The Interaction of the Transforming Growth Factor-βs with Heparin/Heparan Sulfate Is Isoform-specific*

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We have undertaken a comparative study of the interaction of the three mammalian transforming growth factor-βs (TGF-β) with heparin and heparan sulfate. TGF-β1 and -β2, but not -β3, bind to heparin and the highly sulfated liver heparan sulfate. These polysaccharides potentiate the biological activity of TGF-β1 (but not the other isoforms), whereas a low sulfated mucosal heparan sulfate fails to do so. Potentiation is due to antagonism of the binding and inactivation of TGF-β1 by α2-macroglobulin, rather than by modulation of growth factor-receptor interactions. TGF-β2-α2-macroglobulin complexes are more refractory to heparin/heparan sulfate, and those involving TGF-β3 cannot be affected. Comparison of the amino acid sequences of the TGF-β isoforms strongly implicates the basic amino acid residue at position 26 of each monomer as being a vital binding determinant. A model is proposed in which polysaccharide binding occurs at two distinct sites on the TGF-β dimer: Interaction with heparin and liver heparan sulfate may be most effective because of the ability of the dimer to co-operatively engage two specific sulfated binding sequences, separated by a distance of approximately seven disaccharides, within the same chain.

A large number of growth factors and cytokines, belonging to structurally and biologically diverse protein families, possess affinity for heparin in vitro. Such affinity has often proved to be an experimental indicator of a physiological interaction with the heparan sulfate (HS)† chains of heparan sulfate proteoglycans (HSPGs), a widespread and abundant family of complex glycoconjugates expressed on the surface of all adherent cells (1), and also present within basement membranes and stromal matrices (2). Binding to HSPGs in vivo may have an important role in retaining active growth factors/cytokines within a local sphere of action by protecting them from both diffusional and degradational loss. Importantly, however, in the case of an increasing number of such growth factors, e.g. various members of the fibroblast growth factor family, vascular endothelial growth factor, heparin-binding epidermal growth factor, and hepatocyte growth factor, it has been demonstrated that HSPGs have a specific co-receptor role in directly modulating growth factor activation of the respective cell surface signaling receptors (3, 4). The transforming growth factor-βs (TGF-βs) are important regulators of the growth, differentiation, and adhesion of a wide variety of cells (5). They are believed to have an important role in natural repair processes, but overexpression or dysregulation can lead to the development of various fibrotic disorders. TGF-β1 has been demonstrated to possess strong heparin binding activity in vitro (6). Such interaction protects TGF-β1 from proteolytic degradation in vitro (7), and also prevents the formation of inactive complexes with α2-macroglobulin (α2M) (8). The physiological significance of an interaction restricted to heparin, a GAG released only upon the degradation of activated mast cells, is unclear. Although TGF-β does bind to a number of proteoglycan species in vivo, namely the cell surface HS-containing proteoglycan, betaglycan (the type III TGF-β receptor) (9), as well as various members of the family of small secreted chondroitin/dermatan sulfate (CS/DS) proteoglycans (i.e. decorin, biglycan, and fibromodulin) (10, 11), these associations are mediated principally, if not solely, by protein-protein rather than protein-GAG interactions.

In addition to TGF-β1, there are two other mammalian isoforms, TGF-β2 and -β3, and also two distinct, but poorly characterized, non-mammalian isoforms, TGF-β4 and -β5 (identified in chick and Xenopus, respectively). TGF-β1–3 possess very high levels of amino acid sequence homology within the mature bioactive molecule (>70% amino acid identity between isoform pairs with the majority of changes being conservative), including conservation, in both number and position, of the eight cysteine residues that contribute to the compact folding of the monomer, as well as the single cysteine residue involved in disulfide-bonded dimerization. Interestingly, however, there is little if any interspecies variation within each individual isoform. This has led to the proposition that the individual isoforms have closely related tertiary structures, which have diverged sufficiently to behave as functionally distinct species. Indeed, there is substantial evidence pointing to significant differences in biological behavior between the mammalian isoforms, both in vitro and in vivo. There are differential patterns of isoform expression during fetal development (12, 13) and in the adult (14, 15), and indeed the existence of distinctive regulatory elements in the promoter regions of the respective genes (16) is suggestive of a capacity for independent isoform regulation. The isoforms also display marked specificities or differential potencies in various in vitro biological systems. For example, TGF-β3 is more potent than the -β1 or -β2 isoforms in inhibiting DNA synthesis in human keratinocytes in vitro (17). While TGF-β2, but not -β1, can stimulate mesoderm induction in Xenopus embryos (18), TGF-β2 specifically has little or no

