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Importance of 2-O-Sulfate Groups of Uronate Residues in Heparin for Activation of FGF-1 and FGF-2

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Complete drying of heparin with various concentrations of NaOH by lyophilization caused specific 2-O-desulfation to various degrees without detectable depolymerization or other chemical changes. In order to assess the importance of 2-O-sulfate groups in uronate residues to promote FGF-1 and FGF-2 activities, various 2-O-desulfated (2-O-DS-) heparins were quantitatively examined for their effects on FGF-1- and FGF-2-induced proliferation of BALB/c3T3 clone A31 (A31) cells and the chlorate-treated cells. Twenty-seven percent or less loss of the 2-O-sulfate groups had no effect on the ability to activate both FGF-1 and FGF-2, while 44% loss resulted in a significant loss of the ability. Complete loss of the ability was observed in 2-O-DS-heparins with 75% or more 2-O-desulfation. These results suggest that a high content of 2-O-sulfate groups in uronate residues of heparin is required for activation of both FGF-1 and FGF-2.

Key words: desulfation, FGF-1, FGF-2, heparan sulfate, heparin.

The fibroblast growth factor (FGF) family of growth factors currently has nine members with a broad target-cell specificity (*1*). Acidic and basic FGF were the first two members of this family to be described. These growth factors are now referred to as FGF-1 and FGF-2. These factors bind to heparin with high affinity and are thus termed "heparin-binding growth factors." This binding is considered to reflect an ability to bind to heparan sulfate (HS) present in proteoglycans at the cell surface and in the extracellular matrix. In connection with this, various roles have been suggested, including local retention and stabilization of the growth factors in the extracellular matrix and participation of heparan sulfate proteoglycans (HSPGs) as co-receptors at the cell surface together with their high-affinity transmembrane receptors that include tyrosine kinase domains. The latter role has been well demonstrated. Specific binding of FGF-2 to the FGF-receptor and its mitogenic activity are abolished if the binding of the growth factor to cellular HSPG is precluded by blocking the sulfation step in the biosynthesis of HS (*2, 3*), or by utilizing HS-deficient cells (*4*). Addition of soluble heparin, HS, or heparin fragments can enhance the specific binding of FGF-2 to the FGF-receptor and restore its mitogenic activity. This finding for FGF-2 has also been extended to FGF-1 and FGF-4 (*5, 6*).

Recent structural studies on heparin/HS have shown that *N*-sulfated fragments derived from heparin/HS that specifically bound to FGF-2 were enriched in 2-*O*-sulfated iduronate [IdoA(2-*O*-S)] residues (*7-10*). In contrast, our previous studies have shown that a high content of 6-*O*-sulfate groups in *N*-sulfated glucosamine (GlcNS) residues is required for activation of FGF-1, but not FGF-2. However, complete 6-*O*-desulfation of trisulfated disaccharide units in heparin caused a loss of ability to promote the mitogenic activity of FGF-2 (*11*). Thus, some content of 6-*O*-sulfate groups in GlcNS residues appears to be required for activation of FGF-1 and FGF-2, although FGF-1 differs from FGF-2 in requiring a higher content of 6-*O*-sulfate groups.

In the present study, heparin was selectively 2-*O*-desulfated at uronate residues to various extents. The importance of 2-*O*-sulfate groups of uronate residues of heparin for activation of the mitogenic activities of FGF-1 and FGF-2 was investigated using BALB/c 3T3 (A31) cells, as employed in a previous study (*11*).

MATERIALS AND METHODS

Preparation of 2-O-DS-Heparins and Analysis of Disaccharide Compositions—For preparation of 2-*O*-DS-heparins, a modification of the procedure of Jaseja *et al.* (*12*) was employed. Briefly, 0.5 g of bovine intestinal heparin (Syntex) was dissolved in 10 ml of 0.4, 0.2, 0.1, 0.05, 0.025, and 0.01 N NaOH. The solutions were then frozen and lyophilized to dryness. The crusty yellow residues were dissolved in 10 ml of 1 N NaOH and adjusted to pH 9 with 20% aqueous acetic acid. The solutions were then dialyzed against water for 2 days and the dialysates were lyophilized. Each desulfated heparin was then *N*-acetylated by the previously described procedure (*10*). Briefly, 100 mg of each desulfated heparin was dissolved in

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Abbreviations: FGF, fibroblast growth factor; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; 2-*O*-DS-, 2-*O*-desulfated; 6-*O*-DS-, 6-*O*-desulfated; IdoA, iduronate; GlcNS, *N*-sulfated glucosamine; DMEM, Dulbecco's minimal essential medium; PBS, phosphate-buffered saline.

