

# Heparan Sulfate Chain Valency Controls Syndecan-4 Function in Cell Adhesion<sup>\*S</sup>

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Fibroblasts null for the transmembrane proteoglycan, syndecan-4, have an altered actin cytoskeleton, compared with matching wild-type cells. They do not organize  $\alpha$ -smooth muscle actin into bundles, but will do so when full-length syndecan-4 is re-expressed. This requires the central V region of the core protein cytoplasmic domain, though not interactions with PDZ proteins. A second key requirement is multiple heparan sulfate chains. Mutant syndecan-4 with no chains, or only one chain, failed to restore the wild-type phenotype, whereas those expressing two or three were competent. However, clustering of one-chain syndecan-4 forms with antibodies overcame the block, indicating that valency of interactions with ligands is a key component of syndecan-4 function. Measurements of focal contact/adhesion size and focal adhesion kinase phosphorylation correlated with syndecan-4 status and  $\alpha$ -smooth muscle actin organization, being reduced where syndecan-4 function was compromised by a lack of multiple heparan sulfate chains.

Syndecans are an ancient group of transmembrane heparan sulfate proteoglycans, with four members in every mammal examined to date (1–3). Syndecans associate with the actin cytoskeleton, and syndecan-4 in particular, is known as a focal adhesion component (3). In fibroblast adhesion studies (4), it has been shown that the HepII domain of fibronectin will interact with the heparan sulfate chains of syndecan-4 to promote a signaling response that includes binding and activation of protein kinase C $\alpha$  and downstream to the Rho family of G proteins (4–6). Together with integrins,

syndecan-4 then promotes assembly of focal adhesions (3–7). Of the syndecan family, much focus has been on syndecan-4 because of its role in adhesion, and also because key elements of its structure and signaling have been elucidated. The cytoplasmic domain of syndecan-4 forms twisted clamp dimers, stabilized by the presence of phosphatidylinositol 4,5-bisphosphate (PtdIns4,5P<sub>2</sub>), which in turn bind to protein kinase C $\alpha$ , becoming persistently activated (8, 9). It is likely that the dimer is the basal state of all syndecans, because the transmembrane domains of all syndecans form SDS-resistant homodimers by virtue of a highly conserved GXXXG motif (10, 11).

One report suggests that heparan sulfate chains are not required for focal adhesion promotion (12), in this case where syndecan-4 cDNA was transfected into a CHO cell mutant incapable of glycosaminoglycan synthesis. However, syndecan-4 does also possess a site in its external core protein, including an NXIP motif, which triggers integrin-mediated cell adhesion (13). A similar principle has been demonstrated for syndecan-1, where  $\beta$ 3 or  $\beta$ 5 integrins are influenced directly by a portion of the ectodomain, now known as synstatin (14). However, most studies have indicated a need for heparan sulfate substitution on syndecan-4, and some fine structure requirements of the chains for interaction with fibronectin, have been elucidated. These include N-sulfation (but not 2-O-sulfation) and sulfated subdomains of >12 sugar residues (15).

The syndecan-4 knock-out mouse has vascular repair and cell migration defects (16). It also has defects in skeletal muscle regeneration (17). Cell migration defects are not corrected by selected growth factors, suggesting that there are altered cell adhesion properties (16). This is consistent with *in vitro* studies showing a role for the proteoglycan in directionally persistent migration (18) and zebrafish studies demonstrating a similar role in neural crest migration (19). One report suggests that only under limited circumstances is focal adhesion assembly compromised in null fibroblasts (20), but a second report indicates that a difference in  $\alpha$ -smooth muscle actin ( $\alpha$ SMA)<sup>6</sup> organization is observable in knock-out cells (21). Whereas wild-type mouse embryo fibroblasts have a high proportion with this actin isoform incorporated into stress fibers, syndecan-4-null cells do not, though this can be corrected by exogenous transforming growth factor- $\beta$  (21). This difference in

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<sup>S</sup> The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Fig. S1 and Table S1.

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<sup>6</sup> The abbreviations used are:  $\alpha$ SMA,  $\alpha$ -smooth muscle actin; FAK, focal adhesion kinase; HA, hemagglutinin; PBS, phosphate-buffered saline; MEF, murine embryonic fibroblast; GFP, green fluorescent protein.

## Syndecan-4 and the Cytoskeleton

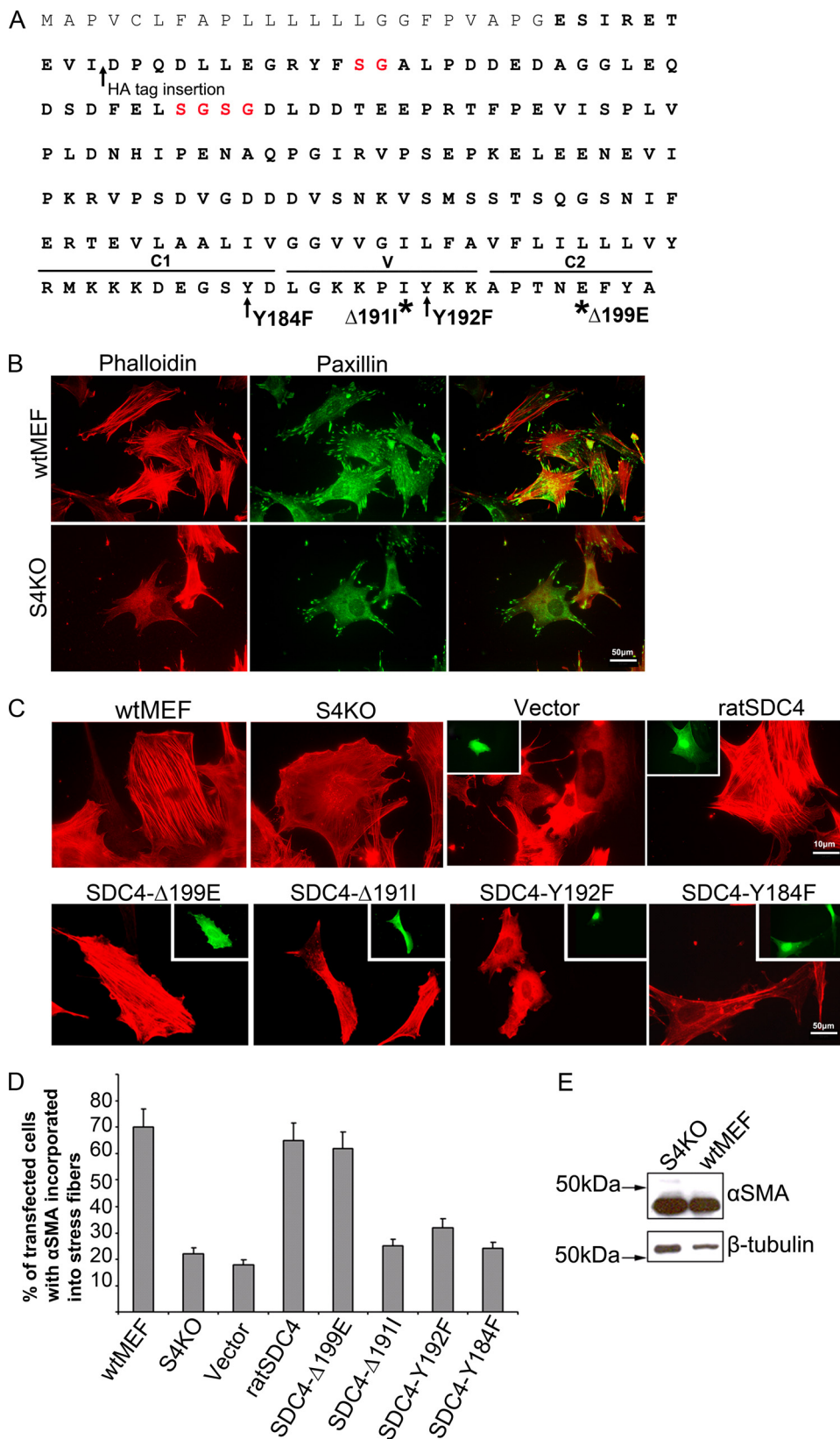
cytoskeletal organization is used here as an assay for syndecan-4 functionality.

