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Endothelial Heparan Sulfate Proteoglycans That Bind to L-Selectin Have Glucosamine Residues with Unsubstituted Amino Groups*

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We earlier reported calcium-dependent, heparin-like L-selectin ligands in cultured bovine endothelial cells (Norgard-Sumnicht, K. E., Varki, N. M., and Varki, A. (1993) *Science* 261,480–483). Here we show that these are heparan sulfate proteoglycans (HSPGs) associated either with the cultured cells or secreted into the medium and extracellular matrix. Activation of the endothelial cells with bacterial lipopolysaccharide (LPS) does not markedly alter the amount or distribution of this material. A major portion of the glycosaminoglycan (GAG) chains released from these HSPGs by alkaline β-elimination rebinds to L-selectin in the presence of calcium, indicating that these saccharides alone can mediate the high affinity recognition. Heparin lyase digestions indicate that these GAG chains are enriched in heparan sulfate, not heparin sequences.

Current understanding of the biosynthesis of heparan sulfate chains indicates that all glucosamine amino groups must be either *N*-acetylated or *N*-sulfated. However, nitrous acid deamination at pH 4.0 suggests the presence of some unsubstituted amino groups in these L-selectin-binding GAG chains from endothelial cell HSPGs. This is confirmed by chemical *N*-reacetylation and by reactivity with sulfo-*N*-hydroxysuccinimide-biotin. These unsubstituted amino groups are also found on HSPGs from human umbilical vein endothelial cells, but are not detected in those from Chinese hamster ovary cells. In both bovine and human endothelial cells, these novel groups are enriched for in the HS-GAG chains which bind to L-selectin. Despite this, studies with *N*-reacetylation and nitrous acid deamination do not show conclusive evidence for the direct involvement of the unsubstituted amino groups in L-selectin binding. This may be because the chemical reactions used to modify the amino groups do not go to completion. Alternatively, the unsubstituted amino groups may only be indirectly involved in generating binding, by dictating the biosynthesis of another critical group. Regardless, these studies shown that HSPGs from cultured endothelial cells which can bind to L-selectin are enriched with unsubstituted amino groups on their GAG chains. The possible biochemical mechanisms for generation of these novel groups are discussed.

The selectins are a family of glycoproteins responsible for the initial recognition and binding events in both the normal exit or "homing" of leukocytes from the blood stream and in leukocyte emigration into inflamed tissues (1–7). All three members of this family, E-, P-, and L-selectin, contain the following common domains: an amino-terminal calcium-dependent carbohydrate recognition domain, an epidermal growth factor-like domain, variable numbers of complement-regulatory repeat domains, a transmembrane segment, and a cytoplasmic domain (1–7). Each selectin has been shown to recognize carbohydrate ligands on the opposing cells during various biologically important recognition processes. Partly because of the therapeutic potential for interrupting abnormal leukocyte emigration into tissues in pathological situations, much work has been done to identify the carbohydrate ligands involved in selectin recognition. Interactions of all three of the selectins can be blocked by high concentrations of the tetrasaccharide sialyl-Lewis^x (SLe^x-Siaα2–3Galβ1–4(Fucα1–3)GlcNAc)[1] (8–10). The SLe^x epitope has been detected on some of the specific ligands for the selectins: on the PSGL-1 ligand for P-selectin (11–14), on the ESL-1 ligand for E-selectin (15), and on the GlyCAM-1 ligand for L-selectin, on which it appears in a modified form as 6′-sulfated sialyl-Lewis^x (16). While SLe^x and its isomer SLe^a can block selectin-mediated interactions, these terminal structures are commonly found on many glycoproteins (17–19), and their apparent affinity for the selectins is quite poor (3, 6, 10). Thus, these structures appear to be biologically relevant only when presented in the context of certain intact glycoprotein ligand structures, and/or perhaps with further modification, such as sulfation (20–23). In this regard, we have suggested that SLe^x (and/or other sialylated or sulfated oligosaccharides) generate high-affinity ligands by forming unique "clustered saccharide patches" on heavily glycosylated molecules such as mucins (6, 12).

We have previously reported the isolation of heparin-like molecules from calf pulmonary artery endothelial (CPAE) cells which bind to L-selectin in a calcium-dependent manner (24). In independent work, Nelson *et al.* (25) reported that fragments from commercial heparin as small as tetrasaccharides could block L- and P-selectin binding to SLe^x-bovine serum albumin, at concentrations far lower than those required for SLe^x itself. Thus, heparin-related structures may be another type of high affinity natural ligand for the selectins. Also, with regard to use as therapeutic blockers for selectins, these molecules might potentially be more useful than the sialylated, fucosylated polylactosamines. Here, we report the initial characterization of these heparin-like ligands from CPAE cells, aimed at under-

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[1] The abbreviations used are: SLe^x, sialyl Lewis-x; HSPG, heparin/heparan sulfate proteoglycan; GAG, glycosaminoglycan; HS, heparin/heparan sulfate; CPAE, calf pulmonary artery endothelial cell; CHO, Chinese hamster ovary cell; HUVEC, human umbilical vein endothelial cell; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; LS-Rg, L-selectin receptor globulin; MOPS, 3-[*N*-Morpholino]propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; HONO, nitrous acid; FPLC, fast protein liquid chromatography.

standing if unusual structure(s) present on the glycosaminoglycan (GAG) chains might account for their specific affinity for L-selectin. Our initial approach assumed the accuracy of current models for biosynthesis of heparin/heparan sulfate (HS) chains, which indicate that unsubstituted amino groups should not occur on the glucosamine residues of naturally occurring HS-GAG chains (26–28). However, we show that these unusual residues do indeed exist in the L-selectin binding HS-GAGs of cultured endothelial cells. The involvement of the free amino groups in binding to L-selectin is also explored.

EXPERIMENTAL PROCEDURES

Materials

Most of the materials used were obtained from Sigma. The following materials were obtained from the sources indicated: *Arthrobacter ureafaciens* neuraminidase (sialidase), Calbiochem; Proteinase K, Life Technologies, Inc.; [^{35}S]sodium sulfate, ICN; trypsin-treated L-1-tosylamido-2-phenylethyl chloromethyl ketone, Worthington; diisopropylfluorophosphate, Aldrich; sulfo-NHS-biotin (an *N*-hydroxysuccinimide ester of biotin) and immuno pure avidin coupled to agarose, Pierce; and Centricon-3 Concentrators, Amicon. Purified heparin lyases (I, II, and III) and *O*-sialoglycoprotease were kind gifts from Dr. Robert Linhardt, University of Iowa, and Dr. Alan Mellors, University of Guelph, Canada, respectively. Tritium end-labeled heparin octasaccharide standards were kindly provided by Dr. Magnus Höök, Texas A & M University. All other chemicals were of reagent grade or better and were from commercial sources.

Cell Lines

CPAE cells, a calf pulmonary artery endothelial cell line, was from American Type Culture Collection (CCL 209) and were used at or before passage 23; HUVEC cells, human umbilical vein endothelial cells, were from Clonetics, San Diego, CA (CC-2008) and were used within the first three passages; CHOK1 cells were from ATCC (CCL 9618).

Enzyme Digestions

Heparin lyase digestions were done in 10–20 μl total volume of 20 mM Tris-HCl, pH 7.2, 1 mM $CaCl_2$ using: 50 milliunits of heparin lyase I, 5 milliunits of heparin lyase II, or 25 milliunits of heparin lyase III/reaction, at 37 °C for 1 h. *O*-Sialoglycoprotease digestions were done in a total volume of 10 μl of 20 mM Tris-HCl, pH 7.2, and 2 μl of enzyme, at 37 °C for 1 h (the specific activity of this enzyme is defined by glycophorin A as a substrate; 1 μl cleaves 5 μg/h).[2] Sialidase digestions were done in a total volume of 10–15 μl of 100 mM sodium acetate pH 5.5 with 5 milliunits of *A. ureafaciens* neuraminidase (sialidase), at 37 °C for 1 h. Proteinase K digestions were done with 0.65 mg of proteinase K in a total volume of 10 μl of 20 mM Tris-HCl, pH 7.2, overnight at 37 °C.

SDS-PAGE

7.5% polyacrylamide gels were poured using a Bio-Rad Mini-Gel Apparatus as per the manufacturer's instructions. Samples were boiled in the presence of SDS and 2-mercaptoethanol (reduced), loaded in a total volume of 40 μl, and gels run at 100 V until the dye front reached the bottom of the gel. Prestained high molecular weight standards from Life Technologies, Inc. were included. Following fixation of the gel in 10% methanol, 10% acetic acid for 30 min and exposure to En^{3}hance for 1 h as per the manufacturer's instructions, the gel was dried and exposed to X-MAT Kodak film at −80 °C.

Pulse-Chase Experiments

CPAE cells at passage 10 were grown in 2 × 6-well plates to subconfluence (80%). Labeling with [^{35}S]sodium sulfate was done in sulfate-limited media (media without cysteine and methionine and a total of 10 uM sodium sulfate as reported earlier (24, 29)). Some wells from each plate were also stimulated by addition of 1 μg of LPS (Sigma L-6018 from *Escherichia coli* 055:B5 γ-irradiated) at the time of addition of the [^{35}S]sulfate (30, 31). After an 8-h pulse, cells to be chased (1 plate) were washed three times with cold PBS and replenished with α-minimal essential media containing 10% heat-inactivated fetal calf serum for an overnight chase period. Cells from each of the four groups ("pulse" pulsed only; and "pulse/chase" pulsed with overnight chase; each with and without LPS stimulation) were either trypsinized or saponin-permeabilized.

Trypsin Treatment—Cells were washed three times with ice-cold PBS, and 0.5 ml of PBS, 0.1% sodium azide containing 0.5 mg of trypsin-TPCK was added and incubated at room temperature for 15 min. The trypsin was inactivated by addition of 0.5 μl of 1 M diisopropylfluorophosphate (in a fume hood). The cells were then scraped into the buffer and the entire mixture centrifuged at 2000 revolutions/min for 15 min. The supernatant was analyzed as the trypsin-released fraction, and the remaining pellet was extracted with Triton-X as described below.

