Abnormal Glycosylation of Human Fibronectin Secreted in the Presence of Monensin*

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Detailed studies of the effects of the ionophore monensin upon the glycosylation of secreted fibronectin have been carried out. Human fibroblasts in culture were incubated in 1 μM monensin for several hours, following which radiolabeled glucosamine or mannose was added to the cultures. Parallel incubation and labeling of control cultures were done. Labeled fibronectin was isolated from the culture media by gelatin-Sepharose chromatography, from cell surfaces by urea extraction, and from intracellular locations by cell lysis followed by immunoprecipitation. Detailed comparison of the glycopeptides released from fibronectin by pronase and of the oligosaccharides liberated by hydrazinolysis was carried out, particularly focusing on the secreted fibronectin, using gel filtration, high performance liquid chromatography, and concanavalin A chromatography, in conjunction with the use of endoglycosidase H and specific exoglycosidases. We demonstrate that fibronectin in the medium of monensin-treated cultures differs in its glycosylation pattern from the control fibronectin. High mannose oligosaccharides are abundant in the monensin-derived fibronectin, whereas the control protein contains primarily complex oligosaccharides. Monensin apparently does not alter the initial glycosylation of fibronectin since the high mannose oligosaccharides are present on both control and monensin-treated intracellular fibronectin. We suggest, therefore, that monensin, by impairing intracellular translocation through the Golgi region, allows incompletely processed forms of fibronectin to reach the cell surface and to be released into the culture medium.

EXPERIMENTAL PROCEDURES

Cell Culture and Metabolic Labeling—Normal adult human skin fibroblasts (American Type Culture Collection, CRL 1220) were maintained in Dulbecco's modified Eagle's medium (Gibco) containing 50 mg/ml of ascorbate and 10% horse serum (Gibco). For experiments, fully confluent cell layers were split 1:2 and used 3-4 days later when each dish was overconfluent and contained about 2 × 10⁶ cells. Preincubation of cultures was in 3 ml of Dulbecco's modified Eagle's medium containing 2% dialyzed horse serum with or without monensin at 1 μM. After 3-4 h, this medium was exchanged for Dulbecco's modified Eagle's medium containing 10% of the normal glucose concentration, 2% dialyzed horse serum, 1 μM monensin as required, and radiolabeled sugars. The latter, obtained from ICN and New England Nuclear, were [6-3H]glucosamine/HCl (20 Ci/mmol), [1-14C]glucosamine/HCl (10 mCi/mmol), [2-3H]mannose (10 Ci/mmol), or [1-14C]mannose (50 Ci/mmol). Tritiated compounds were used at 15 μCi/ml and 14C-labeled sugars at 5 μCi/ml. To permit the eventual mixing of oligosaccharides derived from control and treated cultures, controls with the tritiated sugar, Affinity-purified fibronectins were then mixed, prior to enzymatic or chemical digestion, such that the H counts per min were at least twice as high as 14C counts per min. In one series of experiments, labeled medium was removed from control and monensin-treated cultures under sterile conditions, mixed, and incubated at 37 °C for 8 h. Fibronectin was then isolated from this mixture and parallel preparations of unmixed culture media.

Isolation of Fibronectin—Medium was collected after 12 h incu-
bation, chilled, and brought to 1 mM phenylmethylsulfonyl fluoride, 5 mM N-ethylmaleimide, and 10 mM EDTA (=protease inhibitors).

After centrifugation of the medium (10,000 x g, 10 min), fibronectin was isolated by gelatin affinity chromatography as described elsewhere (13). Intracellular fibronectin was obtained as follows. Cells layers were rinsed once in ice-cold Ca^++/Mg^{++}-free phosphate-buffered saline and 1 ml of 0.25% trypsin in phosphate-buffered saline was added to each dish. Plates were gently shaken for 5 min at room temperature by which time cells could be suspended by pipetting. Cells thus prepared were rinsed several times in ice-cold phosphate-buffered saline and added. After no precipitation, the cells were lysed in 200 

urea extraction method of Yamada et al. (14) as described in Ref. 13. Glycopeptides and Oligosaccharides—Gel filtration of GlcN-labeled control glycopeptides derived from monensin-treated cells was also found when control and monensin glycopeptides (Fig. 2a).