A continuing mystery is how ligand binding to heparan sulfate on the cell surface triggers cytoplasmic signaling events in syndecan-4. Because this proteoglycan becomes clustered into focal adhesions, an obvious possibility is that lateral association of syndecan-4 dimers is sufficient to drive the process. At the same time, all syndecans are potentially multivalent, given that each core protein has at least three Ser-Gly motifs suitable for substitution with glycosaminoglycans (3, 6, 8). Where studied, it appears that glycanation is maximal, *i.e.* if three sites are available, each tends to be substituted with a chain (22). If this applies to syndecan-4 on the cell surface, then up to six heparan sulfate chains per core protein dimer may be available. The question arises, however, whether multiple chains are in fact required to promote a signaling response. To address this, we prepared all possible variants of syndecan-4, ranging from the wild type with three Ser-Gly dipeptides, to two, one, and no Ser-Gly dipeptides, eight variants in total. In mutant forms, key serine residues were mutated to alanine. These cDNA constructs were introduced into syndecan-4-null cells and assessed for their ability to restore a wild-type actin cytoskeleton. The data suggest that a functional syndecan-4 dimer requires a minimum of four heparan sulfate chains, underlining the importance of valency, and suggesting that under normal circumstances, two syndecan-4 dimers must associate in the presence of ligand, to form a signaling unit.

### EXPERIMENTAL PROCEDURES

**Cell Culture and Transfection**—Syndecan-4-null (S4KO) and matching wild-type murine embryonic fibroblasts (MEFs) (20) were grown and maintained in  $\alpha$ MEM (Lonza) supplemented with 10% v/v fetal bovine serum (FBS). COS7 cells were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO) containing 10% FBS and 2% glu-

tamax. Syndecan-4-null fibroblast and COS7 transfections were performed using Lipofectamine (Invitrogen) or Lipofectamine LTX (Invitrogen) respectively, according to the manufacturers' instructions.



**Antibodies**—Antibodies used included monoclonal anti- $\alpha$ -smooth muscle actin antibody (Clone 1A4; Sigma), anti-paxillin antibody (clone Z035; Zymed Laboratories Inc.), monoclonal anti-HA (Clone HA.11; Covance), polyclonal goat anti- $\alpha$ -actinin (C-20; Santa Cruz Biotechnology), polyclonal rabbit antibodies against focal adhesion kinase (FAK; BD Pharmingen), and tyrosine 397-phosphorylated FAK (Cell Signaling), monoclonal mouse anti-Rac (Clone 23A8; Millipore), polyclonal rabbit anti-RhoA (119; Santa Cruz Biotechnology), polyclonal goat anti-ezrin (C-19; Santa Cruz Biotechnology), polyclonal rabbit anti-HA (SG. 77; Zymed Laboratories Inc.), and monoclonal anti- $\beta$ -tubulin (Clone TUB 2.1; Sigma). Syndecan-4 antibody was raised in chickens against a synthetic peptide corresponding to the N terminus of the syndecan-4 core protein as described (15).

**Preparation of Fibronectin 110-kDa (III<sub>3</sub>–III<sub>11</sub> Repeats) Fragment and HepII Domain**—Fibronectin was purified from fresh human plasma by adapting the protocol of Miekka *et al.* (23). The enzymatic cleavage and purification of the central, integrin-binding 110-kDa fibronectin fragment was as previously (15). Recombinant His-tagged HepII (FN repeats III<sub>12</sub>–15) domain was expressed in *Escherichia coli* BL21 cells and purified with His select<sup>®</sup> cobalt affinity gel (Sigma) according to the manufacturer's instructions. The purity of the protein was confirmed by SDS-PAGE with Coomassie Blue staining. The construct in pQE-30 was a kind gift from Dr. Jean Schwarzbauer (Princeton University).

**GAG Chain Mutagenesis**—The three serine residues that serve as syndecan-4 glycosaminoglycan attachment sites were mutated using a two step overlap PCR approach as follows. During the first step 5' and 3' PCR products were generated from full-length rat syndecan-4 cDNA using primers complementary to the 5' and 3' non-coding regions of syndecan-4 and reverse and forward primers containing point mutations so that one serine residue (44, 65, or 67) was mutated to alanine (supplemental Table S1). The resultant two PCR products were combined and used as template in a second reaction using S4for and S4rev primers to generate full-length syndecan-4 cDNA. EcoRI and BamHI restriction sites were incorporated into these primer sequences, and PCR products were digested with these enzymes and ligated into the corresponding sites of pIRES2-EGFP (Clontech) using standard procedures. Using the primers described in supplemental Table S1 it was not only possible to generate single Ser → Ala mutations at each position but also to generate all combinations of the three mutations (Fig. 2).

A similar approach was used to fuse the HA epitope sequence between isoleucine 32 and aspartate 33 in the syndecan-4 constructs. Primers S4for and S4rev were used in conjunction with primers containing the HA coding sequence to generate 5' and 3' PCR products from each of the 6 mutated syndecan-4 cDNAs and the wild-type sequence. Full-length products were obtained for each cDNA, and these were digested and ligated into pIRES2-EGFP as described. The expression of each cDNA was analyzed in COS7 cells by Western blots with anti-HA antibody followed by horseradish peroxidase-conjugated goat anti-mouse antibody (Dako). In control experiments, cell layers were treated with chondroitinase ABC (Sigma Aldrich) before performing the SDS-PAGE to remove chondroitin and dermatan sulfate chains from syndecan-4 core protein. Syndecan-4 mutant cDNAs encoding truncated cytoplasmic domains were prepared as previously (24), and the HA coding sequence was inserted as described above. To create single amino acid substitution of either Tyr-184 or Tyr-192 with phenylalanine in syndecan-4 cytoplasmic domain (25), syndecan-4 cDNAs encoding those sequences in pIRES2-EGFP were constructed using overlap PCR extension methods. The cell surface expression of recombinant syndecan-4 was confirmed in COS7 by flow cytometry. Cells expressing wild-type and recombinant syndecan-4 were harvested using cell dissociation buffer (Invitrogen), resuspended in 1% bovine serum albumin (BSA) in PBS; then the live cells were stained using anti-HA antibody in 1% BSA and rabbit anti-mouse Alexafluor 647 IgG (Molecular Probes). Dead cells were identified by staining with 20  $\mu$ g/ml propidium iodide solution (BD Bioscience). Controls were prepared by staining with Alexafluor 647-conjugated anti-mouse IgG without primary antibodies. Samples were analyzed using a FACS Calibur (Becton Dickinson) and analyzed with CellQuest Pro software.

**Immunofluorescence Microscopy and Western Blotting**—Transfected syndecan-4-null mouse fibroblasts were seeded onto coverslips in growth medium for 24 h prior to serum starvation for a further 24 h. Cells were fixed in 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 min; then stained using conventional procedures using a primary antibody to  $\alpha$ SMA and Alexafluor 568-conjugated goat-anti mouse IgG (Molecular Probes). Samples were analyzed on a Zeiss Axioplan-2 microscope ( $\times 40$  objective), and images were processed using Metamorph and Adobe Photoshop. For focal adhesion area measurement, the structures were stained with paxillin antibody,

