A material of Mₙ 24,000 has been isolated from a cachexia-inducing mouse tumor (MAC16) and shown to initiate protein degradation in isolated gastrocnemius muscle. Biological activity was destroyed by preincubation with peptide N-glycosidase F (PNGase F) and endo-α-N-acetylgalactosaminidase (O-glycosidase) but not by neuraminidase or trypsin. Antibody reactivity was destroyed by treatment with periodate, indicating carbohydrate moieties to be the antigenic determinants. Antigenic activity was also reduced by treatment with PNGase F and O-glycosidase and was completely destroyed by treatment with chondroitinase ABC but was unaffected by treatment with either trypsin or chymotrypsin, confirming that the N- and O-linked sulfated oligosaccharide chains were both the antigenic and biological determinants.

Biosynthetic labeling of MAC16 cells using a combination of [35S]sulfate and [6-3H]GlcN gave a single component of Mₙ 24,000 containing both radiolabels. Similar material could not be isolated from a cell line (MAC13) originating from a tumor that does not cause cachexia in vivo. Digestion of [3H]/[35S] material with PNGase F produced two fragments of Mₙ 14,000 and 10,000 containing both radiolabels, and digestion with O-glycosidase produced three fragments of Mₙ 14,000, 6,000, and 4,000, the first two contained both radiolabels and the third contained only [3H]. Digestion of the fragment of Mₙ 14,000 released by PNGase F with O-glycosidase also gave fragments of Mₙ 6,000 and 4,000. The products from both digestions were acidic as determined by anion exchange chromatography on DEAE-cellulose. The negative charge on the fragment of Mₙ 4,000 was removed by treatment with alkaline phosphatase. This suggests that the charge originated from phosphate residues, and this has been confirmed by biosynthetic labeling of MAC16 cells with [32P]orthophosphate, where radiolabel was incorporated into material of Mₙ 24,000 and into the fragment of Mₙ 4,000 after treatment with O-glycosidase. To determine the size of the polypeptide core MAC16 cells were biosynthetically labeled with [3H][2,5-3H]His which after chemical deglycosylation produced a major component of Mₙ 4,000. These results suggest a model for the Mₙ 24,000 material consisting of a central polypeptide chain of Mₙ 4,000 and with phosphate residues that may be attached to the polypeptide or a short oligosaccharide chain containing GlcN, one O-linked sulfated oligosaccharide chain containing GlcN, and of Mₙ 6,000 and one N-linked sulfated oligosaccharide chain of Mₙ 10,000 also containing GlcN. Neither chain was cleaved into disaccharides with chondroitinase ABC, suggesting that the material is a sulfated glycoprotein.

Depletion of skeletal muscle is an important factor contributing to the decreased survival of cancer patients with loss of cardiac and respiratory muscles being most important. A decreased nutrient intake plays an important role in wasting of lean body mass in cancer cachexia, but it appears that it does not fully account for the changes observed (1). Although protein synthesis is decreased (2), an accelerated protein breakdown accounts in large part for the muscle wasting observed (3).

Several factors have been postulated as signals for this increased muscle proteolysis including tumor necrosis factor-α and interleukins 1 and 6 (4, 5). While continuous infusion of the cytokines in vivo has been shown to increase protein degradation in skeletal muscle, none of the cytokines produced a direct stimulation of proteolysis when incubated in vitro (4, 5).

A novel material of Mₙ 24,000 that appears to fulfill the function of triggering muscle proteolysis during the process of cancer cachexia (6, 7) has been purified from a cachexia-inducing mouse tumor (MAC16). This material was capable of inducing muscle protein degradation in isolated gastrocnemius muscle preparations and of inducing weight loss in vivo and will be referred to as proteolysis inducing factor (PIF). Similar, if not identical, material was isolated from the urine of patients with pancreatic carcinoma and weight loss (7).

Structural studies of PIF indicated a short peptide chain of Mₙ 2,000, which was extensively glycosylated at both Asn and Ser residues (7). Enzymatic degradation suggested that some of the carbohydrate chains contained sulfate residues, whereas lectin blotting (6) indicated the presence of GlcNAc. The material bound strongly to albumin to form a species of Mₙ 69,000, probably through the sulfate residues which would produce a strong electrostatic linkage. Recent studies (8) show that cell-bound albumin binds peptidoglycan, heparin, and sulfated heparinoids as a complex of Mₙ 70,000.

The purpose of the present investigation was to characterize PIF in terms of the number, type, and attachment of the carbohydrate chains to the peptide backbone as well as determining the role of the carbohydrate chains in antibody reactivity and protein degradative activity in isolated gastrocnemius muscle.

**EXPERIMENTAL PROCEDURES**

**Materials**—RPMI 1640 tissue culture medium with and without L-histidine and fetal bovine serum were from Life Technologies, Inc.,

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‡ To whom requests for reprints should be addressed.

1 The abbreviations used are: PIF, proteolysis inducing factor; PNGase F, peptide N-glycosidase F; O-glycosidase, endo-α-N-acetylgalactosaminidase; PMSF, phenylmethylsulfonyl fluoride; PBS, Ca²⁺- and Mg²⁺-free phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]glycine.
all animals were sacrificed between 9 and 10 a.m. The muscles were killed by cervical dislocation, and their gastrocnemius muscles were obtained from our own breeding colony. Female NMRI mice were purchased from Oxford Glycosystems, Oxford, United Kingdom. Pure mouse serum served with normal mouse serum. Tyrosine release when trypsin was added to serum. Using this procedure tyrosine release when trypsin was added to serum did not differ significantly from that observed with normal mouse serum.