4 ml of 10% methanol, containing 50 mM Na_2CO_3. The solution was kept on ice and 200 μl of acetic anhydride was added. The reaction mixtures were agitated while the pH was kept at pH 7 to 8 by additions of 10% methanol saturated with Na_2CO_3. The reactions were continued for a total of 2 h, with repeated additions of 200 μl of acetic anhydride every 30 min. The solutions were then dialyzed against water for 2 days. The dialysates were lyophilized to recover the sodium salts of various 2-*O*-DS-heparins. No depolymerization of any 2-*O*-DS-heparin was detected by gel-filtration chromatography.

Compositional analyses of 2-*O*-DS-heparins were performed as described previously (*11, 13, 14*). Briefly, the polysaccharides (0.1 mg) were treated with a mixture of heparinase (50 mU), heparitinase I (20 mU), and heparitinase II (20 mU) (Seikagaku) in 220 μl of 2 mM calcium acetate and 20 mM sodium acetate (pH 7.0) at 37°C for 2 h. The disaccharide composition was analyzed by ion-exchange chromatography of the reaction mixture with Dionex CarboPac PA-1.

Assay for Inhibition of COS-1 Cell Binding to FGF-2-Coated Wells—The ability of the various 2-*O*-DS-heparins to inhibit the binding of COS-1 cells to immobilized FGF-2 was examined as described previously (*11, 15, 16*). Briefly, each well of a 96-well tissue culture plate was coated with 50 μl of 10 μg/ml human recombinant FGF-2 (Promega) overnight at 4°C. The contents were aspirated, and the wells were rinsed twice with PBS, and blocked by incubation with 5% (v/v) fetal bovine serum in PBS for 1 h at room temperature. A pellet of COS-1 cells was prepared by incubation with 2 mM EDTA in PBS for 20 min at 37°C, followed by trituration, and collection by centrifugation. The cells were then resuspended at a cell density of 5×10^5 cells per milliliter with 5% fetal bovine serum in PBS. A 2-*O*-DS-heparin was added, and the cell suspension (0.1 ml) was immediately applied to the coated wells and incubated for 10 min at room temperature. Each well was washed twice with PBS, and then 20 μl of 5% SDS was added to lyse the bound cells. Two hundred microliters of Micro BCA protein assay reagent (Pierce) was added to each well and the protein concentration of the lysate was determined with bovine serum albumin as a standard by measuring the absorbance at 520 nm of each well.

Cell Proliferation Assay—The abilities of various 2-*O*-DS-heparins to inhibit FGF-1- and FGF-2-induced A31 cell (BALB/c3T3, clone A31) proliferation and to restore FGF-1- and FGF-2-induced proliferation of chlorate-treated A31 cells were examined as described previously (*11*). Briefly, for the assay of non-chlorate-treated A31 cell growth, the cells were seeded at an initial density of 4,000 cells/well in 96-well tissue culture plates and were grown for 3 days in 100 μl of DMEM supplemented with ITS+ (Collaborative Research; insulin [6.25 μg/ml], transferrin [6.25 μg/ml], selenium [6.25 ng/ml], bovine serum albumin [1.25 mg/ml], and linoleic acid [5.35 μg/ml]), 2 ng/ml human recombinant FGF-2 (Promega) or 5 ng/ml human recombinant FGF-1 (Promega), 50 μg/ml gentamicin, and the various 2-*O*-DS-heparins to be tested. For testing of the ability of the 2-*O*-DS-heparins to restore the FGF-induced proliferation of chlorate-treated A31 cells, A31 cells were grown for 3 days in DMEM lacking sulfate but supplemented with ITS+, 2 ng/ml FGF-2 or 5 ng/ml FGF-1, 30 mM sodium chlorate, 100 units/ml penicillin G, and the 2-*O*-DS-heparins to be tested. After the incubation, 20 μl of MTS/PMS solution (Cell titer 96 aqueous non-radioactive cell proliferation assay kit, Seikagaku) was added into each well. After 3 h incubation at 37°C, the absorbance of each well at 490 nm was directly measured from the 96-well tissue culture plate. The background absorbance at zero cells/well was subtracted to obtain the presented data.

