Novel Heparan Sulfate Structures Revealed by Monoclonal Antibodies*

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The sulfated glycosaminoglycan heparan sulfate (HS) is found ubiquitously on cell surfaces, in the extracellular matrix, and intracellularly as HS proteoglycans. Because of the structural heterogeneity of HS, tissue-derived HS preparations represent a mixture of HS chains originating from different cell types and tissue loci. Monoclonal anti-HS antibodies have been employed to detect the localization of specific HS epitopes in tissues, but limited information has been available on the saccharide structures recognized by the antibodies. We have studied the saccharide epitope structures of four anti-HS antibodies, HepSS1, JM13, JM403, and 10E4, which all recognize distinct HS species as demonstrated by different patterns of immunoreactivity upon staining of embryonic rat and adult human tissues. The epitopes recognized by JM13 and HepSS1 were found almost exclusively in basement membrane HS, whereas JM403 and 10E4 reacted also with cell-associated HS species. The binding of HepSS1, JM403, and 10E4 to HS was dependent on the GlcN N-substitution of the polysaccharide rather than O-sulfation. HepSS1 thus interacted with N-sulfated HS domains, JM403 binding was critically dependent on N-unsubstituted GlcN residues, and 10E4 bound to "mixed" HS domains containing both N-acetylated and N-sulfated disaccharide units. By contrast, JM13 binding seemed to require the presence of 2-O-sulfated glucuronic acid residues.

Heparan sulfate (HS)[1] proteoglycans on cell surfaces and in the extracellular matrix are implicated in developmental, regenerative, and pathological processes because of their interactions with multiple proteins (1–5). These interactions are mediated mainly via the HS components of the proteoglycans, which bind to growth factors/cytokines, matrix components, enzymes, and enzyme inhibitors and thereby regulate the tissue localization and biological activities of the proteins. Characterization of HS oligosaccharides with affinity to proteins such as antithrombin (6) and peptide growth factors (7) has led to identification of specialized protein binding HS domains with ligand-specific structural distinctions. These functional domains derive from enzymatic modification in the Golgi apparatus of the primary polymerization product of HS/heparin biosynthesis, composed of alternating glucuronic acid and N-acetylglucosamine units ((GlcUA-GlcNAc)$_n$). The nascent polymer is first subject to partial N-deacetylation/N-sulfation of the GlcNAc residues. The modification occurs in a regioselective fashion, giving rise to (i) consecutively N-sulfated regions, (ii) regions of alternating N-acetylated and N-sulfated disaccharide units, and (iii) domains that escape modification and remain N-acetylated (5, 8). Sometimes, the N-deacetylation/N-sulfation reaction remains partial, with N-unsubstituted GlcN units as a result (9, 10). The further modification reactions, C5 epimerization of GlcUA residues into iduronic acid (IdoUA) residues and O-sulfation, all occur in the vicinity of previously incorporated N-sulfate groups. O-Sulfation is frequently found at C2 of IdoUA units and at C6 of GlcN units. Rarely, O-sulfation occurs also at C2 of GlcUA units and at C3 of GlcNSO$_3$ residues, the latter sulfate substitution providing a hallmark for the antithrombin binding HS/heparin sequence.

In tissues, spatially and temporally coordinated expression of protein binding HS domains might entail mechanisms to control the protein activity above the level of protein expression. Remarkable specificity has indeed been demonstrated with regard to the ability of HS to form complexes with fibroblast growth factors (FGFs) and FGF receptors in mouse embryo tissues (11), presumably because of structural distinctions between the HS species found in the distinct tissue loci. Moreover, immunohistochemical studies of tissues with antibodies against different HS epitopes suggest structural differences in HS in distinct cellular environments, such as the tubular and glomerular epithelia and basement membranes of the kidney (12, 13).

Antibodies have become important tools to assess the occurrence and localization of HS in tissues. Potentially, antibodies can also provide structural information of tissue HS, but proper assessment of such data obviously requires sufficient knowledge of the antibody epitope structure. So far, relatively few anti-HS antibodies have been studied with regard to their cognate epitopes. We have previously characterized the epitope to antibody JM403 (12), in which the key determinant appears to be an N-unsubstituted GlcN residue (GlcNH$_3^+$ or "free amino

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[1] The abbreviations used are: HS, heparan sulfate; CHO, Chinese hamster ovary; EHS, Englebreth-Holm-Schwarz; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; FGF, fibroblast growth factor; GlcUA, glucuronic acid; HEK, human embryonic kidney; IdoUA, iduronic acid; K5PS, capsular polysaccharide from *E. coli* K5; NDST, N-deacetylase/N-sulfotransferase; OST, O-sulfotransferase; PBS, phosphate-buffered saline.

group"). More recently, characterization of the epitopes recognized by the antibody 10E4 (14) and a panel of 10 anti-HS antibodies constructed by the phase display technology (13) have been reported. The 10E4 epitope, occurring in multiple HS species but enriched in the brain lesions of scrapie-infected mice, has been proposed to encompass a tetrasaccharide sequence with an essential $GlcNH_3^+$ residue (15). The phage display antibodies recognized a number of different epitopes that were all characterized by the presence of *N*- and *O*-sulfate substituents both in HS and heparin preparations (13).

We have studied two previously uncharacterized epitopes to monoclonal antibodies against HS, JM13 (16) and HepSS1 (17), and attempted to define further the epitopes recognized by JM403 and 10E4. Two of the antibodies (10E4 and HepSS1) are commercially available and have been widely used as research reagents. Moreover, we have examined the immunoreactivity of these antibodies in rat and human tissue. Our results suggest that HepSS1 and JM13 bind to rare saccharide epitopes that occur predominantly in basement membrane HS. HepSS1 reacts specifically with an epitope of several consecutive *N*-sulfated disaccharide units with little or no *O*-sulfation, whereas JM13 binds to an *O*-sulfated epitope containing GlcUA. 10E4 and JM403 both recognize more common but distinct epitopes. Our results suggest a mixed *N*-sulfated and *N*-acetylated epitope for 10E4 and support the earlier data (12) indicating a critical role for $GlcNH_3^+$ residues in JM403 binding.