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‡The abbreviations used are: HS, heparan sulfate; TGF-β, transforming growth factor-β; HSPG, heparan sulfate proteoglycan; CS, chondroitin sulfate; DS, dermatan sulfate; GAG, glycosaminoglycan; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; α2M, α2-macroglobulin; LRP, low density lipoprotein receptor-related protein.
antiproliferative effect on vascular endothelial cells in vitro (19). Also, exogenously added TGF-β3 appears to have a specific ability to reduce scar formation during wound healing in the adult animal (20). These differences in biological activity may be due, in part, to the known differences in the receptor binding properties of the isoforms (e.g., the reduced affinity of TGF-β2 for the type II receptor protein (21)), although other interactive specificities may also be important.

In the light of these known isoformic differences, together with the previously described affinity of TGF-β1 for heparin, we were interested in further elucidating the nature and extent of GAG-TGF-β interactions.

EXPERIMENTAL PROCEDURES

Materials—Human platelet TGF-β1, recombinant human TGF-β2, and mouse anti-human pan TGF-β monoclonal antibody were purchased from Genzyme (West Malling, United Kingdom (UK)). Recombinant human TGF-β3 was a generous gift from Oncogene Science (Uniondale, NY). α1-Macroglobulin was obtained from Boehringer Mannheim (Lewes, UK). Normal rat kidney fibroblasts (NRK 49F) and mouse anti-human pan TGF-β polyclonal antibody were kindly provided by Dr. H. McCaffrey (Institute for Cancer Research, Potters Bar, Herts., UK). Decorin proteoglycan was generously provided by Dr. H. F. Pearson (University of Alberta, Edmonton, Canada). De-N-sulfated, re-N-acetylated heparin, and selectively de-6-O-sulfated heparin were kindly provided by Dr. B. Mullol (National Institute for Biological Standards and Control, Potters Bar, Herts., UK). Selectively de-2-O-sulfated heparin was a gift from Dr. B. Casu (Istituto Chimica e Biochimica "G. Ronzini," Milan, Italy).

Preparation of Rat Liver HSPG and HS Chains—Cell surface HSPGs were purified from rat liver by the method of Lyon and Gallagher (22). HS chains were liberated by alkaline elimination using 50 mM NaOH, 1 mM sodium borohydride at 45 °C for 48 h. After neutralization with acetic acid the HS was precipitated by the addition of four volumes of 95% (v/v) ethanol at −20 °C overnight. HS concentrations were quantified using an Alcian Blue-binding microassay (23), relative to a standard curve obtained with bovine kidney HS.

Disaccharide Composition Analysis of Heparin and Heparan Sulfate Species—Samples were exhaustively digested with heparinases II, and III. The resulting disaccharide products were then resolved by acetic acid the HS was precipitated by the addition of four volumes of strong anion-exchange chromatography on a 5-m particle size Spherisorb SAX-HPLC column (Technicol, Stockport, UK), essentially as described by Lyon et al. (24). Disaccharides were detected by UV absorbance at 252 nm.

Assay of TGF-β Activity—Batches of TGF-β were routinely assayed for their biological activity through their ability to inhibit the incorporation of [3H]thymidine into mink lung epithelial cells. Briefly, CCL-64 cells were plated out at 104 cells/well of a 24-well plate (Costar, High Wycombe, UK) in Dulbecco’s modified Eagle’s medium/F12 medium (Life Technologies, Inc., Paisley, UK) containing 2% fetal calf serum. After 4 h, cells were treated with a range of TGF-β concentrations and then pulsed with 0.5 µCi/ml [3H]thymidine (NEN Life Science Products, Stevenage, UK) for 2 h. The incorporation of [3H]thymidine into trichloroacetic acid-insoluble cellular material was then determined.

