Biosynthetic Mechanisms for the Addition of Polylactosamine to Chondrocyte Fibromodulin*

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The cartilage matrix glycoprotein fibromodulin contains four N-linked glycosylation sites which act as acceptors for the addition of sulfated polylactosamine (keratan sulfate). In the present study, we examined the biosynthetic processing of these N-linked oligosaccharides for subsequent addition of polylactosamine. Chondrocytes were treated with castanospermine, 1-(+)-deoxymannojirimycin, and swainsonine, radiolabeled with [3,4,5,6-3H]leucine, [2-3H]mannose, or [6-3H]glucosamine, and newly synthesized fibromodulin was immunoprecipitated for analysis. Castanospermine and 1-(+)-deoxymannojirimycin inhibited polylactosamine addition, whereas swainsonine was not effective. This indicated that the linkage regions must be processed to GlcNAc(Man)2(GlcNAc)2Asn but do not require further modification to GlcNAc(Man)3(GlcNAc)2Asn. In both control and swainsonine-treated cells, one or two N-linked oligosaccharides per molecule were modified with polylactosamine containing 4–6 repeating disaccharide units. Moreover, a single short chain was added either to the C-3 or the C-6 branch in control cultures, whereas only the C-3 branch was substituted in the presence of swainsonine. Analysis of endo-β-galactosidase and keratanase II digestion products of the polylactosamine chains synthesized in both culture conditions showed that only about 25% of the hexosamine residues and less than 5% of the adjacent galactose residues were substituted with sulfate. These findings are discussed in relation to the regulation of fibromodulin glycosylation and the likely influence of polylactosamine structure on the extracellular interactions and turnover of fibromodulin.

Fibromodulin (FM)1 is a collagen-binding glycoprotein widely distributed in connective tissues (1). Its 39-kDa core protein (2) contains five N-linked glycosylation sites, at least four of which can be modified in a developmentally regulated manner with polylactosamine (Galβ1-4GlcNAcβ1-3), of variable length and sulfation (3, 4). In general, the unsulfated polylactosamine chains appear to be short (about 2–6 disaccharide units), whereas chains containing sulfated galactose and/or glucosamine (keratan sulfate, KS) are longer (about 14–27 disaccharide units) (3). The majority of the FM in cartilages from fetal and growing animals is substituted with KS, whereas it appears that only a minor portion of the molecules present in adult cartilages are in this sulfated proteoglycan form (4).

The capacity of the FM core protein to interact with collagen fibrils (5) probably indicates a critical function for this molecule in the organization of collagenous matrices. FM is closely related to lumican, a small KS proteoglycan, found associated with the collagen network, in both the cornea (6–9) and aorta (10); the two molecules are similar in core protein structure, in the number and location of the N-linked oligosaccharides (11), and in the presence of N-linked KS (18). The proposed function of lumican in the regulation of collagen fibril size in the cornea is not influenced by the presence of KS chains, but apparently resides solely in the core protein (32). On the other hand, certain corneal dystrophies have been identified which appear to be linked to the absence of sulfate or altered sulfation patterns of lumican KS (9).

The influence of unsulfated polylactosamines on the interaction of cells with matrix has now been widely studied in the areas of embryogenesis (15, 16) and metastasis (17). For example, N-linked oligosaccharides on lamp-1 and -2 (31, 32), laminin (34), fibronectin (25, 35), and band-3 erythrocyte glycoprotein (23) all carry modified polylactosamines which appear to act as ligands for cell adhesion molecules such as the selectins; moreover, alterations in their structures have been shown to influence these interactive properties (36, 37). Structural analyses have shown that polylactosamine extensions on these molecules can occur on both the C-3 and C-6 branches of complex type N-linked oligosaccharides, whereas structures such as the extended Lewis antigens appear to be confined to the C-6 branch (23, 38). Addition of branched unsulfated polylactosamines to N-linked oligosaccharides has been demonstrated with cultured cells from a range of sources (21–23, 29), and in many cases the synthesis of polylactosamine appears to be critically dependent on the action of the glucosaminyl transferase V (19), which modifies the processed C-6 branch to a form suitable for such polymer addition (38).

Information on the structure and biosynthesis of KS on lumenic and fibromodulin is very limited. Structural analyses of the linkage region of purified KS from monkey corneal lumican has indicated that the linear polymer extensions are present on both the C-3 and C-6 branches (12). In contrast, Stuhlflatz and colleagues have proposed a model for pig (13) and bovine (14) lumican, in which only the C-6 branch is substituted with KS. Biosynthetic data has been difficult to obtain in this area, due to the general finding that culture of mammalian and chick corneas results in a rapid decrease in the synthesis of lumican substituted with KS (9). In a recent paper (20) corneal stromal fibroblasts in culture were shown to synthesize lumican on which the N-linked oligosaccharides were extended with polylactosamine; however, no details of N-linkage processing or polymer structure were reported.

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‡ The abbreviations used are: FM, fibromodulin; KS, keratan sulfate; CSP, castanospermine; DMJ, 1-(+)-deoxymannojirimycin; SWN, swainsonine; TM, tunicamycin.

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The present study describes calf chondrocyte cultures in which FM, substituted with polylactosamine, continues to be examined over prolonged culture periods. This system has allowed an examination of branch specificity for the addition of polylactosamine structure on chondrocyte FM, and an assessment of the effects of variable glycosylation on the deposition of FM in the newly formed collagen rich extracellular matrix.