RESULTS

Disaccharide Analysis of 2-O-DS-Heparins—Specific 2-*O*-desulfation by lyophilization of heparin with various concentrations of NaOH was confirmed by the analyses of disaccharide fractions obtained by heparinase/heparitinase digestion of the intact and the treated heparins. As shown in Table I, the treatment, depending on the concentration of NaOH, caused various extents of decrease in trisulfated disaccharide [ΔUA(2-O-S)-GlcNS(6-O-S)] and disulfated disaccharide [ΔUA(2-O-S)-GlcNS], and corresponding increases in disulfated disaccharide [ΔUA-GlcNS(6-O-S)] and monosulfated disaccharide (ΔUA-GlcNS), respectively, in essentially equal amounts, where ΔUA refers to 4,5-unsaturated hexuronate residue. In contrast, these treatments did not significantly affect *N*-sulfate or 6-*O*-sulfate groups. Furthermore, no depolymerization or other chemical changes were detectable after these treatments (data not shown). Thus, these treatments appear to cause specific 2-*O*-desulfation of 2-*O*-sulfated hexuronate residues. Heparin treated with 0.4 N NaOH lost 81.6% of the 2-*O*-sulfate groups, as shown in Table I, and 94.3% loss of the 2-*O*-sulfate groups was achieved by a second treatment with 0.4 N NaOH (data not shown).

TABLE I. **Disaccharide composition of 2-*O*-desulfated heparins.**

	Disaccharides (%)									Total desulfation[a] (%)		
	OS	NS	6S	US	(6,N)S	(U,N)S	(U,6)S	(U,6,N)S	Total	US	6S	NS
Heparin	4.2	2.2	0.5	1.3	6.7	26.6	0	55.2	96.7	0	0	0
0.4 (N NaOH)	5.0	24.5	1.7	2.1	49.7	5.6	0	8.0	96.2	81.6	4.8	3.2
0.2 (N NaOH)	6.2	20.5	2.9	0	43.1	6.4	0	14.4	93.5	75.0	3.2	6.9
0.1 (N NaOH)	10.6	14.3	2.2	1.6	23.8	12.2	1.8	30.8	97.3	44.2	6.1	10.6
0.05 (N NaOH)	10.8	8.6	2.3	1.6	14.6	19.8	0.6	38.6	96.9	27.1	10.1	10.0
0.025 (N NaOH)	7.8	5.1	2.2	1.6	10.2	21.3	0.7	45.6	94.5	16.7	5.9	9.4
0.01 (N NaOH)	4.8	4.4	0.9	1.4	8.6	25.8	0.2	50.4	96.5	6.4	3.7	1.7

OS, UA-GlcNAc; NS, UA-GlcNS; 6S, UA-GlcNAc(6-O-S); US, UA(2-O-S)-GlcNAc; (6,N)S, UA-GlcNS(6-O-S); (U,N)S, UA(2-O-S)-GlcNS; (U,6)S, UA(2-O-S)-GlcNAc(6OO-S); (U,6,N)S, UA(2-O-S)-GlcNS(6-O-S). [a]Total 2-*O*-desulfation (%) was calculated by use of the following equation. $(1-S/H)\times100$ (S: total 2-*O*-, 6-*O*-, and *N*-sulfates in the disaccharides of each heparin treated with NaOH. H: total sulfates in the disaccharides of intact heparin).