Fig. 1. Analysis of purified fibronectin. Fluorogram of a sodium dodecyl sulfate-7.5% polyacrylamide gel resolving fibronectin immunoprecipitated from control (a) and monensin-treated (b) cells or isolated by gelatin affinity chromatography from the medium of control (c) and monensin-treated (d) cultures. Upper and lower arrowheads indicate top of the gel and the migration position of bromophenol blue, respectively.
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indictive of endo H sensitivity. Control oligosaccharides were not cleaved by endo H.

Analysis by HPLC showed further differences between control and monensin-derived oligosaccharides. Separation of intact oligosaccharides in phosphate buffer demonstrated their relative degree of sialylation (Fig. 3) (23). Integration of the data indicated that in controls, 96% of GlcN label was present in non-sialylated oligosaccharides, 28% in monosialylated, and 15% in disialylated forms. Corresponding values for oligosaccharides from monensin-treated cells were 73% non-sialylated, 20% monosialylated and 6% disialylated. These values varied between preparations, but the relative undersialylation of oligosaccharides from monensin-treated cells was consistently seen. After desialylation by mild acid hydrolysis, all oligosaccharides behaved as non-sialylated species (not shown). HPLC of these desialylated oligosaccharides in the acetonitrile/H2O system resolved the control samples into three major peaks (Fig. 4). Under the same conditions, monensin samples were resolved into numerous components (Fig. 4).

A further difference between control and monensin oligosaccharides was revealed by chromatography on ConA-Sepharose (Fig. 5). Control samples of oligosaccharides derived from medium fibronectin contained some components (ConA-I) that did not bind to ConA and some that could be eluted with 10 mM α-methyl-D-glucoside (ConA-II). Samples from monensin-treated cells contained an additional component that was eluted with 0.5 M α-methyl-D-mannoside (ConA-III). Furthermore, in these samples, the elution with 10 mM α-methyl-D-glucoside consistently produced a peak that slowly trailed off, unlike the sharp peak observed in controls. For both control and monensin samples, the nonbinding components did not bind to the column when reapplied. Recovery for control and experimental samples averaged 90%, and no additional label could be eluted with 1.0 M NaCl. Pronase-produced glycopeptides and desialylated or intact oligosaccharides from the same preparation all produced similar elution patterns on ConA-Sepharose. Also, the differences between control and monensin samples were always found in such experiments, confirming the results in Fig. 5.

**FIG. 3.** HPLC analysis of intact oligosaccharides. GlcN-labeled hydrazine-released oligosaccharides were chromatographed on a Micro Pak AX-10 ion exchange column. Nonsialylated (Peak I) and monosialylated (Peak II) oligosaccharides eluted in 12.5 mM KH2PO4, pH 4.0. The KH2PO4 concentration was linearly increased to 500 mM between fractions 51 and 100. Disialylated oligosaccharides eluted at the start of this gradient (Peak III). -- --, 14C, Control oligosaccharides; ---, 3H, monensin oligosaccharides.

**FIG. 4.** HPLC analysis of desialylated oligosaccharides. GlcN-labeled oligosaccharides were desialylated by mild acid treatment and chromatographed in acetonitrile/H2O on a Micro Pak AX-10 column. Elution was initially in 65% acetonitrile, 35% H2O. After 10 min, the H2O content was increased to 46% over 90 min at which time it was increased to 70% over a further 10 min. This gradient is indicated by the dotted line. -- --, 14C, Control oligosaccharides; ---, 3H, monensin oligosaccharides.