MATERIALS AND METHODS


d(2-[3H]mannose (15.4 Ci/mmol) was from Amersham Corp., d-[6-3H]glucosamine (40.4 Ci/mmol) and t-[3,4,5-3H]leucine (155 Ci/mmol) purchased from DuPont NEN. Protein A-Sepharose was from Sigma. Freund’s complete adjuvant was from CalBiochem and Freund’s incomplete adjuvant from Difco. CSP, DMJ, and SWN were from Genzyme, Cambridge, MA. Endo-β-galactosidase (Escherichia freundii) was from ICN, Irvine, CA; keratanase II and KS (bovine cornea) were from Kresse, University of Miinster, Germany. Antisera Production—FM was purified from calf epiphyseal cartilage as previously described (3). Rabbits were injected with 200 µg of core protein equivalents (determined by the Micro BCA Protein Method (Pierce Chemical Co.) in complete Freund’s adjuvant. Booster injections of 100 µg in incomplete adjuvant were given at two 3-week intervals. Antiserum with the highest titer were obtained in 2 months following the final booster injection. These were used in the experiments described in this report.

Specificity of the antisera (B’P 10.90) was assessed by Western blotting of 4 µg guanidine HCl extracts of calf and steer cartilage and immunoprecipitation of [3H]leucine-labeled chondrocyte medium and cell extracts (see Fig. 1). Additional antisera [1209, 1210] showed no reactivity in direct enzyme-linked immunosorbent assay using chondroitin-ABC treated or naive bovine aggrecan, bovine decorin, or biglycan.

Cell Culture and Radiolabeling—Chondrocytes were isolated and cultured from the metacarpalphalangeal joints of 3-4-week-old calves as described earlier (3). Inhibitors were added to the cultures on day 6 at the following concentrations: 200 µg/ml CSP, 790 µg/ml DMJ, 10 µg/ml SWN, or 3 µg/ml TM. After 8 h of pretreatment with CSP, DMJ, or SWN, and 3 h with TM, cultures were changed into media containing 1.3 mg/ml leupeptin, 0.25 µg/ml antipes antipes, and 60 µg/ml d-[3H]glucosamine, 80 µCi/ml d-[3H]mannose, or 60 µCi/ml [3H]leucine, and incorporation was allowed to proceed for 16 h.

Immunoprecipitation of Radiolabeled FM from Chondrocyte Cultures—Following incubation with radioisotopes, medium was removed and the cell layer was washed with 2 ml of medium and kept on ice. All samples were adjusted to contain 0.4% (v/v) SDS, 100 mM HEPES, and protease inhibitors (3), heated at 100 °C for 15 min, then diluted to a final volume of 4.5 ml with 20 µl Tris-HCl, 0.5 mM sodium chloride, pH 7.5, and adjusted to 1% (v/v) Nonidet P-40. 500 µl portions of medium or cell extracts were incubated with 10 µl of antiserum at 4 °C for 18 h. Protein A-Sepharose (200 µl of a 50% suspension in 20 ml Tris-HCl, 0.5 mM sodium chloride, pH 7.5, 1% (v/v) Nonidet P-40) was added and incubated for 2 h at 4 °C. Supernatants were removed and the Protein A pellets were washed five times with 2 ml of 20 mM Tris-HCl, 0.5 mM sodium chloride, pH 7.5, 1% (v/v) Nonidet P-40 and twice with 2 ml of water. Reincubation of supernatants with antisera did not result in any additional precipitation of fibromodulin. Decorin was immunoprecipitated from medium samples using the above protocol with an antiserum to bovine decorin (gift from Dr. H. Kresse, University of Miinster, Germany).

Analysis of Released Oligosaccharides—FM was immunoprecipitated from the medium of [2-3H]mannose-labeled cells. The Protein A beads were washed and digested under sterile conditions for 36 h at 37 °C with 2 units of N-glycanase in 0.2 ml of 0.2 mM sodium phosphate, pH 8.6. Beads were pelleted and washed once with 1 ml of water. Portion of FM immunoprecipitated from radioactivity was then associated with the beads following incubation with N-glycanase, whereas after incubation in the absence of enzyme, greater than 98% of the radioactivity was recovered with the beads.

Released oligosaccharides were concentrated and applied to a Bio-Gel P-10 column (0.6 x 120 cm, in 0.5 mM ammonium acetate, pH 7.0). Portions of the collected fractions were assayed for radioactivity, and appropriate peaks were pooled. These were dried in a Speedvac and resuspended in 0.2 ml of 0.1 M ammonium acetate from Difco. CSP, DMJ, and SWN were from Genzyme, Cambridge, MA. Endo-β-galactosidase (Escherichia freundii) was from ICN, Irvine, CA; keratanase II and KS (bovine cornea) were from Seikagaku America Inc., Rockville, MD; α-mannosidase (jack bean) was from Oxford Glycosystems, Rosedale, NY; neuraminidase (Arthrobacter ureafaciens), N-glycanase (peptide N-glycosidase F), and endo-betahexosaminidase H were from BioScience Mannheim. Bio-Gel P-6 (200-400 mesh) and P-10 (200-400 mesh) were from Pharmacia LKB Biotechnology Inc., TyoPearl HW40S from Toyobo, Tokyo, Japan, and the CarboPac column from Dionex, Sunnyvale, CA. All other chemicals were of the highest chemical purity available.