2-O-DS-Heparins as Inhibitors of COS-1 Cell Binding to FGF-2-Coated Wells—A cell-based assay to measure the ability of selectively 2-O-desulfated heparins to inhibit the binding of COS-1 cells to FGF-2-coated wells was performed as reported (*11*). We have previously demonstrated that COS-1 cells bind to FGF-2-coated plates through a specific interaction between the cell surface heparan sulfate proteoglycan (HSPG) and immobilized FGF-2 (*13, 14*). The interactions were markedly reduced in the presence of 0.75-6 μg/ml intact heparin (Fig. 1).

Various 2-O-DS-heparins showed reduced potency as inhibitors of cell binding with increasing 2-O-desulfation. The 81.6 and 75% 2-O-DS-heparins exhibited no activity to inhibit the cell binding at concentrations up to 6 μg/ml, whereas 6.4% 2-O-DS-heparin strongly inhibited the cell binding at a comparable concentration to intact heparin. The 16.7-44.2% 2-O-DS-heparins showed intermediate activity in inhibiting the cell binding. These results suggest that a high content of 2-O-sulfate groups is required for specific binding of heparin and HS to FGF-2.

Effect of 2-O-DS-Heparins on the Proliferation of Chlorate-Treated A31 Cells—We previously reported that sodium chlorate, which inhibited sulfation of HSPG, blocked the ability of both FGF-1 and FGF-2 to stimulate the growth of A31 cells (*11*), and that the chlorate blockage of FGF-mediated growth could be overcome by exogenous heparin. In this work, we examined the ability of various 2-O-DS-heparins to restore the activities of FGF-1 and FGF-2 on chlorate-treated A31 cells.

A31 cells grown in sulfate-free medium containing 20 mM sodium chlorate and either 5 ng/ml FGF-1 or 2 ng/ml FGF-2 exhibited $A_{492}=0.15$ after 3-day incubation, whereas the addition of 2 μg/ml intact heparin increased cell proliferation to 0.9 (with FGF-1, Fig. 2A) and 0.6 (with FGF-2, Fig. 3A). The ability of heparin to restore the FGF-1- and FGF-2-induced proliferations of chlorate-treated A31 cells decreased in accordance with the decrease of 2-O-sulfate groups in heparin. The 6.4, 16.7, and 27.1% 2-O-DS-heparins were quite effective for restoring both the FGF-1- and FGF-2-induced cell proliferations (Figs.

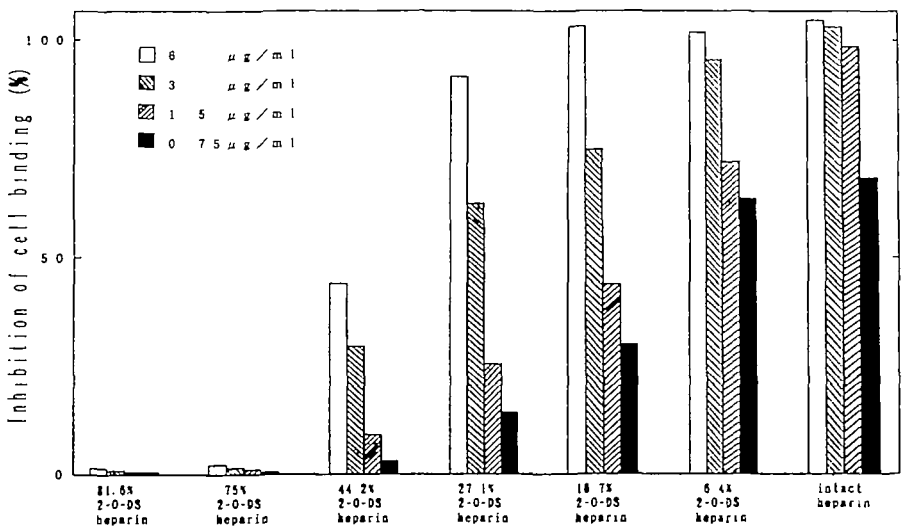

Fig. 1. **Inhibitory effect of 2-O-DS-heparins on COS-1 binding to FGF-2-coated plates.** The COS-1 cells were collected and resuspended in PBS containing 5% fetal bovine serum including various 2-O-DS-heparins at the indicated concentrations. The cells were applied to FGF-2-coated plates and allowed to attach for 10 min, and protein of the bound cells was measured as described under "MATERIALS AND METHODS." The results represent the means of triplicate determinations.

