Multiple Domains Contribute to Heparin/Heparan Sulfate Binding by Human HIP/L29†

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ABSTRACT: Human heparin/heparan sulfate interacting protein/L29 (HIP/L29) is thought to be involved in the promotion of cell adhesion, the promotion of cell growth in the cancerous state, and the modulation of blood coagulation. These activities are consistent with the proposed function of HIP/L29 as a heparin/heparan sulfate (Hp/HS) binding growth factor that has a preference for anticoagulantly active Hp/HS. Previous studies showed that a peptide derived from the C terminus of human HIP/L29 (HIP peptide-1) can selectively bind anticoagulant Hp and support cell adhesion. However, a murine ortholog does not have an identical HIP peptide-1 sequence, yet still retains the ability to bind Hp, suggesting that there may be additional Hp/HS binding sites outside of the HIP peptide-1 domain. To test this hypothesis, a systematic study of the domains within human and murine HIP/L29 responsible for Hp/HS binding activity was undertaken. Using deletion mutants, proteolytic fragments, and protease protection of HIP/L29 by Hp, we demonstrate that multiple binding domains contribute to the overall Hp/HS binding activity of HIP/L29 proteins. Furthermore, a conformational change is induced in human HIP/L29 upon Hp binding as detected by circular dichroism spectroscopy. These studies demonstrate the multiplicity of Hp/HS binding sequences within human and murine HIP/L29.

Human heparin/heparan sulfate interacting protein/L29 has been implicated in the modulation of blood coagulation (*1*) and the promotion of growth (*2*−*4*) in cancer cells. These activities are putatively linked to the ability of HIP/L29[1] to bind Hp/HS. HIP/L29 is hypothesized to regulate blood coagulation by competing with antithrombin III (ATIII) for Hp. In this regard, a peptide domain within the C terminus of human HIP/L29 (HIP peptide-1) binds Hp species containing anticoagulantly active sequences, although the sequences recognized by HIP peptide-1 may not be identical to those recognized by ATIII (*1*). ATIII acts in blood coagulation by binding to a specific Hp pentasaccharide, making it a more potent inhibitor of thrombin and factor Xa (*5*). Fibroblast growth factor receptor also binds a similar Hp sequence (*6*). The ability of 17-amino acid HIP peptide-1 to bind anticoagulant Hp is thought to contribute to the proposed in vivo activities of human HIP/L29.

Previous studies with native and recombinant human HIP/L29 suggested that there were additional Hp/HS binding domains outside of the HIP peptide-1 sequence (*7*). First, a Scatchard analysis of native human HIP/L29 indicated the presence of at least two Hp binding sites. Second, a polyclonal antibody raised against HIP peptide-1 was only able to reduce the Hp binding ability of human HIP/L29 by a maximum of 50%. Other evidence for the presence of non-HIP peptide-1 Hp/HS binding domains came from the analysis of a murine ortholog of human HIP/L29 (*8*). Murine HIP/L29 does not have an identical HIP peptide-1 sequence, yet it retains the ability to bind Hp. Murine HIP/L29 is 84% similar to human HIP/L29 over 159 amino acids and identical over the 78 N-terminal amino acids. The sequence identity between human and murine HIP/L29 suggests a conserved function at the N terminus.

This study uses a systematic approach to identify regions within human and murine HIP/L29 involved in Hp/HS binding. First, using recombinant human and murine HIP/L29, recombinant HIP/L29 truncation mutants, and proteolytically derived fragments of recombinant human HIP/L29, the domains within HIP/L29 proteins were tested for Hp/HS binding in solid-phase and affinity chromatography assays. Second, Hps of three different lengths were used to protect recombinant human HIP/L29 from proteolytic digestion. Last, circular dichroism studies were undertaken to see if significant structural changes took place in recombinant human HIP/L29 upon Hp binding. These studies show that large portions of the human HIP/L29 protein are involved in Hp/HS binding, including the highly conserved N terminus of human and murine HIP/L29 proteins. Additionally, a conformational change in human HIP/L29 occurs upon Hp binding that alters protease susceptibility.

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[1] Abbreviations: IMAC, immobilized metal affinity chromatography; Hp, heparin; HS, heparan sulfate; CS, chondroitin sulfate; HIP/L29, heparin/heparan sulfate interacting protein/L29; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence; PCR, polymerase chain reaction; ATIII, antithrombin III; PBS, phosphate-buffered saline; BSA, bovine serum albumin; dBSA, denatured bovine serum albumin; PAT, PBS/azide/Tween 20.

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EXPERIMENTAL PROCEDURES

Materials. Heparin, 6 kDa mean molecular mass Hp, 3 kDa mean molecular mass Hp, chondroitin sulfate C, sodium chloride, Tris base, glycine, heparin−agarose, tricine, Triton X-100, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Endoproteinase Glu-C (V8 proteinase) was purchased from Boehringer Mannheim (Indianapolis, IN). [^{3}H]Hp (0.44 mCi/mg) was purchased from DuPont/NEN (Boston, MA). Sodium dodecyl sulfate (SDS), β-mercaptoethanol, goat anti-rabbit horseradish peroxidase (HRPO)-conjugated antibody, goat anti-mouse HRPO-conjugated antibody, and Tween 20 were purchased from Bio-Rad. Talon affinity resin was purchased from Clontech (Palo Alto, CA). Enhanced chemiluminescence (ECL) reagents were purchased from Amersham/Pharmacia Biotech (Piscataway, NJ). [^{125}I]Protein A (45 mCi/mg) was purchased from ICN Biochemicals (Irvine, CA). Antibodies generated against HIP peptide-1 (CRPKAKAKAKAKDQTKA) and HIP peptide-2 (CMRFAKKHNKKGLKKMQ) corresponding to human HIP amino acids 119−136 and 43−58, respectively, are as described in refs *8* and *9*. An antibody generated against four sequential histidines was acquired from Qiagen (Valencia, CA).