**FIG. 5.** ConA chromatography of desialylated oligosaccharides. GlcN-labeled control and monensin oligosaccharides were applied to a column (0.8 x 1.5 cm) of ConA-Sepharose. The column was washed with 10 ml of TBS, followed by 20 ml of 10 mM α-methyl-D-glucoside (αMG) in TBS and 20 ml of 0.5 M α-methyl-D-mannoside (αMM) in TBS. Portions of the 1-ml fractions were examined for radioactivity and Peaks I, II, and III were pooled as indicated by the horizontal bars. -- --, 14C, Control oligosaccharides; ---, 3H, monensin oligosaccharides.
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The ConA and Bio-Gel P-6 elution profiles of glycopeptides from cell surface and medium fibronectins were identical. Also, the monensin-induced differences were found in oligosaccharides from both sources.

Monensin is known to affect the release of lysosomal hydrolases from fibroblasts (29), and so the monensin-induced differences in glycopeptide structure might result from the action of glycosidases released into the culture medium. However, the behavior of glycopeptides on Bio-Gel P-6 and ConA was not altered by the incubation of a mixture of conditioned media from control and monensin-treated cultures. Elution profiles of control samples were identical, and the monensin-induced differences were evident whether or not mixed incubation of samples was performed (data not shown).

Enzymatic Analysis of ConA Fractions—ConA chromatography demonstrated that oligosaccharide preparations of medium fibronectin were mixtures of several species. Also, the degree of sialylation of oligosaccharides varied slightly between preparations (see above). Thus, to minimize potential heterogeneity and to facilitate subsequent analysis, oligosaccharides were desialylated by mild acid treatment before fractionation on ConA. The total oligosaccharide preparations showed differences (Fig. 6a) in the size distribution of control and monensin samples when chromatographed on Bio-Gel P-4. Unlike the glycopeptide preparations (Fig. 2a), these differences were free of possible influences of the peptide portion and thus were entirely due to carbohydrate differences.

The ConA pools were desalted on Bio-Gel P-6 and analyzed by chromatography on Bio-Gel P-4 prior to and after various enzymatic treatments. On Bio-Gel P-4, ConA-I, the fraction that did not bind to ConA, presented a complex elution profile including oligosaccharides up to 15 glucose equivalents in size. Although differences between control ConA-I and monensin ConA-I were seen, this fraction was not evaluated further (see "Discussion").

Control ConA-II oligosaccharides (Fig. 6b) eluted as a major peak centered around the elution position corresponding to 13 glucose equivalents. Monensin ConA-II oligosaccharides overlapped this peak but also included later eluting components (Fig. 6b). The results of treating GlcN-labeled ConA-II oligosaccharides with exoglycosidases are shown in Fig. 6c-f. Digestion with β-galactosidase (Fig. 6e) caused label to be released from both monensin and control samples, and in the case of controls, 48% of the total counts eluted at the position of 2 glucose equivalents (Fig. 6e). A smaller proportion (36% of total) was released from monensin ConA-II. Treatment of GlcN-labeled samples with α-mannosidase from culture medium as described under "Experimental Procedures" and subjected to gel filtration on Bio-Gel P-4. α, desialylated oligosaccharides. Also indicated in this panel are the elution positions of glucose oligomers (numbered arrowheads) and [3H]dextran (large arrowhead).

Fig. 6. Comparison of control and monensin oligosaccharides and their susceptibility to digestion by exoglycosidases. GlcN-labeled desialylated oligosaccharides, the ConA-II fraction, and enzymatic digests of ConA-II were prepared from fibronectin isolated from culture medium as described under "Experimental Procedures" and subjected to gel filtration on Bio-Gel P-4. α, desialylated oligosaccharides. Also indicated in this panel are the elution positions of glucose oligomers (numbered arrowheads) and [3H]dextran (large arrowhead). b, the ConA-II fraction derived from the oligosaccharide mixture shown in a. ConA-II was then subjected to digestion by the following exoglycosidases and the products chromatographed on Bio-Gel P4: c, β-galactosidase; d, β-N-acetylgalactosaminidase; e, β-galactosidase and β-N-acetylgalactosaminidase; and f, α-mannosidase.

- - - C, Control; --- 3H, monensin.
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idase (Fig. 6f) had little effect on control oligosaccharides, but the monensin ConA-II fraction showed a markedly altered elution profile and yielded a prominent new peak eluting at ~5 glucose equivalents.