Saponin Treatment—Cells were washed three times with ice-cold PBS and 0.5 ml of PBS, 0.1% sodium azide containing the following: 5 mg of Saponin, 30 μg of pepstatin, 6% (v/v) aprotinin, and 1 mM phenylmethylsulfonyl fluoride was added to each well. After incubation on ice for 30 min, the cells were scraped into the buffer, and the entire mixture centrifuged at 2000 revolutions/min for 15 min. The supernatant was analyzed as the saponin-released fraction, and the remaining pellet was further extracted with Triton X-100 as described below. Each of the supernatants described above, as well as the chase media from each group, was analyzed by applying equivalent amounts of radioactivity (50,000 counts/min) onto an L-selectin affinity column (described below).

The remaining pellets from the saponin and trypsin treatments were extracted with 0.5 ml of ice-cold PBS with 2% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1% (v/v) aprotinin, 10 μg/ml pepstatin, 0.02% NaN_3 on ice for 1 h with periodic vortexing. These samples were then transferred to ultracentrifuge tubes and spun at 13 K in a Ti 50.3 rotor (9500 × *g*) for 1 h. These supernatants (radioactivity remaining after saponin or trypsin) were taken into account when calculating the total radioactivity associated with cells, matrix, and media.

To examine the extracellular matrix material only, a separate experiment was performed. A P-100 dish of CPAE cells at passage 10 was labeled with [^{35}S]sodium sulfate as described above. After an 8-h pulse, the cells were washed with ice-cold PBS and the cells scraped from the plate using a rubber policeman. The plate was inspected to ensure that no cells remained on the plate and washed a further time with cold PBS. The remaining extracellular matrix was removed by adding 3 ml of 2% SDS in PBS and scraped and collected into a 15-ml glass conical tube. The GAG chains were then released as described below using method 1.

Preparation of Free GAG Chains

Method 1—Free GAG chains were released and purified using a modification of a previously reported method (32). Briefly, the samples, either before or after application to a L-selectin column, were digested overnight at 55 °C with 1 mg/ml proteinase K in a buffer containing 50 mM Tris-HCl, pH 7.5, 1% SDS, 0.1 M 2-mercaptoethanol. The SDS was precipitated by adding 1/100th volume of saturated KCl and the mixture left at 4 °C overnight. The potassium SDS precipitate was spun out at 2000 revolutions/min for 15 min. The supernatant fluid was boiled for 15 min and DNase (final concentration 3 μg/ml) and $MgCl_2$ (final concentration 100 mM) was added, and the mixture incubated at 37 °C overnight. After boiling for 15 min, the mixture was extracted with 10 volumes of $CHCl_3$/MeOH (2:1). The aqueous phase which contained the GAG glycopeptides was extensively dialyzed against water (M^r cutoff = 3500) and the sample lyophilized to dryness. For reductive β-elimination, 22.4 mg of $NaBH_4$ was dissolved in 2 ml of 0.4 M NaOH, and 0.5 ml of this mixture was added to each sample (33). After incubation at room temperature overnight, the reaction was quenched by acidification with 1 M HCl followed by neutralization using 1 M NaOH. Excess borates and unreacted products were then removed by desalting using a Bio-Gel P-2 column pre-equilibrated and run in 10 mM sodium acetate, pH 5.5. The free GAG chains from the void volume of this column were then fractionated on an L-selectin affinity column as described below.

Method 2—A second method of GAG chain preparation and purification was employed as a check for the possibility of artifactual production of unsubstituted amino groups and is based on the protocol described by Bame and Esko (34). Briefly, CPAE, HUVEC, and CHOK1 cells were labeled for 4 days with [6-^{3}H]$GlcNH_2$ in complete α-minimal essential media. Cells were then washed and solubilized with 0.1 M NaOH at room temperature for 15 min. The solubilized cell mixture was added to the labeling media and the pH adjusted to 7.0. Proteinase K was added at a final concentration of 0.134 mg/ml to each sample and incubated at 37 °C overnight. The samples were then diluted 5-fold with water and the salt concentration lowered to 0.1 M (as checked with a Vapor Pressure Osmometer (5500XR, Wescor) against calibrated salt solutions). The samples were then applied to 1-ml DEAE-Sephacel columns and washed with 30 ml of 0.1 M NaCl, 20 mM Tris-HCl, pH 7.4. The GAG

[2] A. Mellors, personal communication.

peptides were then eluted with 4 ml of 1 M NaCl, 20 mM Tris-HCl, pH 7.4. Reductive β-elimination was then done similarly to that described above. An equivalent volume of a 2 × $NaOH/NaBH_4$ solution (2 × = 44.8 mg $NaBH_4$, 2 ml of M NaOH) was added to each sample and the pH checked to be at 11.5. The samples were allowed to incubate 24 h at room temperature. They were then quenched by the addition of glacial acetic acid dropwise until bubbling stopped and left at room temperature for an additional 2 h for completion of quenching. The samples were then dialyzed extensively against H_2O in dialysis bags with a molecular weight cutoff of 6,000–8000.

L-selectin Affinity Chromatography

As described previously (35), the L-selectin receptor globulin (LS-Rg) consists of the entire extracellular domain of a human L-selectin molecule attached to a human IgG_2 Fc COOH-terminal domain. The construct is expressed in 293 cells and isolated from the culture media using protein A-Sepharose. Typical affinity columns were constructed using 0.4–2.7 mg of purified LS-Rg immobilized by passage over a prepoured column of 2 ml of protein A-Sepharose. Ligands were bound to the columns in 100 mM NaCl, 20 mM MOPS, pH 7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.02% sodium azide. Bound ligands were specifically eluted using the above binding buffer in which the $CaCl_2$ and $MgCl_2$ were replaced with 5 mM EDTA. Under all of these conditions, the LS-Rg itself remains attached to the protein A-Sepharose column.

Superose 12 FPLC Fractionation

A Pharmacia Superose 12 HR 10/30 FPLC column was used both to size samples as well as to remove unreacted small products and reagents. The column was run isocratically in the same buffer used for the LS-Rg affinity column (see above), allowing for direct application of any isolated material to the latter. A Pharmacia FPLC system (P-LKB-Pump P-500; P-LKB-Controller LCC-500 Plus) was used to elute the column at 0.4 ml/min flow rate with an on-line Pharmacia LKB-Frac 100 fraction collector programmed to collect 1-min fractions after 15 min from the beginning of the run. Fractions that were to be studied analytically were collected directly into scintillation vials, 4 ml of Liquiscint scintillation mixture added, and the radioactivity determined. The elution profile of this column was highly reproducible from run to run, as determined by the markers blue dextran (void volume), [^{35}S]sodium sulfate (total volume) and [^{3}H]heparin octasaccharide standards (partially included).

Nitrous Acid Degradation

Nitrous acid degradation was performed by the method of Conrad *et al.* (36–39). Samples to be treated were either first dialyzed extensively against water or desalted using a Centricon-3 concentrator unit (spun with repeated applications of water washes 2–3 × 2 ml), dried completely, and chilled on ice. 30 μl of either the chilled nitrous acid reagent (see below) at pH 1.5 or pH 4.0 was then added, and the reaction brought to room temperature for 10 min, after which the solution was neutralized by addition of 5 μl of saturated Na_2CO_3, and the final volume adjusted to 200 μl with water. The sample was immediately applied onto the Superose 12 column as described above. For preparation of the pH 1.5 nitrous acid reagent, 0.5 M H_2SO_4 and 0.5 M $Ba(NO_2)_2$ were prepared fresh and cooled separately on ice. Equal volumes were then mixed together and spun in a microfuge to pellet the $BaSO_4$ formed. The supernatant was aspirated and used immediately. For the pH 4.0 nitrous acid reagent, 5.5 M $NaNO_2$ and 0.5 M H_2SO_4 were prepared fresh and cooled separately on ice. Five volumes of $NaNO_2$ and 2 volumes of H_2SO_4 were then mixed on ice and used immediately. pH values of the nitrous acid reagent were checked before addition to the sample. For controls, 30 μl of the nitrous acid solutions actually used were first quenched with 5 μl of saturated Na_2CO_3, and the final volume adjusted to 200 μl with water before addition of the samples, which were then applied immediately to the Superose 12 columns.

N-Reacetylation of Amino Groups

The samples to be *N*-reacetylated were desalted by either extensive dialysis against H_2O or by centrifugation using a Centricon-3 concentrator unit (spun with repeated applications of water washes 2–3 × 2 ml), brought up in a total of 100 μl of H_2O, and 12.5 μl of saturated $NaHCO_3$ was added followed by 12.5 μl of freshly prepared, ice-cold 5% aqueous acetic anhydride while the mixture was vortexed. At 15-min intervals, additional aliquots of 12.5 μl of saturated $NaHCO_3$ and 12.5 μl of fresh, ice-cold 5% aqueous acetic anhydride were added for 60–90 min. The sample was then directly injected onto the Superose 12 FPLC column as described above.

Sulfo-NHS-Biotin Coupling and Purification

Samples to be coupled to sulfo-NHS-biotin were extensively dialyzed against H_2O to remove salts. Coupling was performed in 50 mM sodium borate in volumes ranging from 10 to 27 μl total. The sample was first brought up in the borate solution and then added to vials which had 0.1 mg of sulfo-NHS-biotin dried in them. (Because sulfo-NHS-biotin tends to degrade in aqueous solutions, the fresh solutions of the compound were quickly aliquoted into individual Eppendorf tubes, dried on a Savant Speed Vac apparatus, and then stored refrigerated with desiccation until use). Coupling was allowed to proceed overnight at 4 °C. Two methods were used to separate unreacted sulfo-NHS-biotin from the reacted product. Small oligosaccharides or monosaccharides were spotted directly on Whatman no. 1 paper and run for 16 h in ethyl acetate/pyridine/acetic acid/water (5:5:1:3). The paper was then cut into 1-cm strips, eluted with H_2O, and radioactivity determined. Large oligosaccharides were separated from unreacted sulfo-NHS-biotin by spinning the unreacted sulfo-NHS-biotin reagent through Centricon-3 concentrator units. The samples were washed with repeated applications of water (2–3 × 2 ml).

Avidin Binding of Biotinylated GAG Chains

Samples which had been coupled with sulfo-NHS-biotin and freed of excess reagents were allowed to interact with immobilized avidin-agarose beads. 200 μl of avidin-agarose was added to the sample brought up in 50 μl of sodium borate (50 mM final borate concentration). After incubation with rotation at 4 °C for 1 h, the sample mixture was then applied to 1-ml pipette tips packed with a small amount of glass wool. Unbound material was washed through with 3 × 0.5 ml of 50 mM borate (under slight air pressure). The run through and wash were counted together as unbound material, and the beads were counted as bound material.