Expression and Isolation of Human HIP/L29, Murine HIP/L29, and Human HIP/L29 Truncation Mutant Proteins. Procedures for the expression and purification of recombinant human and murine HIP/L29 are described elsewhere (*7*, *8*). PCR primers with *Bam*HI adapters were used to make the four human HIP/L29 deletions. For the N40 deletion, the following primers were used: forward 5′GGGGATCCGAGGAACATGCGCTTTGCC3′ and reverse 5′GGGGGGGGGATCCAGATATTTACTCTGA3′ corresponding to amino acids 41−159. For the N80 deletion, the following primers were used: forward 5′GGGGATCCGGTTAAGCCCAAGATCCC3′ and reverse 5′GGGGGGGGGATCCAGATATTTACTCTGA3′ corresponding to amino acids 81−159. The C41 deletion was made with the following primers: forward 5′GGGGATCCCATGGCCAAGTCCAAG3′ and reverse 5′GGGGGGGGGATCCTTACCTGAGCCCCTT3′ corresponding to amino acids 1−118. Primers for the C79 deletion were as follows: forward 5′GGGGATCCCATGGCCAAGTCCAAG3′ and reverse 5′GGGGATCCTTACTCCTTGGGCTTTACGAG3′ corresponding to amino acids 1−80. A 25-cycle PCR with denaturing at 94 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 1 min was performed to make the N40del, C41del, and N80del cDNA inserts. The C79del cDNA PCR product was created by a 25-cycle PCR with denaturing at 94 °C for 30 s, annealing at 45 °C for 30 s, and elongation at 72 °C for 1 min. All inserts were created from a human HIP/L29 template, clone 36-1 (*10*). The PCR products were cut with *Bam*HI and subcloned in-frame into the *Bam*HI site of the oligo-His vector, pV2A, as previously described (*11*). The product of this fusion added 27 N-terminal amino acids containing the oligo-histidine motif. Expression and purification of recombinant proteins are as described previously (*7*, *8*). Expression of each protein was checked by Western blot techniques using antibodies against HIP peptide-1 and/or HIP peptide-2. Proteins from one-step IMAC purification were used in a solid-phase [^{3}H]Hp binding assay and affinity chromatography over Hp−agarose. For all other assays, proteins were subjected to a second affinity chromatography over Hp−agarose. Recombinant human HIP/L29 purified by IMAC was applied to a 1 mL bed volume of Hp−agarose at room temperature. The column was washed with 10 column volumes of 50 mM sodium phosphate (pH 7.8) and 0.15 M NaCl. Bound material then was eluted with 50 mM sodium phosphate (pH 7.8) and 2 M NaCl. Protein-containing fractions were identified by Coomassie blue staining of SDS−PAGE gels and pooled. The pooled fractions then were desalted by repeated dilutions and concentrations with 50 mM sodium phosphate (pH 7.8) in a Centricon-10 apparatus. These proteins, purified by two-step affinity purification, were used in the creation of HIP/L29 proteolytic fragments, protease protection studies, and circular dichroism spectroscopy studies.

Heparin−Agarose Affinity Chromatography. Recombinant human HIP/L29, recombinant murine HIP/L29, or recombinant human HIP/L29 truncation mutants were loaded onto a 1 mL bed volume of Hp−agarose and allowed to bind. The column was washed with 5 column volumes of 0.15 M NaCl in PBS and then exposed to a gradient of NaCl in PBS. The gradient was from 0.15 to 2 M, and 500 μL fractions were collected. The salt concentration of each fraction was determined by conductivity measurements. These fractions were immediately dot-blotted onto nitrocellulose. The resulting nitrocellulose blot was blocked with 1% (w/v) BSA in PBS, 0.01% (w/v) sodium azide, and 0.05% (v/v) Tween 20 (PAT buffer) overnight. Primary antibodies against HIP peptide-1 or HIP peptide-2 were used for probing the blots with detection by [^{125}I]protein A. The blot then was exposed to a Molecular Dynamics phosphoimager screen and the resultant blot scanned by a Molecular Dynamics Storm 840 machine and quantified by the programs bundled with it. A plot of arbitrary units (*y*-axis) from the densitometry program versus the salt concentration (*x*-axis) was made to obtain an elution profile for each of the proteins. A single graph that was typical of multiple runs was used for recombinant human HIP/L29 (run in duplicate), N80del (run in duplicate), and C79del (run in triplicate). The results achieved from multiple runs of the previous experiments yielded average peak salt molarities that had standard deviations no greater than 0.06 M NaCl. Therefore, it was determined that all other graphs could be reasonably determined in a single experiment.

Human HIP/L29, Murine HIP/L29, and Human HIP/L29 Truncation Mutant Affinity Chromatography. HIP affinity columns were made by the following method. Recombinant proteins, previously purified by immobilized metal affinity chromatography (IMAC), were subjected to a second step of affinity chromatography over Hp−agarose and reapplied to Talon affinity resin. One-half microliter of [^{3}H]Hp or 5 μL of [^{35}S]HS from RL95 cell surface, obtained as described in ref *12*, was added to HIP/L29−Talon columns pre-equilibrated in 20 mM Tris-HCl (pH 8.0), 0.15 M NaCl, and 20% (v/v) glycerol and allowed to bind for at least 5 min. The column was washed with 5 times the bed volume with 20 mM Tris-HCl (pH 8.0), 0.15 M NaCl, and 20% (v/v) glycerol and then eluted with a gradient of NaCl in 20 mM Tris-HCl (pH 8.0) and 20% (v/v) glycerol. HIP/L29−Talon columns used in these experiments retained similar activity through multiple column runs. One-half milliliter fractions were collected, and a fixed aliquot was counted on a Beckman scintillation counter. The salt content for each fraction was determined on a conductivity meter. A graph

of radioactivity (*y*-axis) versus fraction number (*x*-axis) was drawn for each column run. The data from these graphs were then manipulated as described above. A single graph that was typical of multiple runs was used for [^{3}H]Hp chromatography over N40del matrixes (run in duplicate), [^{35}S]HS chromatography over recombinant human HIP/L29 matrixes (run in triplicate), and [^{35}S]HS chromatography over murine HIP/L29 matrixes (run in duplicate). The results achieved from multiple runs of the previous experiments yielded average peak salt molarities that had standard deviations no greater than 0.03 M NaCl. Therefore, it was determined that all other elution profiles could be reasonably determined in a single experiment.

