Cell surface heparan sulfate (HS) proteoglycans are required in development and postnatal repair. Important classes of ligands for HS include growth factors and extracellular matrix macromolecules. For example, the focal adhesion component syndecan-4 interacts with the III12–14 region of fibronectin (HepII domain) through its HS chains. The fine structure of HS is critical to growth factor responses, and whether this extends to matrix ligands is unknown but is suggested from in vitro experiments. Cell attachment to HepII showed that heparin oligosaccharides of ≳14 sugar residues were required for optimal inhibition. The presence of N-sulfated glucosamine in the HS was essential, whereas 2-O-sulfation of uronic acid or 6-O-sulfation of glucosamine had marginal effects. In the more complex response of focal adhesion formation through syndecan-4, N-sulfates were again required and also glucosamine 6-O-sulfate. The significance of polymer N-sulfation and sulfated domains in HS was confirmed by studies with mutant Chinese hamster ovary cells where heparan sulfation was compromised. Finally, focal adhesion formation was absent in fibroblasts synthesizing short HS chains resulting from a gene trap mutation in one of the two major glucosaminoglycan polymerases (EXT1).

Several separate, specific properties of cell surface HS are therefore required in cell adhesion responses to the fibronectin HepII domain.

Interactions involving both the integrin- and heparin-binding domains of fibronectin contribute to a complete adhesion response in normal fibroblasts (1) and in melanoma (2) and neuroblastoma cells (3). The primary receptor for adhesion to fibronectin commonly involves the RGD motif of repeat III10 through integrins such as α5β1 (4), but this integrin-ligand interaction is only sufficient for attachment and spreading. Additional signaling through the cell surface proteoglycan syndecan-4 is required for focal adhesion formation and rearrangement of the actin cytoskeleton into bundled stress fibers (5, 6).

Heparan sulfate binding occurs primarily via the HepII domain (containing the FN type III repeats 12–14) in the C-terminal region of fibronectin. Solution interaction studies have shown that this domain may contain two distinct HS/heparin-binding sites (13) of which the III13 module is the main binding site for the initiation of focal adhesion formation (6) with the III14 module possibly providing a secondary binding site (14–16). Molecular modeling studies with mutational analysis of III13 suggested the existence of a cluster of six positively charged residues that form a “cationic cradle” binding site for heparin (17), and this has been confirmed in the crystal structure of the FN type III repeats 12–14 (18). Since then, NMR studies have shown that the dominant binding site of heparin in HepII is within the first 29 residues of the III13 module (19). Heparan sulfate, unlike its structural analogue heparin, displays a high degree of structural heterogeneity. The HS polysaccharide has alternating hexuronic acid (D-glucuronic acid or L-iduronic acid) and D-N-acetylgalcosamine residues. The two major heparan sulfate polymerases (EXT1 and −2) combine together to synthesize the heparan sulfate polysaccharide (20), which is subsequently modified by sulfation and uronic acid epimerization. The first modification is mediated by N-deacetylation/N-sulfotransferases but in a block pattern and not to completion so that there are regions of N-sulfate groups (NSO,) interspersed with those rich in N-acetyl groups (NAc). Disaccharides

**Cellular Adhesion Responses to the Heparin-binding (HepII) Domain of Fibronectin Require Heparan Sulfate with Specific Properties**

Received for publication, May 23, 2006, and in revised form, November 24, 2006. Published, JBC Papers in Press, November 27, 2006, DOI 10.1074/jbc.M604938200

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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* The work was supported by Program Grant 065940 from the Wellcome Trust (to J. R. C.) and by a Cancer Research UK program grant (to J. T. G.).

§ The abbreviations used are: HSPG, heparan sulfate proteoglycan; HS, heparan sulfate; BSA, bovine serum albumin; MEF, mouse embryo fibroblast; DNS, de-N-sulfated; DNRAc, de-N-sulfated, re-acetylated; WT, wild type; FAC5, fluorescence-activated cell sorter; CHO, Chinese hamster ovary; REF, rat embryo fibroblast; FN, fibronectin; WT, wild type; PBS, phosphate-buffered saline; NAc, N-acetyl 54Ko, syndecan-4 knock-out; CompDes, completely desulfated; DE6S, de-6-O-sulfated; DE2S, de-2-O-sulfated.