RESULTS

HSPG L-selectin Ligands Are Present Both as Cell-associated and Secreted Molecules—We earlier reported the calcium-dependent intracellular staining of L-selectin ligands in CPAE cells, which were thought to represent the $^{35}SO_4$ metabolically labeled material that was subsequently shown to bind to an L-selectin affinity column (24). These molecules are susceptible to proteases and heparin lyases, indicating that they are heparan sulfate proteoglycans (HSPGs). We next asked whether all of these HSPG L-selectin ligands were cell associated, or if any were associated with the extracellular matrix, and/or secreted into the media. We were also interested to know if LPS stimulation could induce increased expression, in a manner similar to the L-selectin ligand reported by Spertini *et al.* (30, 31). In a series of pulse-chase labeling experiments, we found that the $^{35}SO_4$-labeled L-selectin ligands of CPAE cells were associated with the intracellular pools (released by saponin), with the cell surface and extracellular matrix (released by trypsin), as well as with cell secretions (for an example of the data, see Table I). Saponin extraction of intracellular pools yielded only a minority of labeled material capable of binding to L-selectin, while trypsin treatment yielded a substantially higher amount (Table I). Since trypsin can both dissociate material from the cell surface as well as degrade the matrix, we also tried to first scrape the $^{35}SO_4$-labeled cells from the plate, and then removed the residual matrix with SDS. Indeed, we found that the SDS-released labeled proteoglycan matrix material carried HS-GAG chains which bound to L-selectin (data not shown), showing that under these tissue culture conditions, some of the ligands are deposited into the extracellular matrix. Addition of LPS did not markedly change the total amount of material which was able to bind to the L-selectin column in any fraction, although some increase was seen in the trypsin-released fraction during the chase period. Also, during the chase period, some of the labeled ligand shifts from an intracellular location (saponin releasable) into the chase media (Table I), indicating secretion of a portion of the ligand. To avoid potential problems with cellular proteases and detergents, most of the subsequent studies were performed with the HSPG ligand that was se-

TABLE I
Pulse-chase analysis of [^{35}S]sulfate-labeled CPAE cells for expression of L-selectin ligand and induction by LPS stimulation

CPAE cells (passage 10) were grown to subconfluence in 6-well plates. Each well was labeled with 500 μCi of $Na_2{}^{35}SO_4$. Half of the wells also received 1 μg/ml LPS at the time of addition of the label. After an 8-h pulse labeling, half of the samples were processed, while fresh media without label was added to the others for an overnight chase period. Washed cells from different wells were treated with saponin or trypsin followed by Triton X-100 extraction of the cell pellet, as described under "Experimental Procedures." Equal amounts (50,000 counts/min) of saponin-released, trypsin-released, or secreted material was loaded onto a L-selectin affinity column, which was washed and eluted with EDTA to detect calcium-dependent L-selectin ligands.

Labeling protocol	Extraction procedure	LPS	% of total radioactivity[a]	% bound to L-selectin[b]
Pulse only	Saponin	−	33	35
		+	36	41
	Trypsin	−	86	57
		+	83	67
Pulse-chase	Saponin	−	12	39
		+	11	45
	Trypsin	−	47	77
		+	73	76
	Chase media	−	15	42
		+	14	34

[a] Represents the total radioactivity incorporated into the cells and extracellular matrix (amount released by trypsin plus that left in the cell pellet after trypsin treatment; in the case of the chased samples, the radioactivity found in the medium was included as well). The total radioactivity recovered in the "pulse" experiments was 179–199 × 10^6 counts/min without LPS and 153–248 × 10^6 counts/min with LPS. The corresponding values for the "pulse-chase" samples were 121–222 × 10^6 counts/min and 224–268 × 10^6 counts/min.

[b] Represents the percent of the 50,000 counts/min applied to the L-selectin affinity column, which bound and was eluted with EDTA.

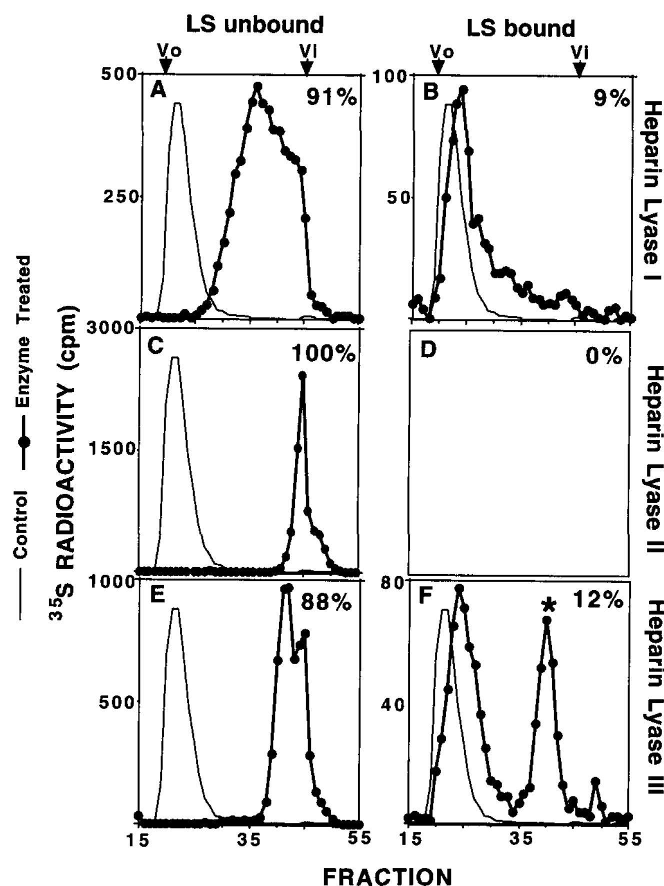

FIG. 1. **Size analysis of ^{35}S-labeled CPAE HS-GAG L-selectin ligands after heparin lyase treatment and rebinding to an L-selectin column.** [^{35}S]Sulfate-labeled-free GAG chains from CPAE cells that originally bound to L-selectin were digested with the individual heparin lyases (*heparin lyase I-III*) (40, 42–44) and reapplied to an L-selectin (*LS*) column. The run through (*Unbound*) and EDTA-eluted (*Bound*) material was then sized using a Superose-12 FPLC column as described under "Experimental Procedures." The percentages of unbound and bound label in each case are indicated in the *upper right hand corner* of each panel. The *arrows* mark the V_o and V_i of the column. [^{3}H]Heparin octasaccharide standards elute at fractions 37–39. The *light line* (control) shown in each panel is the elution profile of undigested material. *Panel D* is empty because there was no L-selectin binding material surviving after heparin lyase II digestion. The peak labeled with an *asterisk* in *panel F* represents ~4% of the starting material subjected to heparin lyase III digestion.

creted into the culture medium, and purified by affinity chromatography on an L-selectin column, with EDTA elution.

Effects of Heparin Lyases on the Structure of the Glycosaminoglycan Chains, and Their Binding to L-selectin—While the intact ligand from CPAE cells appears to be an HSPG, ~50% of the free GAG chains released from these molecules by alkaline β-elimination continue to bind to L-selectin affinity columns (24). Further studies were therefore carried out with these released $^{35}SO_4$-labeled GAG chains. To gain insight into what type of heparin/heparan sulfate (hereafter referred to collectively as HS) oligosaccharide units might be interacting with L-selectin, we digested the CPAE HS-GAG ligands with heparin lyases of defined specificity and checked to see if any of the resulting fragments retained their ability to rebind to L-selectin. Following digestion, the labeled material was separated on an L-selectin affinity column into a non-binding fraction, and an L-selectin-bound fraction. These were each then sized on an FPLC Superose-12 column which gives rapid elution and highly reproducible size exclusion profiles. As seen in Fig. 1, the untreated HS-GAG chains eluted almost exclusively near the void volume of this column (exclusion limit of 2 × 10^6 kDa for globular proteins). Heparin lyase II completely degraded the ligand, and none of the resulting material was capable of rebinding to the L-selectin column. As expected from the broad spectrum of action of this enzyme (40–45), all of the digested material was substantially reduced in size (size about 1–4 disaccharide units, based on calibration of the column with known heparin standards). In contrast, heparin lyase I-digested material still contained some molecules that could rebind, but this fraction remained very large in size. Notably, many of the fragments that no longer bound are still relatively large in size (~60% are >4 disaccharide units in size). This indicates that while the highly sulfated and epimerized sequences recognized by heparin lyase I are present in the GAG chains, the regions that mediate L-selectin binding are unaffected by these treatments. Rather, the heparin lyase I sensitive regions appear to flank both the L-selectin binding and non-binding regions. As seen in Fig. 1, heparin lyase III treatment substantially degraded the ligand, but still produced some fragments which could rebind to the L-selectin column. Taken together, the results indicate that although these GAG chains contain both heparin and heparan sulfate sequences, the majority of the sequences belong to the heparan sulfate class, and the latter probably include the binding motifs of the L-selectin ligand. Furthermore, most of the high affinity ligand surviving after lyase digestions is large (greater than four disaccharides). The only binding fragments small enough for further analysis are those derived by heparin lyase III digestion (see the peak labeled with an *asterisk* in Fig. 1, *panel F*). Unfortunately, these represent a very small fraction (~4%) of the material subjected to treatment.

Nitrous Acid Deamination Suggests the Presence of Unsubstituted Amino Groups on the Glycosaminoglycans—To ascertain whether *N*-acetyl groups or *N*-sulfoamino groups might be

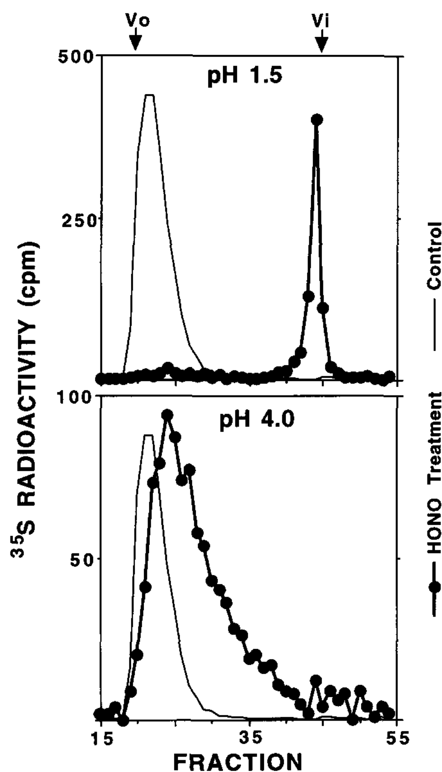

FIG. 2. **Sizing of ^{35}S-labeled CPAE HS-GAG L-selectin ligands after nitrous acid degradation.** [^{35}S]Sulfate-labeled-free GAG chains from CPAE cells that bound to L-selectin were treated with nitrous acid (*HONO*) either at pH 1.5 (*upper panel*) or at pH 4.0 (*lower panel*), and then sized on a Superose-12 FPLC column as in Fig. 1. The *light lines* show controls for each run. [^{3}H]Heparin octasaccharide standards elute at fractions 37–39.