MATERIALS AND METHODS

Glycosaminoglycans

HS (preparation HS-II) isolated from human aorta essentially according to Iverius (18) was provided by Dr. W. Murphy (University of Monash, Melbourne, Australia). Chemical modifications to HS-II (cleavage with HNO_2 at pH 1.5 or 3.9, *N*-deacetylation, *N*-acetylation, or *N*-sulfation) were made as described previously (12). HS preparations from bovine aorta, lung, intestine, and kidney and the murine Englebreth-Holm-Schwarz tumor (EHS-HS) were isolated and characterized as described before (19, 20). The chemically modified derivatives of bovine kidney HS were provided by Dr. A. Tawada (Seikagaku Corp.). The *Escherichia coli* K5 capsular polysaccharide (K5PS) with the same $(GlcUA-GlcNAc)_n$ structure as the HS/heparin precursor polysaccharide, *O*-sulfated K5PS, *N*-desulfated heparin, and *N*-desulfated, *N*-acetylated heparin were supplied by Dr. G. van Dedem (AKZO-Nobel, Oss, The Netherlands). *N*-Sulfated K5PS was a gift from Dr. B. Casu (Instituto di Chimica e Biochimica G. Ronzoni, Milan, Italy). Porcine intestinal heparin was from Leo Pharmaceutical Products (Weesp, The Netherlands).

Biotinylation of HS

HS-II, EHS-HS, and bovine kidney HS were biotinylated by derivatization of the carboxyl groups using biotin LC hydrazide (Pierce Chemical Company) according to the instructions of the manufacturer. HS-II was also biotinylated via its *N*-unsubstituted GlcN units using NHS-LC-biotin (Pierce).

Monoclonal Antibodies

The four murine monoclonal antibodies against HS were all of the IgM isotype. JM403 was generated by immunization with rat glomerular HS proteoglycans (21), JM13 was obtained from immunization with human glomerular HS proteoglycans (16), 10E4 by immunization with liposome-intercalated membrane HS proteoglycans from human fetal lung fibroblasts (14), and HepSS1 after immunization with murine methylcholanthrene-induced fibrosarcoma cells (17). The 10E4 and HepSS1 antibodies are commercially available at Seikagaku Corp. (www.acciusa.com/seikagaku/products/product.asp).

Immunocytological and Immunohistological Analyses

Embryonic Rat Tissues—Methanol-fixed 10-μm cryostat sections of embryonic rat heads were used for immunohistochemical analysis. Nonspecific binding sites were blocked by incubating the sections in PBS containing 1% (w/v) bovine serum albumin and 0.05% (v/v) Tween 20 for 30 min followed by a 40-min incubation with the primary antibodies (diluted 1:10,000). The sections were washed with PBS and 0.05% Tween 20 (three times for 10 min/wash) and incubated for 40 min

with the secondary antibody (Cy3-conjugated goat anti-mouse IgM, 1:600 dilution; Sigma). The slides were again washed with PBS and Tween as above and mounted with Vectashield mounting medium (Vector Laboratories). All incubations were performed at room temperature. The slides were examined using a Zeiss Axiophot microscope.

Human Uterine Cervix—Slide-mounted, paraffin-embedded 6-μm sections of human uterine cervix (generously provided by Dr. Frej Stenbäck, University of Oulu) were pretreated for antigen retrieval[2] in citrate buffer, pH 6.0, in a microwave oven, followed by blocking of unspecific antibody binding sites with 2% normal horse serum and incubation with the primary antibodies JM403 (1:10,000), JM13 (1:20,000), HepSS1 (1:20,000), and 10E4 (1:10,000). The immunoreactivity was amplified using biotin-conjugated anti-mouse IgM (1:250; Vectastain, Vector Laboratories) and detected with streptavidin-conjugated horseradish peroxidase using 3,3'-diaminobenzidine tetrahydrochloride (Sigma) as substrate. Sections were counterstained with Meyer's hematoxylin (Sigma) and examined using an Olympus BX60 microscope. Images were taken with Olympus U-CMAD-2 CCD camera.

Some tissue sections were treated with heparitinase (25 milliunits/ml; EC 4.2.2.8; Seikagaku Corp.) for 1 h at 37 °C in PBS containing 5 mM calcium acetate to assess the specificity of the staining.

Immunocytochemistry—The cells used for immunostaining included human embryonic kidney (HEK293) cells, either mock-transfected or transfected with HS 2-*O*-sulfotransferase (2-OST), 6-*O*-sulfotransferase-3 (6-OST3) (kindly provided by Marion Kusche-Gullberg, Uppsala), or *N*-deacetylase/*N*-sulfotransferase-2 (NDST-2) (a gift from Lena Kjellén, Uppsala) and parental Chinese hamster ovary (CHO) cells (CHO-K1), the HS-deficient strain (CHO677) and the 2-OST-deficient strain (CHO-F17). The cells were maintained as described previously (23, 24), fixed with 4% paraformaldehyde, and processed for indirect immunofluorescence. The primary antibodies were visualized with fluorescein isothiocyanate-conjugated anti-mouse IgM secondary antibodies. The coverslips were mounted with Vectashield mounting medium containing DAPI nuclear stain (Vector Laboratories). Fluorescence images were analyzed with Zeiss LSM 510 confocal microscope.

For FACS analysis, HEK293 cells were incubated with the anti-HS antibodies for 20 min at 4 °C followed by incubation with fluorescein isothiocyanate-conjugated anti-mouse IgM. The cells were washed twice and fixed with 1% paraformaldehyde. All incubations and washes were performed in PBS containing 2% fetal calf serum and 1 nM sodium azide. The immunoreactivity of the cells was analyzed using a FACS Calibur instrument (BD Biosciences).

ELISA—The binding of the antibodies to various polysaccharides was assessed by inhibition ELISA. Two different protocols were used. In Protocol 1, antibodies were immobilized to polystyrene flat bottom microtiter plates (Nunc Maxisorp) precoated with goat anti-mouse IgM antibodies (Sigma). Biotinylated HS-II was added together with increasing concentrations of unlabeled competitor glycosaminoglycans. Biotinylated HS-II remaining on the plates after washes was detected by peroxidase-labeled streptavidin (Amersham Biosciences) and developed using 3,3',5,5'-tetramethylbenzidine (SFRI Laboratories, Berganton, France) as peroxidase substrate. The color reaction was stopped with sulfuric acid, and absorption was measured at 450 nm. In Protocol 2, biotinylated HS from the EHS tumor was immobilized to streptavidin-coated plates followed by the addition of antibodies and the competitor saccharides. Bound antibody was detected using horseradish peroxidase-labeled anti-mouse IgM antibodies using 3,3',5,5'-tetramethylbenzidine as peroxidase substrate. In both protocols, competition was calculated as $(1 - (A_{450} \text{ with competitor}/A_{450} \text{ without competitor})) \times 100\%$. The results from experiments according to Protocol 1 were normalized relative to the inhibition accomplished by HS-II, which was set to 1.00.