Recombinant Human HIP/L29 Fragment Affinity Chromatography. Four hundred microliters of recombinant human HIP/L29 purified by two-step affinity chromatography (74 μg/mL) was incubated with 5 μL of 500 μg/mL V8 protease for 5 min at 37 °C. At the end of this incubation, 20 μL was diluted 3:1 with 4× reducing sample buffer and immediately boiled. The remaining 380 μL was loaded onto a Hp−agarose column, allowed to bind, and eluted with a 0.15 to 3 M NaCl gradient as described above. Fractions were collected in 4 drops per fraction. A portion of the fractions was boiled in reducing sample buffer and analyzed by SDS−PAGE using the tricine gel system (*13*) with the resultant gel stained with Coomassie blue.

Derivation of Human HIP/L29 Fragment Sequences. Human HIP/L29 fragments were generated as described above by incubation with V8 protease for 15 min at 37 °C and separated by affinity chromatography on Hp−agarose with gradient elution. Aliquots of the fractions eluting from the column were immediately diluted 3:1 in 4× sample buffer and boiled. Another aliquot was immediately dot-blotted onto a PVDF membrane pre-equilibrated with 100% methanol and then water. An SDS−PAGE gel was run and stained with Coomassie blue to identify a fraction that contained a mixture of peptides with molecular masses of 5 and 4 kDa and a fraction that contained a peptide with a molecular mass of 16 kDa. These two fractions were subjected to 11 cycles of N-terminal sequence analysis. The fraction containing fragments of 5 and 4 kDa yielded the following sequences: LDMAKSKNHTT corresponding to amino acids −2 and −1 of the oligohistidine affinity tag and human HIP/L29 amino acids 1−9 and SLKGVDPKFLR corresponding to human HIP/L29 amino acids 31−41. The fraction containing the 16 kDa yielded the sequence AIKALVKPKEV corresponding to human HIP/L29 amino acids 71−81. It was determined from a time course experiment that exhaustive digestion of human HIP/L29 with V8 protease yielded three peptides with apparent molecular masses of 16, 5, and 4 kDa and that a 13 kDa fragment is the precursor to the two smaller 5 and 4 kDa fragments (data not shown). Antibody reactivities were also obtained from the products of human HIP V8 digestion (data not shown). Anti-HIP peptide-1 antibody reacted to the 16 kDa peptide, while anti-HIP peptide-2 antibody reacted to the 13 and 5 kDa peptides. None of the peptides were recognized by anti-His(4) Ab. The 4 kDa peptide was found to be unreactive with the panel of antibodies that were used. The combination of these data led to the determination of the peptide products from V8 digestion. The 16 kDa fragment corresponds to amino acids 71−159. The 13 kDa fragment corresponds to two amino acids from the histidine tag adjacent to the starting methionine of human HIP extending to position 70. The 5 kDa fragment is amino acids 31−70. The 4 kDa fragment contains two amino acids N-terminal from the starting methionine of HIP extending to position 30. All the peptides that were generated were the products expected of V8 cleavage at glutamic acid residues.

Protection of Recombinant Human HIP/L29 from Proteolytic Digestion by Hp. Recombinant human HIP/L29 (1.23 μg) was incubated with 2 μg of V8 protease in the absence or presence of Hp with mean molecular masses of 3, 6, or 12 kDa. The Hp was added in 17-, 12-, and 6 -fold molar excess for the 3, 6, and 12 kDa mean molecular mass Hp, respectively. Other reactions included HIP/L29 without the addition of V8 protease or HIP/L29, V8 protease and 12 kDa mean molecular weight Hp in 1.33M NaCl. All reactions were assembled without V8 protease and allowed to incubate for 2 min at 37 °C. V8 protease or 50mM $NaPO_4$ then was added and the reactions incubated at 37 °C for 30 min. At the end of this incubation, the reactions were boiled in sample buffer for 10 min. The reactions then were subjected to SDS−PAGE using the tricine gel system in triplicate. The gels were transferred to nitrocellulose and individually probed with anti HIP peptide-1 antibody, anti HIP peptide-2 antibody or anti His(4) antibody.

Circular Dichroism (CD) Spectroscopy of Recombinant Human HIP/L29 in the Absence or Presence of Hp

CD spectra were collected on a Jasco J-720 spectropolarimeter (Tokyo, Japan) using a round, 0.5 mm quartz cell. Recombinant human HIP/L29 that was purified by two step affinity chromatography was used in the experiments. Recombinant human HIP/L29 in 50mM $NaPO_4$, pH 7.8, 0.22M NaCl, at a concentration of 225 μg/mL was mixed with 10 μL of 2.275 mg/mL 12 kDa mean molecular weight Hp; 10 μL of 1 mg/mL 3 kDa mean molecular weight Hp; or used alone, to obtain HIP/L29, or (HIP/L29)/Hp spectra. The CD data were collected over the wavelengths 195−260 nm at a scan speed of 20 nm/min, a time constant of 1 s, and a bandwidth of 1 nm averaging four scans for the final data. All spectra were corrected for buffer contributions and are presented in units of millidegrees.

RESULTS

Expression of Human HIP/L29 Truncation Mutant Proteins in Escherichia coli. To locate domains within human HIP/L29 that contribute to Hp/HS binding activity, a series of truncation mutant proteins were made (Figure 1A) and analyzed. These proteins are sequentially truncated from the N and C termini of human HIP/L29 by 40, 80, 41, and 79 amino acids, respectively. Collectively, these proteins are deficient in every domain of human HIP/L29. All truncations contained an oligo-histidine affinity tag fused to the N terminus. The proteins were expressed in *E. coli* and purified to >90% homogeneity by immobilized metal affinity chromatography (IMAC). For solid-phase assays, and relative affinity index assays entailing gradient elution from Hp−agarose, this material purified in one step was used. In all other assays, a second step of purification over Hp−agarose was used, yielding proteins with >95% homogeneity (Figure 2). Protein identity was confirmed by Western blot analysis with antibodies against HIP peptide-1 and HIP peptide-2