Some GlcN-labeled ConA-II was digested with β-galactosidase, β-N-acetylglucosaminidase, and α-mannosidase to potentially produce the Man β→GlcNAc α(±Fuc α)→GlcNAc core. Material pooled from the expected elution position of this structure was then rechromatographed with or without prior α-fucosidase digestion (Fig. 7). Without digestion, control material eluted with a size difference equivalent to one saccharide larger than monensin oligosaccharides (Fig. 7a). This difference was abolished by α-fucosidase treatment (Fig. 7b).

ConA fractions of desialylated oligosaccharides were also obtained from preparations labeled with mannose. The ConA-II fraction (Fig. 8a) showed a different size distribution for monensin and control oligosaccharides. Differences between the elution profiles of Man- (Fig. 8a) and GlcN- (Fig. 8b) labeled oligosaccharides were attributed to the relative abundance of labeled saccharides in particular oligosaccharides.

Using the Man-labeled ConA-II fraction, sensitivity to α-mannosidase (Fig. 8b) was illustrated by the release of label, which eluted at the one glucose position, as did a mannose standard. Release of label from control ConA-II was negligible (Fig. 8b).

Digestions were also performed on ConA-III fractions, which were unique to monensin-treated cells (Fig. 5). Digestion of Man-labeled ConA-III (Fig. 8c) with α-mannosidase (Fig. 8d) caused 80% of the label to elute at the one glucose position. GlcN-labeled ConA-III (Fig. 9a) ran as a narrow peak centered around the elution position of 12 glucose equivalents. Digestion with β-N-acetylglucosaminidase caused the appearance of a peak which eluted in the position of GlcNAc but contained only 12% of the radioactivity (Fig. 9b). An identical elution profile was generated by treatment with β-N-acetylglucosaminidase after β-galactosidase digestion (not shown). α-Mannosidase drastically altered the elution profile of ConA-III and left a major GlcN-labeled peak at 5 glucose equivalents (Fig. 9c).

The endo H sensitivity detected in monensin glycopeptides (Fig. 2b) was further investigated in ConA-fractionated oligosaccharides. GlcN-labeled monensin ConA-II showed some endo H sensitivity (Fig. 10a), as was evidenced by the appearance of label at the 2-glucose position, corresponding to cleavage of a single GlcNAc residue from the reducing end of the oligosaccharide (31). A distinct peak also appeared at about the 9-glucose position. A greater effect was seen in the endo H digest of GlcN-labeled ConA-III (Fig. 10b). Label appeared at the 2-glucose position, and the remaining oligosaccharide peak was almost entirely shifted from its previous position (about 12 glucose equivalents, Fig. 9a) to between 9 and 10 glucose equivalents. Endo H sensitivity was also detected in the Man-labeled ConA fractions I and II (not shown).
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Fig. 9. Exoglycosidase digestion of GlcN-labeled ConA-III. Desialylated, GlcN-labeled ConA-III oligosaccharides were chromatographed on Bio-Gel P-4 (a), b, ConA-III after digestion with β-N-acetylglucosaminidase; c, after digestion with α-mannosidase. The elution positions of the 12-glucose oligomer and the 5-glucose oligomer are indicated in a and c, respectively.

Fig. 10. Endo H sensitivity of ConA-II and ConA-III. Bio-Gel P-4 chromatography of ConA-II (a) and ConA-III (b) after digestion with endo H. The elution positions of 9- and 2-glucose oligomers are indicated. The nondigested starting materials for a and b are shown in Figs. 6a and 7a, respectively. ---, Control; --, H, monensin.