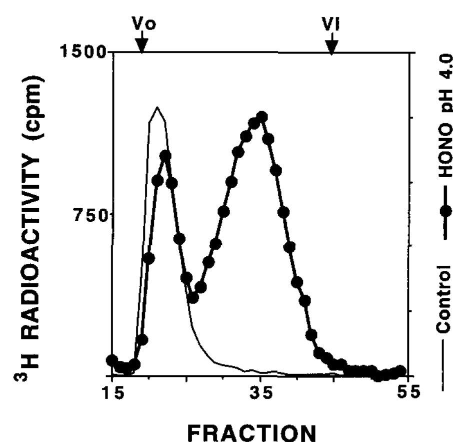

FIG. 3. **Sizing of [^{3}H]glucosamine-labeled CPAE HS-GAG L-selectin ligands after nitrous acid pH 4.0 degradation.** [^{3}H]Glucosamine-labeled released GAG chains from CPAE cells that bind to L-selectin were treated with nitrous acid, pH 4.0, and sized on a Superose-12 FPLC column as in Figs. 1 and 2. The *light line* shows the control run for untreated ligand.

involved in L-selectin recognition, we carried out pH-controlled nitrous acid treatment, which can deaminate certain glucosamine residues and subsequently cleave the adjacent glycosidic linkage. Treatment at pH 1.5 or 4.0 are well known to cause cleavage at *N*-sulfoamino groups or at unsubstituted amino groups respectively (*N*-acetyl groups are not sensitive at either pH) (39). Fig. 2 demonstrates the extent of fragmentation of the $^{35}SO_4$-labeled CPAE HS-GAG ligands produced by each of these treatments. After the pH 1.5 treatment, nearly all of the sulfate label elutes near the totally included region of the column. This indicates that almost all of the sulfate label was either in *N*-sulfoamino groups or on small fragments (1–2 disaccharide units) produced from the deamination/glycosidic cleavage of such groups. Furthermore, this fragmentation pattern indicates either that most of the GAG chains have *N*-sulfoamino groups throughout their entire length or that any stretches with primarily *N*-acetylated regions do not have any other associated *O*-sulfate groups. The latter would not be surprising, based upon the work of others and current understanding of the biosynthetic pathway for HS-GAGs (28, 34, 46).

Since nitrous acid treatment at pH 4.0 will not affect molecules with *N*-sulfated or *N*-acetylated glucosamine residues, this procedure is usually done after chemical *N*-deacetylation, *e.g.* with hydrazine (38, 47). We performed deamination at pH 4.0 without prior *N*-deacetylation primarily as a negative control for the pH 1.5 treatment. Surprisingly, a major portion of the CPAE HS-GAG chains were cleaved to smaller fragments by this treatment. This indicated that unlike most reported HS structures, this ligand might contain a significant amount of unsubstituted amino groups. As mentioned earlier, the FPLC Superose-12 column used for sizing gives highly reproducible elution profiles, and the shifts observed are due to degradation and not to differences between individual column runs. Previous studies have shown that under the conditions used for pH 4.0 nitrous acid deamination, small quantities of sulfoamino groups can be deaminated depending on the amount of reagent used, the type of acid used to create the nitrosating reagent (HCl or H_2SO_4), and the length of time of exposure of the sample with the reagent (48). However, if this was indeed the explanation for all of the fragmentation seen here, one would have expected to see the release of free [^{35}S]sulfate following the nitrous acid treatment at pH 4.0, which was not observed (see Fig. 2).

Unsubstituted Amino Groups Are Not the Result of Growing Cells in Sulfate-depleted Medium—The $^{35}SO_4$-labeling studies described above were performed in sulfate-depleted media (concentration 15 μM) to enhance uptake of the label (29). This could theoretically result in the appearance of unsubstituted amino groups because of an imbalance in biosynthetic processes of *N*-deacetylation and *N*-sulfation. In practice, this has not been observed in the past, probably because the two reactions have been shown to be catalyzed by a single enzyme (49–54), and are therefore coordinately regulated. However, to be certain that sulfate depletion is not the cause of these findings, CPAE cells were also labeled with [6-^{3}H]GlcNH_2 in complete medium (sulfate concentration 810 μM), and the HS-GAG ligands for L-selectin isolated and studied as above. Again, substantial cleavage of the labeled HS-GAG chains by deamination at pH 4.0 was seen (see Fig. 3). The relative difference between the profiles in Fig. 2 (*lower panel*) and Fig. 3. is likely to due to the fact that the latter molecules are labeled with [^{3}H]glucosamine thoughout the entire molecule, while the former are labeled with [^{35}S]sulfate, and therefore label only regions of the polymer which are sulfated. Such regions are known to be non-uniformly distributed in heparan sulfates (26–28). The present results would suggest that the free amino groups may be equally common in the sulfated and non-sulfated regions of the polymer.

Human Umbilical Vein Endothelial Cells Also Have HSPG Ligands Which Bind to L-selectin—The presence of unsubstituted amino groups could be an unusual anomaly of this particular cell line (CPAE cells) when grown in culture. We previously reported that both HUVECs and a second type of bovine endothelial cell (aortic endothelial cells, ATCC AG08132) produced $^{35}SO_4$-labeled material which bound to L-selectin (24). The material from the latter two cell types appeared very large when analyzed by SDS-PAGE. To explore if the HUVEC ligand

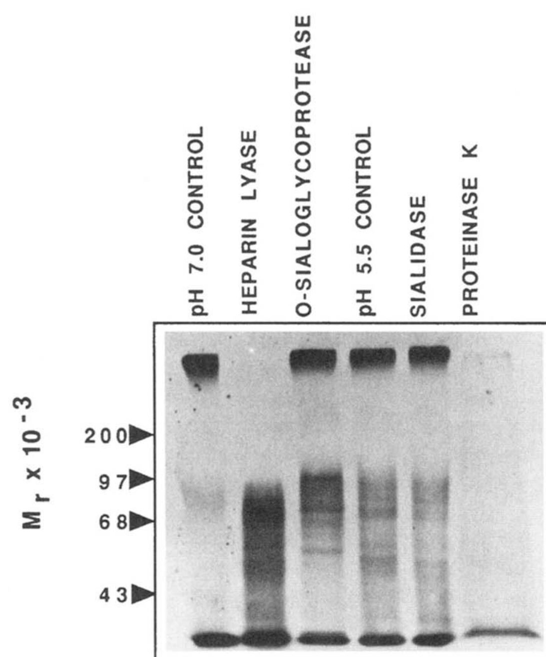

FIG. 4. **SDS-PAGE of L-selectin ligand(s) from [^{3}H]GlcNH$_2$-labeled HUVECs.** [^{3}H]GlcNH$_2$-labeled ligands from the culture medium of HUVEC cells were purified by affinity chromatography on an L-selectin column. Bound material was eluted specifically with 5 mM EDTA, and aliquots (8500 counts/min each) were subjected to the enzyme digestions indicated in the figure and described in detail under "Experimental Procedures." The enzymes were inactivated by heating in sample buffer with 2-mercaptoethanol (reducing conditions) at 100 °C for 10 min and loaded on 7.5% SDS-PAGE. The gel was processed with En^{3}hance for fluorography. Controls were incubated at the appropriate pH values (pH 7 for all reactions except the sialidase digestion, which was done at pH 5.5).

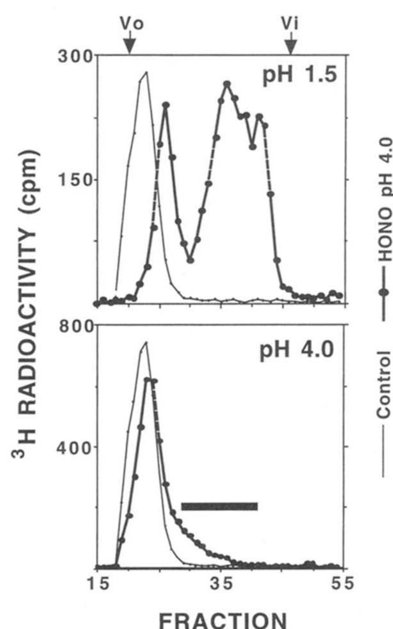

FIG. 5. **Sizing of ^{3}H-labeled HS-GAG L-selectin ligands from HUVECs after nitrous acid degradation.** [^{3}H]GlcNH$_2$-labeled released GAG chains from HUVEC HSPGs that bound to L-selectin were treated with either nitrous acid at pH 1.5 (*upper panel*) or pH 4.0 (*lower panel*) and then sized on a Superose-12 FPLC column as in Fig. 2. The *light lines* show the profiles obtained with controls for each treatment. In the case of pH 4.0 treatment, the percentage of total radioactivity in fraction 28–40 (see *bar*) was 4% for the control and 21% for the treated sample.

was similar to the CPAE ligand and whether it too contained unsubstituted amino groups, the media from [6-^{3}H]GlcNH$_2$-labeled HUVEC cells were subjected to L-selectin affinity chromatography. When the labeled material eluted with EDTA was treated with a variety of enzymes and the products analyzed on SDS-PAGE (Fig. 4), the most obvious change was produced by heparin lyase digestion, indicating that this material also carried HS chains. The ability of proteinase K to digest the material indicates the presence of core protein(s), showing that the native ligand is an HSPG. Notably, *O*-sialoglycoprotease, an enzyme that specifically recognizes mucin-type glycoproteins and destroys several other previously recognized selectin ligands (12, 55–57) produced only small changes in the SDS-PAGE profile. Minor shifts were also produced by sialidase treatment, but these were present also in the pH 5.5 control for this particular enzyme. Thus, the HUVEC ligand is similar to the one from CPAE cells and very different from the mucin-type ligands previously described for L- and P-selectin (reviewed in Refs. 6, 7).