RESULTS

All Four Heparan Sulfate Epitopes Show Distinct Tissue Localization

Developing Rat Tooth—The four antibodies studied demonstrated a different pattern of HS staining in the developing rat molar tooth. The HepSS1 and JM13 epitopes were largely confined to the basement membrane, whereas JM403 and 10E4

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[2] Tissue sections were pretreated with trypsin or incubated in a microwave oven or pressure cooker prior to the immunohistochemical staining, and the results were compared with similarly stained sections without pretreatment. Treatment in a microwave oven (10 min) was employed in further studies because it resulted in distinct antigen visualization and well preserved tissue architecture.
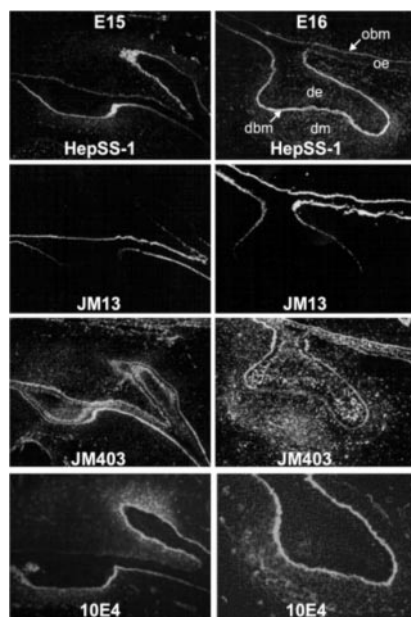
Fɪɢ. 1. **Immunohistochemical localization of HS epitopes in developing tooth.** 10-μm cryostat sections of rat molar tooth were immunostained with HepSS1, JM13, JM403, and 10E4 followed by detection of the bound antibody with fluorescent secondary antibodies. *E15* and *E16* refer to the days 15 and 16 of embryonic development. The specialized tissue structures shown in the *uppermost right panel* are as follows: *obm*, oral basement membrane; *dbm*, dental basement membrane; *oe*, oral epithelium; *de*, dental epithelium; *dm*, dental mesenchyme.

staining was seen both in the basement membrane and the dental epithelium and/or mesenchyme (Fig. 1 and Table I). JM403 staining was restricted to the epithelium during early development (embryonic day 15), but at embryonic day 16 a distinct staining was seen also in the mesenchymal tissue (Fig. 1). The appearance of the mesenchymal JM403 reactivity thus coincided with the mesenchymal condensation, which reflects the commencement of active morphogenesis in the mesenchyme. By contrast, 10E4 staining was confined to the mesenchyme, whereas the dental epithelium remained negative throughout the development (Fig. 1; embryonic days 17 and 18, data not shown). The surrounding oral epithelium stained positively for JM403 but did not contain the 10E4 epitope (Fig. 1). Tissue sections pretreated with heparitinase were not stained by any of the antibodies (data not shown).

All four HS epitopes were found in the basement membrane HS but with marked differences in their occurrence in the oral *versus* dental basement membranes. HepSS1 epitope was thus expressed weakly in the oral basement membrane but strongly in the dental basement membrane that separates the morphogenetically active epithelial and mesenchymal tissues (Fig. 1). Immunostaining of adjacent tissue sections with JM13 revealed a largely opposite expression pattern, such that intense expression was seen in the oral basement membrane but faint expression in the dental basement membrane. The JM403 and 10E4 epitopes were found in both basement membranes, the former epitope being expressed preferentially in the oral and the latter in dental basement membrane (Fig. 1). The different antibodies thus showed distinct reactivities in the dental *versus* oral basement membrane and mesenchyme *versus* epithelium.

Human Uterine Cervix—We proceeded to immunostain sections of human uterine cervix to elucidate whether the same HS epitopes are found in adult human tissue (Fig. 2). Both JM13 and JM403 showed distinct staining of the basement membrane but little or no staining in the ectocervical epithe-

lium or in the underlying stroma. By contrast, 10E4 showed intense cell surface staining at the suprabasal layers of the stratified squamous epithelium, but no staining in the basement membrane. The human cervix tissue was not immunoreactive to HepSS1 with any of the antigen retrieval procedures employed in our study. These data suggest different structures of basement membrane and epithelial cell HSs in adult human tissue.

Characterization of the Polysaccharide Epitopes Recognized by the Antibodies

To characterize the saccharide epitopes recognized by the antibodies, we assessed the ability of different saccharides to inhibit the binding of biotinylated HS to immobilized anti-HS antibodies in ELISAs. All four anti-HS monoclonal antibodies (JM13, HepSS1, 10E4, and JM403) bound efficiently to biotinylated HS-II, a low sulfated HS species from human aorta, which was used in the first series of ELISA studies made according to Protocol 1 (see "Materials and Methods"). To compare the binding properties of the antibodies, the results were normalized by setting the inhibition obtained by unbiotinylated HS-II to 1.00. The inhibitor saccharides included various bovine HS species, heparin, and K5PS from *E. coli*, as well as various derivatives of these saccharides obtained by chemical modification (Table II). Further assessment (Table III) of the 10E4 epitope specificity was made in a second series of experiments (Protocol 2; see "Materials and Methods") involving immobilized EHS-HS, particularly to evaluate the role of GlcN *N*-substitution in 10E4 binding (see below).

JM13—None of the bovine HS preparations competed for the binding of JM13 to biotinylated HS-II, suggesting that the epitope occurs rarely in HS (Table II). Treatment of HS-II with HNO_2 at pH 1.5 abolished binding, pointing to the presence of *N*-sulfate in the JM13 epitope. The finding that treatment of HS-II with HNO_2 at pH 3.9, which cleaves the polysaccharide at *N*-unsubstituted GlcN residues, also abolished antibody reactivity, would reflect the presence of free amino groups in the JM13 epitope. However, because *N*-acetylated HS-II bound JM13 as effectively as native HS-II, it is more likely that $GlcNH_3^+$ residues in HS-II are present vicinal to the JM13 epitope but not a part of the antibody binding site. Removal of *N*-acetyl groups from HS-II as well as their replacement with *N*-sulfate groups increased JM13 binding, suggesting an inhibitory role for the *N*-acetyl groups in the interaction (Table II). Alternatively, the finding may also reflect generation of new epitopes containing either $GlcNH_3^+$ or $GlcNSO_3^-$ residues.