In addition, 50-µg portions of corneal KS in 50 µl of 50 mM sodium acetate, pH 6.8, were digested sequentially with 5 units of endo-β-galactosidase and 5 units of keratanase II. The ultrafiltered digestion products were reduced as described above for the cell products but in the absence of NaBH4 (specific activity, 124.5 Ci/mmol). Reduced radiolabeled oligosaccharides were applied to TyoPearl HW40S (HR 16/50) eluted at 0.8 ml/min in 0.5 M ammonium acetate, pH 7.0, 0.5 ml fractions were collected and assayed for radioactivity, and appropriate fractions were combined (see Figs. 5-6). Ammonium acetate was removed by Speedvac evaporation before further analysis of these oligosaccharides on CarboPac PA1. The HW40S column was calibrated by determining the elution positions, by uronic acid assay (43) of reduced preparations of chondroitin-4-sulfate disaccharide (chondroitinase ACII digest of rat chondrosarcoma aggrecan), and hyaluronan tetra- and hexasaccharides (hyaluronan digest of Healon®).

High Performance Liquid Chromatography Analysis of Oligosaccharides on CarboPac PA1—Portions of oligosaccharides separated on Toyopel HW40S were eluted in 50 µl of water and applied to CarboPac PA1, equilibrated in 20 mM NaOH, 1 mM NaCl. A 10-min wash with the starting buffer, a linear gradient of NaCl from 1 mM to 200 mM was applied over 50 min followed by a 10-min wash with 1 mM NaCl in 20 mM NaOH. The column was eluted at 1 ml/min, and 1-ml fractions were analyzed for radioactivity. Recovery of radiolabeled oligosaccharides was in excess of 95% for all analyses.

RESULTS

Synthesis of Fibromodulin by Articular Chondrocytes Maintained in Monolayer Culture—High density chondrocyte cultures were labeled for 16 h with [3H]leucine after 2, 7, 14, and 21 days of culture, and [3H]FM was quantitated by immunoprecipitation from both medium and cell extracts. Total synthesis of FM core protein was maintained at a high rate (about 0.85 x 10⁹ cpm/35-mm dish culture) over this period, and at all times greater than 90% of the newly synthesized FM was present in the culture medium. Immunoprecipitates from both medium (Fig. 1A) and cell layer (Fig. 1B) were separated by electrophoresis and visualized by fluorography. The fluoro-
grams show that at all times, up to 21 days of culture, the chondrocytes continued to synthesize FM which migrated as a diffuse band with an apparent molecular mass between about 60 and 95 kDa. This is similar to the electrophoretic properties of FM extracted from calf epiphyseal cartilage which is substituted with KS (3). In addition, FM synthesized at all culture times was sensitive to endo-\(\beta\)-galactosidase digestion, as shown by the change in electrophoretic properties (Fig. 1, lane 8 compared to lane 7), confirming that polylactosamine addition to FM is maintained for at least 21 days in this culture system.

**Processing of N-linked Oligosaccharides for Addition of Polylactosamine**—To assess whether the addition of polylactosamine to FM takes place after the processing of N-linked oligosaccharides by established intracellular pathways (28), chondrocytes were cultured in the presence of CSP, DMJ, and SWN, inhibitors of specific N-linked oligosaccharide processing enzymes (29). Additional cultures were treated with TM, which had previously been shown to completely prevent KS addition to FM (3). Inhibitor treated cultures were radiolabeled with \(^{3}H\)leucine, \(^{3}H\)glucosamine, and \(^{3}H\)mannose prior to immunoprecipitation of FM with polyclonal antibody BF10.90. Table I shows the effect of the inhibitors on the 16-h incorporation of \(^{3}H\)leucine, \(^{3}H\)glucosamine, and \(^{3}H\)mannose into total FM (cell layer plus medium). CSP, DMJ, and SWN did not markedly alter the rate of \(^{3}H\)leucine incorporation into FM by these cells, consistent with their specificity of action and absence of any cytotoxic effects at the concentrations used here. TM, however, inhibited \(^{3}H\)leucine incorporation into FM by about 35%, presumably due to known toxic effects of this compound on cells (29).

Incorporation of \(^{3}H\)mannose into FM was decreased to less than 1% of the control values in the presence of TM, consistent with the expected profound inhibition of formation of dolichol phosphate-bound intermediates. In the presence of CSP and DMJ, the incorporation of the radioisotope was 131% and 100% of control, respectively, so that the labeling ratio of \(^{3}H\)mannose to \(^{3}H\)leucine was slightly elevated or similar to control in the presence of these compounds (Table I). In the presence of SWN, incorporation of \(^{3}H\)mannose was slightly elevated, resulting in a 1.07-fold increase in the \(^{3}H\)mannose:\(^{3}H\)leucine ratio over control. From the studies of Foddy et al. (31) who examined the structure of N-linked oligosaccharides secreted in the presence of SWN, the \(^{3}H\)mannose:\(^{3}H\)leucine ratio should have been increased 1.66-fold, to account for the presence of (Man\sb{3})\sb{4} hybrid on FM synthesized by SWN-treated cells compared to the (Man\sb{3})\sb{4} complex structure on controls. This discrepancy was explained in the present work by monosaccharide analysis of the \(^{3}H\)mannose-labeled oligosaccharides (Table II). This showed that in culture controls about 25% of the radioactivity incorporated from \(^{3}H\)mannose into FM was actually present in \(^{3}H\)glucose residues; further, this value was reduced to about 4% in the presence of SWN. When the extent of fucose labeling was taken into account, the true \(^{3}H\)mannose:\(^{3}H\)leucine ratio in the presence of SWN was found to be 1.51-fold higher than control, which agrees well with the theoretical value (31).