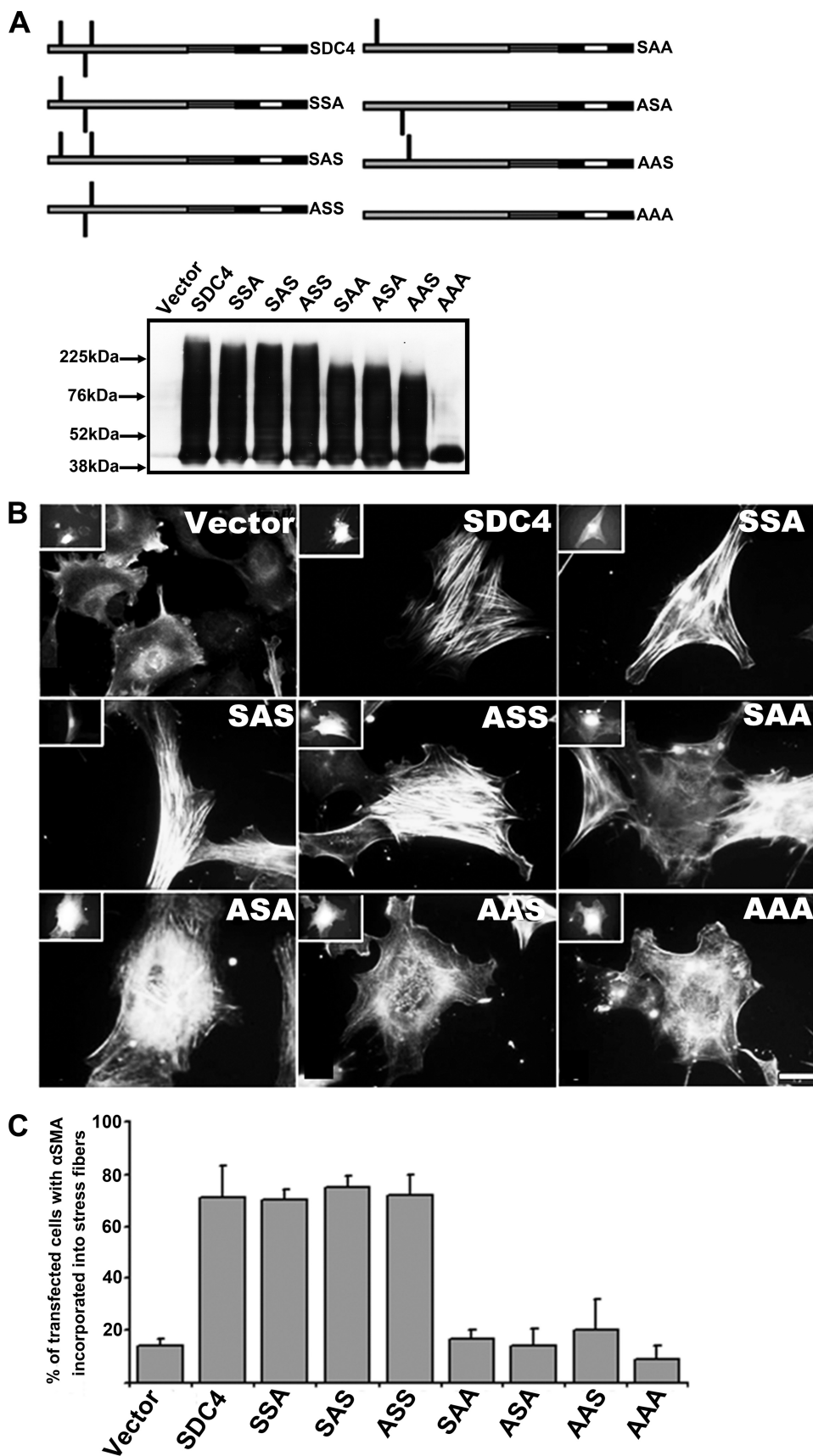
**FIGURE 1. Syndecan-4 regulates  $\alpha$ SMA incorporation into fibroblast stress fibers.** *A*, rat syndecan-4 protein sequence showing the signal peptide in *normal text* and the mature protein in *bold*. Serine residues 44, 65, 67 in the serine-glycine consensus sequences (*red text*) are the putative sites of glycosaminoglycan chain substitution. Sites of cytoplasmic domain truncation  $\Delta 199E$  (terminating at Glu-199) and  $\Delta 191I$  (terminating at Ile-191) are marked by an *asterisk*. The Y192F and Y184F mutations are marked with an *arrow*. The site of HA tag insertion is shown, as are the subdomains of the cytoplasmic domain, C1, V, and C2. *B*, wild-type and syndecan-4-null murine fibroblast showed differences in the stress fiber and focal adhesion phenotype when stained with phalloidin and anti-paxillin antibodies. *C*, similarly, immunostaining shows incorporation of  $\alpha$ SMA into stress fibers of wild type, but not syndecan-4-null fibroblasts. The wild-type  $\alpha$ SMA phenotype of stress fibers in syndecan-4-null MEF is restored by the reintroduction of rat syndecan-4 (*SDC4*) but not transfection of the equivalent empty vector (*pIRES2-EGFP*). A series of mutant syndecan-4 cDNA transfections shows that the C2 region is dispensable, but the V region and tyrosine residues 184 and 192 are essential for restoration of a wild-type  $\alpha$ SMA phenotype. For all transfections except *SDC4- $\Delta 199E$*  and *SDC4- $\Delta 191I$* , the transfected cells were identified by expression of GFP encoded in the pIRES-EGFP plasmid. For *SDC4- $\Delta 199E$*  and *SDC4- $\Delta 191I$*  the transfected cells are distinguished from untransfected cells by immunostaining for syndecan-4 expressed on cell surface. *D*, for each transfection represented in *C*, >100 cells were counted, and the data are means  $\pm$  S.E. from triplicate experiments. *E*, Western blots show that protein levels of  $\alpha$ SMA are equivalent in wild-type and syndecan-4-null fibroblasts. The scale bar is marked in each panel.

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followed by Alexafluor 568-conjugated goat anti-mouse IgG. The area of each focal adhesion was measured and calibrated using Metamorph and ImageJ imaging software. For each transfection a minimum of 100 cells was analyzed for focal adhesion area. Transfected cells were identified by the expression of GFP from the bicistronic vector or by immunological detection of the HA insert in the syndecan-4. For fibronectin domain experiments, transfected syndecan-4-null fibroblasts were seeded onto coverslips coated with fibronectin (1  $\mu$ g/coverslip) or FN 110 (1  $\mu$ g/coverslip) in growth medium, followed by incubation for 3–4 h and HepII (1–10  $\mu$ g/ml) solution was added to the medium. Cells were stained for focal adhesion components, and the percentage of cells containing these organelles was assessed, with a minimum of 100 cells per condition. For all experiments transfected cells were identified by the expression of GFP or by immunological detection of the HA tag in the syndecan-4 core protein. To compare the expression of different focal adhesion proteins in wild-type and syndecan-4-null murine fibroblasts, Western blots were performed with antibodies against paxillin,  $\alpha$ -actinin, Rac 1, Rho A, and ezrin.

**Analysis of Focal Adhesion Kinase**—Syndecan-4-null MEFs were transfected with cDNAs corresponding to wild type, and glycosaminoglycan chain substitution mutants of syndecan-4 and allowed to express the protein for 36 h. The expression of FAK and the level of its phosphorylation was analyzed by Western blotting with anti-FAK and anti-phospho-FAK (tyrosine 397) antibodies followed by horseradish peroxidase-conjugated goat anti-rabbit antibody. Western blots were calibrated by quantitation of  $\beta$ -tubulin as a loading control.

**Syndecan-4 Clustering Experiments**—Transfected syndecan-4-null fibroblasts were seeded on glass coverslips in the presence of serum



for 24 h prior to serum starvation for a further 24 h. Syndecan-4 clustering was achieved by first incubating cells with 10  $\mu\text{g}/\text{ml}$  of anti-syndecan-4 polyclonal antibody in serum-free medium (15) for 15 min at 37 °C prior to washing twice with PBS. Cells were then treated with Alexafluor 568-conjugated goat anti-chicken IgY (Invitrogen; 1  $\mu\text{g}/\text{ml}$  in serum-free medium) for 15 min at 37 °C prior to fixation. The cells were then conventionally stained for  $\alpha\text{SMA}$  as described as above.

**Syndecan-4 Glycosaminoglycan Analysis**—COS7 cells expressing wild-type and recombinant syndecan-4 mutants were washed with PBS and DMEM was supplied without FBS. Cultures were metabolically labeled with  $^{35}\text{S}$  ( $\text{Na}_2\text{SO}_4$ ; specific activity 43Ci/mg sulfur; MP Biomedicals) at a concentration of 140–150  $\mu\text{Ci}/\text{ml}$ . The cells were incubated for 48 h at 37 °C in a  $\text{CO}_2$  incubator. Syndecan-4 was purified by immunoprecipitation with a polyclonal anti-HA antibody (Zymed Laboratories Inc.) using protein-A beads. Radiolabeled glycosaminoglycan chains were cleaved from syndecan-4 core protein by alkaline elimination (0.1 M  $\text{NaBH}_4$  in 0.1 M NaOH) for 24–30 h at 4 °C, followed by neutralization with 4% HCl. For gel filtration chromatography, the heparan sulfate chains (~8000 cpm) were applied to a Superdex 200 10/300GL gel filtration column (GE Healthcare) pre-equilibrated in 50 mM  $\text{NaH}_2\text{PO}_4$  (pH 7.0) containing 150 mM NaCl. The heparan sulfate chains were eluted in the same buffer at a flow rate of 0.4 ml/min. The column was calibrated with blue dextran (2000 kDa), ovalbumin (42 kDa), and vitamin B12 (1.35 kDa). Eluted fractions were collected and analyzed for radiolabel content by scintillation spectroscopy. For anion-exchange chromatography, metabolically labeled heparan sulfate chains were applied to a MonoQ10/100GL column (GE Healthcare). The column was pre-equilibrated with 20 mM Tris-HCl containing 0.5 M NaCl. The unbound radioactivity was removed by washing column with 5 column volumes of 20 mM Tris-HCl containing 0.5 M NaCl. Bound radioactivity was eluted in a linear gradient of NaCl (0.5–2.0 M) in 20 mM Tris-HCl buffer (10 column volumes). The eluted fractions were collected and analyzed for radiolabel content.

