaccharide abbreviations are as described in legend to Fig. 6. ND, not detectable (>1%).

**TABLE II**

**Heparan Sulfate-binding Peptide of Uterine Epithelia**

<table>
<thead>
<tr>
<th>Disaccharide fraction</th>
<th>Secreted</th>
<th>Cell surface</th>
<th>Intra cellular</th>
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<tbody>
<tr>
<td></td>
<td>RT</td>
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<tr>
<td>ΔUA → GlcNAc</td>
<td>66</td>
<td>45</td>
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</tr>
<tr>
<td>ΔUA → GlcNS</td>
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<td>17</td>
<td>ND</td>
</tr>
<tr>
<td>ΔUA → GlcNAc-6S + ΔUA-2S → GlcNAc</td>
<td>11</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>ΔUA → GlcNS-6S</td>
<td>4</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>ΔUA-2S → GlcNS + ΔUA-2S → GlcNAc-6S + ΔUA-2S → GlcNS-6S</td>
<td>16</td>
<td>18</td>
<td>55</td>
</tr>
</tbody>
</table>

RL95 cells were metabolically labeled with [3H]glucosamine, HS fractions prepared, digested with heparinas, and analyzed by strong anion exchange liquid chromatography described under “Experimental Procedures.” The numbers in the table reflect the percentage of H recovered in each respective fraction. Some disaccharides were not clearly resolved when collected into scintillation vials for counting. These unresolved components were group for the calculations as indicated. Abbreviations: RT, run-through from peptide affinity column; LA, material eluted from peptide affinity column with 0.45M NaCl; HA, material that bound and that remained bound to the peptide affinity column at 0.45 M NaCl and eluted with 3 M NaCl; disaccharide abbreviations are as described in legend to Fig. 6. ND, not detectable (>1%).

**FIG. 7.** JAR cells attach but do not spread on HIP peptide. JAR cells were added to wells coated with fibronectin (FN), HIP peptide (HIP Pep), or heat-denatured BSA (D-BSA), respectively, and incubated at 37 °C for 24 h. Unbound cells were removed with PBS at the end of the experiment, and cell attachment was determined by hexosaminidase assay. JAR cells attached to both HIP peptide and FN, but only spread on FN. JAR cells did not attach to denatured BSA. JAR cell attachment to HIP peptide in the presence of 1 mg/ml HP showed the similar pattern as that of denatured BSA (data not shown). Original magnification, ×200.

**TABLE III**

**Heparan Sulfate-binding Peptide of Uterine Epithelia**

<table>
<thead>
<tr>
<th>Coating material</th>
<th>Percentage of control</th>
<th>Percentage of control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−HP</td>
<td>+HP (1 mg/ml)</td>
</tr>
<tr>
<td>HIP peptide</td>
<td>100 ± 5</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>D-BSA</td>
<td>3 ± 3</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>180 ± 3</td>
<td>182 ± 7</td>
</tr>
<tr>
<td>CT-1 peptide</td>
<td>6 ± 2</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>3 ± 1</td>
<td>2 ± 1</td>
</tr>
</tbody>
</table>

**TABLE IV**

**Percentage of control**

<table>
<thead>
<tr>
<th>Competitor</th>
<th>Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>HIP peptide, 100 μg/ml</td>
<td>15 ± 4</td>
</tr>
<tr>
<td>Scrambled peptide, 100 μg/ml</td>
<td>94 ± 8</td>
</tr>
<tr>
<td>Scrambled peptide, 500 μg/ml</td>
<td>99 ± 4</td>
</tr>
</tbody>
</table>

JAR cells (1 × 10⁵ cells/well) were added to the wells coated with HIP peptide-BSA, as described under “Experimental Procedures,” and incubated at 37 °C for 5 h. Unattached cells were rinsed off with PBS at the end of the experiment, and cell attachment was determined by hexosaminidase assay (28). Data are expressed as percentages of untreated control, i.e. attachment to HIP peptide in the absence of HP. Results represent the means ± S.D. of triplicate determinations in each case.

**HP Sensitivity and Glycosaminoglycan Selectivity of JAR Cell Attachment to HIP Peptide—GAG selectivity of JAR cell attachment to HIP peptide was determined using different GAGs as competitors.** Fig. 8 shows that HP was the most effective inhibitor among all GAGs tested. Dermatan sulfate and keratan sulfate also displayed inhibitory activity at a concentration of 200 μg/ml, but other GAGs did not significantly inhibit cell attachment to HIP peptide. Further studies on the inhibitory effect of different concentrations of HP, dermatan sulfate, and keratan sulfate demonstrated that both of the latter polysaccharides failed to inhibit JAR cell attachment activity at concentrations below 50 μg/ml, i.e. about 100-fold higher than that required for HP (Fig. 8, inset). In fact, very low concentrations (1 μg/ml) of dermatan or keratan sulfate consistently stimulated JAR cell attachment to HIP peptide, although the reason for this is unclear. Additional studies demonstrated that JAR cell attachment to HIP peptide was inhibited more than 90% in the presence of 0.5 μg/ml (approximately 50 nM) of HP. Therefore, JAR cell attachment to HIP peptide is highly selective for HP versus other GAGs.