TM, CSP, and DMJ also profoundly inhibited the incorporation of \(^{3}H\)glucosamine into FM, resulting in a marked reduction (from 0.51 to 0.0014, 0.1, and 0.054, respectively) in the \(^{3}H\)glucosamine:\(^{3}H\)leucine labeling ratios (Table I). In contrast, SWN produced a minimal decrease (from 0.51 to 0.44) in the \(^{3}H\)glucosamine:\(^{3}H\)leucine ratio.

To interpret the variable effects of these inhibitors on structure of the newly synthesized FM-oligosaccharides, \(^{3}H\)leucine-labeled and immunoprecipitated FM from the medium and cell layers was examined by electrophoresis and fluorography. Polylactosamine-substituted FM from both culture compartments migrated as a diffuse band between 60 and 90 kDa (Fig. 2, a and b, lanes 7). The marked reduction in polylactosamine synthesis in the presence of both CSP and DMJ (Table I) was reflected in the finding that the products isolated from CSP-treated (Fig. 2, a and b, lanes 1) and DMJ-treated (Fig. 2, a and b, lanes 3) cultures were considerably smaller than the molecules secreted from control cultures (Fig. 2, a and b, lanes

| Table I | Incorporation of radiolabeled precursors into FM synthesized in the presence of N-linked oligosaccharide processing inhibitors |
|---------|------------------------------------------------------------------------------------------------|
| **Assay** | **\(^{3}H\)Leucine** | **\(^{3}H\)Mannose** | **\(^{3}H\)Glucosamine** | **\(^{3}H\)Mannose/[\(^{3}H\)Leucine** | **\(^{3}H\)Glucosamine/[\(^{3}H\)Leucine** |
| Control | 1.70 ± 0.03 | 0.16 ± 0.01 | 0.87 ± 0.01 | 0.094 | 0.510 |
| Tanicamycin (3 μg/ml) | 0.83 ± 0.07 | 0.009 ± 0.001 | 0.0012 ± 0.0001 | 0.001 | 0.0014 |
| CSP (200 μg/ml) | 1.90 ± 0.07 | 0.21 ± 0.01 | 0.19 ± 0.009 | 0.110 | 0.100 |
| DMJ (700 μg/ml) | 1.78 ± 0.10 | 0.16 ± 0.011 | 0.10 ± 0.011 | 0.089 | 0.054 |
| SWN (10 μg/ml) | 1.88 ± 0.06 | 0.19 ± 0.017 | 0.82 ± 0.021 | 0.101 | 0.441 |

* The data shown are the sum of radiolabeled immunoprecipitable FM present in the medium and cell extract. These results represent the mean ± S.D. from triplicate plates from two separate cell preparations.
7). In addition, digestion with endo-β-galactosidase only slightly altered the electrophoretic properties of the products formed in the presence of CSP (Fig. 2, a and b, lanes 2) and DMJ (Fig. 2, a and b, lanes 4). Inhibition of glucosidase I (by CSP) and mannosidase I (by DMJ) clearly had occurred in these cultures since the majority of the secreted FM contained only partially processed intermediates (probably (Glcp)Man(GlcNac) or (Man)g(GlcNac)z) which were completely removed by digestion of the intact molecule with endoglycosidase H resulting in the production of a 40-kDa core protein (Fig. 2c, lanes 2 and 4). This presumably represents the completely deglycosylated core protein, since this was also the species secreted by tunicamycin-treated cells (Fig. 2c, lane 9), and it could be generated by N-glycanase digestion of the control product (Fig. 2c, lane 10).

It is, however, evident that CSP was only partly inhibitory under the conditions used since some polylactosamine-substituted FM was detected in its presence (Fig. 2, a and b, lanes 1). This was presumably present on fully processed N-linked oligosaccharides as shown by their resistance to digestion with endoglycosidase H (Fig. 2c, lanes 2 and 4). This presumably represents the completely deglycosylated core protein, since this was also the species secreted by tunicamycin-treated cells (Fig. 2c, lane 9), and it could be generated by N-glycanase digestion of the control product (Fig. 2c, lane 10).

In contrast to the effects of CSP and DMJ, FM immunoprecipitated from SWN-treated cultures apparently carried a full complement of polylactosamine, since the molecules migrated as a diffuse band between 69 and 90 kDa and were essentially indistinguishable from the control product both in size (Fig. 2, a and b, compare lanes 5 and 7) and sensitivity to endo-β-galactosidase (Fig. 2, a and b, compare lanes 6 and 8). Moreover, only a minimal decrease in the [3H]GlcNAc2:[3H]leucine labeling ratio in the SWN cultures relative to control cells was observed (Table I). Similar results were obtained over a range of SWN concentrations between 1 and 50 μg/ml (data not shown). The lack of effect of SWN on polylactosamine addition to FM was not due to insufficient inhibition of the target enzymes, Golgi α-mannosidase II, since all N-linked oligosaccharides on FM secreted in the presence of SWN were removed by endoglycosidase H treatment (Fig. 2c, lane 6). On the other hand, oligosaccharides on FM from control cultures were totally resistant to endoglycosidase H treatment (Fig. 2c, lane 8), strongly suggesting that under control conditions all N-linked oligosaccharides on FM are fully processed to the complex type.