As with the CPAE ligand, a significant fraction (~50%) of the free GAG chains released from the HUVEC ligand by β-elimination can rebind to L-selectin in a calcium-dependent manner (data not shown). Again as with the CPAE cells, both [^{35}S]sulfate- and [^{3}H]GlcNH$_2$-labeled ligands from HUVECs were found associated with the cell and/or extracellular matrix, as well as secreted into the media, and heparin lyase treatments gave similar profiles (data not shown). Fig. 5 illustrates the fragmentation of [^{3}H]GlcNH$_2$-labeled HUVEC ligand produced by nitrous acid treatments at pH 1.5 and 4.0. Treatment at pH 1.5 produces substantial fragmentation, although to a lesser extent than seen with the [^{35}S]sulfate-labeled CPAE ligand. Fragmentation by pH 4.0 nitrous acid treatment was clearly seen (Fig. 5), again to a somewhat lesser extent than with the CPAE material. (Interestingly, in some other labelings more unsubstituted amino groups were detected in the HUVEC ligands, see Fig. 10.) Regardless, these data indicate that the HSPG ligands from CPAE and HUVECs have generally similar properties, including the apparent presence of unsubstituted amino groups.

Unsubstituted Amino Groups Are Not Caused by Chemical Degradation during β-Elimination—The nitrous acid treatments presented thus far were performed on free GAG chains released by β-elimination and then repurified on an L-selectin affinity column. This has the advantage of enriching for the GAG chains capable of binding to L-selectin. However, because β-elimination is carried out under alkaline conditions, we considered the possibility that chemical degradation might account for small amounts of unsubstituted amino groups seen. We therefore studied the intact proteoglycans from the culture medium that bind L-selectin without any prior chemical or enzymatic manipulation. Fig. 6 illustrates the fragmentation profiles of the intact [^{3}H]GlcNH$_2$-labeled HUVEC HSPG ligand with heparin lyase treatment or nitrous acid treatment done at pH 4.0. As expected, the great majority (80%) of the label was digested to smaller sized fragments with heparin lyase II. Nitrous acid treatment at pH 4.0 also produced a significant amount of degradation in comparison to incubation with a pH-adjusted control (about 21% of the label was found in the included volume, in comparison to 5% in the control). This provides independent confirmation that the GAG chains sensitive to pH 4.0 nitrous acid treatment are present in the native HSPG ligand and are not generated as a chemical artifact during subsequent processing.

Confirmation of the Presence of Unsubstituted Amino Groups on the HS-GAGs with N-Reacetylation—As discussed earlier, it is unlikely that the fragmentation seen with the pH 4.0 nitrous acid deamination is due to a small fraction of cleavage at sulfoamino residues. To further demonstrate the presence of

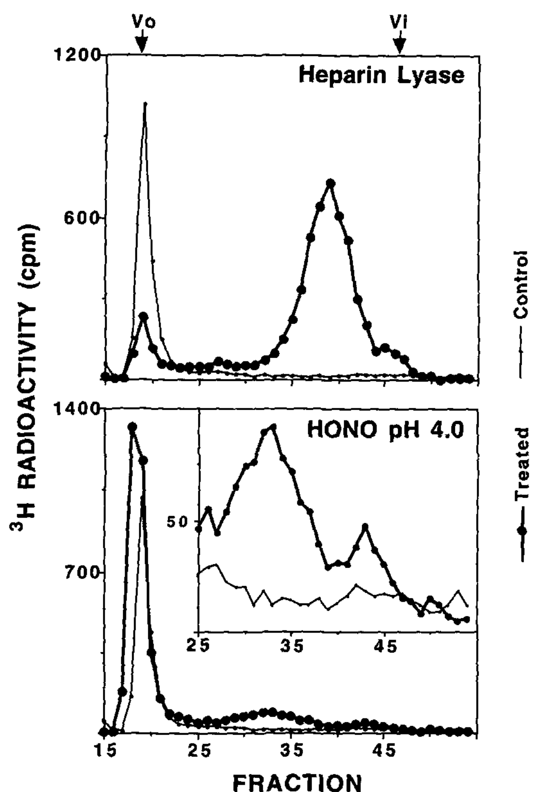

FIG. 6. **Effects of digestion with heparin lyase II or nitrous acid treatment at pH 4.0. on the size of the intact L-selectin-binding HSPG from ^{3}H-labeled HUVECs.** Intact HSPGs from the culture medium of [^{3}H]GlcNH$_2$-labeled cells were bound to an L-selectin affinity column and eluted with EDTA. Aliquots were subjected to heparin lyase II digestion or nitrous acid treatment at pH 4.0, and then sized on a Superose-12 FPLC column. Samples from control incubations were also sized and are shown in each *panel* with the *light line*. The *inset* in the *lower panel* shows the "released" region in an expanded scale. With the nitrous acid treated material, 21% of the label was found in this region, in comparison to 5% in the control.

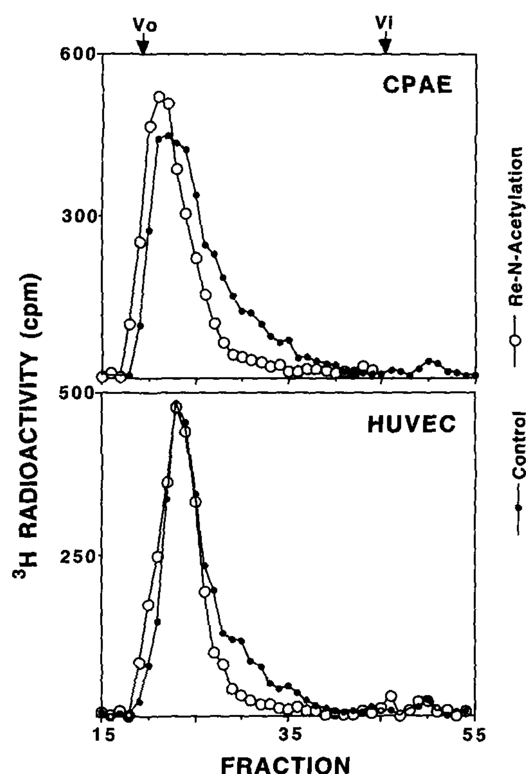

FIG. 7. **Protection of ^{3}H-labeled L-selectin HS-GAG ligands from nitrous acid deamination after chemical *N*-reacetylation.** L-selectin-binding HS-GAG chains from both HUVEC and CPAE cells were chemically *N*-reacetylated as described under "Experimental Procedures." After *N*-reacetylation, the ligands were desalted by dialysis, subjected to deamination/cleavage with nitrous acid at pH 4.0, and sized using the Superose-12 FPLC system. In each panel, profiles of ligands subjected to sham *N*-reacetylation reactions are shown as a control. Even though the *N*-reacetylation provided significant protection from nitrous acid degradation, there is still some detectable shift compared to the completely non-treated material (see Fig. 10), indicating that the *N*-reacetylation is incomplete.

naturally occurring unsubstituted amino groups, we attempted to protect the ligands by chemical *N*-reacetylation of such groups. *N*-reacetylation is predicted to prevent deamination and glycosidic linkage cleavage by blocking the reaction with nitrous acid at pH 4.0. As seen in Fig. 7, *N*-reacetylation did indeed give substantial protection of the HS-GAG ligands of both CPAE and HUVEC cells from nitrous acid deamination/cleavage at pH 4.0.

Confirmation of the Presence of Unsubstituted Amino Groups on the HS-GAGs by Coupling with Sulfo-NHS-Biotin—As an independent approach to demonstrate the presence of unsubstituted amino groups, we reasoned that they should react with sulfo-NHS-biotin (an *N*-hydroxysuccinimide ester of biotin), a reagent commonly used for coupling biotin to the unsubstituted amino groups of proteins (58). If this reaction occurred with the HS-GAGs, it would not only confirm the presence of the unsubstituted amino groups, but would also provide a biotin tag which could be used to retrieve the coupled GAG chain, using immobilized avidin. Furthermore, by subsequently digesting the tagged ligand with heparin lyases, it should be possible to estimate the minimum fraction of disaccharides that carry unsubstituted amino groups. Since this procedure has not been previously used with oligosaccharides, we first sought to validate and optimize it by demonstrating the reaction of sulfo-NHS-biotin with the monosaccharide glucosamine. After treatment of [6-^{3}H]GlcNH$_2$ and [^{14}C]*N*-acetyl-mannosamine (ManNAc) with sulfo-NHS-biotin under various conditions, we studied the products by paper chromatography. As shown in Fig. 8, nearly 90% coupling of the GlcNH$_2$ was achieved under optimized conditions, as evidence by a shift in migration of the molecule. Under identical conditions there was no reactivity of sulfo-NHS-biotin with the control ManNAc, which does not have an unsubstituted amino group.

Although a coupling efficiency of ~90% was possible with unsubstituted GlcNH$_2$, unsubstituted amino groups within an intact HS chain might be less susceptible to coupling due to steric hindrance, charge effects, or salt bridges. However, if sulfo-NHS-biotin coupling protected the ligand from subsequent deamination/cleavage by pH 4.0 nitrous acid treatment, then a reasonably high efficiency of coupling could be assumed. As seen in Fig. 9, sulfo-NHS-biotin coupling of the HUVEC HS chains did indeed provide a substantial, although incomplete protection from nitrous acid degradation. Interestingly, when HUVEC HS-GAG chains which had been reacted with sulfo-NHS-biotin were then digested with heparin lyase, the sulfo-NHS-biotinylation actually seemed to enhance the degradation by heparin lyase, as detected by a sharpened peak profile, slightly more included than the uncoupled HS-GAG chains (Fig. 9, *lower panel*). This could be because the heparin lyase action is hindered by the presence of the unsubstituted amino groups, and restored by their substitution with the biotinyl groups. The intact and heparin lyase-degraded HS chains were then allowed to interact with avidin immobilized on agarose beads, and bound and unbound fractions were determined. As shown in Table II, a substantial fraction of the intact HS chains from HUVECs were biotinylated by this procedure. Even

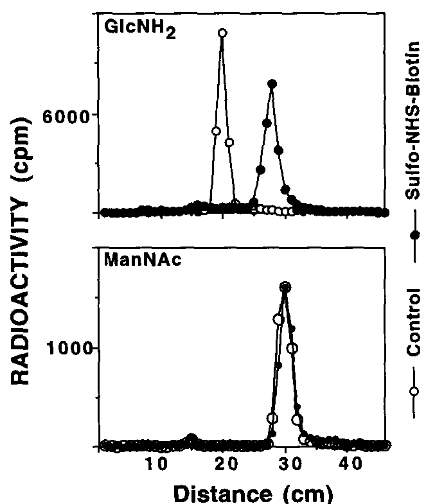

FIG. 8. **Paper chromatography of monosaccharides with and without prior reaction to sulfo-NHS-biotin.** [^{3}H]GlcNH$_2$ or [^{14}C]ManNAc were reacted or sham reacted with sulfo-NHS-biotin as described under "Experimental Procedures." The samples were spotted on Whatman No. 1 paper and developed in an ethyl acetate/pyridine/acetic acid/water (5:5:1:3) solvent system for 16 h at room temperature. The paper was dried, cut into 1-cm strips, and radioactivity determined.