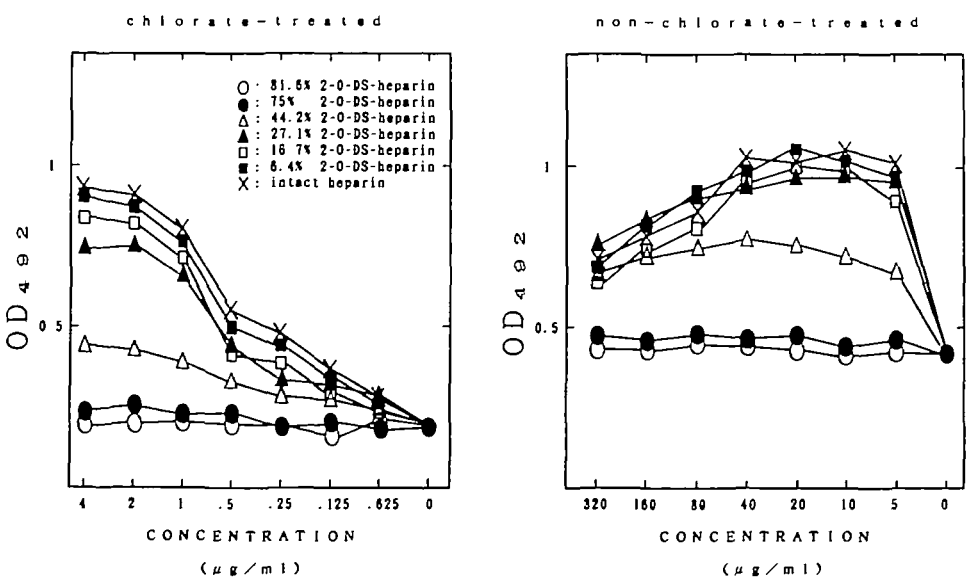

Fig. 2. **Ability of 2-O-DS-heparins to potentiate FGF-1 activity on chlorate-treated and non-chlorate-treated A31 cells.** The chlorate-treated cells were grown for 3 days in sulfate-free DMEM containing 5 ng/ml hrFGF-1, ITS+, 100 units/ml penicillin G, 20 mM sodium chlorate, and various 2-O-DS-heparins at the indicated concentrations. The non-chlorate-treated cells were also grown for 3 days in DMEM containing 5 ng/ml hrFGF-1, ITS+, 100 units/ml penicillin G, and the various 2-O-DS-heparins. Cell proliferation was assessed as described under "MATERIALS AND METHODS." The results represent the means of triplicate determinations.