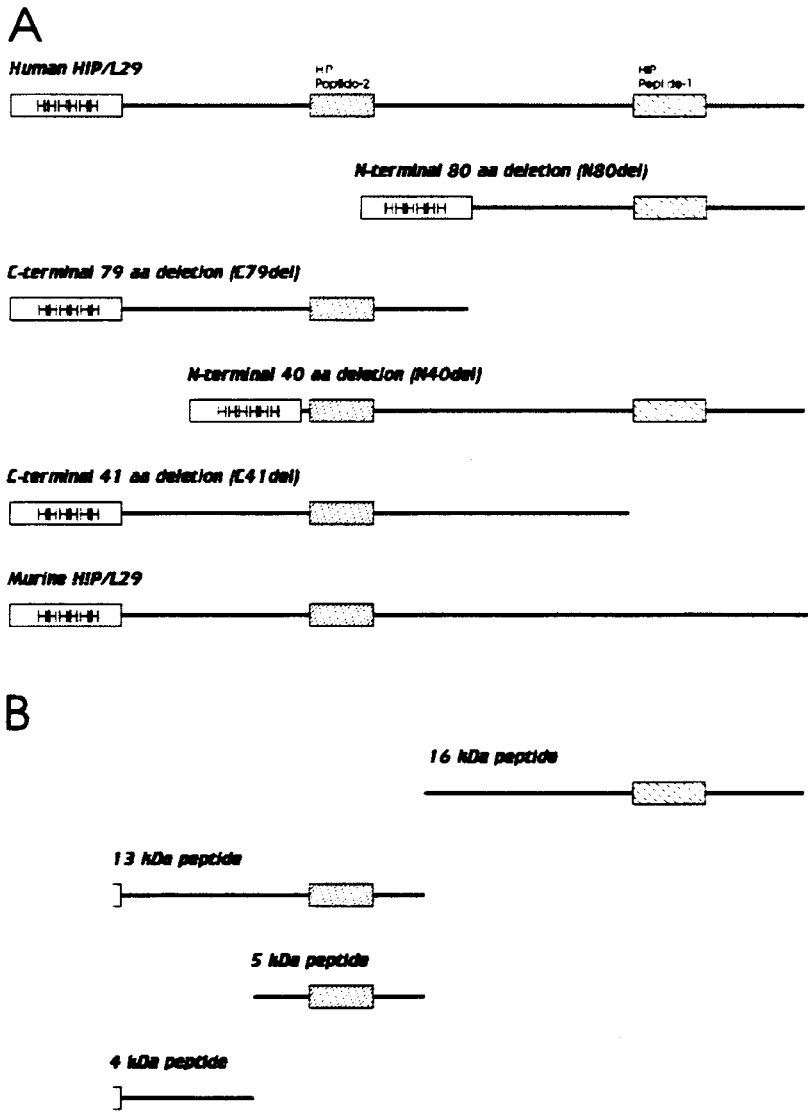

FIGURE 1: Panel of recombinant HIP/L29 proteins and proteolytic peptides. (A) The six proteins used in this study are shown in schematic form. Collectively, these proteins are deficient in each HIP/L29 domain. Each protein has an N-terminal histidine tag denoted by the boxes with HHHHHH. The boxes labeled HIP peptide-1 and HIP peptide-2 are shown for each of the proteins and indicate epitopes for antibodies used in this work. (B) The four peptides derived from V8 digestion of recombinant human HIP/L29 are shown in schematic form.

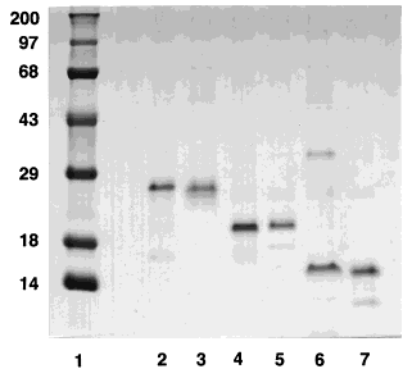

FIGURE 2: Recombinant HIP/L29 proteins expressed in *E. coli* and purified by IMAC followed by precipitation with Hp−agarose. The six recombinant HIP/L29 proteins from Figure 1 were separated by SDS−PAGE and stained with Coomassie blue: lane 1, molecular weight markers with molecular weights in kilodaltons given at the left of each marker; lane 2, recombinant human HIP/L29; lane 3, recombinant murine HIP/L29; lane 4, N40del; lane 5, C41del; lane 6, N80del; and lane 7, C79del.

(data not shown). In addition to using truncation mutant proteins of human HIP/L29 to examine Hp/HS binding, murine HIP/L29 was used. Murine HIP/L29 contains significant sequence divergence in the C terminus compared to human HIP/L29 (*8*). This series of proteins was used as an initial screen for HIP function in Hp/HS binding.

HIP/L29 Truncation Mutants Bind [^{3}H]Hp in a Solid-Phase Assay. Initially, Hp binding activity of each protein was tested in an established solid-phase assay (*8*; Figure 3). Negative controls were dBSA and contaminating proteins from IMAC purification of *E. coli* transformed with an expression vector containing the HIP/L29 cDNA in the reverse orientation. In all cases, equimolar amounts of recombinant human HIP/L29, recombinant murine HIP/L29, N40del, N80del, C41del, and C79del were used to coat the wells, and all protein truncations were found to have at least 10-fold higher [^{3}H]Hp binding activity over dBSA and reverse vector-purified proteins. A direct relationship was noted between the amount of [^{3}H]Hp bound and the size of the protein used to coat the surface.