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ridges can be further O-sulfated at the C-6 and/or C-3 positions of the d-glucosamine and at the C-2 position of the iduronic acid (21) but predominantly in N-sulfated regions. The HS domain structure then results in sulfate-rich regions (S-domains) interspersed with relatively unsulfated N-acetyl-rich sequences (Nac-domains); transition zones of intermediate sulfation lie at the interface of the S- and Nac-domains (22). The length of the S-domains in HS and its pattern of sulfation are critical for optimum binding to protein ligands and their subsequent biological functions (23, 24). For example, an octasaccharide library subjected to affinity chromatography for interactions with various fibroblast growth factors suggested that FGF-10 required 6-O-sulfate but not 2-O-sulfate groups (25). Another study reported with x-ray scattering using region-selectively desulfated heparin samples that high affinity FGF-2 interactions required 2-O sulfation (26).

A previous in vitro study demonstrated that the interaction between fibroblast-derived HS and the HepII region of fibronectin occurred within the S-domains and that the smallest S-domain length with measurable affinity at physiological ionic strength was an octasaccharide (27). Since then, a further cell-free study of the structural features of HS in this interaction indicated that there was a prominent role for N-sulfate groups, but the presence of 6-O-sulfate groups enhanced affinity as did increasing the oligosaccharide length to a tetradecasaccharide (28). However, cell adhesion experiments examining the specificity of sulfation in interactions with the HepII domain have been lacking. The hypothesis here was that cell attachment and focal adhesion formation in response to the fibronectin HepII domain depend on specificity of sulfation and that the two cellular events may not be equivalent in their requirements.

It is now shown that although N-sulfation of glucosamine residues is essential for both responses, focal adhesion formation has an additional glucosamine 6-O sulfation requirement. Furthermore, there are minimal oligosaccharide length requirements for cell adhesion, and focal adhesion formation also depends on a substantial chain length, indicative of complex specific and multiple interactions with the HepII domain of fibronectin.

EXPERIMENTAL PROCEDURES

Materials and Antibodies—For cell adhesion assays, heparin (Sigma), heparan sulfate (Seikagaku, Tokyo), chondroitin sulfate C (Seikagaku), and variously desulfated heparin and heparan sulfate oligosaccharides (see below for production methods) were used as competitors. Alexa Fluor 586-conjugated phalloidin (Invitrogen) and anti-vinculin (clone hVIN1; Sigma) antibody, together with Alexa Fluor 488-conjugated goat antimouse IgG were used for focal adhesion assembly assays. Gelatin-agarose (Sigma), Ultrigel-4% agarose (Sigma), DEAE-Sephasel (Amersham Biosciences), and heparin-agarose (Sigma) were used for the purification of fibronectin and derivation of the 110-kDa cell-binding fragment. For purification of the recombinant HepII fragment of fibronectin, TALON beads (BD Biosciences) were used. Total RNA was extracted using RNAzol™ B (AMS Biotechnology, Abingdon, UK).

The polyclonal chicken anti-syndecan-4 antibody (Harlan Sera-Lab, UK) was raised against 20 amino acids of the N-terminal human syndecan-4 ectodomain sequence, and antibodies were affinity-purified from plasma using standard procedures. This antibody together with Alexa Fluor 488-conjugated goat anti-chicken IgY (Invitrogen) was used for FACS analysis.

Cell Culture—Rat embryo fibroblasts (29) were cultured in α-minimum Eagle’s medium (Cambrex, Wokingham, UK) containing 5% fetal calf serum (Biowest, Ringmer, UK) and 1% glutamine (Invitrogen). Wild type and syndecan-4 knockout mouse embryo fibroblasts (30) were also cultured in α-minimum Eagle’s medium but with 10% fetal calf serum and 1% glutamine. CHO-K1, CHO-761 (31), and CHO-606 (32) cells were cultured in Ham’s F-12 medium supplemented with 10% fetal calf serum and 1% glutamine. Wild type and exon-1 gene trap (EXT1) mouse embryo fibroblasts (kindly donated by Dr. Marion Kusche-Gullberg (33)) were cultured in Dulbecco’s modified Eagle’s medium (containing high glucose and glutamine) with 10% fetal calf serum. All cell lines were tested for mycoplasma and were negative.