To determine if HS on JAR cell surfaces mediated JAR cell attachment to HIP peptide, JAR cell attachment to HIP peptide was performed in the presence of different GAG lyases. GAG lyases were added to HIP peptide-coated wells in the presence of protease inhibitors (Fig. 9). Heparinase ABC inhibited JAR cell attachment to HIP peptide by approximately 90%. In contrast, chondroitinase ABC inhibited attachment to a lesser extent (55%); however, chondroitinase AC was ineffective in this regard. Consequently, consistent with previous studies (16), cell surface dermatan sulfate, but not chondroitin sulfate, appeared to participate in JAR cell binding to HIP peptide as well as HS. In addition, Fig. 9 shows that soluble HIP peptide as well as HP both effectively inhibited JAR cell attachment to HIP peptide. Collectively, these observations indicate that cell surface HS and, to a lesser extent, dermatan sulfate expressed by JAR cells mediate binding to HIP peptide.
absence of GAG competitor. Abbreviations are as described for Fig. 2. Data are expressed as percentages of cell binding observed in the coated wells, and cell attachment was determined as described above.

Inset: JAR cells were added in the presence of the indicated concentrations of GAGs to wells coated with HIP peptide, and incubated at 37 °C for 5 h. At the end of the experiment, unattached cells were removed with PBS, and cell attachment was determined by hexosaminidase assay. Results represent the means ± S.D. of triplicate determinations in all cases. In the inset, JAR cells were added in the presence of the indicated concentrations of HP (●), keratan sulfate (KS, □), and DS (▲) to HIP peptide-coated wells, and cell attachment was determined as described above. Data are expressed as percentages of cell binding observed in the absence of GAG competitor. Abbreviations are as described for Fig. 2.

**DISCUSSION**

Multiple lines of evidence support the hypothesis that HSPGs and their corresponding binding sites participate in the initial attachment of murine blastocyst and human trophoblastic cell lines to the uterine epithelial cells and cell lines. Highly specific cell surface HS/HP-binding sites have been identified on RL95 cells, and a partial amino-terminal amino acid sequence was obtained for HP binding, tryptic peptides derived from RL95 cell surfaces (17). A full-length cDNA has been obtained corresponding to one of these fragments using approaches of reverse transcription-polymerase chain reaction and library screening (1). Structural analysis of the predicted peptide sequence derived from this cDNA revealed a region that is composed of highly positively-charged amino acids separated by hydrophobic amino acids, a motif found in various HS/HP-binding proteins and suggested to function as a HS/HP-binding domain (18). This region is hydrophilic and likely to be exposed at the exterior surface of the protein, where it might participate in HS/HP-binding events. Antibodies directed to this peptide react with RL95 cell surfaces and recognize a protein that binds HP in an 125I-HP overlay assay and binds to HP-agarose with high affinity (2). In the present studies, a synthetic peptide was synthesized corresponding to this putative HP/HS-binding region for functional studies. HP/HS binding to this simple peptide also was highly selective for certain forms of HP and HS and of comparable affinity to various intact HP/HS-binding proteins (32, 33). The results confirm the prediction that this sequence binds HS/HP.

[^3H]HP binding to HIP peptide was both time- and concentration-dependent as well as saturable. More than 90% of this binding was blocked by unlabeled HP at concentrations as low as 10 µg/ml. HIP peptide bound bulk[^3H]HP with an apparent KD of 300 nM. Among all the GAG competitors tested, HP was the most effective inhibitor of[^3H]HP binding to HIP peptide and was approximately 50-fold more effective than dermatan sulfate, the next most active GAG. Therefore, it appears that the interaction of HP with the HIP peptide is not simply due to generic interactions of negatively charged polymers with the highly positively charged peptide. This contention is supported further by the observation that[^3H]HP binds relatively poorly to both lysozyme and a scrambled sequence of the HIP peptide.