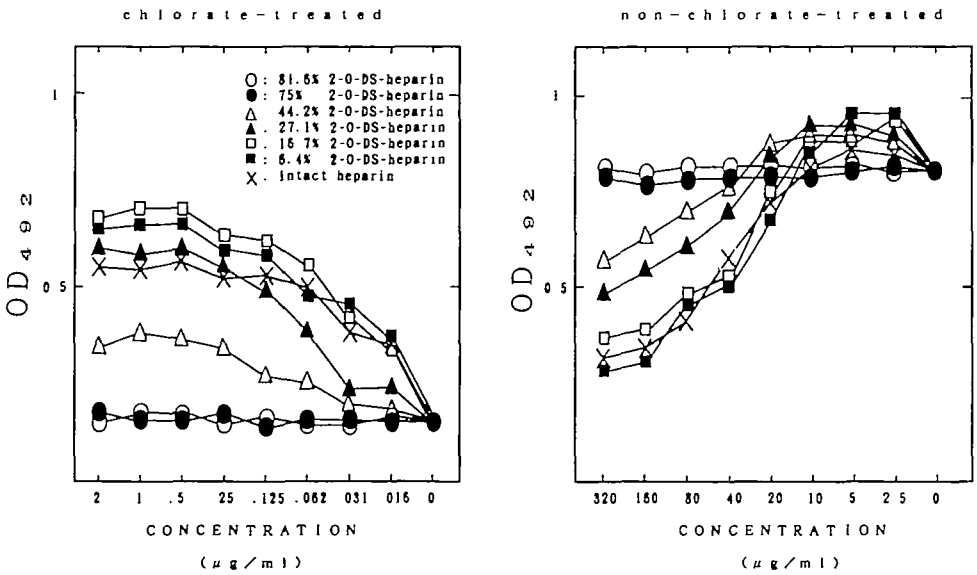

Fig. 3. **Ability of 2-*O*-DS-heparins to modulate FGF-2 activity on chlorate-treated and non-chlorate-treated A31 cells.** Ability of 2-*O*-DS-heparins to modulate FGF-2 activity was determined as described in Fig. 3, except for use of 2 ng/ml hrFGF-2 instead of 5 ng/ml hrFGF-1.

TABLE II. **Abilities of 2-*O*-desulfated heparins and 6-*O*-desulfated heparins to promote FGF-1 and FGF-2 activities on chlorate-treated A31 cells.**

	Promotion of FGF-1-induced proliferation of chlorate-treated A31 cells	Promotion of FGF-2-induced proliferation of chlorate-treated A31 cells
Intact heparin	+	+
2-*O*-desulfated heparins		
81.6% 2-*O*-desulfated	−	−
75% 2-*O*-desulfated	−	−
44.2% 2-*O*-desulfated	+/−	+/−
27.1% 2-*O*-desulfated	+	+
16.7% 2-*O*-desulfated	+	+
6.4% 2-*O*-desulfated	+	+
6-*O*-desulfated heparins		
88.8% 6-*O*-desulfated	−	−
66.8% 6-*O*-desulfated	−	+
62.2% 6-*O*-desulfated	−	+
44.8% 6-*O*-desulfated	+/−	+
24.4% 6-*O*-desulfated	+	+

+, active; −, inactive; +/−, partially active.

2A and 3A), whereas the 75 and 81.6% 2-*O*-DS-heparins had no activity. The 44.2% 2-*O*-DS-heparin showed intermediate activity in restoring both the FGF-1 and FGF-2 activities.

Table II presents a comparison of the abilities of the various 2-*O*-DS-heparins and 6-*O*-DS-heparins that were described in our previous paper (*11*), to restore the mitogenic activities of FGF-1 and FGF-2 on chlorate-treated A31 cells.

Effect of 2-O-DS-Heparins on the Proliferation of Non-Chlorate-Treated A31 Cells—We previously reported that FGF-2-induced A31 cell growth was inhibited by high concentrations of heparin, but similar concentrations of heparin potentiated FGF-1-induced A31 cell growth (*11*). In this work, various 2-*O*-DS-heparins were examined for the ability to modulate the mitogenic activities of FGF-1 and FGF-2 on non-chlorate-treated A31 cells. Native heparin and 2-*O*-DS-heparins (6.4, 16.7, and 27.1% 2-*O*-DS-heparins) were effective in potentiating the mitogenic activity over a wide range of concentrations (50–80 μg/ml), whereas 75 and 81.6% 2-*O*-DS-heparins had no activity (Fig. 2B). The 44.2% 2-*O*-DS-heparin exhibited intermediate activity. Thus, heparin-like structure including a relatively high content of 2-*O*-sulfate groups is required for maximal mitogenic activity of FGF-1.

In contrast, maximal growth stimulation by FGF-2 was achieved with FGF-2 alone, suggesting that endogenous HS is quantitatively and qualitatively sufficient enough to potentiate the mitogenic activity of FGF-2. Intact heparin inhibited FGF-2-induced A31 cell growth at high concentrations (>20 μg/ml), while 2-*O*-DS-heparins had weaker inhibitory effect on FGF-2-induced A31 cell growth (Fig. 3B). More than 75% 2-*O*-desulfation resulted in complete loss of the inhibitory effect.