Preparation of Selectively Desulfated Heparin/HS Species—Chemically desulfated heparin oligosaccharides and heparan sulfate polymers were essentially prepared as described previously (34). Briefly, these oligosaccharides and polymers were prepared from low molecular weight heparin (Innhoep) or heparan sulfate produced by partial heparinase cleavage. The saccharides were then separated by high resolution gel filtration on Bio-Gel P-10 and subjected to the various chemical desulfurations. The selectively desulfated heparan sulfates (desulfated at the N-, 2-O, and 6-O positions) were prepared as described previously (28). Based on disaccharide compositions, the de-N-sulfated HS lost 93% of its N-sulfates; the de-2-O sulfated lost 84% of its 2-O-sulfates, and 55% of the 6-O-sulfates were lost from the 6-O-desulfated material. There was negligible unspecific loss of sulfates in these preparations.

Preparation of Fibronectin and 110-kDa Fragment—Fibronectin was purified by adapting the protocol of Meikka et al. (35). Briefly, fresh human plasma was treated with citrate and 0.1 M e-aminoacaproic acid to inactivate plasminogen. Tandem columns of Ultrogel and Ultrogel-gelatin agarose were equilibrated with 50 mM Tris-HCl (pH 7.5), 50 mM e-aminoacaproic acid, 20 mM sodium citrate, 2 mM EDTA (Buffer A). After spinning at 14,000 rpm for 1 h at 4 °C, plasma supernatant was loaded onto the first column and directly onto the affinity matrix. The gelatin column was washed with 1 M NaCl in Buffer A and then eluted with 3 M urea in Buffer A.

The 110-kDa fragment of fibronectin, representing repeats III1–III11, was isolated as described previously (36). Purified human fibronectin was digested with α-chymotrypsin, and fragments were passed through gelatin and heparin-agarose columns in tandem. Columns were previously equilibrated with column buffer: 10 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 2 mM EDTA, 5 mM 2-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride. Pass through fractions were collected and passed over a DEAE ion exchange column equilibrated with 50 mM phosphate buffer (pH 6.0) 50 mM NaCl, 2 mM EDTA, 5 mM 2-mercaptoethanol, and 2 mM phenylmethylsulfonyl fluoride. The 110-kDa polypeptide was eluted with increasing concentrations of NaCl.
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**Fibroblast Adhesion to HepII Domain of Fibronectin Is Mediated by Heparan Sulfate**—Previously it was shown in cross-linking experiments that the heparan sulfate chains of syndecan-4 bound to the HepII domain of fibronectin (1). To examine whether the HepII fragment could support cell attachment only through heparan sulfate in these cells, adhesion assays using HepII-coated surfaces were carried out in the presence of competing heparin, heparan sulfate, or chondroitin sulfate. Fibronectin- and BSA-coated substrates served as positive and negative controls, respectively (Fig. 1). Heparin (Fig. 1A) and heparan sulfate (Fig. 1B) reduced adhesion to the HepII domain in a dose-dependent manner with IC<sub>50</sub> levels of 0.16 and 60 μg/ml, respectively (Table 1). This suggests that heparin, with its higher overall sulfation levels, was the more efficient inhibitor. In contrast, exogenous chondroitin sulfate was unable to block fibroblast adhesion to HepII-coated substrates (Fig. 1C). Moreover, whereas the HepII domain has a binding site for α4β1 integrin (37), this was not a factor in these assays because the primary fibroblasts lack this receptor (not shown). This is consistent with the high sensitivity of cell attachment to HepII in the presence of heparin alone.

**Optimal Inhibition of Fibroblast Adhesion to HepII Requires a Minimal Heparan Sulfate 14-mer**—Previous affinity chromatography data suggested that a minimal heparin octamer was required for binding to the HepII domain but that increased size of oligosaccharide resulted in concomitantly higher affinity (28). To investigate whether these data could be extrapolated to cell adhesion, heparin oligosaccharides of increasing size were used as competitors in fibroblast adhesion to HepII. Detectable inhibition was seen with a hexamer, but this was strongly increased by longer oligosaccharides to yield complete inhibition (Fig. 2A). When 12-mers and 14-mers were more closely compared in dose-response experiments (Fig. 2B), the 14-mer was a more efficient competitor. This is also reflected in the IC<sub>50</sub> values where a 50% reduction in adhesion required 2.5 μg/ml of 12-mers but only 1 μg/ml of 14-mers (Table 1). Also because 14-mer S-domains rich in N- and 6-O-sulfates are much rarer in heparan sulfate, this would partly explain why higher concentrations of heparan sulfate chains are required in competition assays, compared with heparin, which is almost a continuous S-domain. In any case, the previous cell-free experiments correlate well with fibroblast attachment to the HepII domain and suggest that this fibronectin domain can have an extensive interaction site(s) with cell surface heparan sulfate.