The absence of an inhibitory effect of SWN on polylactosamine synthesis in the present work was also not simply due to an increase in the specific activity of the UDP-[3H]hexosamine pool; thus it was shown in a separate experiment that the labeling ratio of [3H]glucosamine to [35S]sulfate in the chondroitin sulfate 4 and 6 S disaccharides (44), isolated from agar-acek synthesized in these cultures, was 0.69 in control cells and 0.56 in the presence of SWN.

α-Mannosidase Digestion of N-linked Oligosaccharides—The finding that polylactosamine continues to be added to FM in the presence of SWN in the presence of SWN in the presence of SWN, since the polymer is present on the C-9 branch of the (Man)₉ hybrid structure, because the inhibition of Golgi α-mannosidase II by SWN would be expected (29, 31) to leave the C-6 branch substituted with (Man)₆ and therefore unsuitable for polymer addition.

To confirm that polylactosamine was indeed present on such hybrid N-linkages, FM secreted into the medium of control and SWN-treated cells radiolabeled with 2-[3H]mannose was immunoprecipitated, and the N-linked oligosaccharides were released by N-glycanase. For both control cells (Fig. 3a, top) and SWN-treated cells (Fig. 3b, bottom) the [3H]oligosaccharides were swelled in Bio-Gel P-10 into two populations (see black bars in Fig. 3). Digestion with endo-β-galactosidase followed by Bio-Gel P-10 chromatography (data not shown) indicated that in both cases only the larger population (fractions 25-43) contained polylactosamine. The smaller population is therefore likely to represent the population of unmodified N-linked oligosaccharides.

A comparison of the elution profiles of both populations of oligosaccharides suggested that those formed in the presence of SWN were somewhat smaller than controls, and this would be expected if the C-6 branch in the controls is fully processed to NeuAcα-(Galβ1-4GlcNAcβ1-3Glc)-Galβ1-4GlcNAcβ1-2Man, whereas it would carry only (Man)₆ in the presence of SWN.

Indeed, consistent with this interpretation, oligosaccharides from the SWN-treated cultures exhibited markedly sensitivity to jack bean α-mannosidase (Fig. 3b, bottom panel) whereas oligosaccharides from the control cultures were completely insensitive to this enzyme (Fig. 3a, bottom panel). This result strongly suggests the presence of unsubstituted α-mannose residues on all oligosaccharides secreted in the presence of SWN. When taken together with the known specificity and the demonstrated effect of endo-hexosaminidase H (Fig. 2c, lanes 5 and 6), as well as the known site of action of SWN, these results confirm that in the presence of SWN chondrocytes secrete FM with polylactosamine present only on the C-3 branch of the hybrid N-linked oligosaccharides.

FM Polylactosamine Digestion with Endo-β-galactosidase and Keratanase II—Given that the polylactosamine added in the presence of SWN was on the C-3 branch only, we next examined the possibility that the position of addition (C-3 or C-6 branch) might influence the structure of the polylactosamine formed with regard to size, sulfation pattern, or nature of the non-reducing terminal of the chains. This involved digestion with endo-β-galactosidase and keratanase II of FM immunoprecipitated from cells incubated with [3H]glucosamine, followed by analysis of the free and core-bound oligosaccharide products. Interpretation of these results was based on the reported specificities of the glycosidases; thus endo-β-galactosidase (24) cleaves the Gaβ1-4GlcNAc bond of polylactosamine unless the galactose in the repeating disaccharide is sulfated or is involved in a β1-6GlcNAc branch point; it is also ineffective on the galactose in the last Galβ1-4GlcNAc disaccharide at the non-reducing terminal of the chain (24, 26). Keratanase II cleaves the GlcNAcβ1-3Gal linkage of polylactosamine but only if the glucosamine is sulfated (27).

Incubation with endo-β-galactosidase of immunoprecipitated [3H]glucosamine-labeled FM from control cells liberated only about 20% of the radiolabel from the Protein A. Since these conditions of digestion had been shown to result in extensive...
the absence of N-linked oligosaccharides substituted with polylactosamine (lanes 1, 3, 5, and 7) and presence (Panel a and b, lanes 2, 4, 6, and 8) of endo-β-galactosidase or absence (Panel c, lanes 1, 3, 5, and 7) and presence (Panel c, lanes 2, 4, 6, and 8) of endohexosaminidase H. [3H]Labeled molecules were electrophoresed on 10% SDS-polyacrylamide gel electrophoresis gels and visualized by fluorography. The products from cultures treated with CSP (lanes 1 and 2), DMJ (lanes 3 and 4), SWN (lanes 5 and 6), and controls (lanes 7 and 8) are shown. The electrophoretic mobility of unglycosylated [3H]FM from the medium of cells treated with 3 μg/ml tunicamycin and control medium digested with N-glycanase is shown in Panel c, lanes 9 and 10, respectively.