Experiments with various K5PS derivatives proved useful in assessing the role of *O*-sulfates in JM13 binding. Although the native or *N*-deacetylated K5PSs did not bind JM13, some reactivity was obtained with *N*- or *O*-sulfated K5PS and very strong reactivity with *O*-sulfated K5PS containing either *N*-sulfate groups or *N*-unsubstituted GlcN units (Table II). These data indicate that *O*-sulfation is essential for the binding and confirm the idea that *N*-acetyl groups inhibit the binding. The finding that *N*-deacetylated, *O*-sulfated K5PS bound JM13 much better than *N*-deacetylated or *N*-desulfated heparin suggests that IdoUA units interfere with JM13 binding because K5PS lacks IdoUA, which is abundant ($>75\%$ of all hexuronic acid residues) in heparin. In conclusion, JM13 appears to recognize a rare HS epitope, characterized by the presence of *O*-sulfate substituents in a GlcUA-rich sequence (Table IV).

HepSS1—The HepSS1 epitope was present in bovine aorta HS but not in other bovine HS preparations (Table II). Treatment of HS-II with HNO_2 at pH 1.5 abolished HepSS1 binding, whereas treatment with HNO_2 at pH 3.9 or *N*-acetylation had no effect. *N*-Deacetylation of HS-II abolished the binding, but

TABLE I
Immunolocalization of anti-HS antibody epitopes in embryonic rat tooth and adult human cervix tissues
Tissue sections of developing rat tooth and human uterine cervix were stained with the anti-HS antibodies. The staining intensity was graded as follows: −, no staining; +, weak staining, ++, moderate staining, +++, strong staining.

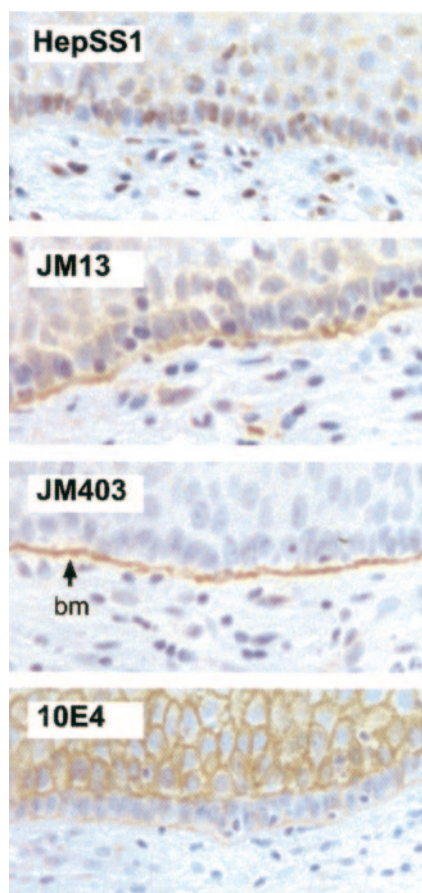| Antibody	Dental BM[a]	Oral BM	Dental epithelium	Oral epithelium	Dental mesenchyme	Uterine cervix epithelium	Uterine cervix BM
HepSS1	+++	++	+	−	+	−	−
JM13	−	+++	−	−	−	−	+++
JM403	+++	+++	++	++	++	−	+++
10E4	+++	+	−	−	++	+++	−

[a] BM, basement membrane.

FIG. 2. **Immunohistochemical localization of HS epitopes in human uterine cervix.** Paraffin-embedded sections (5 μm) of human uterine cervix tissue were subjected to antigen retrieval by treatment in microwave oven followed by immunostaining with HepSS1, JM13, JM403, and 10E4, detection by peroxidase-conjugated secondary antibodies, and counterstaining with hematoxylin. The *arrow* indicates the basement membrane separating the stratified squamous epithelium of the ectocervix and the underlying stroma.

increased binding was seen upon subsequent *N*-sulfation of the *N*-deacetylated polysaccharide. All of these findings suggest a role for the *N*-sulfate substituents in the binding. Notably, a functional HepSS1 epitope could be generated solely by extensive (>95%) *N*-deacetylation/*N*-sulfation of K5PS (Table II). These findings suggest a role for consecutive GlcUA-GlcNSO$_3$ disaccharide units in the interaction. *O*-Sulfate groups or IdoUA did not seem to be involved in HepSS1 binding, as indicated by experiments with *O*-sulfated K5PS species or chemically modified heparin preparations (Table II).

To define the HepSS1 epitope further, we assessed the ability of EHS-HS and partially *N*-sulfated K5PSs to bind HepSS1. Partially (~60%) *N*-sulfated K5PS did not react with HepSS1 (Fig. 3), whereas a completely (>95%) *N*-sulfated preparation was reactive (Table II). Furthermore, HepSS1 displayed strong reactivity toward EHS-HS that was shown to contain,

by compositional disaccharide analysis, an unusually high proportion (~60%) of non-*O*-sulfated HexA-GlcNSO$_3^-$ disaccharide units. Such units are arranged in a consecutive fashion in HS (*N*-sulfated domains) but not in partially *N*-sulfated K5PS in which the chemical *N*-deacetylation/*N*-sulfation gives rise to randomly distributed GlcNSO$_3^-$ residues along the polysaccharide.

10E4—The 10E4 epitope was found in bovine aorta and intestine HS preparations (Table II). The reactivity was lost by treatment of HS-II with HNO$_2$ at pH 1.5, suggesting the presence of *N*-sulfate substituents in the epitope. Treatment of HS-II with HNO$_2$ at pH 3.9 resulted in reduced 10E4 binding. This finding would point to an involvement of GlcNH$_3^+$ units, but because *N*-substitution of such units by *N*-acetylation did not influence the binding, it is more likely that chain breakage at GlcNH$_3^+$ units destroys the 10E4 epitope without GlcNH$_3^+$ units being an integral part of the antibody recognition site. Reactivity was lost after *N*-deacetylation, and it could not restored by subsequent *N*-sulfation of HS-II. These data suggest that *N*-acetyl groups are essential for 10E4 binding.