**Cell Adhesion to the HepII Domain Requires Heparan N-Sulfation**—Sulfation of glucosamine and uronate residues in heparan sulfate is not random but is regulated in a complex manner (38). Binding studies with HS and heparin have shown that the pattern of sulfation in turn can determine the affinity of interactions with fibronectin and its isolated HepII domain. To determine whether sulfation position also regulates cell surface heparan sulfate–HepII interactions, we examined a range of mono–desulfated 14-mer heparin oligomers deficient in N-sulfates (DNS and a recacytlated derivative DNRAc), the 6-O-sulfates of the glucosamine residue (DE6S), or 2-O-sulfate groups of the iduronic acid residue (DE2S) (see "Experimental Proce-
decreased cell adhesion to the HepII substrate in a similar dose-dependent manner and with similar IC_{50} values (Table 1). This suggests that 2-O- and 6-O-sulfates were not critical for inhibition of cell attachment to HepII. However, the DNRAc heparin oligomer was a significantly weaker inhibitor than either the DE2S or DE6S species, and in the absence of reacetylation, the N-desulfated oligomer (DNS) containing N-unsubstituted amines was unable to compete with cell surface HS for attachment to HepII (Fig. 3A and Table 1). These inhibition studies indicate that the N-sulfates are the key functional groups for recognition of the HepII substrate by adherent cells, and they are consistent with previous affinity chromatographic data showing that N-sulfates play a predominant role in heparin interaction with the HepII domain of fibronectin (28).

To determine whether desulfated heparan sulfate polymers would give similar results to the desulfated heparin oligomers, further competition assays were conducted (Fig. 3B). In common with the de-N-sulfated heparin 14-mers, de-N-sulfated HS was a very poor competitor for cell adhesion to the HepII domain. Unsurprisingly, given the lower overall sulfation in heparan sulfate compared with heparin, higher concentrations were required in these assays to obtain similar levels of cell adhesion inhibition. However, DE2S heparan sulfate polymer was a more effective competitor than DE6S heparan sulfate polymer (IC_{50} values of 30 and 850 ng/ml respectively; see Table 1), implying that in contrast to heparin there is a greater reliance on 6-O sulfation in HS for efficient cell attachment to the HepII substrate. As with heparin, the DE2S HS form competed about as effectively as native HS, suggesting a hierarchy of importance with the N-sulfates being the most important, followed by 6-O- and 2-O-sulfate groups.

**Heparan N- and 6-O-Sulfation Are Required for Focal Adhesion Formation**—It is well documented that focal adhesion formation in primary fibroblasts in response to fibronectin requires both integrin and syndecan-4 heparan sulfate proteoglycan (1, 8, 9). In particular, syndecan-4 is recruited in response to the HepII domain of fibronectin (7, 9, 12, 30). The α5β1 integrin interacts with repeats III_{9–10}, including the RGD tripeptide motif, whereas syndecan-4 interacts with III_{12–14}, comprising the HepII domain (14). This system was further studied to determine whether a common pattern of sulfation is required for both cell attachment to HepII and HepII-mediated formation of focal adhesions. Wild type (WT) and syndecan-4 knock-out (S4KO) mouse fibroblasts were seeded on HepII domain substrates in the presence of competing heparin. Both cell types showed dose-dependent decreases in attachment (IC_{50} for WT, 0.05 ng/ml; for S4KO, 0.04 μg/ml; Fig. 1D). Interestingly, spreading of S4KO cells on HepII was limited compared with wild type cells (data not shown). Therefore, attachment to the HepII domain can be mediated by HSPGs other than syndecan-4. However, as shown previously (30), whereas the HepII fragment promoted focal adhesion formation in WT cells pre-spread on the 110 kDa integrin-binding domain of fibronectin, S4KO were unable to do so (supplemental Fig. 1). Having established the essential role of syndecan-4, and its HS chains in focal adhesion formation, rat embryo fibroblasts were spread on fibronectin (Fig. 3C) alone or in the presence of 10
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Cell adhesion to fibronectin in the presence of the variously desulfated heparin 14-mers yielded a distinct pattern of results (Fig. 3C). When 50 or more cells were randomly analyzed for focal adhesion formation, it could be seen that in the presence of CompDes and DNS, 95 and 92% of the cells, respectively, had unimpaired focal adhesion and stress fiber formation. However, partial disruption was reproducibly seen in the presence of DE6S oligosaccharide where ~57% of cells had focal adhesions. 74% of cells in the presence of DNRAc or 75% of cells in the presence of DE6S assembled focal adhesions (Table 1), suggesting once again that there maybe a hierarchical importance of the various sulfate groups with the N-sulfates being the most essential, 6-O-sulfates being of intermediate importance, and 2-O-sulfates being least critical.