TGF-β Binding to Heparin by Affinity Chromatography—Samples of TGF-β1, TGF-β2, and TGF-β3 (50 ng each) were individually applied to 0.3 ml of heparin-agarose and Sepharose CL4B. The resulting disaccharide products were then resolved by strong anion-exchange chromatography on a 5-μm particle size Spherisorb SAX-HPLC column (Technicol, Stockport, UK), essentially as described by Lyon et al. (24). Disaccharides were detected by UV absorbance at 252 nm.
Heparin and Liver HS—

All three TGF-β isoforms stimulate to a similar extent the anchorage-independent proliferation of colonies of NRK 49F fibroblasts in soft agar suspension, primarily through the stimulation of the endogenous synthesis and secretion of extracellular matrix macromolecules, especially fibronectin. Using suboptimal concentrations of the TGF-β isoforms (0.25 ng/ml, equivalent to approximately 10 mU), it was observed that the addition of heparin markedly potentiated the activity of TGF-β1, with the optimal heparin concentration of 1 μg/ml eliciting a 6.6-fold potentiation (Fig. 3A). This effect was specific in that there was no discernible effect on cellular proliferation induced by either TGF-β2 or -β3 over the same range of heparin concentrations (at a higher concentration of 10 μg/ml, a barely significant potentiation of TGF-β2 was seen) (Fig. 3A). Heparin alone, in the absence of TGF-β, has no independent stimulatory effect on colony growth (Fig. 3A).

In contrast to the effects of heparin, a porcine mucosal heparan sulfate preparation failed to potentiate any of the TGF-β isoforms, including TGF-β1 (Fig. 3B). Interestingly, however, HS chains derived from the liver HSPG did markedly potentiate the activity of TGF-β1, at the highest concentrations of heparin (Fig. 3A) and liver HS (Fig. 3C) may be due to a competing direct antagonistic effect on the cells of these GAGs, apparent only at higher concentrations, or an inhibitory sequestration of the TGF-β at very high molar ratios of GAG to TGF-β. Protection assays also indicate that both heparin and liver HS protect TGF-β1 from tryptic degradation, whereas porcine mucosal HS does not (data not shown), implying that their ability to potentiate TGF-β1 activity is positively correlated with direct high affinity binding.
Modulation of the activity of TGF-β isoforms by heparin and HS species. The ability of individual TGF-β isoforms (in 5% (v/v) serum) to support the growth of colonies of NRK 49F cells in soft agar suspension was assayed in the absence and presence of varying concentrations of bovine lung heparin (A), porcine mucosal HS (B), and rat liver HS (C), as described under “Experimental Procedures.” In the light of the results from A and B, only TGF-β1 was used in C. The dashed line in C represents the comparative activity elicited by 1 μg/ml bovine lung heparin. The variation in control TGF-β1 potencies between different experiments primarily reflects batch to batch variations in individual reagents over the time period during which the various experiments were performed. The data represent mean values ± S.E. (n = 5, except for C, where n = 3).

Structural Specificity of the Potentiating Effect of Sulfated GAGs on TGF-β1 Activity—The foregoing data indicate that of the N-sulfated GAGs tested the most potent were found to be heparin and rat liver HS, while the lower sulfated porcine mucosal HS had little effect (see Table I for structural comparisons). Compared with heparin, other classes of sulfated GAGs were relatively ineffective. CS had no discernible effect, while a mucosal DS, although having a potentiating effect at the highest concentration tested (1 μg/ml), was approximately 10-fold less potent (data not shown). However, as this DS preparation was found to be contaminated to at least 3–4% with highly sulfated HS and/or heparin, the specificity of this level of activity is uncertain. Indeed, McCaffrey et al. (8) observed no apparent interaction of TGF-β1 with DS by agarose electrophoresis. However, a weak inherent DS binding activity of heparin/HS-binding proteins is not unusual, presumably because DS also contains both idurionate and variable sulfation which may partially satisfy the binding requirements.