Fig. 2. Fibromodulin synthesized in the presence of N-linked oligosaccharide processing inhibitors. Portions (from about 0.2 × 10⁶ cells) of medium (Panels a and c) and cell extracts (Panel b) from cultures radiolabeled with [3H]leucine in the presence and absence of inhibitors were incubated with antiserum BF10.90 as described under "Materials and Methods." Immunoprecipitates on Protein A pellets were incubated in the absence (Panels a and b, lanes 1, 3, 5, and 7) and presence (Panels a and b, lanes 2, 4, 6, and 8) of endo-β-galactosidase or absence (Panel c, lanes 1, 3, 5, and 7) and presence (Panel c, lanes 2, 4, 6, and 8) of endohexosaminidase H. [3H]Labeled molecules were electrophoresed on 10% SDS-polyacrylamide gel electrophoresis gels and visualized by fluorography. The products from cultures treated with CSP (lanes 1 and 2), DMJ (lanes 3 and 4), SWN (lanes 5 and 6), and controls (lanes 7 and 8) are shown. The electrophoretic mobility of unglycosylated [3H]FM from the medium of cells treated with 3 μg/ml tunicamycin and control medium digested with N-glycanase is shown in Panel c, lanes 9 and 10, respectively.

removal of the polylactosamine with generation of the 60-kDa form of the [3H]leucine-labeled core protein (Fig. 2, a and b, lanes 8), these results showed that about 80% of the [3H]glucosamine would be present in the N-linked oligosaccharide core structures. Taken together with our previous data on tissue FM and data in Fig. 3, it can be concluded that one, or at most two, of the five N-linked oligosaccharides on FM are substituted with polylactosamine. Moreover, since the digestibility of [3H]-labeled FM with endo-β-galactosidase was not influenced by SWN (see also Fig. 2, a and b, lanes 6), the proportion of N-linked oligosaccharides substituted with polylactosamine did not appear to be influenced by the non-availability of the C-6 branch for polymer addition.

Keratanase II digestion alone released only about 7% of the [3H] radioactivity from immunoprecipitates derived from both control and SWN-treated cultures. This indicates the presence of a limited number of sulfated glucosamine residues in the polylactosamine chains synthesized under both culture conditions. In keeping with this was the observation that keratanase II treatment did not markedly affect the electrophoretic properties of [3H]leucine-labeled FM (data not shown).

**Polylactosamine on Fibromodulin**

**Fig. 2. Fibromodulin synthesized in the presence of N-linked oligosaccharide processing inhibitors.** Portions (from about 0.2 × 10⁶ cells) of medium (Panels a and c) and cell extracts (Panel b) from cultures radiolabeled with [3H]leucine in the presence and absence of inhibitors were incubated with antiserum BF10.90 as described under "Materials and Methods." Immunoprecipitates on Protein A pellets were incubated in the absence (Panels a and b, lanes 1, 3, 5, and 7) and presence (Panels a and b, lanes 2, 4, 6, and 8) of endo-β-galactosidase or absence (Panel c, lanes 1, 3, 5, and 7) and presence (Panel c, lanes 2, 4, 6, and 8) of endohexosaminidase H. [3H]Labeled molecules were electrophoresed on 10% SDS-polyacrylamide gel electrophoresis gels and visualized by fluorography. The products from cultures treated with CSP (lanes 1 and 2), DMJ (lanes 3 and 4), SWN (lanes 5 and 6), and controls (lanes 7 and 8) are shown. The electrophoretic mobility of unglycosylated [3H]FM from the medium of cells treated with 3 μg/ml tunicamycin and control medium digested with N-glycanase is shown in Panel c, lanes 9 and 10, respectively.
Polylactosamine chains on FM and in particular to determine whether this is influenced by SWN, a method was developed to separate the oligosaccharide fragments separated on ToyoPearl HW40S in terms of their anion-exchange properties. This separation was achieved by high pH anion exchange chromatography on CarboPac PA1 by a modified version of the method by Shibata et al. (46) used to separate chondroitin sulfate disaccharides and linkage region structures. To establish conditions for the separation of unsulfated, monosulfated, and disulfated species, ^3H-labeled KS disaccharides were prepared from corneal KS (see "Materials and Methods" and Fig. 6a), and their separation on CarboPac PA1 is shown in Fig. 6b. Given the known presence of unsulfated, monosulfated, and disulfated disaccharides in corneal KS (47), and the fact that these species are here being separated in terms of negative charge, it seems reasonable to assume that peaks 1, 2, and 3 are (GlcNAcβ1, 3Gal-0H), (GlcNAcβ1, 3Gal-0H from endo-β-galactosidase digestion), and (Gal6Sβ1, 4GlcNAcβ1-0H from keratanase II digestion), and (Galβ1, 4GlcNAcβ1-0H from keratanase II digestion), respectively. When purified disaccharides from endo-β-galactosidase digests of FM secretion from control and SWN-treated cultures (see Fig. 6, c and e, respectively) were fractionated on CarboPac PA1 (see Fig. 6, d and f, respectively) very similar profiles were obtained. Thus, the majority of the products in both cases (about 75%) was recovered in the position of the unsulfated disaccharide, and the remainder was eluted as the monosulfated disaccharide. This is consistent with the presence of very few sulfated hexosamine residues on polylactosamine chains synthesized under both culture conditions.

Disaccharides from keratanase II digests were fractionated on CarboPac PA1 (see Fig. 7, b and d, respectively), and a very similar proportion of the radioactivity (about 85%) was recovered in the position of the monosulfated disaccharide. The remainder in both cases eluted as the disulfated disaccharide from control and SWN-treated cultures (see Fig. 7, a and c, respectively).