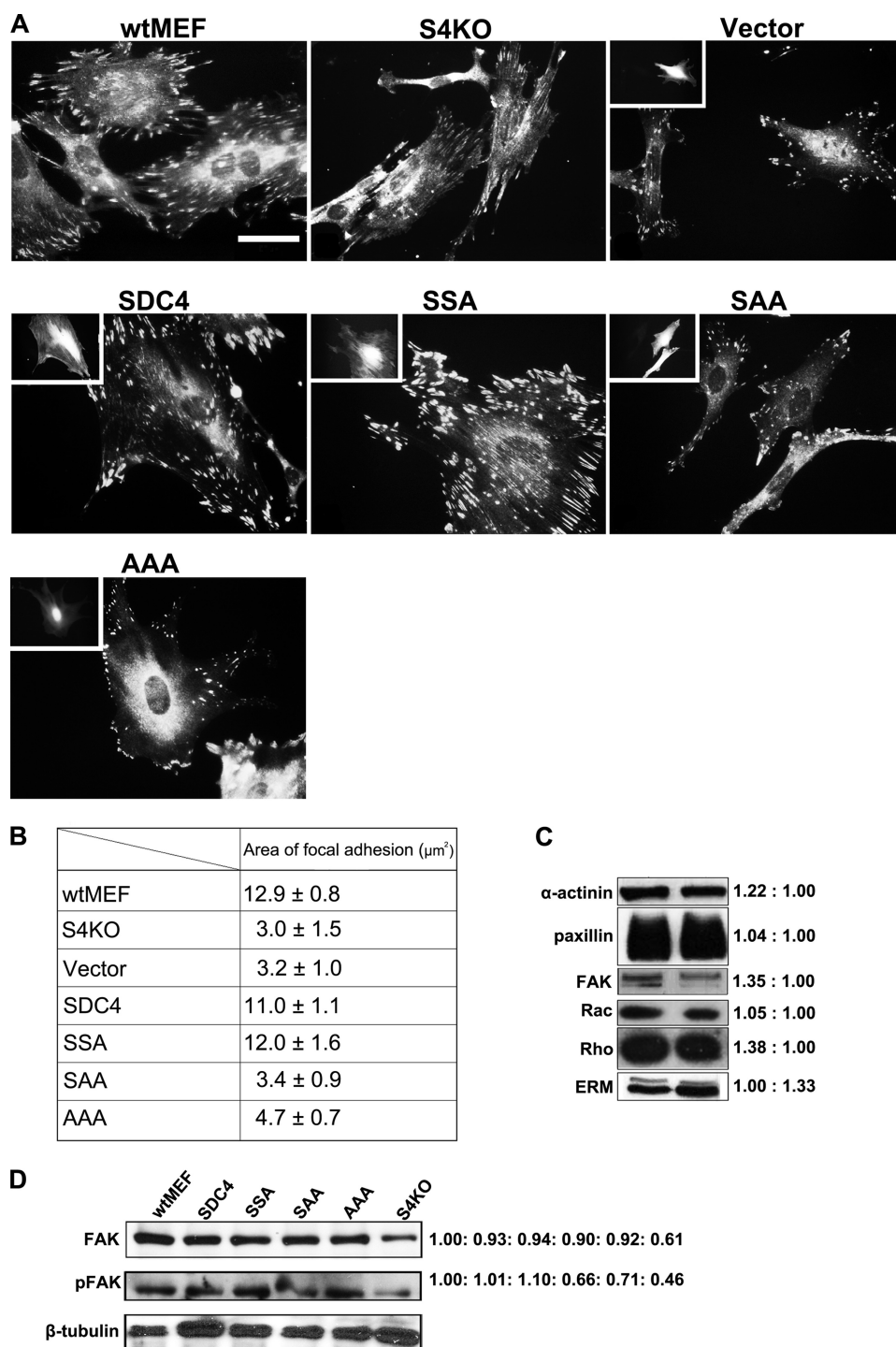
Low pH (1.5) nitrous acid treatment of heparan sulfate chains was performed according to Shively and Conrad (26), to cleave the heparan sulfate chains at *N*-sulfated glucosamine residues. Immunoprecipitated syndecan-4 was also treated with 5 milliunits of chondroitinase ABC (Sigma, EC 4.2.2.4) in 50 mM Tris pH 8.0, 60 mM sodium acetate at 37 °C for 16h. Heparinase III treatments were as described (27). Briefly,  $^{35}\text{S}$ -labeled heparan sulfate chains from purified syndecan-4 were mixed with unlabeled heparan sulfate (0.01 mg/ml final) and incubated with 1.5 milliunits of heparinase III (heparinase EC 4.2.2.8, Seikagaku America) for 6 h at 37 °C in 0.1 M sodium acetate, pH 7.0 containing 0.1 mM

calcium acetate. Another 1.2-milliunit aliquot of the enzyme was added after 6 h, and the mixture was incubated for 16 h. The products from nitrous acid, chondroitinase ABC and heparinase III treatments were applied to Superdex 75 column (GE Healthcare) pre-equilibrated with 50 mM  $\text{NaH}_2\text{PO}_4$  (pH 7.0) containing 150 mM NaCl. The column was calibrated with commercially available protein standards and disaccharide,  $\Delta\text{UA-GlcNS}$  (462 Da; Iduron UK).

## RESULTS

**Syndecan-4-null Cells Have an Altered Actin Cytoskeleton**—Two independent strains of newborn and embryonic syndecan-4-null fibroblasts exhibit the same defect in organization of their actin cytoskeleton. On fibronectin substrates, these cells have  $\beta$ -actin containing microfilament bundles, often oriented circumferentially, and small focal contacts (Fig. 1B). These focal contacts contained several expected marker proteins including paxillin (Fig. 1B), vinculin, talin, and phosphotyrosine. In contrast, wild-type cells have more substantial stress fibers, frequently running from end-to-end, and larger focal adhesions (Fig. 1B). A key difference however, is the organization of  $\alpha\text{SMA}$ . This is a stress fiber component of normal mouse (Fig. 1C), and rat fibroblasts but is not a component of the finer microfilament structures of the null cells (Fig. 1C). Quantitative data are shown in Fig. 1D. This difference is not a result of decreased  $\alpha\text{SMA}$  protein levels in the null cells (Fig. 1E) but its organization. Moreover it can be overcome by transfection of null cells with cDNA constructs encoding wild type syndecan-4 (Fig. 1C). These patterns of  $\alpha\text{SMA}$  organization are the phenotypic marker for subsequent experiments where mutated forms of syndecan-4 are expressed in null cells to determine the parameters necessary for reconstitution of the wild-type phenotype. The role of the syndecan-4 cytoplasmic domain was confirmed by transfection of a series of mutant constructs, shown in Fig. 1A. Deletion of the three C-terminal amino acids, which prevents PDZ proteins from binding, had no effect on syndecan-4 ability to restore a normal actin cytoskeleton (Fig. 1C). However, a truncation to the center of the V region (terminating at Ile-191) prevented a restoration of actin cytoskeleton. Similarly, mutation of either of two tyrosine residues at proximal and distal ends of the V region rendered the syndecan-4 non-functional in these assays (Fig. 1C). Quantitation of these experiments is shown in Fig. 1D. These data suggest that signaling through the cytoplasmic domain, and/or interactions with actin-associated proteins, is critical to the  $\alpha$ -smooth muscle phenotype promoted by syndecan-4. FACS data confirmed that all constructs were expressed on the cell surface of the fibroblasts (supplemental Fig. S1) to broadly equivalent levels.

**FIGURE 2. A minimum of two heparan sulfate chains on syndecan-4 core protein are required to restore a normal actin cytoskeleton in syndecan-4 null fibroblasts.** A, syndecan-4 variants, where the serine residues 44, 65, and 67 were mutated to alanine either individually or in combination, are shown diagrammatically. Each HA-tagged mutant was expressed in COS7 cells and lysates analyzed by Western blot with an HA tag-specific antibody. The heterogeneous products are consistent with GAG substitution. In the AAA mutant with no glycosaminoglycan chains, dimeric core protein was detectable as a 40-kDa polypeptide. B, immunostaining of the syndecan-4-null fibroblasts transfected with wild-type and glycosaminoglycan chain mutant syndecan-4 constructs showed that syndecan-4 can mediate signaling to incorporate  $\alpha\text{SMA}$  into stress fibers if at least two of three serine residues are substituted by heparan sulfate chains. This is quantified in C. The scale bar is 10  $\mu\text{m}$ .