DISCUSSION

The carbohydrate content of fibronectins from several tissues and species has been described. These glycoproteins contain from about 5 (20, 32) to 9.5% (33) carbohydrate in an estimated three (34) to five (35) N-linked oligosaccharides/fibronectin monomer. The most abundant oligosaccharide, described for several fibronectins (17, 20, 30, 35), has the structure (±SA→Galβ→GlcNAcβ→Manα→)3Manβ→GlcNAcβ→GlcNAc. Variations have been noted in the peripheral Galβ→GlcNAc linkages (30) and, more commonly, in the abundance and linkage of terminal SA residues (30, 37). The presence of fucose linked to the proximal GlcNAc of the chitobiose unit is variable and may (33) or may not (21, 34, 36) be present on human fibronectins. Thus, the major fibronectin oligosaccharide is a typical complex biantennary structure similar to that found on transferrin (34). The occurrence on fibronectin of oligosaccharides of greater complexity has also been suggested (34, 35, 37). The intracellular elaboration of fibronectin oligosaccharides appears to follow a known pathway via high mannose intermediates (38).

A major component of control oligosaccharides derived from medium fibronectin is the low affinity ConA-II fraction that is rapidly eluted by 10 mM α-methylglucoside. This behavior (39) and the results of the enzymatic digestions are entirely compatible with the complex biantennary structure cited above. As did Fisher and Laine (34) and Wagner et al. (37), we find an oligosaccharide subfraction that does not bind to ConA. This property (39) and the elution position on Bio-Gel P-4 (data not shown) suggest that this fraction, present in both control and monensin oligosaccharides, may consist of more highly branched complex oligosaccharides. However, due to the lack of more definitive structural information, we have not evaluated this fraction further.

That monensin can have effects on the glycosylation of fibronectin was initially demonstrated by comparison of glycopeptide elution profiles on Bio-Gel P-6 (Fig. 2a). The mean size of glycopeptides derived from medium fibronectin was diminished in the presence of monensin, and a substantial proportion of them were endo H-sensitive, in contrast to the endo H-resistant control glycopeptides (Fig. 2b). This enzyme sensitivity showed that the size difference required the presence of novel oligosaccharides and did not simply reflect a differential abundance of normal oligosaccharides. This conclusion was reinforced by ConA chromatography and by HPLC of desialylated oligosaccharides in monensin samples, unique peaks were present (Figs. 4 and 5).

Assumptions concerning the probable structure of some of these novel forms were made, based on the structure of normal fibronectin oligosaccharides (as summarized above) as well as the sequence of events involved in the synthesis of typical complex oligosaccharides (10). Analysis was facilitated by the initial fractionation of desialylated oligosaccharides on ConA. The ConA-II fraction, which contained the typical fibronectin oligosaccharides, also contained some abnormal forms in the monensin sample. Thus, the ability of β-N-acetylglucosaminidase to remove significant amounts of GlcN label without prior treatment by β-galactosidase (Fig. 6d) is indicative of the incomplete galactosylation of some monensin oligosaccharides. Cleavages from control ConA-II fractions by this enzyme were minimal. However, after initial β-galactosidase exposure, the 48% GlcN label released from controls by β-N-acetylglucosaminidase is close to the theoretical value of 50% (Fig. 6e).

Fucosylation of ConA-II oligosaccharides was investigated by digesting them to a presumptive Manβ→GlcNAcβ→(±Fucar→)GlcNAc core. The resulting mixture eluted from Bio-Gel P-4 with a size difference of one saccharide between control and monensin samples (Fig. 7a). This difference was abolished by α-fucosidase treatment (Fig. 7b), suggesting that in control fibronectin, the majority of oligosaccharides bear an α-fucose on the core structure, whereas in the presence of monensin, fucose is absent or severely reduced.

Impaired fucosylation, galactosylation, and the general reduction of sialylation (Fig. 3) seen in the presence of monensin are all indicative of abnormalities in the “later” events involved in complex oligosaccharide formation. The conclusion that complex oligosaccharides with incompletely formed branches are present on fibronectin secreted into the medium...
in the presence of monensin is supported by the greater abundance of smaller oligosaccharides in the monensin ConA-II fraction (Fig. 6b). Also, we note that Baenziger and Fiete (39) have shown that the sequential removal of peripheral sugars from complex biantennary oligosaccharides results in a progressive increase in their affinity for ConA. This could explain the trailing of the monensin ConA-II peak, in contrast to the sharply eluted control ConA-II (Fig. 5). If they are present at all, structures as small as (Man)\(_4\) \(\rightarrow\) \(\text{Man} \beta\) \(\text{GlcNAc} \beta\) \(\rightarrow\) GlcNAc cannot be abundant, as only a small proportion of monensin oligosaccharides elute from Bio-Gel P-4 at the ~7-glucose position expected for such structures (Fig. 6b). Digestion with \(\alpha\)-mannosidase (Figs. 6f and 8b) shows, nevertheless, that the monensin ConA-II fraction does contain structures with terminal \(\alpha\)-linked mannose residues. However, at least some of these are probably larger high mannose structures because this same fraction does contain a component that is sensitive to endo H and which yields a structure eluting at the 9-glucose position after cleavage with that enzyme (Fig. 10a) (40).