Fibroblasts spread on the proteolytically derived 110-kDa fragment for 2 h developed some thin microfilament bundles but few focal adhesions (Fig. 3D). Following the addition of the HepII fragment for a further 30 min, bundling of the stress fibers and focal adhesions was visible (as indicated by the appearance of vinculin in these adhesions). However, when the HepII fragment was preincubated with either heparin or heparin 14-mer (Fig. 3D) and added to cells pre-spread on the 110-kDa integrin-binding domain of fibronectin, focal adhesion formation was blocked (Table 1). This is consistent with syndecan-4-heparan sulfate interactions with the HepII domain to promote focal adhesion assembly.

When cells pre-spread on the 110-kDa domain were incubated with the HepII domain in the presence of desulfated heparin 14-mers, only cells that had the addition of either CompDes or DNS and to a lesser extent DNRAc were able to bundle their stress fibers and form focal adhesions. Analysis of focal adhesion formation showed that in the presence of HepII and CompDes and DNS or DNRAc, 78, 72, and 62% of cells, respectively, had focal adhesions. Those exposed to HepII in the presence of DE2S could not establish mature focal adhesions, with only 10% of the cells having some small focal complexes. However, 55% of cells exposed to HepII with DE6S assembled focal adhesions (Table 1). All this suggests that N-sulfation is required in syndecan-4 for signaling focal adhesion assembly, but a secondary requirement for glucosamine 6-O-sulfate was also clear.

Table 1: Summary table of IC50 values for competitors of REF attachment to HepII and percentage of REFs containing focal adhesions on either FN or the integrin-binding domain with HepII treatment, in the presence of these competitors (heparin, 10 μg/ml; all others, 30 μg/ml)

| Competitor                      | Average molecular mass (kDa) | IC50 values (μg/ml) | FN, focal adhesion (%) | IBD + HepII, focal adhesion (%) |
|---------------------------------|-----------------------------|---------------------|------------------------|---------------------------------|
| Heparin                         | 15                          | 0.16                | 14                     | 10                              |
| Heparin 14-mer oligosaccharide  | 4.2                         | 1                   | 25                     | 12                              |
| CompDes heparin oligosaccharide | 2.8                         | >10                 | 95                     | 78                              |
| DNS heparin oligosaccharide     | 3.5                         | >10                 | 92                     | 72                              |
| DNRAc heparin oligosaccharide   | 3.8                         | 2.5                 | 74                     | 62                              |
| DE6S heparin oligosaccharide    | 3.5                         | 0.65                | 75                     | 55                              |
| DE2S heparin oligosaccharide    | 3.5                         | 0.23                | 57                     | 10                              |
| Heparin 12-mer oligosaccharide  | 3.6                         | 2.5                 | 60                     |                                 |
| HS polymeric chains             | 25                          | >1000               |                        |                                 |
| DNS HS polymeric chains         | 25                          | 1040                |                        |                                 |
| DNRAc HS polymeric chains       | 25                          | 850                 |                        |                                 |
| DE6S HS polymeric chains        | 25                          | 30                  |                        |                                 |
| DE2S HS polymeric chains        | 25                          | 30                  |                        |                                 |
| Chondroitin sulfate C           | 30                          | >100                |                        |                                 |

FIGURE 2. Optimal fibroblast adhesion to HepII domain requires a minimal heparin 14-mer oligosaccharide. A, REFs were seeded on HepII substrates in the presence of competitive heparin oligosaccharides of increasing sizes (6-20-mers) for 1 h. Oligosaccharide concentration was 30 μg/ml. B, comparison of REF attachment to HepII in the presence of increasing concentration of either 12-mer (white bars) or 14-mer (black bars) heparin oligosaccharides as competitor. Attachment of cells without competitor to whole fibronectin was set at 100%, whereas attachment to BSA served as a negative control for each competitor condition.
CHO Cells Deficient in N-Sulfates Cannot Form Focal Adhesions—A series of Chinese hamster ovary cell mutants has been well characterized (reviewed in Ref. 39), including one (CHO-761) totally deficient in heparan and chondroitin sulfate (40). This line is defective in galactosyltransferase that catalyzes the addition of galactose to xylose in the stem or linkage tetrasaccharide. The CHO-761 cell line cannot assemble focal adhesions (31). Also characterized is the CHO-606 line, defective in \(N\)-deacetylase/\(N\)-sulfotransferase 1 (NDST-1). This is the most widespread of the four vertebrate \(N\)-deacetylase/\(N\)-sulfotransferases, and in this cell line \(N\)-sulfation is 2–3-fold less than wild type CHO-K1 cells with reduced length of the S-domains (32). However, because of the sequential nature of heparan sulfate modification in vivo, there is also a reduction in 2-O- and 6-O-sulfation (41).