To try and elucidate the relative importance of the different sulfate groups in heparin for the binding and subsequent potentiation of TGF-β1 activity, a series of selectively desulfated heparins (see Table I for structural comparisons) were tested for their effect in the soft agar colony growth assay. The results, although complex, suggested that the specific loss of N-sulfates had a greater effect than the selective loss of either 2-O- or 6-O-sulfates. At a heparin concentration of 0.1 μg/ml (giving a 4-fold potentiation of TGF-β1 activity), de-N-sulfation, with replacement by N-acetyl groups, resulted in a 95 ± 3% reduction in activity. Removal of 2-O- or 6-O-sulfates resulted in lesser, although similar reductions of 48.5 ± 10.8% and 51.5 ± 4.5% respectively (although the 2-O-sulfates had been more selectively removed than the 6-O-sulfates; see Table I). In all cases a 10-fold increase in concentration of the modified heparins increased the level of potentiation. De-2-O- and de-6-O-sulfated heparins at 1 μg/ml gave 79.9 ± 1.3% and 88.7 ± 3%, respectively, of the potentiation observed with 0.1 μg/ml heparin, although the de-N-sulfated derivative still only achieved 56.5 ± 9% (all % values being the mean of triplicate determinations ± S.E.). This complex behavior suggests that the TGF-β1 binding site in heparin/liver HS probably involves a combination of specific structural determinants of which N-sulfation is a major contributor.

The TGF-β1 Potentiating Activity of Heparin Only Occurs in the Presence of α2M—The NRK 49F colony growth assay is normally performed in the presence of 5% (v/v) fetal calf serum (25, 26). The requirement for serum can, however, be replaced by a defined protein mixture (comprising insulin, transferrin, high density lipoprotein, and serum albumin) (27) in which the cells remain responsive to stimulation by all three TGF-β isoforms (data not shown). Interestingly, under these serum-free conditions, the potentiating effect of exogenous heparin upon TGF-β1 is lost, and the behavior of TGF-β1 becomes comparable to that of TGF-β2 (Fig. 4A). This suggests that the potentiating effect is not due to a direct heparin-mediated enhancement of TGF-β1 binding to its receptor, but to a modulation by heparin of a TGF-β1 neutralizing activity present in serum. The most likely candidate molecule is α2M, which is known to be the major TGF-β-binding protein in serum (30) and forms non-covalent complexes in which the TGF-β is rendered latent (30, 31). The addition of 750 μg/ml α2M to the serum-free cell system inhibited the activities of all the TGF-β isoforms by between 43% (TGF-β3) and 68% (TGF-β1 and -β2) (Fig. 4B). Heparin alone at 1 μg/ml had no effect (Fig. 4B). However, the combination of heparin and α2M elicited differential responses. Whereas the activity of TGF-β3 remained completely suppressed, that of TGF-β1 was markedly restored by heparin to 84% of control levels, although the activity of TGF-β2 was restored to a significantly lesser extent (57% of the control; Fig. 4B).

DISCUSSION

We have demonstrated that marked differences exist in the interaction of the mammalian TGF-β isoforms with the heparin/HS family. Both TGF-β1 and -β2 possess affinity for heparin and highly sulfated HS, whereas TGF-β3 does not. Heparin and liver HS potentiate TGF-β1 in supporting the “anchorage-independent” growth of NRK fibroblasts. In keeping with its failure to bind heparin, the activity of TGF-β3 was unaffected by it, although, paradoxically, so also was the activity of the heparin-binding TGF-β2. The relative lack of effect of CS and DS on TGF-β1, coupled with the marked reduction in activity of heparin after removal of its N-sulfate groups, indicates a strong requirement for N-sulfated GAGs. However, even among the latter there is a marked selectivity, as liver HS (24) was as potent as heparin, while a mucosal HS preparation was essentially inactive. Liver HS is highly sulfated (Table I), and occupies one extreme end of the HS spectrum (32). Additionally, its marked asymmetric chain structure (24), with the great majority of both N- and O-sulfates concentrated within the distal two-thirds, generates a localized sulfate density approaching that of heparin. The similarity in properties of heparin and liver HS may thus reflect a TGF-β binding requirement for a specific highly sulfated sequence, which is rare or absent in most less sulfated HS species. Selective depletion of either the 2-O- or 6-O-sulfate groups in heparin brings about a more modest reduction in activity, compared with N-sulfate removal.
Heparin Binding to TGF-β Isoforms