DISCUSSION

We previously utilized 6-*O*-DS-heparins, which were selectively 6-*O*-desulfated from GlcNS(6-*O*-S) residues to various extents, to examine the importance of 6-*O*-sulfate groups in promoting mitogenic activities mediated by FGF-1 and FGF-2. Our previous study showed that a high content of 6-*O*-sulfate groups in GlcNS residues was required for activation of FGF-1, but not FGF-2. However, intensive 6-*O*-desulfation (>88.8% 6-*O*-desulfation) resulted in a loss of the ability to activate FGF-2 (*11*). Thus, FGF-1 differs from FGF-2 in requiring a high content of 6-*O*-sulfate groups for specific interaction with polysaccharide. In the present study, we have prepared various 2-*O*-DS-heparins which were selectively 2-*O*-desulfated from IdoA(2-*O*-S) residues to various extents, and we used a similar approach to that of the previous study (*11*) to investigate the importance of 2-*O*-sulfate groups in uronate residues of heparin in promoting the mitogenic activities mediated by FGF-1 and FGF-2. The major conclusion in this study is that a high content of 2-*O*-sulfate groups in uronate residues of heparin is required to promote the mitogenic activities of both FGF-1 and FGF-2, and for strong binding to FGF-2.

In the case of chlorate-treated A31 cells, which do not produce endogenous HSPG (*11*), intact heparin restored

the mitogenic activities of both FGF-1 and FGF-2. Partial 2-O-desulfation from heparin resulted in a decrease of the ability to restore the mitogenic activities of both FGF-1 and FGF-2, and complete loss of the ability was caused by 75% or higher 2-O-desulfation. On the other hand, in the case of non-chlorate-treated A31 cells which produce endogenous HSPG, the mitogenic activity of FGF-2 was inhibited by high concentrations of heparin (>20 μg/ml). Partial 2-O-desulfation from heparin also resulted in a decrease of the ability to inhibit the mitogenic activity of FGF-2 on non-chlorate-treated A31 cells (Fig. 3) and to inhibit COS-1 cell binding to FGF-2-coated wells (Fig. 1). These results suggest that endogenous HSPGs are sufficient for activation of FGF-2, and that they may have stronger ability to activate FGF-2 than native heparin and 2-O-DS-heparins. In contrast with the case of FGF-2, intact heparin was effective in potentiating the mitogenic activity of FGF-1 on non-chlorate-treated A31 cells over a wide range of concentrations (Fig. 2), suggesting that heparin-like structures are required for activation of FGF-1. This effect was also decreased by partial 2-O-desulfation. Thus, a high content of 2-O-sulfate groups in heparin is essential to modify the biological activities of FGF-1 and FGF-2.

The putative endogenous ligand of FGFs, HS, although generally much less sulfated than heparin, contains many distinct types of highly sulfated domains, generated by different patterns of sulfation and epimerization (*17, 18*). HS interacts with numerous molecules, including growth factors, selectins, enzymes, and extracellular matrix proteins, *etc.*, through the highly sulfated domains (*19, 20*). Our present study demonstrates the importance of 2-O-sulfate groups in uronate residues for specific interaction with both FGF-1 and FGF-2 and for their activation. In contrast, a high content of 6-O-sulfate groups in GlcNS residues is required for activation of FGF-1, but not FGF-2 (*11*). The high-affinity binding of heparin/HS to antithrombin III is due to a specific sequence which includes a unique 3-O-sulfate group (*21*). The binding of heparin/HS to hepatocyte growth factor seems to be most closely associated with the content of 6-O-sulfate groups (*22*). Furthermore, highly sulfated domains enriched in both 2-O- and 6-O-sulfate groups are required for specific interaction with lipoprotein lipase (*23*). Thus, the presence of various O-sulfate groups in addition to N-sulfate groups in heparin/HS can provide specific affinity to each heparin-binding protein. The identification of specific protein-binding domains of heparin/HS is important for our understanding of the biological functions. The methodology for specific 2-O-desulfation to various extents reported in this paper, combined with the specific 6-O-desulfations reported in the previous paper, should prove useful for the identification and characterization of the functional domains of heparin/HS in their various biological functions.

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