It was confirmed by FACS analyses that syndecan-4 core protein was present on the cell surfaces of wild type and mutant cell lines (Fig. 4A), whereas CHO-K1 and CHO-606 also contained abundant syndecan-1 mRNA (not shown). The cell attachment assay data observed in Fig. 4B show that CHO-K1 cells were partially sensitive to heparin competition during attachment to HepII. CHO-761 cells, deficient in both heparan and chondroitin sulfate, were unable to adhere to the fibronectin HepII domain, whereas CHO-606 cells could attach to the HepII domain, although not as effectively as CHO-K1 cells, again partially sensitive to heparin. However, Fig. 4C shows that neither CHO-K1 nor CHO-606 cells were sensitive to chondroitin sulfate, suggesting once again that this is not mediating adhesion to the HepII domain of fibronectin. In further complementary experiments to those above, focal adhesion formation on fibronectin substrates was examined in CHO-K1 and CHO-606 cells. CHO-K1 cells plated on fibronectin formed focal adhesions, but this was inhibited by the presence of heparin or 14-mer heparin oligosaccharide competitors (Fig. 4D). CHO-606 cells spread on fibronectin but failed to establish focal adhesions (Fig. 4D, bottom panels). The mutant also displayed diffuse cytoplasmic staining for vinculin. These data support not only the role for cell surface HS in focal adhesion formation but also the importance of \(N\)-sulfation and S-domain length in heparan sulfate.

Heparan Sulfate Chain Size Is Critical to Cellular Responses through Surface HSPG—EXT1 and EXT2 genes encode glycosyltransferases that are involved in heparan sulfate polymerization. Analysis of heparan sulfate synthesized by mouse primary fibroblast cultures (MEF), with a gene trap mutation in the Ext1 gene, revealed that the size of the heparan sulfate chains is critical for cell attachment and focal adhesion formation.

**FIGURE 3.** Cell attachment and focal adhesion formation requires \(N\)-sulfated glucosamine. A, REF attachment to HepII substrates for 1 h with increasing concentrations of either heparin or the variously desulfated heparin 14-mer oligosaccharides CompDes, DNS, DNRAc, DE6S, or DE2S as competitors (concentration range from 0 to 10 \(\mu\)g/ml). B, REF attachment to HepII substrates for 1 h with increasing concentrations of heparan sulfate or the variously desulfated heparan sulfate polymeric chains DNS, DNRAc, DE6S, or DE2S as competitors (concentration range from 0 to 1000 \(\mu\)g/ml). In both cases, cell attachment to whole fibronectin (in the absence of competitor) was set at 100%. C, REFs were spread for 2 h on coverslips coated with either fibronectin only (10 \(\mu\)g/ml), or in the presence of 10 \(\mu\)g/ml heparin, or 30 \(\mu\)g/ml heparin 14-mer oligosaccharide, or the variously desulfated heparin 14-mer oligosaccharides as indicated. D, REFs were spread on coverslips coated with the 110-kDa integrin-binding domain of fibronectin (10 \(\mu\)g/ml) (top right panel) for 2 h only, or were treated for a further 30 min with 1 \(\mu\)g/ml of the HepII domain (top left panel). In the remaining panels, 1 \(\mu\)g/ml HepII was preincubated with either 10 \(\mu\)g/ml heparin or 30 \(\mu\)g/ml 14-mer heparin oligosaccharide or the variously desulfated heparin oligosaccharides for 15 min before adding to cells pre-spread on the 110-kDa integrin-binding domain of fibronectin. Immunofluorescent double staining is for F-actin (red) and vinculin (green). Arrows denote focal adhesions. Scale bar = 50 \(\mu\)m.
gene, showed not only smaller amounts of heparan sulfate than wild type but a significant difference in the molecular size of their heparan sulfate (33). The average chain mass of heparan sulfate in the wild type was estimated to be 70 kDa compared with 20 kDa in the mutant. However, although the size and the spacing of the S-domains in the gene trap cells are unknown, the overall pattern of sulfation was observed to be similar between the wild type and mutant (33).