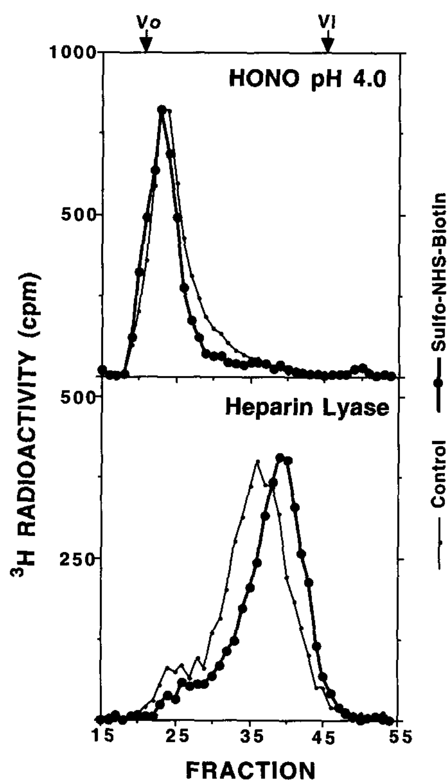

FIG. 9. **Effects of sulfo-NHS-biotin on the size of fragments produced by nitrous acid deamination or heparin lyase digestion of ^{3}H-labeled L-selectin HS-GAG ligand from HUVEC cells.** [^{3}H]GlcNH$_2$-labeled L-selectin binding HS-GAG chains from HUVEC cells were reacted with sulfo-NHS-biotin as described under "Experimental Procedures." Portions of the sample (and appropriate sham-treated controls) were subjected to nitrous acid treatment at pH 4.0 or heparin lyase II digestion and then sized on a Superose-12 FPLC column, as in Fig. 2.

among heparin lyase-digested HS fragments, a small but significant fraction (6.7% as compared with 0.3% in the control) was selectively retained on the avidin beads. These data confirm that unsubstituted amino groups are present on a substantial fraction of the HS-GAG L-selectin ligands. The decrease in the amount of radioactivity binding to avidin following heparin lyase digestion is not surprising, since the label is expected to be distributed throughout the polymer, whereas the amino groups are present only in a limited number of residues. Even in the HUVEC HS-GAG ligands which have lower levels of unsubstituted amino groups than the CPAE ligand, we estimate that 1 in 20 to 1 in 50 glucosamine residues have unsubstituted amino groups. Since we cannot be certain that the reaction with sulfo-NHS-biotin proceeds to completion, these numbers represent minimum estimates.

TABLE II

HS-GAG ligands contain unsubstituted amino groups that react with sulfo-NHS-biotin

L-selectin-binding [^{3}H]GlcNH$_2$-labeled HS-GAGs from HUVEC cells were treated or sham treated with sulfo-NHS-biotin as described under "Experimental Procedures." After removing unreacted sulfo-NHS-biotin by centrifugation through a Centricon-3 concentrator unit, the GAG chains were allowed to bind to avidin-agarose beads, and the ligand-biotin-avidin-agarose complex isolated as described under "Experimental Procedures." Values are reported as the percentage of the total radioactivity recovered. An aliquot of each fraction was also digested with heparin lyase II after sulfo-NHS-biotin coupling and prior to avidin binding. Fig. 9 demonstrates protection from nitrous acid pH 4.0 following biotinylation and the susceptibility of this material to heparin lyase II digestion.

	Binding to avidin	Sulfo-NHS-biotin	
		+	−
		%	
Intact HS-GAG chains	Unbound	58	97
	Bound	42	3
Heparin lyase II treated	Unbound	93.3	99.7
	Bound	6.7	0.3

Unsubstituted Amino Groups Are Enriched on Endothelial HS-GAGs as Compared to Those from CHO Cells—The existing literature on HS-GAGs favors the notion that unsubstituted amino groups do not occur naturally because of the coordinated enzymatic *N*-deacetylation and *N*-sulfation. The unsubstituted amino groups described here are present on only a minor fraction of the glucosamine residues. We therefore carried out a direct comparison of HS-GAGs from these endothelial cells with those from CHO cells, which have been extensively studied in the past and have not been found to contain unsubstituted amino groups in the wild type CHOK1 line (49). Following [6-^{3}H]GlcNH$_2$ labeling under identical conditions, total HS-GAGs from CHO, CPAE, and HUVEC were isolated by protease digestion and ion-exchange chromatography, almost exactly as described previously by Bame and Esko (34). To ensure good comparison, total [6-^{3}H]GlcNH$_2$-labeled material from cells, extracellular matrix, and medium were pooled together and digested with proteinase K, a homogeneous protease of broad specificity. The resulting labeled glycopeptides were desalted and enriched for GAG chains by step-wise elution from an anion-exchange column as described previously (34). The GAG chains were then released with β-elimination/NaBH$_4$ reduction, and the resulting material (which would include other types of GAG chains such as chondroitin sulfate) was treated with nitrous acid at pH 4.0 and analyzed for any resulting change in size. As shown in Fig. 10, the total GAG chains from CHO cells showed a very minor (if any) shift in the profile of elution from the Superose-12 column. In contrast, the CPAE and HUVEC-derived material showed significant shifts, as before, which was almost completely protected by *N*-reacetylation of the material.

Unsubstituted Amino Groups Are Enriched on the HS-GAGs

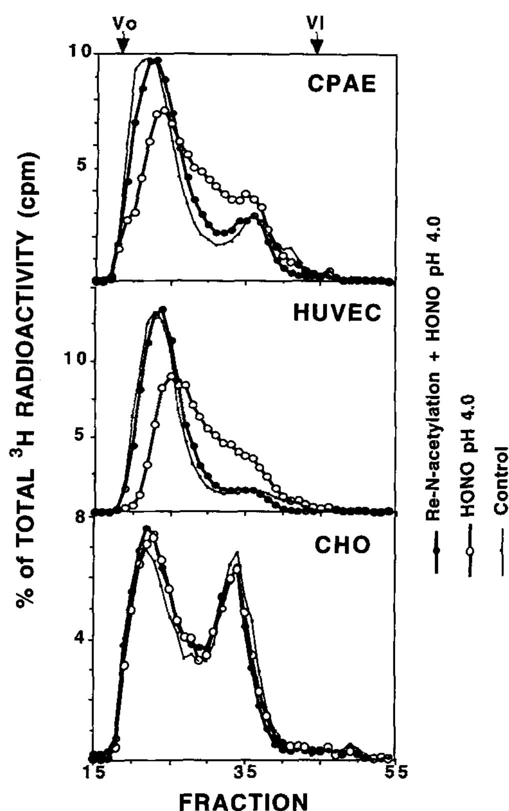

FIG. 10. **Sizing of total ^{3}H-labeled GAG glycopeptides from CPAE, HUVEC, or CHO cells before and after nitrous acid degradation with or without prior *N*-reacetylation.** [^{3}H]GlcNH$_2$-labeled GAG-enriched glycopeptides from CPAE, HUVEC, and CHO cells were prepared with Method 2 as described under "Experimental Procedures." Aliquots were treated with nitrous acid at pH 4.0, with or without prior *N*-reacetylation, and then sized on a Superose-12 FPLC column (similar to Figs. 2 and 7). In each profile, the *Control* trace is the profile of the material injected on the column without any type of treatment. To better compare the three different traces, each data point is presented as percent of total counts/min recovered in the run.

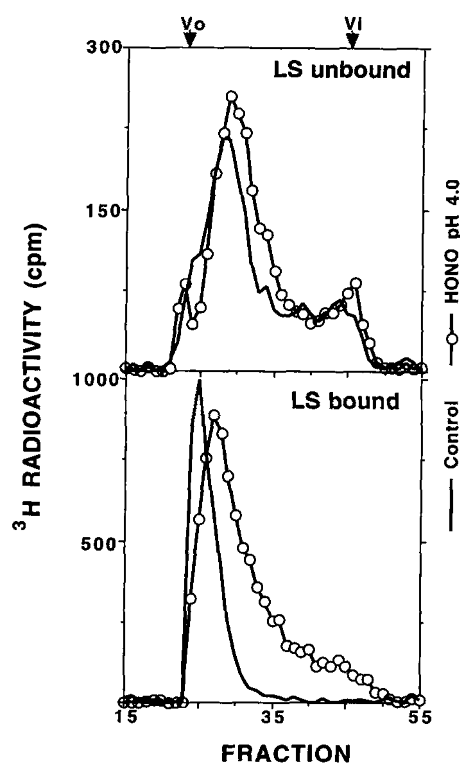

FIG. 11. **Heparan sulfate L-selectin ligands from endothelial cells are enriched in GAG chains with unsubstituted amino groups.** Intact [^{3}H]GlcNH$_2$-labeled HSPGs from CPAE endothelial cells that initially bound to an L-selectin (*LS*) affinity column and eluted with EDTA were subjected to β-elimination, and the products reapplied to the L-selectin column. The unbound (*upper panel*) or bound (*lower panel*) fractions were desalted and aliquots treated with nitrous acid (*HONO*) at pH 4.0. Treated and untreated samples were studied by gel filtration on a Superose-12 FPLC as described under in Fig. 1. *Arrows* mark the V_o and V_i of the column. [^{3}H]Heparin octasaccharide standards elute at fractions 37–39.