It has been postulated previously that the 10E4 epitope would encompass the unsulfated —GlcUA-GlcNH$_3^+$-GlcUA-GlcNAc— sequence. Because the initial data from the current study (Table II) did not support the conclusion that GlcNH$_3^+$ residues play a role in 10E4 binding, a second set of experiments (Table III) was performed to address further the role of GlcN *N*-substitution. For comparison, JM403 was studied in a similar fashion because earlier data (12, 25) as well as the results presented here (Tables II and III; see below) indicate a critical role for GlcNH$_3^+$ units in JM403 binding. The results demonstrated that partially deacetylated K5PS, containing ~40% of *N*-unsubstituted GlcN residues and ~60% of *N*-acetylated GlcN residues, bound well to JM403 but failed to react with 10E4, whereas the 10E4 reactivity was induced upon *N*-sulfation of the GlcNH$_3^+$ units (Table III). Moreover, two of three chemically modified preparations of bovine kidney HS that contained significant proportions (23–39% of all GlcN residues) of GlcNH$_3^+$ units lacked 10E4 reactivity, whereas the same preparations all bound JM403. One preparation (completely desulfated, *N*-sulfated, and partially *N*-deacetylated HS; Table III) showed some 10E4 reactivity, but the reactivity was three times weaker than that of an un-*N*-deacetylated preparation (Table III). The current data thus support the conclusion that both *N*-sulfated and *N*-acetylated disaccharide units are required for 10E4 reactivity. Comparison of the reactivities of native and *O*-desulfated HS as well as partially *N*-deacetylated/*N*-sulfated HS with or without *O*-sulfation suggests that *O*-sulfation is not required for 10E4 binding.

JM403—We have demonstrated earlier that JM403 recognizes an epitope with one or more critically important GlcNH$_3^+$ units (12, 25). The epitope occurred commonly in the bovine HS preparations because all HS species were recognized by JM403 (Table II). Although native K5PS did not bind JM403, strong reactivity was obtained by *N*-deacetylated K5PS. The finding that the *N*-deacetylated K5PS was the most efficient inhibitor of the HS-II-JM403 interaction of all polysaccharides tested

TABLE II

Inhibition of the binding of biotinylated HS to immobilized anti-HS antibodies in ELISA (Protocol 1) by various heparin-like polysaccharides

Results are expressed as inhibitory capacity relative to HS-II (set to 1.00).

Inhibitor	JM13	HepSS1	10E4	JM403
Derivatives of K5PS				
Native K5	<0.01	<0.01	<0.01	<0.01
N-Deacetylated K5	<0.01	<0.01	<0.01	7.50
N-Deacetylated, *N*-sulfated K5	0.50	2.80	<0.01	<0.01
O-Sulfated K5	2.20	<0.01	<0.01	<0.01
N-Deacetylated, *O*-sulfated K5	>100.00	<0.01	<0.01	0.03
N-Deacetylated, *N*-sulfated, *O*-sulfated K5	>100.00	<0.01	<0.01	<0.01
Derivatives of human aorta HS-II				
Native HS-II	1.00	1.00	1.00	1.00
HS-II, HNO_2, pH 1.5	<0.01	<0.01	<0.01	<0.01
HS-II, HNO_2, pH 3.9	<0.01	1.00	0.10	<0.01
N-Acetylated HS-II	1.00	1.00	1.00	<0.01
N-Deacetylated HS-II	10.00	<0.01	<0.01	4.40
N-Deacetylated, *N*-sulfated HS-II	10.00	5.21	<0.01	<0.01
Heparan sulfate preparations from bovine organs				
Aorta	<0.01	0.36	2.56	0.94
Lung	<0.01	0.06	<0.01	0.77
Intestine	<0.01	0.07	6.88	0.91
Kidney (1.1 M)	<0.01	0.04	<0.01	0.27
Kidney (1.25 M)	<0.01	<0.01	<0.01	0.16
Heparin derivatives				
Native heparin	<0.01	<0.01	<0.01	<0.01
N-Deacetylated heparin	<0.01	<0.01	<0.01	<0.01
N-Desulfated heparin	<0.01	<0.01	<0.01	<0.01
N-Desulfated, *N*-acetylated heparin	<0.01	<0.01	<0.01	<0.01
N- and *O*-desulfated, *N*-deacetylated heparin	<0.01	<0.01	<0.01	0.05
N- and *O*-desulfated, *N*-reacetylated heparin	<0.01	<0.01	<0.01	<0.01
N-Resulfated, *O*-desulfated heparin	<0.01	<0.01	<0.01	<0.01

TABLE III

Inhibition of the binding of two anti-HS antibodies to immobilized EHS-HS in ELISA by various HS-related polysaccharides in the fluid phase

Results are expressed as the concentration of inhibitor which gives 50% inhibition in the ELISA system (IC_{50}). The GlcN *N*-substitution and degree of 6-*O*-sulfation of the inhibitor polysaccharides are indicated as a percent of all GlcN residues.

Inhibitor	GlcN substitution			6-*O*-Sulfate	IC_{50}	
	N-Acetyl	*N*-Sulfate	$-NH_3^+$		10E4	JM403
	%	%	%	%	μg/ml	μg/ml
Derivatives of *E. coli* K5PS						
Native K5	100	0	0	0	>100	>100
PDeAc[a] K5	59	0	41	0	>100	0.004
PDeAc K5 followed by NS[b]	42	58	0	0	36.4	>100
Heparan sulfate						
Bovine kidney	65	33	2	25	67.1	5.16
EHS tumor	35	63	2	2	3.0	5.1
Derivatives of HS from bovine kidney						
CDS[c] HS	61	0	39	0	>100	4.64
CDS HS followed by NS	62	38	0	2	20.1	>100
CDS/NS-HS followed by PDeAc	38	37	25	2	62.9	0.45
CDS/NS/PDeAc/NS HS	39	61	0	4	38.9	>100
PDeAc HS	43	34	23	19	>100	0.05
PDeAc/NS HS	36	64	0	30	>100	>100

[a] PDeAc, partially *N*-deacetylated.
[b] NS, *N*-sulfation.
[c] CDS, completely desulfated.

TABLE IV

*Epitope characteristics of four anti-HS antibodies*

Antibody	Occurrence in HS	Required GlcN *N*-substitution	Requirement for		Epitope characteristics
			O-Sulfate	IdoUA	
JM13	Rare	*N*-Sulfate or $-NH_3^+$	Yes	No	2-*O*-Sulfated GlcUA-rich sequence
HepSS1	Rare	*N*-sulfate	No	No	*N*-Sulfated GlcUA-rich sequence
10E4	Common	*N*-sulfate and *N*-acetyl	No	No	Mixed *N*-acetylated/*N*-sulfated sequence
JM403	Common	$-NH_3^+$	No	No	GlcUA-rich sequences with *N*-unsubstituted GlcN units

indicates that the *N*-unsubstituted GlcN units are both necessary and sufficient, in the absence of any other polymer modification, for JM403 binding. IdoUA units seemed to hamper the binding because *N*-/O-desulfated, *N*-deacetylated heparin did not function as an efficient inhibitor.