Reverse transcription-PCR performed on mRNA isolated from the gene trap cells, and their wild type counterparts revealed the syndecan-4 transcripts in both cases (data not shown). Furthermore, FACS analyses confirmed that syndecan-4 core protein was present on the cell surfaces of both cell types (Fig. 5A). This indicates that even though the gene trap cells are deficient in producing “normal” sized heparan sulfate chains, this does not affect their ability to express syndecan-4 on the cell surface. In attachment assays (Fig. 5B), it was noted that although both wild type and gene trap cells adhered well to fibronectin, the wild type attached better to the HepII substrate than the gene trap cells. Both cell types were sensitive to heparin competition yielding similar results to rat embryo fibroblasts (cf. Fig. 1A). Both cell types spread on fibronectin, but although >95% of the wild type cells formed vinculin-containing focal adhesions (Fig. 5C), virtually none of the gene trap cells assembled these structures. Once again, focal adhesion formation by wild type MEF was inhibited by heparin and the 14-mer heparin oligosaccharide. Approximately 78% of the mutant cells formed small, punctate focal complexes containing vinculin, but as these were not located at the termini of stress fibers, they are not focal adhesions (Fig. 5C, bottom panels). Also in the wild type cells, extensive actin bundles terminating with vinculin could be seen, which were not present in the gene trap cells,
instead these had very diffuse F-actin staining or short thin bundles (Fig. 5C).

**DISCUSSION**

Cell surface heparan sulfate proteoglycans are important in many invertebrate and vertebrate developmental processes (reviewed in Refs. 42 and 43). Moreover some, such as syndecan-4, are known to be involved in both development and repair processes (44–48). Although the syndecan-4 null mouse survives and is fertile, there are obvious postnatal wound repair defects. These are characterized by impaired cell adhesion and migration defects (44). A major feature of heparan sulfate proteoglycans is the ability to bind a very large number of growth factors, cytokines, chemokines, and extracellular matrix molecules. In a few cases, it has been shown that the interactions are highly specific in terms of the degree and position of sulfation required in heparan sulfate to achieve high affinity binding. Much of this work still remains to be done, but the classic example is antithrombin III where there is a specific pentasaccharide, including a 3-O-sulfated glucosamine residue that is essential for high affinity interactions (49). Others have shown specific patterns of sulfation correspond with the binding of growth factors from the fibroblast growth factor family (25, 26). It is therefore apparent that the fine structure of cell surface heparan sulfate is critical, and it is known that heparan sulfate modification steps are regulated. Because a very early step in this process is the deacetylation and N-sulfation of glucosamine residues that will, to a large extent, set the pattern for subsequent modification steps, the regulation of N-deacetylase/N-sulfotransferases is important to ascertain. The fine regulation of cell surface heparan sulfate structure is underscored by recent data showing that the expression and action of cell surface sulfatases that remove selective 6-O-sulfate residues from heparan sulfate can influence the affinity of Wnt growth factors (50) and the

**FIGURE 5.** Heparan sulfate chain size is critical for focal adhesion assembly. A, FACS analyses of syndecan-4 with a polyclonal chicken antibody on wild type and Ext1 gene trap cells. *Thin line* represents secondary antibody only control (Alexa Fluor 488 goat anti-chicken), and *thick line* represents primary plus secondary antibody staining. B, wild type embryo fibroblasts (*black bars*) and gene trap embryo fibroblasts (*white bars*) were seeded on the HepII domain (10 μg/ml) of fibronectin in the presence of heparin (0–100 μg/ml) as competitor. As a positive control, cell adhesion on whole fibronectin substrates (10 μg/ml) was set at 100%. Adhesion to BSA (50 μg/ml) substrates was a negative control. C, wild type fibroblasts were plated on fibronectin-coated coverslips for 2 h in the absence or presence of 10 μg/ml heparin or 30 μg/ml 14-mer heparin oligosaccharide as indicated. Gene trap fibroblasts were plated on fibronectin-coated coverslips for 2 h in the absence of competitor. Cells were stained for F-actin (phalloidin) and vinculin. *Scale bar* = 50 μm.
binding of the bone morphogenetic protein inhibitor Noggin to cell surfaces (51).