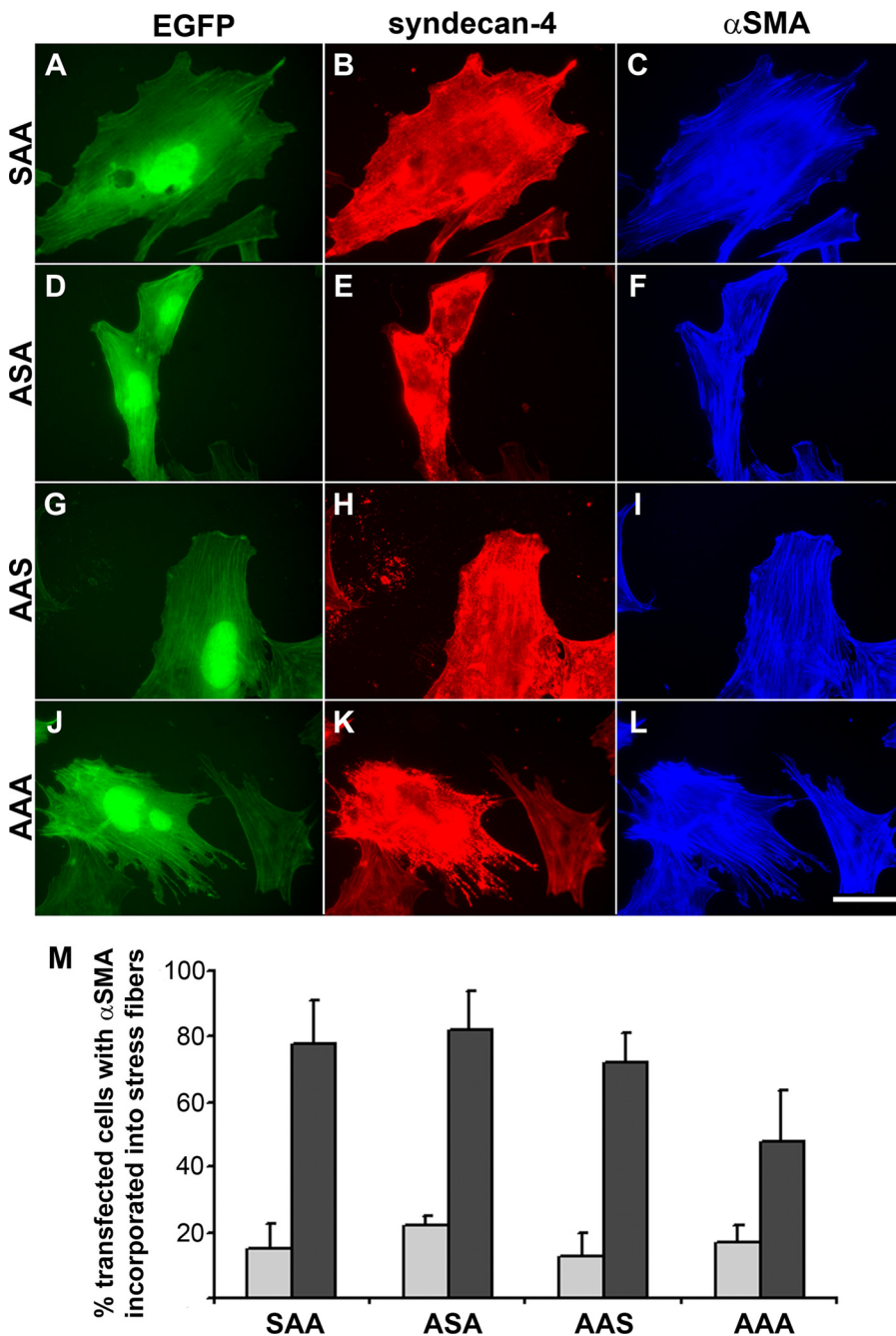


**FIGURE 3. Syndecan-4 with multiple heparan sulfate chains promotes larger focal adhesions with increased FAK activity.** *A*, wild-type, syndecan-4-null, and syndecan-4 re-expressing-null fibroblasts were stained for the focal adhesion component paxillin. In each case focal contacts or focal adhesions were formed, but those of null cells, or those expressing syndecan-4 with one or no heparan sulfate chains were smaller in area than wild-type cells or those expressing syndecan-4 with two or three heparan sulfate chains. Quantification of 75–100 adhesions, from >50 cells per condition is shown in *B*. *C*, Western blot of focal adhesion proteins and cytoskeletal regulators in both wild-type and syndecan-4-null fibroblasts revealed that protein expression levels were mostly comparable, although the latter contain less FAK protein. *D*, FAK activity was assessed from the extent of tyrosine 397 phosphorylation by Western blotting with a specific antibody. Null cells and those expressing syndecan-4 with one chain have lower FAK activity than null cells expressing two or three chains. FAK protein and tyrosine 397 phosphorylation are calibrated to  $\beta$ -tubulin levels. In *C* and *D*, quantitation is the mean of three independent experiments. Scale bar is 50  $\mu\text{m}$ .

*Multiple Heparan Sulfate Chains Are Required for Syndecan-4 Function in Cell Adhesion*—Null cells transfected with wild-type syndecan-4 cDNA become essentially identical to wild-type cells within 48 h of transfection, when examining the distribution of  $\alpha$ SMA (Fig. 2*B*). Correspondingly, focal adhesions were much enhanced in size and number (Fig. 3). In contrast, an empty vector control had no influence on cell morphology or cytoskeleton (Figs. 2*B* and 3). To directly ascertain how many glycosaminoglycan chains were required for the phenotype restoration and whether their position on the core protein was important, all possible combinations of mutations in the three serine residues of the N-terminal region of the core protein were produced (Fig. 2*A*). Complementary DNAs for all six mutants were transfected into COS7 and null mouse embryo fibroblasts and the cytoskeleton examined 48 h later.

Glycanation of each syndecan-4 variant was examined by Western blotting of COS7 cell lysates, into which HA-tagged forms for syndecan-4 cDNA had been transfected (Fig. 2*A*). Where the AAA mutant was expressed, a single prominent polypeptide of  $\sim$ 40 kDa was detected, consistent with an SDS-resistant dimeric core protein (9, 10). Where a single serine was present (SAA, ASA, and AAS), glycanation was apparent, with heterodisperse populations of syndecan-4 visible. This confirmed the presence of glycosaminoglycan in all three variants of syndecan-4. Similar heterogeneous populations of syndecan-4 were detected by Western blotting of SSA, SAS, ASS, and SDC4 (wild-type) forms of syndecan-4, though all extended into higher mass regions of the gel than the “single chain” forms. The SDC4 material had the highest mass range of all, and the data are consistent with syndecan-4 being substituted on each available serine residue, as noted previously (22).

When the syndecan-4 cDNA constructs were transfected into



**FIGURE 4. Clustering of syndecan-4 bearing one or no heparan sulfate chains, with antibodies triggers incorporation of  $\alpha$ SMA into stress fibers.** A–L, syndecan-4-null mouse fibroblasts were transfected with the syndecan-4 cDNAs encoding none or one GAG attachment site. Transfected cells were grown in the presence of serum for 24 h prior to serum starvation for 24 h. Syndecan-4 clustering was achieved with a polyclonal anti-syndecan-4 antibody raised against an N-terminal peptide sequence, followed by an Alexafluor-568 conjugated anti-chicken IgY. Transfected cells were identified by GFP from the bicistronic vector, cell surface syndecan-4 visualized directly, and the  $\alpha$ SMA detected by an Alexafluor 405 antibody (blue). M, percentage of transfected cells with  $\alpha$ SMA incorporated into stress fibers was calculated in syndecan-4-expressing cells that had been clustered as above (black bars) or remained untreated (light gray bars).  $n = 110$ – $130$  cells from each condition. The scale bar is  $10 \mu\text{m}$ .

null fibroblasts, the results showed that at least two of three serine residues were required in order that  $\alpha$ SMA became incorporated into microfilament bundles. Quantitative data are shown in Fig. 2C. However, and importantly, it did not matter which two of three serine residues were present (Figs. 2B and C). In all cases though, where only one serine residue was present, allowing only one heparan sulfate chain

substitution on the core protein, no restoration of normal cytoskeletal phenotype was seen. The data therefore suggest that two or more heparan sulfate chains are required to interact with the matrix substrate for productive syndecan-4 function. In each case, focal adhesion characteristics followed that of the microfilament architecture, being larger and more numerous where  $\alpha$ SMA was incorporated into microfilament bundles (Fig. 3). Quantitative analysis showed that focal adhesions were  $<5 \mu\text{m}^2$  in null cells, and where single chain variants of syndecan-4 were expressed. In contrast, focal adhesions were on average  $>10 \mu\text{m}^2$  in wild-type cells and those null cells expressing syndecan-4 with two or three heparan sulfate chains (Fig. 3B). In control experiments, cell surface expression of each syndecan-4 variant in terms of heparan sulfate chain composition was confirmed by FACS analysis (supplemental Fig. S1). The difference in focal adhesion size between null and wild-type cells was not related to the expression level of protein components such as  $\alpha$ -actinin, paxillin, Rac1, RhoA, or ezrin proteins that were comparable in syndecan-4-null and wild-type cells (Fig. 3C). This suggests that organization, rather than protein expression levels, is relevant to syndecan-4 promotion of focal adhesions and large microfilament bundles containing  $\alpha$ SMA. However, null cells reproducibly expressed  $\sim 30\%$  less FAK protein than wild-type cells (Fig. 3C), and this was further studied by examination of FAK activity.