Anti-HS Antibody Immunoreactivity in Cells with Altered HS Biosynthesis Enzyme Expression—We sought to corroborate the ELISA data above by assessing the immunoreactivity of the antibodies in cells with defined alterations in HS composition, either because of transfectional overexpression of HS biosynthesis enzymes or lack of enzyme activity. HS from human HEK293 cells overexpressing HS 2-OST (24) thus contains a

A

B
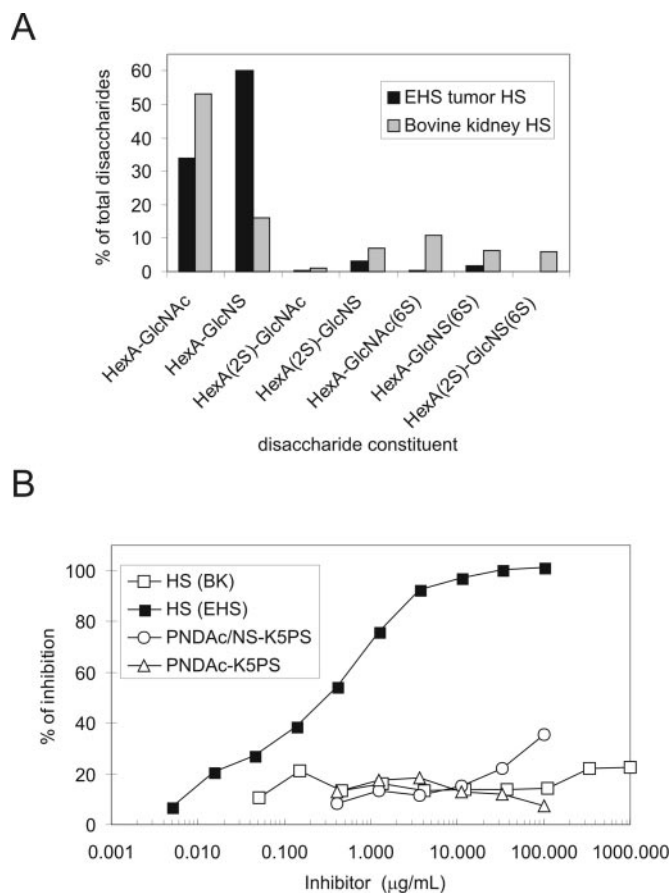
FIG. 3. **HepSS1 binds to EHS-HS containing GlcUA-GlcNSO$_3^-$ sequences with a low degree of *O*-sulfation.** *A*, disaccharide composition of HS from EHS tumor and bovine kidney. Note the high proportion of *N*-sulfated, non-*O*-sulfated disaccharides (*HexA-GlcNS*) in EHS-HS and the low proportion of such units in BK-HS. *B*, inhibition of HepSS1 binding to immobilized EHS-HS by BK-HS, EHS-HS, and partially *N*-deacetylated K5PS with (*PNDAc/NS*) or without (*PDNAc*) *N*-sulfation of the GlcN units. The ability of the saccharides to inhibit HepSS1 binding to biotinylated EHS-HS was assessed by the ELISA Protocol 2 (see "Materials and Methods").

high proportion of 2-*O*-sulfated GlcUA units (19% of total hexuronic acid residues *versus* <4% in untransfected cells), whereas HS from 2-OST-deficient CHO cells (F17) completely lacks GlcUA(2-OSO$_3^-$) (23). Immunostaining of the cells with JM13 indicated markedly increased reactivity in 2-OST-transfected cells compared with untransfected cells, whereas there was no staining in 2-OST-deficient cells (Fig. 4*A*), indicating that 2-*O*-sulfated GlcUA units are critical for JM13 binding. In agreement with this notion, JM13 staining was not altered in HS 6-OST3- (data not shown) or NDST-2- (26) transfected cells (Fig. 4*A*), which show increased GlcN 6-*O*-sulfation[3] and high proportions of non-*O*-sulfated GlcUA-GlcNSO$_3^-$ disaccharide units (26), respectively, but do not differ from untransfected cells in their GlcUA(2-OSO$_3^-$) content. The results were confirmed by FACS analysis, which demonstrated >2-fold increase in JM13 reactivity (Fig. 5).

In contrast to findings with JM13, the NDST-2-transfected cells showed intense immunoreactivity to HepSS1 (Fig. 4*B*), in agreement with the finding that the HepSS1 epitope contains consecutive non-*O*-sulfated HexA-GlcNSO$_3^-$ units. FACS analysis indicated a 5-fold increase compared with mock-transfected cells (Fig. 5). The NDST-2-transfected cells were also reactive to 10E4 (Figs. 4*C* and 5), consistent with the finding

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[3] M. Kusche-Gullberg, personal communication.

that their HS shows increased *N*-sulfation and decreased *O*-sulfation compared with control cells (26), hence higher proportions of non-*O*-sulfated domains containing both *N*-sulfated and *N*-acetylated disaccharide units. Similar findings, *i.e.* increased HepSS1 and 10E4 immunoreactivity, were obtained with the 2-OST-deficient CHO-F17 cells (Fig. 4, *B* and *C*) because these cells also display increased *N*-sulfation and decreased *O*-sulfation compared with parental CHO-K1 cells (23). The antibodies did not bind to the HS-deficient CHO677 cells, indicating that they did not cross-react with chondroitin sulfate or other cell surface components.

Conclusions—The results of the epitope characterization indicate that the four antibodies recognize different HS epitopes, which differ in their abundance in isolated HS species, cultured cells, and tissue specimens. Generally, the results support the idea that the HepSS1 and JM13 epitopes represent rarely occurring HS structures (Table IV). They did not bind heparin and showed little or no binding to HS preparations from bovine aorta, lung, intestine, and kidney. At the tissue level, their expression was confined to the basement membrane HS. By contrast, the 10E4 and JM403 epitopes seemed to occur more commonly in preparations of purified bovine HS as well as in tissues. However, the epitopes were not found in heparin. 10E4 thus recognized both aorta and intestine HS, and JM403 showed some binding to all HS species tested. JM13 was the only antibody that required *O*-sulfate substituents for its binding, presumably in the form of 2-*O*-sulfated GlcUA residues. HepSS1 recognized *N*-sulfated disaccharide sequences in HS, whereas *O*-sulfation had an inhibitory role. The JM403 epitope contains an essential GlcNH$_3^+$ residue, in accordance with our earlier findings (12). The 10E4 epitope appears to involve both *N*-sulfated and *N*-acetylated disaccharide constituents of HS, whereas *O*-sulfate groups are not required for 10E4 binding. All four antibodies seemed to bind to GlcUA-containing sequences, whereas IdoUA units did not appear to have a role in their epitope recognition.