Syndecan-4 has been shown to bind the HepII domain of fibronectin, and this is part of a process, in concert with α5β1 integrin, of focal adhesion formation (11, 12, 30). This is likely to be important in wound healing and tissue repair, because equivalent structures are abundant in granulation tissue for example (52). The crystal structure of HepII domain of fibronectin has been resolved (18), but not the interactions with heparan sulfate, although a cationic cradle has been identified that would approximately accommodate the 14-mer of heparan sulfate. Chromatographic studies of heparin interactions with HepII indicate that high affinity interactions require a 14-mer, although smaller oligosaccharides will bind. Our cell adhesion studies also indicate that oligosaccharides larger than a 14-mer have very high competitive abilities for cell surface heparan surface in cell adhesion assays. Here too, however, smaller oligosaccharides are able to compete, but more weakly.

There are several major conclusions from this study of cell adhesion and signaling mediated by the HepII domain of fibronectin. The previous cell-free experiments examining the interactions of heparan sulfate and HepII domain of fibronectin can be extrapolated to cell attachment. Not only is the size of the optimal heparin oligomer in competition assays equivalent to that seen in vitro for maximal binding, but there is a shared central importance of N-sulfation and long (14 or more sugar residues) HS S-domains. Thus full occupancy of the HS-binding site in HepII seems necessary for optimal cell attachment and triggering of focal adhesions. However, the 2-O-sulfate residues in HS are not required for the effects studied here despite the relative abundance of these substituents in the S-domains of fibroblast heparan sulfate (24). This observation distinguishes heparan sulfate binding to fibronectin from its interactions with the fibroblast growth factors that are strongly dependent on 2-O-sulfate groups (24). It was notable that focal adhesion formation is not entirely equivalent to cell attachment with respect to heparan sulfation requirements. Although N-sulfation is important for focal adhesion formation in fibroblasts, there is a secondary role for 6-O-sulfation that is more significant than in cell attachment. The reasons for the difference in sulfation requirements may be complex. Possibly, several cell surface heparan sulfate proteoglycans can take part in cell attachment, whereas syndecan-4 is the only cell surface proteoglycan known to contribute to focal adhesion formation (1). On the other hand, a previous study of the same cell type indicated no marked differences in the overall pattern of sulfation between syndecans and glypicans on the surface of the cells (53). Because syndecan-4 signaling is likely to involve clustering, it may be that multiple interactions are important between heparan sulfate and HepII domain of fibronectin, and these may require a more complex pattern of sulfation.

Finally, our findings clearly indicate that heparan sulfate chain length is a critical factor in focal adhesion formation. The gene trap cells used here, with a mutation in the ExtI gene, one of the two major heparan polymerases, show that chains of around 20 kDa are not sufficient to promote focal adhesion assembly even though they mediate cell attachment, albeit with less efficiency than the normal fibroblast heparan sulfate chains that are about 70 kDa in size. The lack of focal adhesion formation in the gene trap cells was not because of the lack of syndecan-4 expression on the cell surface but rather a specific defect associated with short heparan sulfate chains. Although there may be disruption of heparan sulfate modifications in the mutant cells, overall, sulfation is similar in the gene trap mutants as to the wild type (33). In principle, based on current knowledge of heparan sulfate organization (24), a 20-kDa heparan sulfate chain is large enough to contain at least one 14-mer S-domain capable of interacting with the HepII. Therefore, the data further suggest that multiple interactions between heparan sulfate and HepII domain of fibronectin are important in focal adhesion formation. However, these cells may provide important clues for understanding the relationship of matrix molecule binding to cell surface carbohydrates that trigger cytoplasmic signaling events. A detailed molecular study is certainly warranted because there is no understanding of the precise molecular interactions of heparan sulfate and the HepII domain on the cell surface or subsequent recruitment to focal adhesions. These studies represent a first step in understanding the requirement in heparan sulfate fine structure that is necessary in cell adhesion signaling.

Acknowledgments—We thank Dr. Jean Schwarzauer (Princeton University) for the HepII domain plasmid, Dr. Marion Kusche-Gullberg (University of Bergen) for the ExtI gene trap mutant and corresponding wild type fibroblasts, Prof. Takashi Muramatsu for the syndecan-4 null mice (Nagoya University) and Dr. Anne Woods (University of Alabama) for establishing fibroblast cultures from them, and N. Gasiunas (University of Manchester) and Jon Deakin (University of Manchester) for preparing the desulfated heparan oligosaccharides and HS polysaccharides. We are also grateful to Dr. Hinke Multhaupt (Imperial College London) for the affinity-purified syndecan-4 polyclonal chicken antibody and for help with the figures.

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