Determining Relative Affinity Indices for Recombinant Human HIP/L29, Recombinant Murine HIP/L29, and Human HIP/L29 Truncation Mutants. To determine the relative Hp/

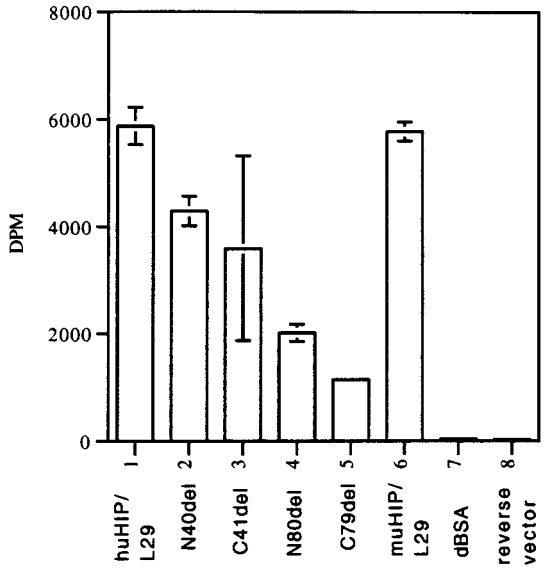

FIGURE 3: Recombinant HIP/L29 proteins bind [^{3}H]Hp in a solid-phase assay. Approximately equimolar amounts (24−29 pmol) of the panel of recombinant HIP/L29 proteins were adsorbed to wells of a 96-well plate and subjected to a solid-phase [^{3}H]Hp binding assay as described previously (*8*). [^{3}H]Hp was allowed to bind to 600 ng (29 pmol) of recombinant human HIP (lane 1), 450 ng (28 pmol) of N40del (lane 2), 450 ng (27 pmol) of C41del (lane 3), 300 ng (26 pmol) of N80del (lane 4), 300 ng (24 pmol) of C79del (lane 5), 600 ng (29 pmol) of recombinant murine HIP/L29 (lane 6), 1 μg of dBSA (lane 7), or an equal volume of proteins, purified from *E. coli* transformed with human HIP/L29 cDNA in the reverse orientation in the expression vector (lane 8). All bars were derived from triplicate values, and the means ± the standard deviation of the mean are shown accordingly.

HS binding affinity of the panel of proteins, three assays were employed (Figure 4). In the first assay, HIP/L29 proteins were chromatographed on Hp−agarose (panel A). In the second and third assays, [^{3}H]Hp or [^{35}S]HS from RL95 cell surfaces were chromatographed on HIP/L29 affinity matrixes (panels B and C, respectively). From these approaches, it was determined that intact, recombinant human HIP/L29 was the strongest binder in all assays. Both intact human and murine HIP/L29 as well as all truncation mutants bound Hp−agarose. In contrast, three unrelated His-tagged proteins did not bind Hp−agarose, indicating that the presence of the histidine tag motif was not sufficient to confer Hp binding activity (data not shown). Also, [^{3}H]Hp and [^{35}S]-HS bound to HIP/L29 matrixes over background levels, determined by applying [^{3}H]Hp over the affinity matrix column substrate alone (data not shown). Intact murine and human HIP/L29 generally bound well to Hp and HS. In general, each successive truncation of human HIP/L29 resulted in a decrease in salt molarity needed for peak elution of HIP/L29 proteins from Hp−agarose or [^{3}H]Hp and [^{35}S]-HS from HIP/L29 matrixes. In all cases, recombinant murine HIP/L29 displayed a decrease in molarity needed for peak of elution, when compared to that of recombinant human HIP/L29. Deletion of the 79 C-terminal amino acids appeared to reduce the affinity more profoundly than deletion of the 80 N-terminal amino acids.

Four Proteolytically Derived Fragments of Recombinant Human HIP/L29 Retain the Ability To Bind Hp−Agarose. Another set of human HIP/L29 truncations was generated by cleaving recombinant human HIP/L29 with endoproteinase Glu-C (V8 protease). A limited digestion of recombinant human HIP/L29 yielded four major cleavage products as detected by Coomassie blue staining of the SDS−PAGE-separated reaction mixture. These peptides migrated at apparent sizes of 16 000, 13 000, 5000, and 4000 (M_r), respectively, and were mapped along the HIP/L29 polypeptide as shown in Figure 1B. A mixture of the four peptides was fractionated by affinity chromatography on Hp−agarose. Fractions were collected, aliquots run on SDS−PAGE in the tricine gel system, and the resultant gels stained with Coomassie blue (Figure 5). From this gradient elution, a relative affinity index was formulated. All four peptides bound to Hp−agarose after extensive washing with 0.15 M NaCl in PBS. The two smaller peptides eluted between 0.3 and 0.6 M NaCl. The 13 000 M_r cleavage product eluted between 0.48 and 0.86 M NaCl. The 16 000 M_r peptide eluted between 0.61 and 0.92 M NaCl. Thus, the affinity order is as follows: 16 000 > 13 000 > 5000 = 4000. This is similar to the results obtained with the truncation mutants that indicated that larger HIP/L29 fragments generally bind Hp−agarose more tightly than smaller fragments.

Recombinant Human HIP/L29 Is Protected from Proteolytic Digestion by Hp. The interaction of Hp with domains of human HIP/L29 also was tested by exposing recombinant human HIP/L29 to V8 protease in the absence or presence of Hp with average molecular masses of 12, 6, and 3 kDa in solution (Figure 6). The resultant reaction mixtures were transferred to nitrocellulose and probed with a panel of antibodies against the histidine tag, HIP peptide-2, and HIP peptide-1. The reaction of recombinant human HIP/L29 with V8 protease in the absence of Hp generated an anti-HIP peptide-1 reactive band with an M_r of 16 000 and an anti-HIP peptide-2 reactive band with an M_r of 5000. Various peptide fragments obtained in the presence of Hp with average molecular masses of 12, 6, and 3 kDa displayed the expected antibody reactivities as peptides of these sizes described above. A protected peptide with an apparent size (M_r) of 27 000 was reactive with both anti-HIP peptide-1 and anti-HIP peptide-2 antibodies, but not to the anti-oligo-histidine antibody, indicating that the histidine tag was not protected by Hp. To demonstrate that Hp protection is derived from interaction with Hp, human HIP/L29-Hp binding was inhibited by including a high level of salt in the binding buffer. This reaction mixture was incubated with V8 protease, resulting in the same fragmentation pattern observed when recombinant human HIP/L29 was cleaved in the absence of Hp, indicating that the interaction of HIP/L29 with Hp confers protection. Only recombinant human HIP/L29, migrating at 29 000 in this gel system, without the addition of V8 protease reacted with antibodies against the oligo-histidine tag. Collectively, these data indicate that a large portion of HIP/L29 participates in Hp binding in solution.