Interestingly, dermatan sulfate is the only GAG other than HP that contains iduronic acid in its structure and has a small percentage of disulfated disaccharide units (34). It has been suggested that the presence of iduronic acid in the polysaccharide chains provides more flexibility in polysaccharides which may facilitate their association with corresponding binding sites (34, 35). HIP peptide binding species also are enriched for di- and trisulfated disaccharides as compared with nonbinding HS species. HS species that bound to HIP peptide with higher affinity (HA-HS) also were more enriched, in this regard, than the species that interacted with lower affinity (LA-HS). The enrichment with di- and trisulfated disaccharides is consistent with the higher negative charge density observed for LA-HS and HA-HS compared with RT-HS by anion exchange chromatography. Collectively, the present studies demonstrate that HIP peptide binds HP with a high degree of selectivity and suggest that the presence of iduronic acid in and/or regions with a high degree of sulfation contribute to HP binding to HIP peptide.

A small fraction (approximately 1–5%) of[^3H]HP isolated from commercially available[^3H]HP by HIP peptide affinity chromatography binds to HIP peptide with a substantially higher affinity (KD(app) = 10 nM) than bulk HP (23). Since HP/HS polysaccharides possess the highest degree of sequence heterogeneity among GAGs, this result further suggests that HIP peptide recognizes only a subset of structures resident within HP/HS chains. In the present study, it was further demonstrated that certain forms of HS found on cell surfaces and extracellular matrix of RL95 and JAR cells specifically bound to HIP peptide. In contrast, very little secreted HS bound to HIP peptide at physiological salt concentrations (0.15 M NaCl). It is possible that released forms of HS are depleted of HIP peptide binding motifs due to the action of heparanase, an
enzyme that destroys these sequences (36). Collectively, these observations strongly suggest that specific polysaccharide structures recognized by HIP peptide reside both in commercial HP preparations and certain forms of cell-expressed HS. Previously, we found that HA-HP binds to antithrombin III with particularly high affinity (23), suggesting that HIP peptide recognizes the same well defined HP polysaccharide structure specifically recognized by antithrombin III (37). Thus, HIP peptide can account, at least in part, for the HP/HS binding activity of the parental HIP protein. The HP/HS binding activity of the HIP peptide coupled with the cell surface disposition of HIP (2) and the affinity of cell surface and extracellular matrix forms of HS for HIP peptide (Ref. 30, and present studies) strongly suggest that HIP participates in aspects of HS-dependent cell adhesion.

JAR cell attachment to HIP peptide was HS-dependent and not simply due to nonspecific charge effects. JAR cell attachment was much more sensitive to HP than the other GAGs tested with maximal inhibition achieved at HP concentrations of 0.5 μg/ml. This concentration is approximately 50 nm, assuming an average molecular weight of 10,000 for HP chains and is consistent with the Kd obtained for the high affinity fraction of HP. HS removal from JAR cell surfaces most efficiently (>90%) inhibited JAR cell attachment to HIP peptide, indicating that cell surface HS mediated this binding; however, dermatan sulfate displayed on JAR cell surfaces also may play a role. Previous studies demonstrated the presence of dermatan sulfate bearing molecules on JAR cell surfaces and indicated that this class of GAGs participated to some extent in adhesion between JAR cell surfaces and IL95 cells (16). In the present studies, selective removal of dermatan sulfate, but not chondroitin sulfate, as well as HS from JAR cells also inhibited binding to HIP peptide. Partial (55%) inhibition was observed after digestion of JAR cells with chondroitinase ABC, which removes dermatan sulfates from JAR cell surfaces. In contrast, heparinasases reduced binding by 90% or more. Soluble dermatan sulfate also inhibited JAR cell attachment, but was much less effective than HP. Collectively, these results demonstrate that HIP peptide specifically supports JAR cell attachment by binding HS expressed on JAR cell surfaces. Dermatan sulfate participates in this process, albeit to a lesser extent.

As discussed above, a number of studies in both mouse and human model systems indicate that HS PGs and their corresponding binding proteins participate in blastocyst attachment to the uterine epithelium during the initial phases of implantation. HIP is expressed by human uterine epithelium throughout the menstrual cycle; moreover, the HIP peptide motif studied here has been shown to be accessible at cell surfaces (2). Previous studies demonstrated that intact HIP supports the attachment of human trophoblastic cell lines (20). More recently, we have determined that human cytotrophoblast not only express, but also appear to utilize this protein for aspects of trophoblast invasion as well as attachment to the HSPG, perlecan (19). These same studies indicate that antibodies to this sequence inhibit trophoblast invasion in vitro. The interactions of the HIP peptide sequence with HS motifs important for susceptibility to heparanase action, anticoagulant activity and trophoblast attachment indicate that HIP has the potential to play a key role in HS-dependent processes in developing and adult tissues.

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A Peptide Sequence of Heparin/Heparan Sulfate (HP/HS)-interacting Protein Supports Selective, High Affinity Binding of HP/HS and Cell Attachment
Shouchun Liu, JoAnne Julian and Daniel D. Carson

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