That Bind to L-selectin—We wanted to see if the unsubstituted amino groups which we had detected were associated only with the GAG chains capable of binding to L-selectin or whether they occurred in all of the GAG chains isolated from the CPAE cells. To examine this, the intact [^{3}H]glucosamine-labeled CPAE HSPG fraction that bound to L-selectin was submitted to β-elimination, the products desalted, and reapplied to the L-selectin affinity column. The fraction of the radiolabeled material that no longer bound to the column (50–75%) contains HS-GAG chains, but also may include some conventional *O*-linked chains and *N*-linked glycopeptides arising from the β-elimination reaction on the intact HSPG. However, the latter are much smaller in size than the HS-GAG chains and are discernable on the Superose-12 sizing column as included material. As shown in Fig. 11, while the L-selectin-bound HS-GAGs showed extensive cleavage with pH 4.0 nitrous acid treatment, negligible fragmentation was seen among the non-binding chains. Thus, the unsubstituted amino groups appear to be highly enriched for in those HS-GAG chains which bind to L-selectin, suggesting that they may play a role in recognition.

N-Reacetylation of the HS-GAG Chains Does Not Diminish Their Ability to Interact with L-selectin—In using chemical *N*-reacetylation to demonstrate the presence of unsubstituted amino groups, we could protect most, but not all, of the ligand from nitrous acid degradation at pH 4.0 (Fig. 7). To see if these chemically *N*-reacetylated ligands maintained their ability to bind to L-selectin, they were reapplied to an L-selectin affinity column. Although some experiments showed that a fraction of the ligand no longer interacted with L-selectin (data not shown), most experiments gave results such as those shown in the *upper panel* of Fig. 12. Since the majority of the unsubstituted amino groups are successfully *N*-reacetylated by this procedure, these data indicate that all of the free amino groups are not required for high affinity recognition of HS-GAGs by L-selectin.

Nitrous Acid Deamination at pH 4.0 Destroys the Ability of Some of the HS Chains to Interact with L-selectin—In contrast to the results with *N*-reacetylation, nitrous acid treatment of the HS-GAGs at pH 4.0 (which cleaves at the position of unsubstituted amino groups) does cause a major portion of the ligand (~75%) to lose binding to L-selectin (see Fig. 12, *lower panel*). When the non-binding and binding material obtained after nitrous acid treatment are studied by sizing analysis (Fig. 13), the material which no longer binds has been degraded to smaller-sized fragments, while that which still binds remains large in size (compare with Figs. 2 and 3, and their discussion above). These data are subject to different explanations. On the one hand, the survival of some binding following fragmentation suggests that some or all of the interactions of the intact molecules is due to regions free of unsubstituted amino groups. Alternatively, these groups could be critically important for the majority of the binding, with the remaining binding being explained either by other types of sequences, or by unsubstituted amino groups that escaped cleavage and/or *N*-reacetylation. Because it is likely that neither treatment (*N*-reacetylation or nitrous acid deamination) went to completion, both explanations remain viable.

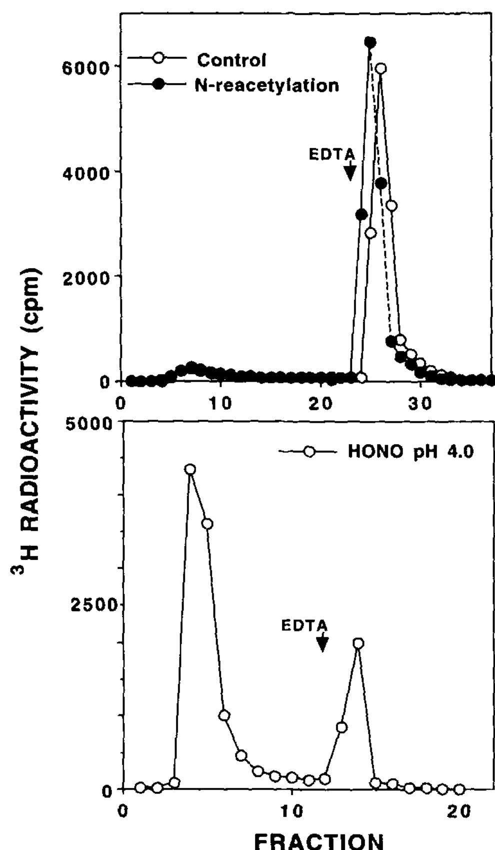

FIG. 12. **Effects of *N*-reacetylation and nitrous acid deamination on the rebinding of HS-GAG ligands to L-selectin.** Samples of [^{3}H]GlcNH$_2$-labeled HS-GAGs from CPAE cells that had previously bound to L-selectin were treated by *N*-reacetylation or nitrous acid (*HONO*) at pH 4.0, as described under "Experimental Procedures." After removal of all salts by dialysis against water and re-equilibration in column running buffer, the samples were reapplied to an L-selectin affinity column. The *arrow* indicates the position of elution with 5 mM EDTA. A control run of untreated sample is shown in the *upper panel*.

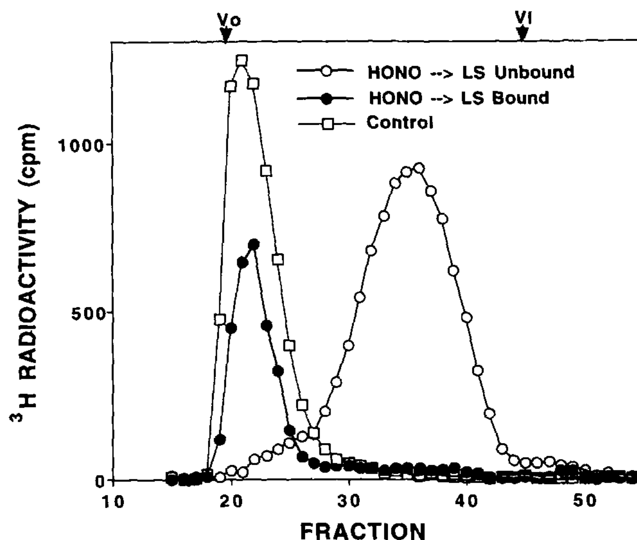

FIG. 13. **Size fractionation of nitrous acid deamination products following the rebinding of HS-GAG ligands to L-selectin.** After nitrous acid deamination at pH 4.0 (see *lower panel* of Fig. 12) HS-GAG products that bound or no longer bound to L-selectin (*LS*) were size fractionated on a Superose-12 FPLC column as described in Fig. 1. The profile obtained with a control (untreated) sample is shown for comparison.

DISCUSSION

In this study, we have further characterized the heparin-like ligands for L-selectin reported earlier in CPAE endothelial cells (24). While our report noted that L-selectin staining of CPAE cells was primarily intracellular in nature (24), we have not further explored the precise intracellular localization here. However, these ligand(s) are shown to be HSPGs found associated with the cells and/or extracellular matrix, as well as secreted into the medium. These observations of secretion and matrix association must of course be regarded with caution, since cultured cells may have patterns of expression and/or secretion that are altered relative to their normal counterparts *in vivo*. For this reason, it is also difficult to know what the specific role of this ligand might be in the normal biology of L-selectin. Since HSPGs are known to be exposed on the luminal surface of endothelial cells *in vivo* (26, 59–62), it is possible these L-selectin ligands play an initial role in recognition by L-selectin bearing leucocytes. In this regard, it has been noted that cytokine-stimulated endothelial cells display an altered basement membrane structure and a glycosaminoglycan-rich pericellular matrix (63, 64). Alternatively, the L-selectin binding HS-GAG sequences might be primarily expressed in the extracellular basement membrane. If so, they may become important only when endothelial cell junctions become separated in injury, or in inflammation. Lastly, secreted ligands might act in a negative manner to inhibit leucocyte adhesion to a normal endothelium. Notably, we could not induce a major alteration in synthesis or subcellular distribution of the HS ligand by LPS stimulation of CPAE cells. Thus, this ligand must be distinct from the inducible L-selectin ligand of HUVECs reported earlier with either interleukin-1 (30, 31) or interleukin-4 (65) stimulation. Furthermore, the latter requires new protein synthesis, and recognition is evidently dependent upon sialic acids (30, 31).

Heparin disaccharides are highly sulfated and contain many 2-*O*-sulfated iduronic acids, while heparan sulfate disaccharides contain the less sulfated disaccharide units and glucuronic acid residues. To explore which type of HS chains were involved in L-selectin binding, we used a panel of heparin lyases (I-III) with well-known substrate specificities (40–45). The complete abolition of binding by the broad spectrum heparin lyase II confirms that recognition does indeed involve HS chains and that with use of heparin lyases, fragments larger than octasaccharides seem to be required for high affinity binding. Heparin lyase I digestion did not abolish binding of most of the labeled HS-GAGs, indicating that the regions involved do not contain the highly sulfated and epimerized heparin-like sequences recognized by this enzyme. The limited fragmentation seen indicates that some of these sequences are present on the flanking ends of large segments which continue to bind to L-selectin. In contrast, heparin lyase III digestion caused a substantial loss of recognition. However, it did not completely abolish binding, and some small fragments were produced which were still able to bind (see the peak indicated with an *asterisk* in Fig. 1, which contains fragments with ~4 disaccharide units). This indicates that while this enzyme was not able to cleave all the regions which bind L-selectin, it did cleave many regions very near by, suggesting that the regions which are binding contain heparan sulfate type sequences. Unfortunately, the amounts of the small fragments that continue to bind have been too little for more detailed study.

It is known that the HS-GAG chains can contain many distinct types of disaccharide units, generated by different patterns of sulfation and/or epimerization (46). The pentasaccharide sequence responsible for the high affinity binding of heparin to antithrobin III (66, 67) is a specific sequence which

includes an uncommon 3-*O*-sulfate group. The heparan sulfate sequence which binds to basic fibroblast growth factor predominantly contains IdoA(2-*O*-SO_3)α1,4-$GlcNSO_3$ disaccharides with few 6-*O*-sulfate groups and it appears that both the 2-*O*-sulfate and *N*-sulfate groups are essential for the binding activity (68–70). In contrast, binding of heparan sulfate to hepatocyte growth factor seems to involve domains with predominantly non-sulfated iduronic acids, and the highest affinity seems to be most closely associated with 6-*O*-sulfated $GlcNSO_3$ residues (71). Because HS-GAG-L-selectin binding could be a highly regulated biological event, we considered the possibility that a unique sequence and/or unique modification of the HS chains might be present to endow these ligands with a high affinity. In examining this possibility, we made the surprising finding that there were a significant amount of unsubstituted amino groups on glucosamine residues. Given the novelty of this finding, we chose to focus more attention to it, regardless of whether or not it was related to L-selectin binding. Interestingly, we found that the number and ratios of *N*-sulfo and *N*-acetyl groups in the HS ligands from CPAE and HUVEC cells vary considerably, but both cell types have in common the presence of these unsubstituted amino groups.