Recombinant Human HIP/L29 Undergoes a Conformational Change upon Hp Binding. A circular dichroism scan of recombinant human HIP/L29 in the absence or presence Hp with average molecular masses of 12 and 3 kDa was performed to determine if Hp binding influences the conformation of human HIP/L29. Scans over the wavelengths of 195−260 nm were performed. All spectra were baseline subtracted against the buffer diluent (Figure 7). Hp-alone spectra were added to HIP/L29-alone spectra with little difference from HIP/L29-alone spectra, showing that Hp has

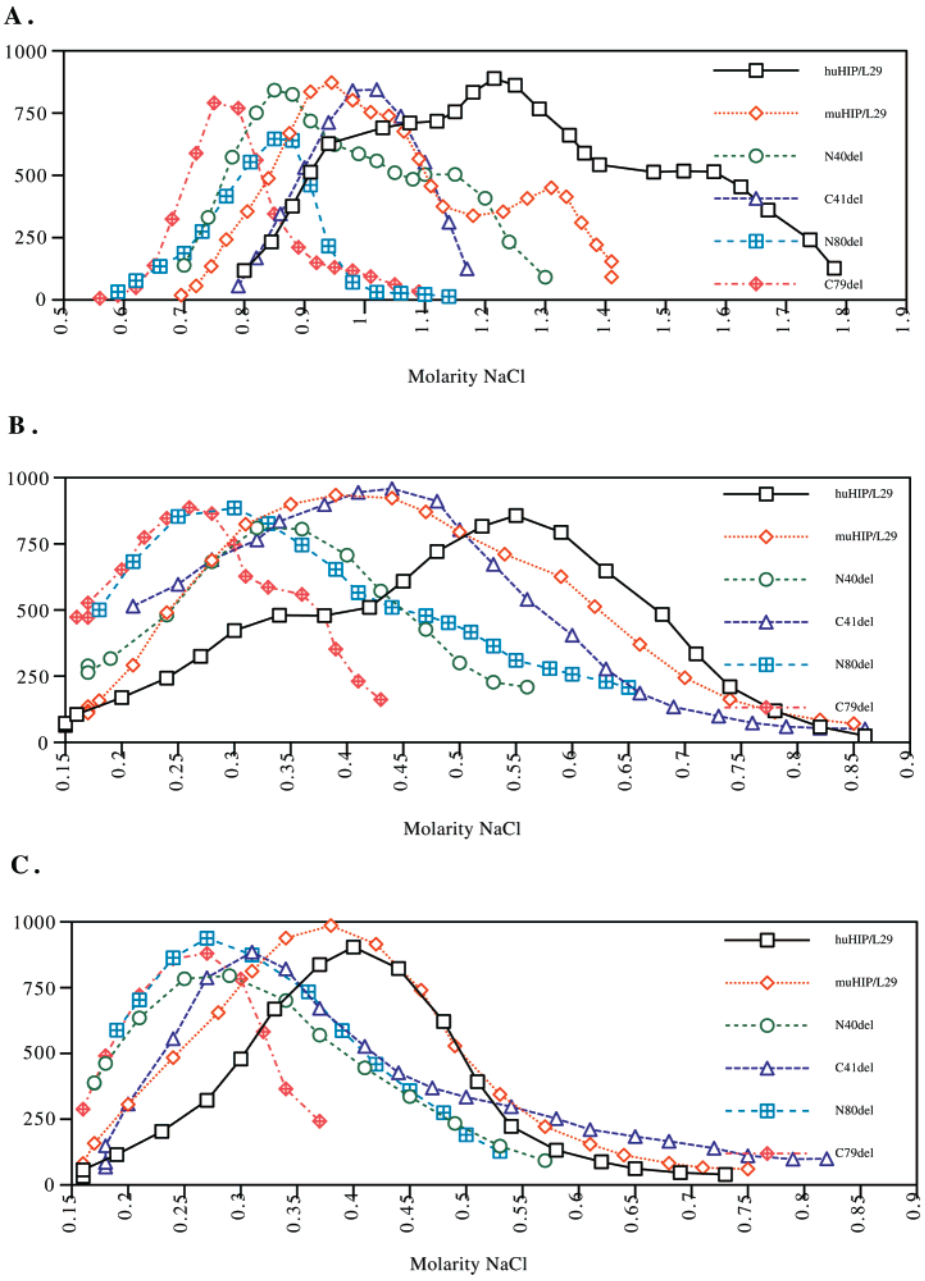

FIGURE 4: Elution profiles for affinity chromatography experiments. In panel A, recombinant HIP/L29 proteins are applied to Hp−agarose and eluted with a NaCl gradient. Elution positions were determined by Western blotting of individual fractions as described in Experimental Procedures. In panel B, [^{3}H]Hp was applied to recombinant HIP/L29 matrixes and eluted with a NaCl gradient. In panel C, [^{35}S]HS was applied to recombinant HIP/L29 matrixes and eluted with a gradient of salt. In panels B and C, elution was monitored by liquid scintillation counting of portions of each fraction. The *x*-axis for all chromatograms is NaCl molarity. The *y*-axes are arbitrary units that have been normalized and smoothed.

little contribution to absorbance at this range of wavelengths. A significant increase in the molar ellipticity of HIP/L29 was found in the 195−220 nm range upon the addition of both molecular masses of Hp. These observations indicated that the binding of Hp induced a significant conformational change in human HIP/L29.