When FM secreted by control and SWN-treated cells was immunoprecipitated, digested sequentially with neuraminidase, endo-β-galactosidase, and keratanase II, and the products were analyzed on ToyoPearl HW40S, products of the size of di-, tri-, tetra-, and hexasaccharides were obtained in both cases (Fig. 8a). The presence of these larger fragments (tetra- and hexasaccharides) which were insensitive to both endo-β-galactosidase and keratanase II suggested that substituted galactose residues might also be present in these preparations. To assess whether glycosidase insensitivity was due to sulfation of galactose, the resistant oligosaccharides (Fig. 8a, Pools I and II) were fractionated on CarboPac PA1. Greater than 95% of the oligosaccharides in both pools from control and SWN-treated cells were only weakly bound to the column, due to the absence of sulfated residues. This would suggest that the resistance of the larger fragments to digestion is not simply due to sulfated galactose residues, but indicates other substitutions on the galactose, or adjacent to the galactose, which prevent the action of the endo-β-galactosidase used in this study.

The non-reducing terminal trisaccharide (Galβ1-4Glc-
FIG. 4. Size fractionation on ToyoPearl HW40S of oligosaccharides released by endo-β-galactosidase. FM present in the medium of [3H]glucosamine-labeled cultures (from about 10^6 cells) was immunoprecipitated, and Protein A beads from control and SWN-treated cultures were incubated with either endo-β-galactosidase alone (a and b) or endo-β-galactosidase following neuraminidase (c and d), and the liberated oligosaccharides were fractionated on ToyoPearl HW40S. The elution positions of hyaluronate, hyaluronate, and chondroitin sulfate disaccharides are indicated in the top panels.

NAcβ1–3Gal-OH) in these digest (Fig. 8a) was recovered from CarboPac PA1 before the start of the salt gradient (data not shown), indicating the absence of sulfated residues in these oligosaccharides.

Effects of SWN on Fucosylation of N-linked Oligosaccharides—Whereas SWN clearly influenced the processing of the C-6 branch of the N-linked oligosaccharides on FM, it apparently had no marked effect on the size or sulfation of the polylactosamine added. However, monosaccharide analysis (Table II) of the total population of [3H]mannose-labeled oligosaccharides released by N-glycanase showed a greatly reduced content of [3H]fucose on the oligosaccharides formed in the presence of SWN. Thus, the estimated molar content of fucose per N-linked oligosaccharide was about 1.0 in controls but this value was only 0.19 under SWN treatment. Supporting evidence for this effect of SWN was obtained when the same analysis was done on [3H]mannose-labeled oligosaccharides isolated from immunoprecipitated decorin; again a marked reduction in the [3H]fucose labeling was observed.

To examine the location of the fucose on these polylactosamine chains, [3H]mannose-labeled FM from control cultures was immunoprecipitated and pre-digested with endo-β-galactosidase or keratanase II, and the [3H]mannose/[3H]fucose ratio was determined on remaining oligosaccharide structures. Since removal of the polylactosamine did not markedly alter this ratio the results show that fucose is present within the linkage structure, presumably linked α1–6 to the asparagine-bound GlcNAc (31). This would therefore indicate that SWN either directly inhibits the fucosyl transferase or that the hybrid structure formed in the presence of SWN is a poor substrate for this enzyme.

Effect of FM Glycosylation on Distribution between Cell Layer and Medium—At all times under the standard culture conditions, greater than 90% of the FM synthesized over a 16-h labeling period was released into the culture medium, and this distribution toward the medium compartment remained essentially unchanged in the presence of CSP, DMJ, and SWN (Fig. 2). However, supplementation of the culture medium with 50 μg/ml ascorbate resulted in >85% retention of newly synthesized FM in the cell layer (data not shown); this is consistent with the idea that FM specifically associates with the fibrillar collagens (5) since only in the presence of ascorbate do these cultures deposit measurable collagen in the cell layer.

The degree of retention of FM within such a newly formed collagenous matrix was not markedly influenced by treatment of cells with CSP, DMJ, or SWN. Thus, although the compounds had the expected inhibitory effects on the glycosylation of FM in the presence of ascorbate, in all cases greater than 85% of the newly synthesized FM was found in the cell layer (data not shown). Alterations in the structure of the N-linked oligosaccharides on FM and marked inhibition of polylactosamine addition did not influence the distribution of fibromodulin between the cell layer and the medium in these experiments.

DISCUSSION

Data are presented here which show that chondrocytes from calf cartilage maintained for 21 days in culture continue to synthesize and secrete FM which is substituted with polylactosamine. Although a small proportion of the FM was secreted as a 59-kDa species, presumably without polylactosamine addition, the majority of the molecules contained extended polymer. However, since only about 30% of the [3H]mannose-labeled oligosaccharides on the FM core protein was substituted with polylactosamine (Fig. 3, top panel) it appears that polymer is added to one, or at most two, of the five potential N-linked oligosaccharide acceptors (2, 3). Supporting evidence for this

B. Johnstone, personal communication.
also comes from the finding that extensive removal of polylactosamine with endo-β-galactosidase from \[^{3}H\]glucosamine-labeled FM liberated only about 20% of the incorporated radioactivity; thus a greater proportion of the \[^{3}H\]glucosamine is in the N-linked oligosaccharides than in the \((\text{Galβ1-4 GlcNAcβ1-3})^n\) repeats. Partial substitution of N-linked oligosaccharides on FM was also observed for molecules purified from cartilage (3); additionally, biosynthetic studies in other cell systems with lysosomal membrane glycoproteins have shown a variable substitution of different N-linked oligosaccharides with polylactosamine (22, 36).