Tyrosine 397 phosphorylation of FAK is an index of kinase activity (28). Syndecan-4-null fibroblasts together with transfectants encoding one, two, or all three (wild type) were examined by Western blotting with a tyrosine 397 FAK phosphospecific antibody (Fig. 3D). The results were consistent with focal adhesion size; lower levels of phosphorylation were detected in null cells and those expressing syndecan-4 with only one glycosaminoglycan chain. Higher levels were present in cells expressing wild-type syndecan-4 and a mutant

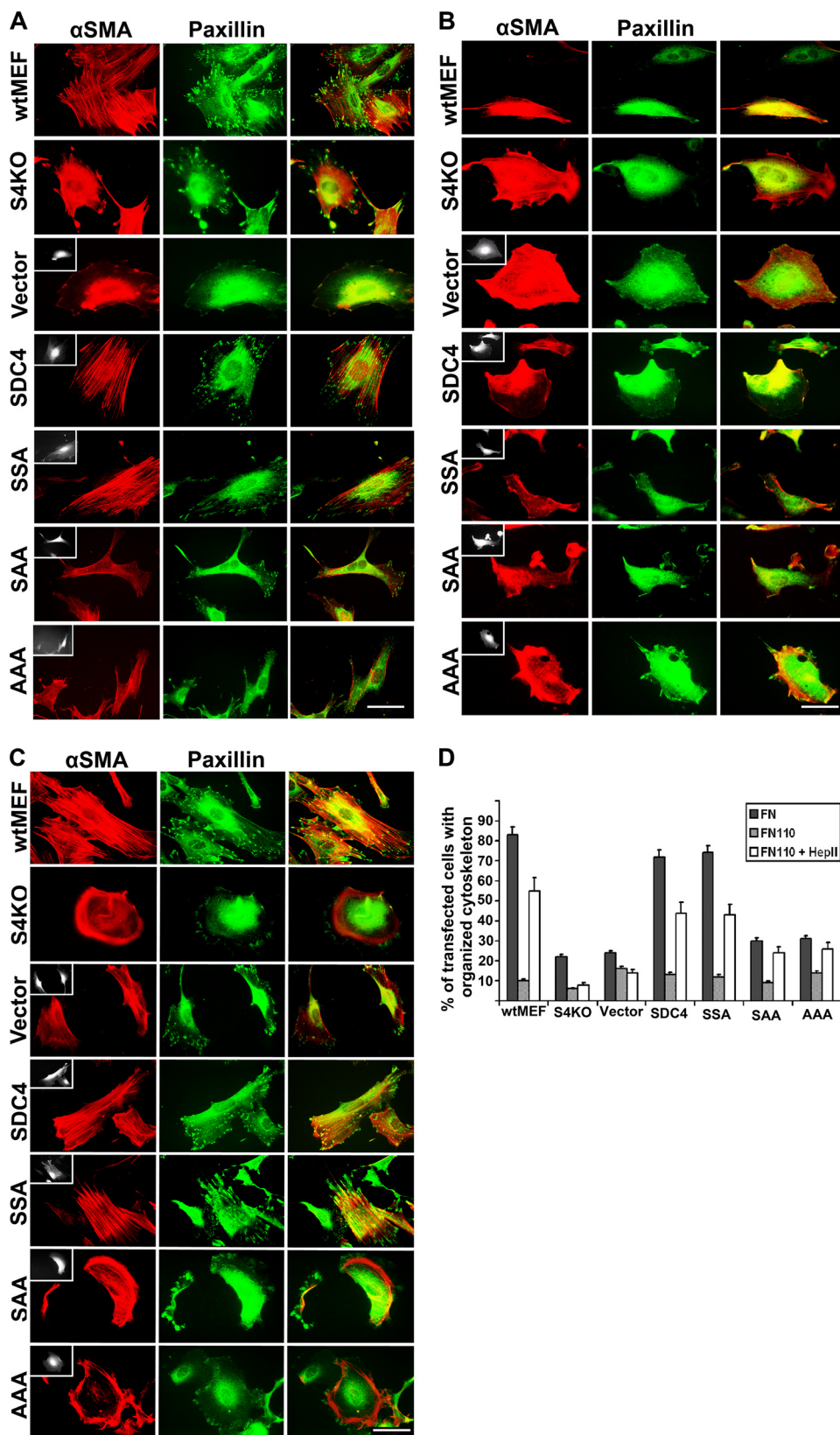
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expressing two chains, these having larger focal adhesions (Fig. 3).

As a further test of heparan sulfate chain number and valency in cell-matrix interactions, mouse fibroblasts expressing only one chain per syndecan-4 core protein (*i.e.* SAA, ASA, and AAS forms) were clustered with a core protein-specific polyclonal antibody, directed to the N-terminal region, before fixation and staining. In no case was a wild-type phenotype reconstituted. Moreover, the syndecan-4 remained diffusely present on the cell surface with no evidence of clustering (not shown). In contrast, where both a primary anti-syndecan-4 antibody and a secondary antibody were sequentially added to cells expressing single serine syndecan-4 constructs, a restoration of cytoskeletal phenotype was seen in many cells (Fig. 4, A–I and M). Here, syndecan-4 was clustered by the double layer of antibody. Once again, it did not matter in which position the serine residue was present,  $\alpha$ SMA incorporation into microfilament bundles was induced by the clustering treatments. Because it has been reported that clustering of syndecan-4 expressed in a CHO mutant lacking heparan sulfate synthesis could lead to microfilament bundle and focal contact formation (12), a similar experiment was carried out with a AAA mutant form of syndecan-4 transfected into null cells. Whereas the effects were weaker, nevertheless, some focal adhesion and  $\alpha$ SMA-positive microfilaments were observed (Fig. 4, J–M).

It has been reported previously, that syndecan-4-null cells, when spread on the central 110-kDa integrin-binding portion of fibronectin, are unresponsive to the addition of the HepII domain (20). We have shown that this heparin-binding domain of fibronectin can promote focal adhesion assembly through syndecan-4 (4). As a further test of the heparan sulfate requirements in syndecan-4, cDNAs encoding syndecan-4 with varying heparan sulfate chain number were transfected into null cells, and then seeded on fibronectin integrin-binding do-

main. Their ability to promote focal adhesion assembly and  $\alpha$ SMA incorporation into stress fibers in response to the HepII domain was assessed. Consistent with data shown above, syndecan-4 with only one serine-glycine site for heparan sulfate





substitution was not able to restore a wild-type phenotype in null cells (Fig. 5), while wild-type syndecan-4 or mutants lacking only one substitution site, were responsive to the HepII domain.

*Heparan Sulfate Chain Length Is Unaltered Where Chain Number Is Reduced, but Sulfation Density Can Vary*—An important factor where glycosaminoglycan chain number was manipulated, was to ascertain heparan sulfate chain size, because this might affect migration on SDS-PAGE as well as the biological responses of cells expressing the chains. This was determined for each mutant and wild-type syndecan-4 expressed in COS7 cells by immunoprecipitation with HA-specific antibodies, then gel chromatography of the glycosaminoglycan chains released by alkaline elimination (Fig. 6). Regardless of whether the chains were derived from wild-type or mutant forms of syndecan-4 with reduced chain number, chain size was consistent and within the range of 36–42 kDa. This corresponds well with previous data from cell surface proteoglycan analyses (29).

The properties of the heparan sulfate chains from each of the expressed syndecan-4 molecules were also analyzed by gradient anion exchange chromatography. Here differences in elution profile were noted, indicating variation in sulfation density that depended on chain number and their origin. Chains from wild-type syndecan-4 transfectants had the highest charge density, equaled by chains from the AAS mutant. Those with the lowest charge density originated from the SAA mutant form of syndecan-4, with others being intermediate. This interesting variation in chain characteristics appeared to have two underlying themes. Generally, chain number was proportional to charge density. For example SAS-derived chains had higher charge density than those from SAA, while SDC4 had the highest density. The other consideration is position of the chain on the core protein. Chains closer to the N terminus, particularly serine 44, had lower charge density than more membrane-proximal chains, e.g. on serine 67. Charge density was in the order SAA < ASA < AAS. However, it was also the case that chains from SAS, for example, were intermediate in charge density between SAA and SDC4 (or AAS), but only present as a single population, not two populations of lower and higher charge density.