DISCUSSION

The study presented here establishes the multiplicity of Hp/HS binding domains within HIP/L29. Using recombinant HIP/L29 proteins as a model of native HIP/L29 function is justified in previous reports where HIP/L29 derived from a human cell line and recombinant human HIP/L29 purified from *E. coli* retain identical biological activities (*7*). Deletion constructs of human HIP/L29 expressed in *E. coli* were made that produce proteins that are deficient in each HIP/L29 domain. Each truncation of human HIP/L29 retained Hp/HS binding activity over background levels. Even though it was found that all truncation mutants bound Hp/HS, significant differences in the apparent affinity of each protein relative to that of the intact protein were found. The hypothesis that multiple domains within human HIP/L29 are involved in Hp binding is further supported by the observation that four fragments of recombinant human HIP/L29, generated by limited digestion with V8 protease, also bind Hp−agarose and elute between 0.30 and 0.92 M NaCl. The molarity of salt needed for fragment elution directly correlated with the size of the fragment. In addition to these

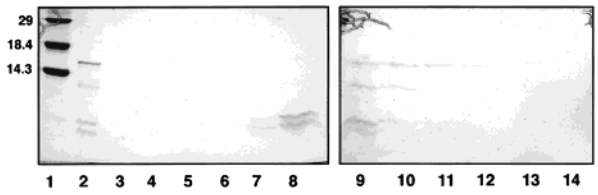

FIGURE 5: Affinity chromatography of recombinant human HIP/L29 fragments on Hp−agarose. Fragments of recombinant human HIP/L29, generated by limited proteolytic cleavage with V8 protease, are applied to Hp−agarose and eluted with a gradient of salt. Fractions are collected and subjected to SDS−PAGE and proteins detected by Coomassie blue staining. Lane 1 contained molecular weight markers with molecular weights in kilodaltons given to the left of each marker. Lane 2 contained the product of recombinant human HIP/L29 digestion for a 5 min incubation with V8 protease before the proteins were subjected to Hp−agarose affinity chromatography. Lanes 3−6 contained representative fractions collected from elution with low salt concentrations. Lanes 7−14 contained fractions collected as the salt gradient is applied. The salt molarities of the fractions in lanes 3−14 were as follows: 0.17 (lane 3), 0.17 (lane 4), 0.17 (lane 5), 0.19 (lane 6), 0.30 (lane 7), 0.48 (lane 8), 0.61 (lane 9), 0.75 (lane 10), 0.86 (lane 11), 0.88 (lane 12), 1.04 (lane 13), and 1.14 M (lane 14).

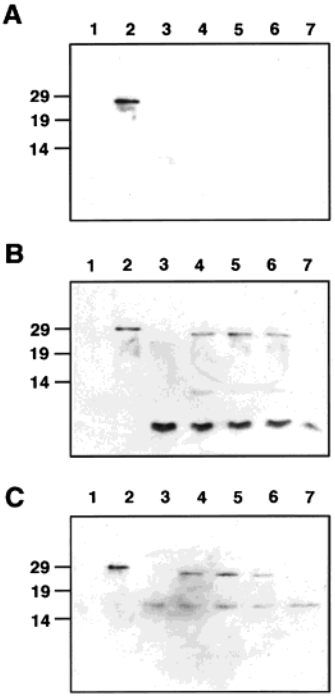

FIGURE 6: Heparin of three different sizes protects human HIP/L29 from proteolytic cleavage by V8 protease. Recombinant human HIP/L29 was incubated with V8 protease in the presence of three different lengths of Hp. Reactions were run and the products separated by SDS−PAGE. Blots were made in triplicate and probed with a panel of three antibodies reactive to three separate epitopes on recombinant human HIP/L29. In panel A, the blot was probed with an antibody generated against the His tag. In panel B, the blot was probed with an antibody generated against HIP peptide-2. In panel C, the blot was probed with an antibody generated against HIP peptide-1. The proteins and/or reaction mixtures in lanes 1−7 were as follows: 1.7 μg of V8 protease (lane 1), recombinant human HIP/L29 (lane 2), recombinant human HIP/L29 with V8 protease (lane 3), recombinant human HIP/L29 with V8 protease and 12 kDa Hp (lane 4), recombinant human HIP/L29 with V8 protease and 6 kDa Hp (lane 5), recombinant human HIP/L29 with V8 protease and 3 kDa Hp (lane 6), and recombinant human HIP/L29 with V8 protease and 12 kDa Hp in 1.33 M NaCl (lane 7).

studies, it was found that Hps of three different lengths protected a large portion of recombinant human HIP/L29

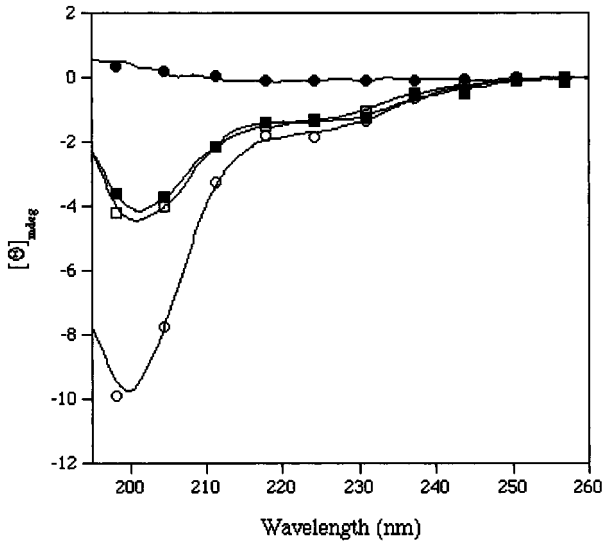

FIGURE 7: Circular dichroism spectra of recombinant human HIP/L29 in the absence or presence of 12 or 3 kDa average molecular mass Hp. All samples were in 50 mM $NaPO_4$ (pH 7.8) and 0.22 M NaCl. The ● line is the spectrum obtained for 11 μM Hp (12 kDa). The ■ line is the spectrum of 10 μM recombinant human HIP/L29 and 11 μM Hp (12 kDa). The □ line is the spectrum of 10 μM recombinant human HIP/L29 and 37 μM Hp (3 kDa). The ○ line is the sum of the independently obtained spectra of 10 μM recombinant human HIP/L29 and 11 μM Hp (12 kDa), showing that these spectra are dominated by protein absorbance.

from proteolytic digestion by V8 protease. Finally, circular dichroism studies indicate that recombinant human HIP/L29 undergoes a large conformational change upon addition of Hp. These studies show that multiple domains within recombinant human HIP/L29 can bind Hp/HS. Furthermore, within the context of the intact protein, these domains act in concert, resulting in the Hp/HS binding activities of human HIP/L29.