A far greater proportion of such high mannose forms are present in the ConA-III fraction, which is unique to monensin oligosaccharides. Digestion of this fraction with \(\alpha\)-mannosidase reduces most Man label to the single saccharide (Fig. 8d) or the GlcN label to a structure eluting at the 5-glucose position expected for Man\(\beta\) \(\text{GlcNAc} \beta\) \(\rightarrow\) GlcNAc (Fig. 9c). Consistent with this is the endo H sensitivity of almost the entire ConA-III fraction (Fig. 10b). The elution position of ConA-III on Bio-Gel P-4 suggests that structures as large as (Man)\(_{16}\) \(\rightarrow\) (GlcNAc)\(_2\) may be present. The occurrence of a small amount of terminal (\(\beta\)-N-acetylgalactosaminidase-releasable) GlcNAc in the GlcN-labeled ConA-III (Fig. 9b) is probably due to spillover of some ConA-II components. This possibility was avoided in the Man-labeled preparation by extending the elution with 10 mM \(\alpha\)-methylglucoside. Further support for the high mannose nature of ConA-III is its high affinity for ConA, which is characteristic of such oligosaccharides (41, 42). Also, ConA chromatography of oligosaccharides from intracellular fibronectin yielded high affinity ConA-III peaks for both control and monensin samples (not shown). This is consistent with the expected presence of high mannose intermediates on intracellular control fibronectin (39). Why some apparently high mannose oligosaccharides should be present in the monensin ConA-II fraction is unclear. ConA chromatography may not provide absolute separation of identical forms, or subtle differences in similar (but not identical) high mannose structures may modulate their affinity for ConA.

We conclude that fibroblast fibronectin secreted in the presence of monensin bears abnormal oligosaccharides which fall into two types. The first are those in which the final stages of complex oligosaccharide formation are incomplete and so are deficient in their sialylation, galactosylation, and fucosylation. This more rigorously illustrates, at the structural level, the monensin effects inferred by Tartakoff and Vassalli (9) from studies of immunoglobulin M. We have also explained our previous observation that monensin apparently increased the incorporation of mannose into secreted fibronectin (11) by the demonstration that high mannose oligosaccharides, usually restricted to intracellular fibronectin, are found on fibronectin secreted in the presence of monensin. Other investigators have described the intracellular accumulation of high mannose glycoproteins under such conditions (43), but no information was given about their secretion. The release of molecules bearing high mannose oligosaccharides resembles the situation seen with the alkaloid, swainsonine (44). This compound has, however, been shown to specifically inhibit an \(\alpha\)-mannosidase involved in oligosaccharide processing (45).

The pleiotropic effects of monensin can be viewed differently; viz. the monensin-induced disruption of Golgi elements described in many cell types (1, 3, 5) may be a manifestation of incorrect fission-fusion of membrane systems, of which reduced secretion is a prominent effect.

At another level, one can invoke current paradigms of glycoprotein biosynthesis to explain our observations. These are the sequential nature of the multienzyme systems involved in oligosaccharide processing (46) and the concept of functionally and topologically distinct Golgi subcompartments (47, 48). Thus, membrane-bound packages of secretory molecules emerging from the endoplasmic reticulum may be denied access to the complete range of oligosaccharide-processing enzymes in the monensin-treated cell. However, whether the incompletely processed fibronectin molecules completely bypass particular compartments or whether they pass through functionally incompetent compartments remains to be determined.

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