In further experiments, the glycosaminoglycans from SAA, ASA, AAS, ASS, and wild-type syndecan-4 from 35sulfate-labeled cultures were treated with chondroitinase ABC, heparinase III, or nitrous acid (pH 1.5). The results showed that chains from AAS and wild-type syndecan-4 were close to 100%

heparan sulfate, but the others had varying chondroitin/dermatan sulfate content (Fig. 7). All populations contained heparan sulfate cleaved by nitrous acid, generating mostly disaccharides and tetrasaccharides, therefore indicating the presence of N-sulfate. The heparinase III degradation profiles were broadly similar for chains from each form of syndecan-4. However, tetrasaccharides and larger saccharides were not as abundant in chains derived from the SAA and ASA mutants of syndecan-4 compared with heparan sulfate from other mutants or wild type (Fig. 7).

This has implications for the regulation of heparan sulfate synthesis, but does not, in fact, bear on syndecan-4 function in regulation of the cytoskeleton. This is because two-chain forms of syndecan-4 were functional, even though the sulfation was less than in the wild type. Similarly, AAS forms of syndecan-4 were not functional, unless clustered by antibodies, yet had similar charge density and heparan sulfate characteristics to chains derived from the SDC4 (wild type).

## DISCUSSION

Syndecan-4 is unique among the four mammalian family members in being a focal adhesion component and promoting their assembly in response to heparin-binding domains of fibronectin (3, 4). All syndecans have been reported to associate with the actin cytoskeleton, and interactions with  $\alpha$ -actinin, cortactin and ezrin have been reported (3, 6, 8). Moreover, signaling to second messengers has been demonstrated, most notably in syndecan-4 where in the presence of PtdIns4,5P<sub>2</sub> and protein kinase C $\alpha$ , downstream signaling to RhoGTPases occurs (3, 5, 6, 8). Evidence from *in vivo* and *in vitro* studies suggests a role for syndecan-4 in cell adhesion and particularly, cell migration (18, 19, 24). This is coupled to the fact, shown before and here, that there is a distinction in actin cytoskeleton organization when wild-type and syndecan-4-null cells are compared (21). The incorporation of  $\alpha$ SMA into stress fibers appears to be syndecan-4 dependent, and may indicate a decreased ability of null cells to exert tension on extracellular matrix substrates (8, 31). Furthermore, previous data (32), and that presented here, suggest that syndecan-4 can influence FAK activity. Wild-type syndecan-4 when expressed in null cells had higher levels of FAK tyrosine 397 phosphorylation than the parent null cells, or those cells expressing syndecan-4 with only one glycosaminoglycan chain. FAK activity data were commensurate with the extent of focal adhesion assembly. How syndecan-4 regulates FAK activity is not known, but may be indirect,

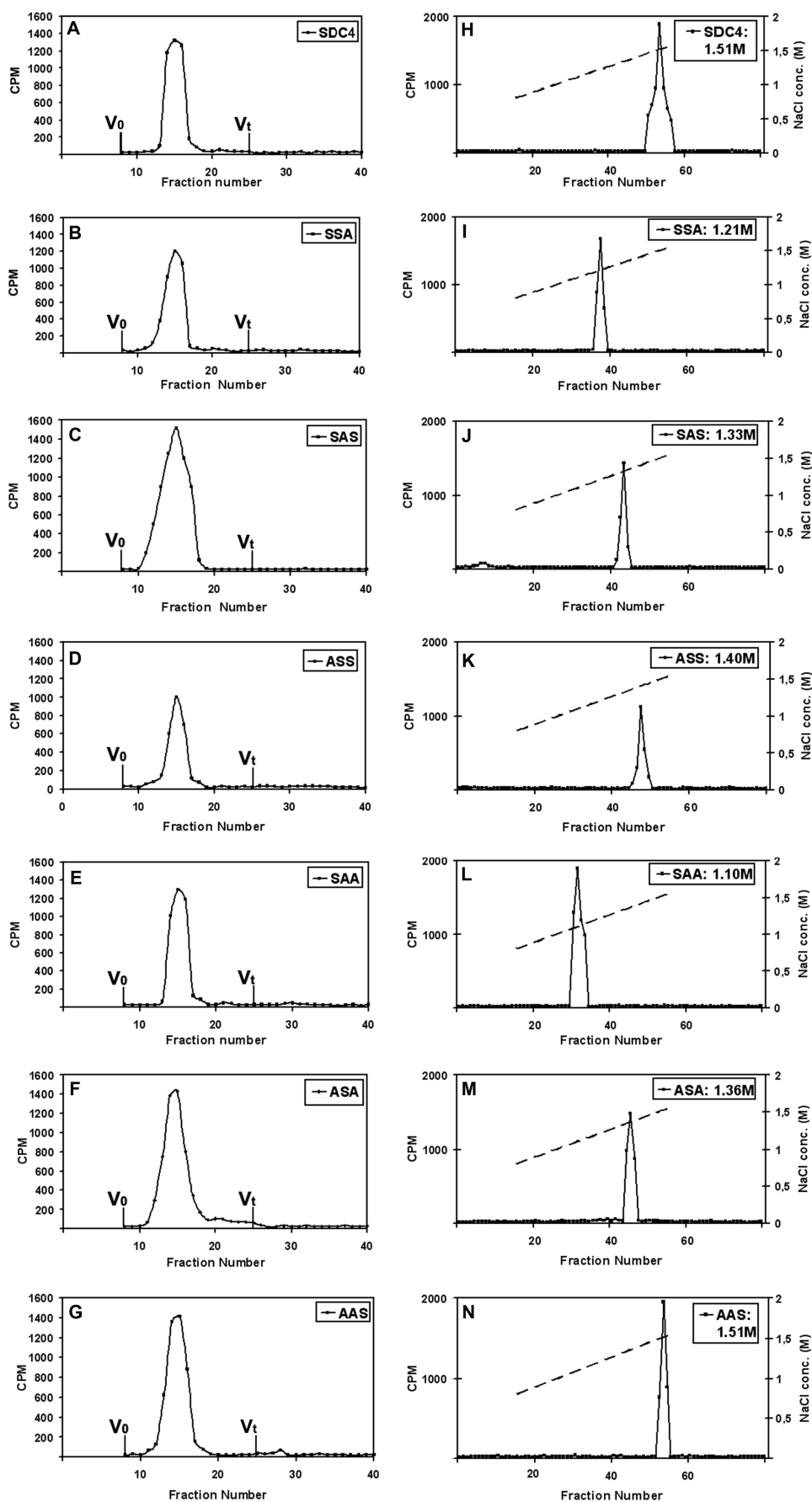
**FIGURE 5. Syndecan-4 with multiple heparan sulfate chains can signal focal adhesion assembly in response to the HepII domain of fibronectin.** Wild-type and untransfected or transfected syndecan-4-null mouse fibroblasts were seeded on coverslips coated with whole FN (A) or its integrin-binding 110-kDa fragment (FN110; B) incubated for 3–4 h and stained for paxillin and  $\alpha$ SMA. On fibronectin, the wild-type mouse fibroblasts (*wtMEF*) and syndecan-4 knock-out MEFs transfected with multiple heparan sulfate chain forms of syndecan-4 (SDC4 and SSA) had well developed focal adhesions with  $\alpha$ SMA incorporated into stress fibers. The syndecan-4-null cells (*S4KO*) and those expressing syndecan-4 with a single (SAA) or no (AAA) heparan sulfate chains had reduced  $\alpha$ SMA organization. B, none of the fibroblasts plated on FN110 alone were able to assemble a well organized actin cytoskeleton. C, same fibroblast populations were seeded on coverslips coated with FN110 fragment but after 3–4 h, fibronectin HepII domain was added for a further 1–2 h. Cytoskeletal organization was analyzed by staining for paxillin and  $\alpha$ SMA. The addition of HepII enabled the wild-type fibroblasts (*wtMEF*) and the null cells expressing syndecan-4 with two (SSA) or three heparan sulfate chains (SDC4) to organize the actin cytoskeleton and substantial focal adhesions. Syndecan-4-null cells and those expressing syndecan-4 with one or no heparan sulfate chains responded weakly or not at all. For all experiments a control transfection was performed using empty vector. Where applicable, transfected cells are shown as insets by their GFP expression from the bicistronic vector. Quantitative analysis is shown in D where >100 cells from each condition were counted,  $\pm$  S.E. from triplicate experiments. The scale bar is 50  $\mu$ m.