DISCUSSION

It is thought that specialized domain structures in HS can modulate the functions of various effector proteins. The elucidation of such structures has been pursued by biochemical methods employing isolated HS oligosaccharides, which, however, have to be purified close to homogeneity to allow detailed analysis such as sequencing (7, 27–29). Although these techniques provide detailed data of HS structure they do not yield information on the localization of domains in tissues because the "HS starting material" unavoidably represents a mixture of different HS species. On the other hand, protein binding studies made *in situ* with cultured cells or tissue specimens have provided useful in assessing the localization of functional HS domains (11, 30), but structural information is not readily obtained. Antibodies recognizing structurally defined HS epitopes should therefore represent a useful complement to other methods of HS analysis. Detection of HS by monoclonal antibodies is used increasingly in studies addressing the role of the polysaccharide in various biological phenomena and its distribution in cells and tissues. Because of the complex and highly variable structure of HS, such antibodies likely recognize specific epitopes that may be present in some HS species and absent in others instead of recognizing ubiquitous HS structures. This assumption is particularly relevant for antibodies raised by immunization of animals with HS because the most commonly occurring saccharide structures are not likely to elicit an immune response. The general structural features of HS are the same among a number of HS preparations from different cell and tissue sources, and they seem to be shared by a variety of animal species. By contrast, antibodies generated

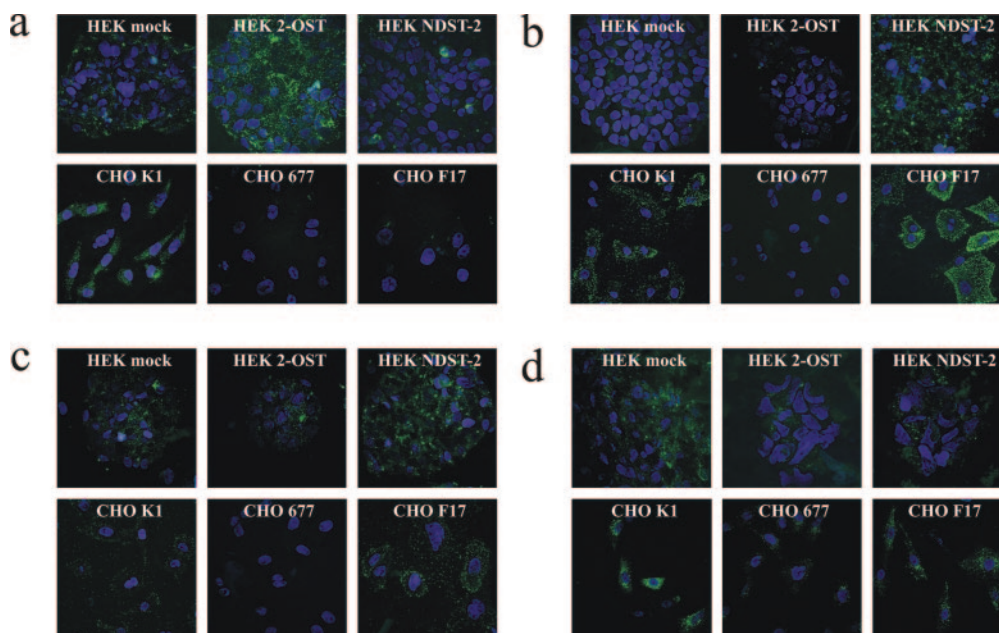*Expression and Structure of Heparan Sulfate Epitopes*

FIG. 4. **Immunocytochemical analysis of antibody binding to cells with altered HS structure.** Mock-transfected (*mock*), 2-OST-transfected, or NDST-2-transfected HEK293 cells and parental CHO cells (K1), HS-deficient CHO677, cells and 2-*O*-sulfate-deficient CHO-F17 cells were grown on glass coverslips, immunostained with JM13 (*a*), HepSS1 (*b*), 10E4 (*c*), and JM403 (*d*) and analyzed by confocal microscopy. The immunoreactivity appears in *green* and the nuclear DAPI stain in *blue*. Note the high JM13 immunoreactivity in 2-OST-transfected HEK293 cells and lack of immunoreactivity in 2-*O*-sulfate-deficient cells.

FIG. 5. **FACS analysis of antibody binding to sulfotransferase-transfected HEK293 cells.** The cells were stained with the various antibodies (as indicated in each *panel*) and analyzed by FACS. The immunoreactivity intensities (measured as mean fluorescence intensity) are expressed as setting the immunoreactivity of the mock-transfected HEK293 cells to 1.
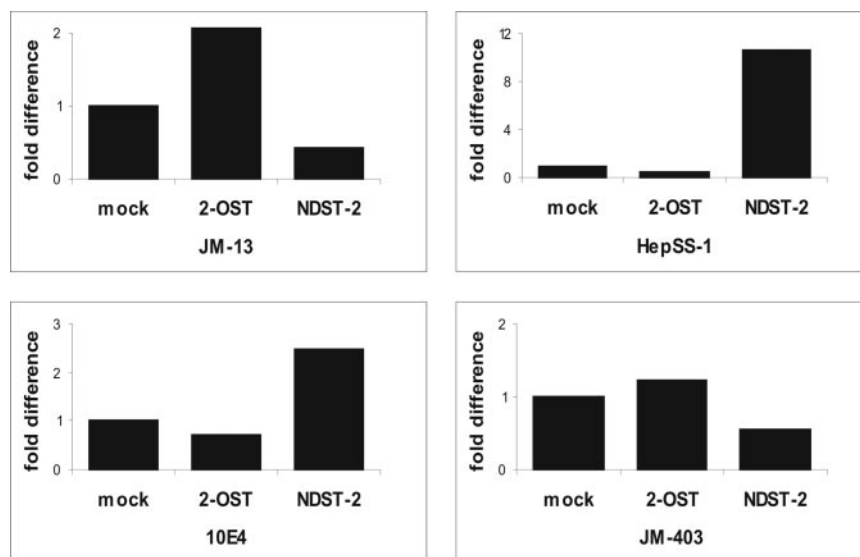
by the phage display technique do not depend on the host immune response and may conceivably bind any HS structure regardless of the frequency of its occurrence in natural HS species. The results of Dennissen *et al.* (13) support this view by demonstrating that many generally occurring saccharide structures found in HS and heparin were recognized by the phage display antibodies.