With the panel of truncation mutants, it was found that all proteins bound [^{3}H]Hp in a solid-phase assay. In addition, this binding was inhibited by unlabeled Hp by >90%, but only by <10% when unlabeled CS was used as a competitor (data not shown). Thus, multiple domains within HIP/L29 all bind Hp selectively. Second, this shows C41del, murine HIP/L29, and C79del proteins, deficient in HIP peptide-1, still retain the ability to bind [^{3}H]Hp. Consequently, domains present within the highly homologous N-terminal regions of murine and human HIP/L29 also serve as Hp/HS binding domains. However, differences in binding kinetics, coating efficiency, and protein presentation of different mutants may affect the total amount of bound [^{3}H]Hp detected in such assays.

Relative affinity indices were obtained by affinity chromatography to gain insights into the relative importance of individual domains in Hp/HS binding. The general order of elution from highest peak salt concentration to lowest was as follows: recombinant human HIP/L29 > recombinant murine HIP/L29 = C41del > N40del > N80del > C79del. Both murine HIP/L29 and C41del lack a previously described selective, Hp/HS-binding motif (*1*, *8*). This indicates that differences in the C-terminal region of human and murine HIP/L29 account for the observed decrease in murine HIP/L29 Hp/HS binding affinity. While the loss of the 41 C-terminal amino acids reduces the strength of Hp/HS binding, other domains also contribute. Deletion of the 40

N-terminal amino acids had an even more profound effect on Hp binding. It is noteworthy that this region is identical in human and mouse proteins. Therefore, the 40 N-terminal amino acids of human and murine HIP/L29 also appear to be involved in Hp/HS binding. Finally, deleting approximately half of human HIP/L29 further decreases the apparent affinity when compared to the smaller deletions made to the N and C termini. While the N40del and C79del proteins have a lower affinity for Hp than the corresponding deletions to the C and N termini, these observations indicate an additive, domain-independent effect of deletion. In general, the affinity for Hp/HS decreases with a decrease in the size of each protein, irrespective of domain. This suggests that each domain examined is involved in the Hp/HS interaction and that overall affinity represents the collective contribution of interactions involving multiple regions.

Four fragments of recombinant human HIP/L29 bound Hp−agarose and eluted between 0.3 and 0.9 M NaCl. The larger fragments eluted with higher salt concentrations. As with the truncation mutant studies, these observations indicate that increased affinity results from the additive contributions of additional domains present in larger HIP/L29 fragments. This is most readily apparent when comparing the elution profile of the 13 kDa peptide to those of the 5 and 4 kDa substituents, where the divided forms have a lower affinity than the intact segment. None of the fragments contained an oligo-histidine tag as indicated by Western blot analysis. Thus, the affinity tag present in all of the truncation mutants does not account for or contribute to Hp/HS binding activities. The affinity of fragments for Hp−agarose again does not appear to map to a specific domain, but rather, the size of the fragment appears to predict the strength of interaction.

It was found that Hp protects recombinant human HIP/L29 from proteolytic digestion by V8 protease. The largest fragment of recombinant human HIP/L29 generated in the presence of Hp was found to be deficient in the oligo-histidine affinity tag. This, demonstrated that V8 protease remained active in the presence of Hp. Moreover, this is further evidence that the affinity tag does not participate in the Hp/HS binding activities of the panel of proteins that were tested. Furthermore, this indicates that multiple domains within human HIP/L29 are involved in Hp/HS binding and, thus, are protected from V8 digestion upon binding to Hp. The fact that a high salt level nullifies the protective effect of Hp once again underlies the electrostatic nature of the interaction of Hp with HIP/L29 and shows that Hp has no inherent inhibitory effect on V8 protease as suggested previously by Liu and Chang (*14*). Hp of three average sizes from 3 to 12 kDa all protected similarly sized HIP/L29 fragments in this assay. A Hp chain of 3 kDa would have an average length of six disaccharides that would span a distance of 50 Å (*15*). Since the translational length of a 33-amino acid α-helix is approximately 50 Å (*16*), it seems that multiple, independent interactions of 3 kDa Hp with the intact 17 kDa HIP/L29 contribute to binding and account for protection of larger HIP/L29 fragments.

Recombinant human HIP/L29 undergoes a conformational change upon binding to Hp with average molecular masses of 12 and 3 kDa. The signature of recombinant human HIP/L29 alone or bound to Hp does not conform to either α-helical or *β*-sheet structure (*17*). This is in contradiction to several structure-predicting algorithms, indicating a probability of human HIP/L29 adopting a high percentage of α-helical structure (D. E. Hoke and D. D. Carson, unpublished data). The significant change in the absorbance spectra of recombinant human HIP/L29 when bound to Hp could be indicative of many domains within human HIP/L29 creating electrostatic interactions with Hp. Perhaps multiple 3 kDa Hp chains cause human HIP/L29 to assume an extended structure by binding along the entire length of human HIP/L29. This would explain the resistance of a large portion of human HIP/L29 to protease cleavage. Another model would predict that HIP/L29 changes shape in the presence of Hp by bringing the electrostatic potential of multiple HIP/L29 domains to bear upon one 3 kDa piece of Hp, forming a tight, globular structure that is resistant to protease cleavage.

These studies demonstrate that most, if not all, portions of human HIP/L29 are involved in Hp/HS binding. While a deletion of 40 N-terminal amino acids results in a decrease in apparent affinity that is greater than the decrease of its counterpart, a deletion of the 40 C-terminal amino acids, the apparent affinity of human HIP/L29 seems to be generally dependent upon the amount of human HIP/L29 present, irrespective of domain. This could be due to the high overall content of lysine and arginine present in human HIP/L29 (29.5%), distributed fairly evenly throughout the protein. Several models are given to explain how a 50 Å length of Hp can protect a large piece of recombinant human HIP/L29 from proteolytic digestion and cause recombinant human HIP/L29 to undergo significant change in structure. This work supports previous models that indicate multiple basic amino acids in the tertiary structure of proteins additively contribute to Hp/HS binding.

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