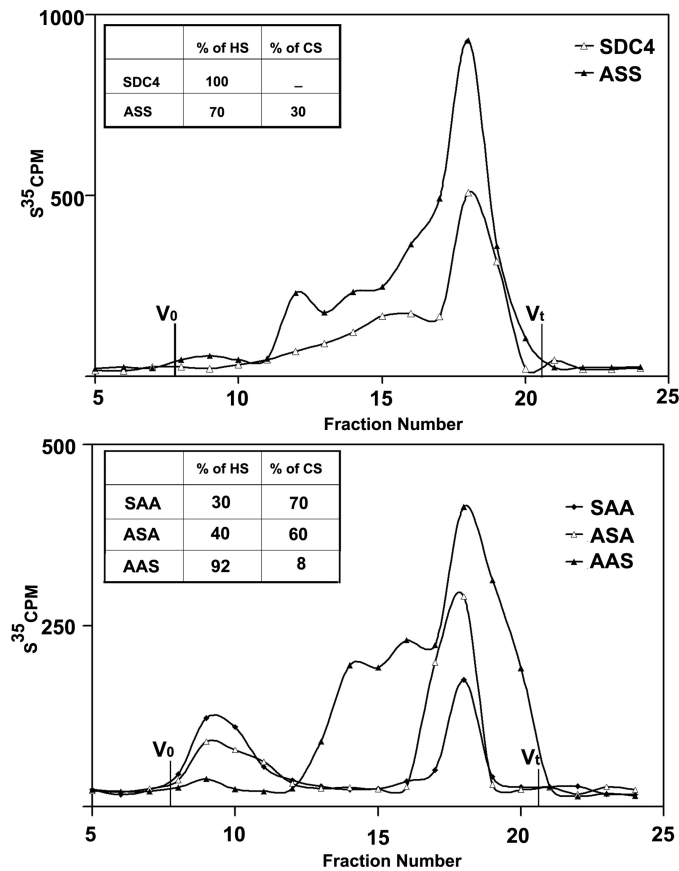
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because no direct interaction between these two molecules has been recorded.

Taken as a whole, the data here support a hypothesis that valency is an important consideration in heparan sulfate-ligand interactions. Forms of syndecan-4 that possess only one chain were unable to reconstitute a wild-type cytoskeletal phenotype when expressed in null cells, unlike wild-type syndecan-4 or forms lacking a single serine for heparan sulfate substitution. The lack of response, could, however, be overcome by antibody-mediated clustering of single chain forms. This suggests that receptor clustering is an important facet of syndecan-4 biology. Whether binding of HepII domains of fibronectin to heparan sulfate is positively co-operative as has been shown for another heparan sulfate-binding protein, FGF-2 (33), is not known but of interest. Previous affinity co-electrophoresis showed that single heparan sulfate chains have a >2-fold lower affinity for fibronectin, than do whole proteoglycans from the same rat fibroblast syndecans (19).

In addition, the native state of syndecan-4 core protein impacts our understanding of the process. Our previous NMR spectroscopy data revealed that the cytoplasmic domain of syndecan-4 forms twisted clamp dimers (34, 35). In addition, the transmembrane domains of all syndecans form homodimers, due in part to the GXXXG motif in the outer half of the putative transmembrane domains (10). As a result, all syndecans when prepared as core proteins resolve as dimers on SDS-PAGE. This very strong tendency to dimer formation probably means that all syndecans are homodimeric in their native state (3, 11). Furthermore, single chain forms of syndecan-4 were not competent to restore the wild-type phenotype, even though they would also form dimers, given that the transmembrane and cytoplasmic domains were unaltered (*i.e.* wild-type). Therefore two chains are





**FIGURE 7. Ectopically expressed wild-type and mutant syndecan-4 possesses heparan sulfate sensitive to heparinase III.** Syndecan-4 purified from transfected COS-7 cells was analyzed for susceptibility to chondroitinase ABC, nitrous acid at pH 1.5 (quantitated and combined in the inset boxes) and heparinase III. In the upper panel, results from the wild-type and ASS mutant are shown, in the lower panel results from the three single chain mutants are shown. The chromatograms are Superdex 75 elution profiles of the <sup>35</sup>S<sub>2</sub>O<sub>4</sub>-labeled heparan sulfate chains after heparinase III treatment. The V<sub>0</sub> and V<sub>t</sub> are marked, as is the elution position of a heparin disaccharide standard. All preparations contained heparan sulfate, sensitive to nitrous acid, and heparinase III. The proportion of chondroitin/dermatan sulfate varies, and is very low in the case of wild-type syndecan-4 and AAS mutant. Most heparinase products elute as disaccharide and tetrasaccharide, with varying amounts of larger products. Most similar to the wild type is the heparan sulfate from the AAS mutant, although the latter does not restore a wild-type phenotype unless clustered by antibody (see Figs. 2–6).

probably insufficient when on separate core proteins, but two chains on a single core protein are functionally competent. These presumably also form dimers which, therefore, have four chains. This would explain why clustering of single chain forms, presumably on dimeric core proteins, into tetramers or higher order oligomers, was functional. Again, a minimum of four chains would be brought into play.

Until structural resolution of the extracellular domains of syndecans is available, it may be difficult to ascertain why a minimum of four chains is required for signal transmission.

The core proteins have no obvious domains or motifs suggestive of their conformation. However, the core protein extracellular domains are functional, supporting integrin-mediated cell adhesion (13, 14). In the case of syndecan-4, a region of the ectodomain, including an NXIP motif can, when used as a substrate (*i.e. in trans*), promote  $\beta$ 1 integrin-mediated adhesion (13). However, this is cell-type restricted, being common to mesenchymal, but not epithelial cells. This and other evidence suggests that syndecan-4 does not interact directly with integrin. In experiments reported here, AAA forms of syndecan-4 do not spontaneously promote  $\alpha$ SMA incorporation into microfilament bundles. Therefore, cis interactions of syndecan-4 appear to require heparan sulfate in responses to fibronectin (3, 36). Also key to these interactions is the syndecan-4 cytoplasmic domain. Truncation and tyrosine mutations show that a wild-type phenotype, even when three heparan sulfate chains are present, cannot be achieved without a functional V region of the cytoplasmic domain. This is consistent with signaling through protein kinase C $\alpha$  (5, 25). Syndecan-4 interactions with PDZ proteins through its C-terminal FYA motif (30) appear, however, to be dispensable, at least for normal fibroblast-matrix adhesion and actin cytoskeletal organization.

It has been shown *in vivo* and *in vitro*, that high affinity interactions between heparan sulfate and the HepII domain of fibronectin require N-sulfation, and S-domains of 12 sugar residues or more (15). Under these conditions, syndecan-4 signaling to the cytoskeleton resulted. Heparan sulfate chains from wild-type and mutant forms of syndecan-4 with reduced HS chain number were analyzed but no significant differences in HS chain length were detected, whether derived from one, two, or three chain variants of syndecan-4. However, there was interesting variation in sulfation, as judged by gradient anion exchange chromatography. The further from the N terminus, and the higher the chain number, the higher the overall sulfation of the chains. However, the heparan sulfate chains from the AAS mutant had as high charge density, and similar elution profiles after nitrous acid and heparinase III treatments as the wild-type chains, so that the results, while intriguing, seem not to bear on the role of multiple heparan sulfate chains in cell adhesion. Moreover, antibody clustering of any of the single chain bearing mutants of syndecan-4 led to increased organization of  $\alpha$ SMA into stress fibers. In this context, we have shown previously that N-sulfate content of heparan sulfate is important in syndecan-4-mediated cell adhesion (15). All the heparan sulfate samples, regardless of which mutant of syndecan-4 from which they were obtained, were degraded similarly by nitrous acid at pH 1.5, confirming the presence of N-sulfate.

**FIGURE 6. Heparan sulfate chains from recombinant syndecan-4 are of similar size but different charge characteristics depending on the number of chains and site of substitution.** Transfected COS7 cells were metabolically labeled with <sup>35</sup>S<sub>2</sub>O<sub>4</sub> and the syndecan-4 purified by immunoprecipitation with anti-HA antibodies. Glycosaminoglycan chains were cleaved from the core protein and applied to gel filtration and ion exchange columns. In each case, the chains are of similar length regardless of whether derived from syndecan-4 bearing 1, 2, or 3 chains or their position on the core protein (A–G). However, ion exchange chromatography showed that the net charge characteristics of the chains on syndecan-4 depends both on their number and their position on the core protein (H–N). Chains substituted toward the N-terminal of core protein (L) are less charged than those more C-terminal (M and N). However, when multiple chains were expressed on the core protein, the charge density of all chains was proportionally higher (H–K). The salt concentrations corresponding to peak elution of each sample are noted on each panel.

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In summary, syndecan-4 has a role in focal adhesion and stress fiber assembly by virtue of at least two important properties, multiple heparan sulfate chains, and a functional cytoplasmic domain. This may regulate migration, but also the strength of cell adhesion, because the loss of  $\alpha$ SMA from stress fibers accompanies the knock-out of this proteoglycan.

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