Comparative sulfate contents of the various heparin, heparan sulphate and modified heparin species

| Sulfates per 100 disaccharides | Rat liver HS | Porcine mucosal HS | Bovine lung heparin | Modified bovine lung heparin
|-------------------------------|-------------|--------------------|-------------------|---------------------------|
|                               |             |                    |                   | De-N-sulfated, re-N-acetylated | De-6-O-sulfated | De-2-O-sulfated |
| N-Sulfates                    | 60.7        | 43.5               | 97.7              | 2.4                       | 98.2           | 91.4            |
| 6-O-Sulfates                  | 34.0        | 19.4               | 92.4              | 85.3                      | 4.2            | 80.2            |
| 2-O-Sulfates                  | 38.4        | 18.2               | 89.3              | 90.5                      | 54.7           | 2.2             |
| Total sulfates                | 133.1       | 81.1               | 279.4             | 178.2                     | 157.1          | 173.8           |

* Data derived from Lyon et al. (24).

b The various modified heparins were obtained from different sources and therefore probably derive from different batches of bovine lung heparin, which may differ slightly in composition.

* Additional minor contributions from 3-O-sulfate groups are not included.

Fig. 4. The specific potentiation of TGF-β activity by heparin is only apparent in the presence of α2M. The effect of heparin on the growth of colonies of NKR 49F cells in soft agar suspension promoted by the individual TGF-β isoforms was compared in the presence or absence of 5% (v/v) serum (A) and under serum-free conditions (B), with or without added α2M, as described under "Experimental Procedures." In A only TGF-β1 and -β2 are shown under serum-free conditions (TGF-β3 being similar), compared with the specific effect seen only with TGF-β1 in the presence of serum. In B the data are presented as a percent of the activity elicited by the respective TGF-β isoform alone. The data represent mean values ± S.E. (n = 3).

Interestingly, all the selectively desulfated heparins are less effective than liver HS, although they retain a higher overall sulfate density (Table I). This also suggests a requirement for a complex binding specificity, rather than just the exceeding of a particular sulfate density.

The potentiation of TGF-β1 by heparin/liver HS, but only in the presence of serum or purified α2M, confirms that the GAGs are acting as antagonists of serum α2M, rather than as direct modulators of receptor binding/activation. The majority of endogenous serum TGF-β is known to be inactive and complexed to α2M in vivo (30, 31). α2M is a proteinase inhibitor, which irreversibly traps circulating proteinases and in the process undergoes a major conformational change. The conformationally activated form of α2M is specifically cleared from the circulation by the low density lipoprotein receptor-related protein (LRP) in the liver. Both forms of α2M can also carry a wide range of cytokines. TGF-β1 possesses a higher affinity for pro-

teo-lytically activated α2M than for the native form (33, 34). In contrast, TGF-β2 possesses a higher overall affinity for α2M, although it displays less selectivity between the two forms (34, 35). TGF-β3 also interacts with α2M in vitro (data not shown), although its comparative affinities are not known. Reflecting these affinity differences, α2M is more effective at counteracting the mitoinhibitory action of TGF-β2, than of TGF-β1, on cultured hepatocytes (36). Heparin’s potentiation of the antiproliferative effect of TGF-β1 on smooth muscle cells (8) may be due to blocking of the α2M-TGF-β1 interaction (note that α2M is not itself a heparin-binding protein; Ref. 37). Electrophoretic analyses have also suggested that α2M/TGF-β2 complexes in vitro are less susceptible to dissociation by heparin than those containing TGF-β1 (33). We have shown that a differential sensitivity of TGF-β1 and -β2 complexes with α2M can be demonstrated with either heparin or liver HS in an in vitro bioassay and, importantly, that TGF-β3 is unusual in that, lacking a heparin/HS-binding site, its inactivation by α2M cannot be counteracted in this way.