In 1982, Höök *et al.* (72) noted that preparations of purified heparins and heparan sulfates which were being labeled by *N*-[^{3}H]acetylation contained significant amounts of *N*-unsubstituted hexosamines. However, they suggested that these unsubstituted amino groups were either artifacts, biosynthetic intermediate structures, or products of degradative enzymes. Since that time, conventional dogma indicates that such amino groups are transient biosynthetic intermediates that are not retained in the final product (26–28). We have presented several lines of evidence that these unsubstituted amino groups are unlikely to be experimental artifacts. Although the initial studies with [^{35}S]sulfate were done using a sulfate-deficient media, the presence of unsubstituted amino groups is still noted when labeling with [^{3}H]$GlcNH_2$ in a sulfate replete medium. In fact, others who have used sulfate-deficient media or studied sulfation-negative mutants have not observed unsubstituted amino groups in HS-GAGs from other cell types (27, 34, 49, 73, 74). In addition, the chemical technique used to release chains (alkaline β-elimination) is a traditional method that has not been previously reported to generate unsubstituted amino groups. Regardless, to ensure that this was not the case in our hands, we also subjected the intact HSPG (obtained directly from the culture medium by L-selectin binding) to nitrous acid deamination at pH 4.0; again, evidence was seen for a small but significant amount of unsubstituted amino groups. Since these molecules were isolated directly from the culture medium of the cells, and maintained at neutral pH in an isotonic buffer, they were not exposed to any harsh chemical conditions that could have artifactually created the unsubstituted amino groups. Another concern is that under the conditions used for nitrous acid deamination at pH 4.0, a small amount of breakdown and release of *N*-sulfoamino groups might occur (48). However, when the [^{35}S]sulfate-labeled HS-GAGs were subjected to this treatment, we did not see a peak of released free [^{35}S]sulfate. Finally, we have compared the HS chains from CPAE and HUVEC cells to those from CHO cells, which have been extensively studied in the past (27, 49, 73–75). HS-GAGs were isolated from these three cell types using identical isolation procedures and were studied for the presence of unsubstituted amino groups. Again the CPAE and HUVEC GAG chains showed evidence for unsubstituted amino groups, whereas those from the CHO cells had few, if any, such groups.

Since the amounts of material isolated from these cultured cells are too small to demonstrate the presence of the unsubstituted amino groups directly by quantitative chemical or physical methods, we devised alternate approaches, showing that chemical *N*-reacetylation as well as a reaction with sulfo-NHS-biotin protected the molecules from subsequent nitrous acid deamination at pH 4.0. In the latter case, the covalent attachment of biotin residues was also confirmed by demonstrating that a major fraction of the chains are then able to bind to avidin. For a variety of reasons, it is difficult to precisely quantitate the number of unsubstituted amino groups/HS chain. A significant limitation is that we do not have sufficient amounts of material to ascertain chemically that the reactions have gone to completion. The sulfo-NHS-biotin coupling reaction can covalently modify as much as 90% of the unsubstituted amino groups on the free monosaccharide glucosamine. Likewise, the *N*-reacetylation method used is expected to completely modify unsubstituted glucosamine. However, we cannot rule out the possibility that these reactions are less efficient when dealing with a large negatively charged polymer. Furthermore, the presence of another modification, such as a *O*-sulfate at the 3-position, could further decrease the efficiency of any one of the chemical treatments which we have employed. Although precise quantitation is not possible, we can state some minimal estimates. In the case of the HUVEC HS-GAGs, at least half of the intact GAG ligands contain at least one unsubstituted amino group. Furthermore, since ~6% of the heparin lyase treated material (fragments between two to four disaccharides in length) seem to have an unsubstituted amino group, one can estimate that between 1 in 20 to 1 in 50 of the disaccharide units can have such groups.

One reason for the persistent dogma that unsubstituted amino groups should not exist on natural HS chains is that the *N*-deacetylation and *N*-sulfation reactions that occur during biosynthesis are catalyzed by the same enzyme (49–54), effectively coupling the two steps. However, much of the structural work and enzymology studies to date have been done using mast cells, CHO cells, or intestinal epithelial cells. These cells may or may not have the identical biosynthetic machinery as endothelial cells. We can speculate on a few different mechanisms by which these unsubstituted amino groups might be generated in endothelial cells. First, the kinetics or the efficiency of the specific enzyme reactions may be altered in different cell types, *i.e.* in endothelial cells; perhaps the *N*-deacetylase activity may be very high and efficient, but the *N*-sulfotransferase activity may be low or inefficient. This could potentially lead to a disproportionate amount of glucosamine residues whose amino groups are not substituted. Interestingly, Orellana *et al.* (53) have reported a 4–8-fold higher ratio of *N*-deacetylase to *N*-sulfotransferase activity in the enzyme complex cloned from a murine mastocytoma cell line as compared to that obtained from rat liver. As a second possibility, endothelial cells may be expressing novel tissue-specific enzymes, such as either an independent *N*-deacetylase or an *N*-sulfatase. Enzymes such as these might be inducible by inflammatory cytokines or triggered to be expressed in tissue culture, where the cells may perceive themselves to be in a "pseudo-inflammatory" state. Lastly, it is conceivable that an internal ester migration event (enzyme-dependent or independent) may be occurring, in which *N*-sulfate groups migrate to another position. In this regard, Uchiyama and Nagasawa (76) have shown that defined chemical conditions can cause a fairly specific migration from the *N*-sulfate groups of heparin to the adjacent 3-hydroxyl groups. Such a migration would cause the creation of an unsubstituted amino group and perhaps an unusual *O*-sulfate group within the heparin chain. Either or both these modifications due to the migration might be responsible for a high affinity interaction with the L-selectin molecule.

We have also shown here that the heparan sulfate GAG chains that bind to L-selectin are enriched in glucosamine residues whose amino groups are unsubstituted. These unsubstituted amino groups could potentially be involved in binding to L-selectin either directly, or indirectly, for instance, by forming a salt bridge that generates an optimal binding conformation in an adjacent part of the GAG chain. Attempts were made to determine if these amino groups were important for binding by masking them via chemical *N*-reacetylation. Although some experiments showed a partial decrease in binding, this was not a consistent finding. Thus, the presence of these unusual amino groups on these GAG chains could be serendipitous. However, other possibilities must be considered. For instance, we have consistently seen that the chemical *N*-reacetylation procedure protects most but not all of the ligand from degradation by nitrous acid at pH 4.0. It is possible that a small subset of residual groups is sufficient to maintain binding. Unfortunately, we cannot obtain enough material from the cells in culture to determine the efficiency of the *N*-reacetylation procedure by other direct chemical techniques. It is also possible that as with anti-thrombin III binding to a highly specific pentasaccharide sequence in heparin (66, 67), an unsubstituted amino group is only one of two or more modifications which direct the high affinity binding to selectins. The unsubstituted amino group might even serve as a signal for a further modification during biosynthesis, such as a specific *O*-sulfation which, in combination with the unsubstituted amino group, promotes the high affinity binding to L-selectin. Such a second modification could also cause a selective difficulty of *N*-reacetylation if it acts to sterically inhibit this reaction, *e.g.* a 3-*O*-sulfate residue, immediately adjacent to the unsubstituted amino group. In this scenario, it is possible that either modification alone could support binding, but that together they yield the highest affinity. If so, the amount and ratio of the two groups present on the HS-GAGs obtained from a given labeling might determine whether subsequent *N*-reacetylation could completely destroy binding. In this regard, it is interesting that commercial heparin (which has very few if any unsubstituted amino groups) contains relatively few structures that can bind with high affinity to L-selectin (24).

In contrast to *N*-reacetylation, nitrous acid degradation at pH 4.0 consistently caused a major loss in the ability of the resulting fragments to rebind to L-selectin. Of the fragments which still bound after the nitrous acid degradation, all were rather large in size. Unfortunately these results are also open to more than one interpretation. First, the large fragments which are still able to bind might contain one or more unsubstituted amino groups that escaped deamination and are responsible for their ability to continue to bind to L-selectin. Indeed, the fact that the great majority of the fragments which no longer bind were small in size suggests that these unsubstituted amino groups may reside at or very near potential L-selectin-binding sites. However, the coupling of the unsubstituted amino groups with sulfo-NHS-biotin never caused more than ~50% of the ligand to be retained on avidin-agarose. This may indicate that there are two types of binding of HS-GAGs to L-selectin, one which is dependent on unsubstituted amino groups and one which is not.

Regardless of the exact explanation for these results, it appears likely that these endothelial HS-GAGs carry specific sequences mediating L-selectin recognition that are not found in high frequency in commercial heparin preparations. It is interesting to note that synthetic SLe^x/SLe^a molecules whose *N*-acetyl-glucosamine residue is replaced with a glucosamine bind better to L-selectin than the native structures (10). Perhaps one motif recognized by L-selectin is an unique arrangement of a negatively charged groups near a positively charged group, which promotes a high affinity interaction. In this regard, it has been recently reported that *Bacteroides fragilis* polysaccharides which induce the formation of intra-abdominal abcesses (collections of neutrophils that originally bear L-selectin) are characterized by repeated disaccharide units which contain alternating positively charged amino groups and neighboring negatively charged groups (77). *N*-Reacetylation of the unsubstituted amino groups in these molecules resulted in a dramatic diminution of neutrophil accumulation (77). We are currently exploring the possibility that such polysaccharides are indeed selectively recognized by L-selectin.

While this work was nearing completion, we became aware of work by others, who have recently used immunohistochemical approaches to demonstrate the natural occurrence of heparan sulfate with unmodified amino groups.[3] Thus, these groups may be more common than previously recognized and represent yet another way in the which the heparin/heparan sulfate family of glycosaminoglycans can achieve diversity, which in turn can mediate specific biological functions.

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