Inhibition by DMJ and CSP of polylactosamine synthesis on FM indicated that processing of N-linked oligosaccharides minimally requires sequential trimming by glucosidases I and II and mannosidase I to the hybrid GlcNAc(Man)\_2(GlcNAc)\_2. These findings with FM are consistent with previous work in other cell systems on the inhibition of polylactosamine synthesis by DMJ (39) and CSP (40). Further, the required action of mannosidase I on the C-3 branch makes it unlikely that the (Man)\_4(GlcNAc)\_2 structure found on corneal lumican (12) represents a major intermediate for polylactosamine addition to FM.

Another question which arises in relation to the intracellular mechanisms of polylactosamine addition to FM is the extent to which polymer can be added to the C-3, C-6, or both branches of the N-linked oligosaccharide. In this study, the unavailability of the C-6 branch for polylactosamine addition in cells treated with SWN did not appear to reduce the total synthesis of polylactosamine on FM core protein. Thus, the \[^{3}H\]glucosamine:\[^{3}H\]leucine ratio in FM secreted by SWN-treated cells was reduced by only about 14% relative to controls, and this was not simply due to an increase in the specific activity of the UDP-[\(^{3}H\)]hexosamine pool in the presence of SWN. Second, the percentage of \[^{3}H\]oligosaccharides which were released from FM by endo-β-galactosidase was identical for both sets of cultures. The lack of effect of SWN in these experiments is in contrast to reports on the control mechanisms for lactosamine polymer synthesis which operate in a range of metastatic and embryonic cell cultures (19, 38). It also appears to distinguish FM produced in the present culture system from the model proposed for monkey lumican (12), in which both the C-3 and C-6 branches are substituted with KS, and also the models for pig and bovine lumican (14), in which only the C-6 branch contains KS.

Maintenance of synthesis in the presence of SWN could be explained if the inhibition of addition to the C-6 branch was compensated for by the synthesis of longer chains on the C-3 branch. However this was not the case, since SWN did not
Fig. 6. Analysis of endo-β-galactosidase generated disaccharides by high pH anion exchange chromatography. Immunoprecipitated FM (see legend to Fig. 4) was digested with endo-β-galactosidase, and the oligosaccharide products were reduced and fractionated on ToyoPearl HW40S (Panels a and c). Disaccharides were pooled as shown and further fractionated on CarboPac PA1 (Panels d and f) eluted with a gradient of NaCl (indicated by the broken line). Calibration of the system with NaβH₄-labeled disaccharides from corneal KS is shown in Panels a and b. Identification of the disaccharides under peaks 1, 2, and 3 (Panel b) is discussed in the text.

Fig. 7. Analysis of keratanase II generated disaccharides by high pH anion exchange chromatography. Immunoprecipitated FM (see legend to Fig. 4) was incubated with neuraminidase followed by keratanase II. The oligosaccharide products were reduced and fractionated on ToyoPearl HW40S (Panels a and c). Disaccharides were pooled as shown and further fractionated on CarboPac PA1 (Panels b and d) eluted by a gradient of NaCl (indicated by the broken line).
markedly alter the size of the polylactosamine chains added to FM, which was about 4–6 repeating disaccharide units in all cultures (data from Fig. 4). This strongly suggests that a single short chain is present on either the C-3 or C-6 branch in control cultures, whereas it is present on only the C-3 branch in the presence of SWN.

The polylactosamine added to FM by chondrocytes, maintained with or without SWN treatment, was poorly sulfated. Indeed, depolymerization of the chains with endo-β-galactosidase and keratanase II and subsequent separation of the digestion products by high pH anion exchange chromatography suggested that only about 25% of the hexosamine residues and less than 5% of the adjacent galactose residues were substituted with sulfate. In this regard we have also now found that tendon fibroblasts in culture secrete FM-containing polylactosamine which is totally resistant to keratanase II but extensively degraded to unsulfated disaccharides with endo-β-galactosidase. This low sulfation of culture products is apparently in contrast to KS on FM extracted from cartilage and tendon, which is strongly reactive with antibody 5D4 (3, 42). This may also be related to the finding (20) that corneal fibroblasts in culture synthesize lumican KS which is poorly sulfated relative to extracted material.

In this regard it may be relevant that in preparations of extracted calf cartilage FM (3) two populations of polylactosamine chains were separated on MonoQ anion exchange. The unbound population was composed of 4–6 disaccharide units, whereas the bound and highly sulfated chains were considerably larger at about 25 disaccharide units. It therefore appears that in culture the cells produce only the short chain population, and this addition is not selective for a specific position on the linkage region.

Experiments on the intracellular processing of FM support the contention that the structure of polylactosamines (chain length, branching, sulfation) is highly variable and can be markedly influenced by both the cell source and culture conditions under study (26). For the unsulfated polylactosamines this variation in structure appears to provide a means for controlling cell-matrix and/or cell-cell interactions via adhesive molecules such as the selectins (45). This may also be the function of the unsulfated short chain species on FM, although data in the present study suggest that deposition of FM within the chondrocyte-associated collagenous matrix does not absolutely require polylactosamine addition. On the other hand, since long chain and highly sulfated polylactosamine sequences are abundant only on FM in embryonic cartilages (4), these structures may confer properties on the collagen network which are essential for controlled growth.

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