McCaffrey et al. (6) identified three potential heparin-binding sites in TGF-β1, corresponding to residues 23–32, 30–41, and 92–99. Residues 23–32 were considered most likely to constitute an in vivo binding site, being most able, as a free peptide, to inhibit the binding of TGF-β1 to immobilized heparin (6). In light of the extensive sequence homologies between TGF-βs, but the specific absence of a heparin/HS-binding site in TGF-β3, we thought it possible that information on the binding site may be indirectly obtained from the conservation of basic amino acids (important for interaction with polyanionic GAGs) across the isoforms (Fig. 5). Two specific basic residues, at positions 26 and 60 in both TGF-β1 and -β2 are shown under serum-free conditions (TGF-β3 being similar), compared with the specific effect seen only with TGF-β1 in the presence of serum. In B the data are presented as a percent of the activity elicited by the respective TGF-β isoform alone. The data represent mean values ± S.E. (n = 3).

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be too short, and/or too far apart (48), to optimally engage both sites.

Selective interaction of the TGF-β isoforms with heparin/HS may have significant biological implications. The specific structure of the HS expressed by a particular cell type or tissue may affect the potency and isoformic specificity of TGF-β activity. In addition, the relatively high levels of TGF-β, mostly TGF-β1, released from activated platelets (49) in response to tissue damage must have their activity restricted to the injury site, and be inhibited from having an undesired systemic effect through entry, in an active state, into the circulation. This is primarily achieved by efficient inactivation by the high levels of serum α2M, although locally this inactivation must be temporarily forestalled. Platelet-derived thrombospondin appears to possess a protective activity (50), and in certain tissues rich in highly sulfated HS, e.g. liver (24) and possibly lung (51), this function may also be served by HSPGs. Interestingly, TGF-β is the most potent chemoattractant factor so far described for mast cells (52), which often accumulate at wound sites. In addition to releasing heparin upon degranulation, mast cells have recently been shown to secrete TGF-β1 in vitro (53, 54). Coordinate release of heparin and TGF-β1 may act to maintain locally active TGF-β1. By comparison, the release of TGF-β2 or -β3 under similar circumstances, with little or no respective protection being afforded by heparin, would be relatively ineffective. All the TGF-βs can also be neutralized in the extracellular matrix by complexation with the core proteins of decorin, biglycan and fibromodulin (11). Preliminary evidence suggests, however, that the interaction of TGF-β1 with decorin is not inhibited by heparin (data not shown).

Overall these issues of potential bioavailability may be particularly important in the evaluation of the different TGF-β isoforms for potential therapeutic use in wound healing and chemoprotection (55–57). For example, in certain circumstances it may be more effective to employ TGF-β1 in combination with an appropriate protective heparinoid species.

TGF-β bound to serum α2M was thought to be cleared from the circulation by liver LRP, the specific receptor for activated α2M. When TGF-β1 is injected intravenously the majority is rapidly sequestered and cleared by the liver, and to a lesser extent the lungs (58, 59). Clearance of TGF-β1 precomplexed with methylamine-activated α2M (which mimics proteinase-activated α2M) is, as expected, even more liver-specific (59). However, the serum concentration of native α2M far exceeds that of the activated form, and consequently the former is considered to be the primary carrier of TGF-β1 in vivo (60, 61). This suggests that a considerable proportion of the rapid clearance may not be LRP-mediated. Indeed, it has been demon-