Polysaccharides are generally regarded as poor immunogens. It is of note that all four antibodies characterized here are of the IgM isotype, the pentameric structure of which might enable multivalent binding to a polymeric antigen such as HS. It is possible that polyvalent binding is required to entail sufficient binding avidity if the binding of an individual Fc moiety to the antigen is weak. According to this hypothesis, the binding of an IgM antibody to HS would require the presence of multiple binding sites conforming to the cognate epitope structure of the antibody, perhaps with appropriate spacing along the HS polymer.

Results from the JM13 epitope characterization point to an important role of *O*-sulfation together with GlcUA residues and either *N*-sulfated or *N*-unsubstituted GlcN units. In JM13-reactive HS, such *O*-sulfate groups may reside either in the 2-*O* position of the GlcUA residues or as 6-*O*-sulfated GlcN units. Although it was difficult to differentiate between these possibilities by the ELISAs (because of the lack of sufficiently specific competitor saccharides), the immunostaining experiments with cells showing either increased 2-*O*- or 6-*O*-sulfation or lack of 2-*O*-sulfate clearly indicated that the critical structural determinant for JM13 binding is 2-*O*-sulfated GlcUA. 3-*O*-Sulfated GlcN units are not likely to be a part of the JM13 epitope because human aorta HS was highly reactive with JM13 but lacks 3-*O*-sulfation (31).

Several lines of evidence suggest a critical role for consecutive, non-*O*-sulfated GlcUA-GlcNSO$_3$ disaccharide units in HepSS1 binding. First, the epitope was generated by extensive but not partial *N*-sulfation of *N*-deacetylated K5PS. Second, the antibody bound to aorta HS, which is rich in *N*-sulfate but

has a low degree of *O*-sulfation (19, 31), and *N*-deacetylation/*N*-sulfation of the polysaccharide further increased its HepSS1 reactivity. Third, HepSS1 showed strong reactivity with HS from the EHS tumor that contains an unusually high proportion of GlcUA-GlcNSO$_3$ disaccharide units ($\sim$60%; Fig. 3), and NDST-2-transfected cells expressing HS with high proportions of such units displayed increased immunoreactivity.

The results reported by Leteux *et al.* (15) suggested that the 10E4 antibody recognizes a HS tetrasaccharide with the structure —GlcUA-GlcNH$_3^+$-GlcUA-GlcNAc—. The *N*-unsubstituted GlcN residue appeared to have a critical role in the sequence because tetrasaccharides with GlcNSO$_3^-$ or GlcNAc residues in the same position failed to react with 10E4. In our studies, the preferred epitope seemed to contain both *N*-sulfated and *N*-acetylated disaccharide units, whereas no direct role for the GlcNH$_3^+$ residues could be demonstrated. The same conclusion can also be drawn from the recent study of Cheng *et al.* (25) in which *N*-acetylation of HS in cultured bladder carcinoma cells did not affect 10E4 reactivity, whereas JM403 binding was lost. The results of the current study further show that 10E4 did not bind completely or partially *N*-deacetylated K5PS and that *N*-acetylation of HS-II did not affect its 10E4 reactivity. However, deaminative cleavage of HS-II at its GlcNH$_3^+$ residues resulted in reduced reactivity, suggesting that in native HS the 10E4 epitope may be found vicinal to the *N*-unsubstituted GlcN units and that such units are tolerated within the epitope (32). Because the HS derivatives with both *N*-sulfated and *N*-acetylated disaccharide units all reacted with 10E4, whereas those containing only a single type of *N*-substitution did not, we conclude that a mixed *N*-sulfated/*N*-acetylated epitope is required for 10E4 binding.

Previous studies suggest that 6-*O*-sulfated GlcN residues may play a role in 10E4 recognition. The extracellularly acting 6-*O*-endosulfatase QSulf1 thus reduces 10E4 staining (33) in cultured cells, and treatment of cultured mouse embryos with sodium chlorate, a metabolic inhibitor of sulfation, diminishes 10E4 reactivity in the developing spinal cord (34). Chlorate treatment preferentially affects *O*-sulfation, especially the 6-*O*-sulfation of GlcN residues, rather than *N*-sulfation (35). The results of the current study do not support these views because cells with increased 6-*O*-sulfation showed weak 10E4 reactivity (data not shown), whereas increased reactivity was seen in NDST-2-transfected cells in which glucosaminyl *N*-sulfation is increased with concomitant decrease of *O*-sulfate substitution.

In agreement with our previous findings, the current study points to the critical role of the GlcNH$_3^+$ residues in JM403 binding. Modification of K5PS by complete or partial *N*-deacetylation thus generated polysaccharides that bound JM403 more efficiently than any other preparation tested, indicating that the —GlcUA-GlcNH$_3^+$— structure is sufficient for efficient JM403 binding. It is of note that JM403 reactivity was decreased in NDST-2-transfected cells because the increased *N*-sulfotransferase activity likely reduces the number of free amino groups.

The developmental processes are critically dependent on the spatially and temporally controlled activity of morphogenic proteins (22, 36, 37). Because many such proteins exert their action at distance from the site of their synthesis, there have to be mechanisms to control not only the expression but also the subsequent tissue localization of the proteins. Tooth development is a well characterized example of organ formation through interactions of the ectodermal epithelium and neural crest-derived mesenchyme. Such interactions require that molecules synthesized in one tissue compartment (epithelium or mesenchyme) and exerting their action in the other must traverse the cellular and basement membrane structures separat-

ing the sites of production and action. For example, epithelial signaling molecules such as FGF8 and bone morphogenetic protein 4 have been shown to regulate gene expression in the mesenchymal cells, and mesenchymally produced FGFs stimulate FGF receptor-expressing cells in the epithelium (37). It is interesting to note that the dental basement membrane HS stained positively for antibodies that recognize epitopes with *N*-sulfation or *N*-unsubstituted GlcN residues, but no *O*-sulfate groups, whereas the basement membrane HS of human uterine cervix contained the *O*-sulfated JM13 epitope. The HS found in the embryonic basement membrane may be more permissive to the molecular traffic between the epithelial and mesenchymal tissues than more highly *O*-sulfated HS species.

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