| Isoform | 9 | 18 | 25 | 26 | 31 | 34 | 37 | 58 | 60 | 94 | 97 | 107 | 110 |
|---------|---|----|----|----|----|----|----|----|----|----|----|-----|-----|
| β1      | S | R  | R  | K  | K  | H  | Y  | K  | R  | K  | R  | K   | K   |
| β2      | R | R  | K  | R  | K  | H  | R  | R  | K  | K  | K   | K   | K   |
| β3      | R | R  | R  | Q  | K  | H  | T  | R  | K  | K  | K   | K   | K   |
| β4      | G | R  | R  | K  | K  | H  | Y  | K  | R  | R  | R   | K   | K   |
| β5      | G | R  | R  | K  | K  | K  | Y  | K  | R  | K  | R   | R   | N   |

FIG. 5. Comparison of the conservation of basic amino acids across the five known TGF-β isoforms. Only those amino acid positions are shown which are occupied by basic residues (enclosed by a box) in at least two of the TGF-β1–3. The numbers refer to positions within the mature 112-amino acid monomer sequences of all the isoforms, with the exception of TGF-β4. The latter is 114 amino acids in length with an additional dipeptide sequence inserted between positions 11 and 12 (ignored for alignment). The sequences of human TGF-β1–3, chick TGF-β4, and Xenopus TGF-β5 were obtained from published sources (38–42).

FIG. 6. Schematic model of the interaction between heparin/HS and the TGF-β1 or -β2 dimer. In the TGF-β dimer the disulfide-bonded monomers partially overlap in an anti-parallel face-to-face mode, with the main association occurring between an α-helix at one end of a monomer and the concave surface of the central β-sheet of its partner. In TGF-β1 and -β2, but not -β3, basic amino acid residues occur at positions 26 and 60 in each monomer. Residue 26 contributes to a strongly positively charged surface (solid shaded area), duplicated toward the opposite poles of the TGF-β dimer, while residue 60 is partially buried in the dimer interface region on the opposite side of the monomer. The two sites containing residue 26 are therefore better placed to interact with heparin/HS, and could interact independently with separate ligands. However, as the side chains of both sites project from the same face of the dimer, the model illustrates a putative co-operative interaction occurring with a single heparin/HS chain running parallel to the long axis of the dimer. Each monomer specifically interacts with a highly sulfated sequence present within an N-sulfated domain (cross-hatched blocks). In liver HS the dimer could bind wholly within a single large sulfated domain, or alternatively could bridge two adjacent, closely spaced domains (as shown). Such interaction may elicit a conformational change unfavorable to α2M binding, and/or sterically hinder α2M docking.

duplicated at opposite poles, but on the same face, approximately 60 Å apart. These may constitute two independent heparin/HS-binding sites (Fig. 6) in which residue 26 is critical. Nevertheless, it is also possible that the highest affinity interaction that most effectively inhibits α2M complexation could be formed by two co-operative interactions within the same GAG chain. As the length of a heparin disaccharide in solution is 8.6 Å (47), the minimum sequence required to span the two sites on the TGF-β dimer would comprise approximately seven disaccharides. This suggests an alternative explanation for the apparent functional selectivity of heparin and liver HS. In addition to possessing specific monomer recognition sequences, they may also effectively present two such sequences at an appropriate spacing to engage both binding sites in the dimer. Heparin, being highly sulfated in a relatively uniform manner throughout, could accommodate dimer binding at multiple positions. Liver HS possesses relatively large sulfated domains (7–9 disaccharides long) separated by only short N-acetylated sequences (24), so interaction may be possible within a single large sulfated domain or involve two adjacent ones. In contrast, the sulfated domains in a low sulfated HS species are likely to
strated that clearance is not affected by preblocking of LRP with activated αM (61). Conceivably, HS expressed on the sinusoidal surface of hepatocytes (and possibly a highly sulfated counterpart in the lungs) may itself function as a major mediator of TGF-β clearance by displacing it from native αM. TGF-β delivered to the liver by this endocrine mechanism may be important in liver regulation and repair, being mito-inhibitory for hepatocytes.

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