Solid MAC16 tumors excised from mice with established cachexia were fractionated on an affinity column containing the MAC16 monoclonal antibody as described (6). The enzyme-linked immunosorbent assay-positive fractions were pooled and concentrated, and portions (0.134 mg/ml) were treated with the enzymes as described under “Experimental Procedures.” All samples were preincubated with serum from non-tumor-bearing mice for 30 min at 37 °C prior to determination of tyrosine release from gastrocnemius muscle as described under “Experimental Procedures.” After incubation with trypsin (0.2 μg/μl of protein) for 20 h, α1-antitrypsin (8.3 μg) was added prior to addition to serum. Using this procedure tyrosine release when trypsin was added to serum did not differ significantly from that observed with normal mouse serum.

| Treatment                  | Tyrosine n mole/mg/2 h  |
|----------------------------|-------------------------|
| Normal mouse serum         | 37 ± 2                  |
| Cachectic mouse serum       | 54 ± 6                  |
| Cachectic mouse serum + antibody | 35 ± 3                |
| Normal mouse serum + Ag     | 67 ± 18<sup>b</sup>     |
| Normal mouse serum + Ag + PNGase F | 28 ± 3<sup>c</sup> |
| Normal mouse serum + Ag + sulfatase | 41 ± 4<sup>d</sup>   |
| Normal mouse serum + Ag + O-glycosidase | 35 ± 4<sup>c</sup> |
| Normal mouse serum + Ag + neuraminidase | 65 ± 3                |
| Normal mouse serum + Ag + trypsin | 62 ± 10                |

<sup>a</sup> p < 0.05 when compared with normal mouse serum.
<sup>b</sup> p < 0.02 when compared with normal mouse serum.
<sup>c</sup> p < 0.02 when compared with normal mouse serum + Ag.
<sup>d</sup> p < 0.05 when compared with normal mouse serum + Ag.

| Treatment                  | Antigen binding activity<sup>a</sup> (A492)  |
|----------------------------|----------------------------------|
| None                      | 0.24 ± 0.031                   |
| Trypsin                   | 0.17 ± 0.021                   |
| Chymotrypsin              | 0.21 ± 0.006                   |
| Neuraminidase             | 0.29 ± 0.017                   |
| O-Glycosidase             | 0.11 ± 0.016                   |
| PNGase F                  | 0.16 ± 0.014                   |
| Keratanase                | 0.26 ± 0.003                   |
| Sulfatase                 | 0.11 ± 0.02                    |
| Chondroitinase AC         | 0.17 ± 0.02                    |
| Chondroitinase ABC        | 0.01 ± 0.01                    |
| Alkaline phosphatase      | 0.13 ± 0.01                    |

<sup>a</sup> Values given are mean ± S.E. for three determinations per point.

Paisley, Scotland, United Kingdom. α-[1,6-<sup>3</sup>H]GlcN hydrochloride (specific activity 422.3 Ci/mmol) was purchased from DuPont Ltd., Hertfordshire, United Kingdom, and Na<sup>35</sup>SO<sub>4</sub> (specific activity 10–100 mCi/mmol) α-[2,6-<sup>3</sup>H]histidine (specific activity 56 Ci/mmol), [<sup>32</sup>P]orthophosphate (10 mCi/ml), and Na<sup>125</sup>I (1 mCi/10μl) were purchased from Amersham Int., Bucks, United Kingdom. The Sephadex G-50 and the MW-GF-70 kit used to construct a calibration curve were purchased from Amersham Int., Bucks, United Kingdom; and sulfatase, alkaline phosphate, chondroitinase AC, keratanase, neuraminidase, PNGase F were from Boehringer Mannheim, East Sussex, United Kingdom; and α1-antitrypsin (8.3 μg). The details of the procedure are given under “Experimental Procedures.” A, autoradiograph of material shown in A after SDS-PAGE electrophoresis. Lane 1, from tissue culture supernatant; lane 2, from MAC16 cells. blotted, weighed, and carefully tied via tendon ligatures to stainless steel incubation supports (9). This prevents contraction and improves protein balance and energy status (10). Protein degradation was measured by tyrosine release, since tyrosine rapidly equilibrates between intracellular pools and the medium and is neither synthesized nor degraded. Muscles were preincubated in RPMI 1640 (3 ml) lacking phenol red in the presence of serum (280 μl) for 30 min at 37 °C in an atmosphere saturated with O<sub>2</sub>:CO<sub>2</sub> (19:1). The muscles were rinsed and incubated for a further 2 h in Krebs-Henseleit bicarbonate buffer, containing 6 mM d-glucose, 1.2 mg/ml bovine serum albumin, and 130 μg/ml cycloheximide with continuous gassing. At the end of the incubation the buffer was removed, deproteinized with ice-cold 30% trichloroacetic acid (0.2 ml), centrifuged at 3000 × g for 10 min, and the supernatant used for the measurement of tyrosine by a fluorometric method (11) at 570 nm on a Perkin-Elmer LS-5 luminescence spectrometer.

**Table I**

| Treatment                  | Tyrosine n mole/mg/2 h  |
|----------------------------|-------------------------|
| Normal mouse serum         | 37 ± 2                  |
| Cachectic mouse serum       | 54 ± 6                  |
| Cachectic mouse serum + antibody | 35 ± 3                |
| Normal mouse serum + Ag     | 67 ± 18<sup>b</sup>     |
| Normal mouse serum + Ag + PNGase F | 28 ± 3<sup>c</sup> |
| Normal mouse serum + Ag + sulfatase | 41 ± 4<sup>d</sup>   |
| Normal mouse serum + Ag + O-glycosidase | 35 ± 4<sup>c</sup> |
| Normal mouse serum + Ag + neuraminidase | 65 ± 3                |
| Normal mouse serum + Ag + trypsin | 62 ± 10                |

**Table II**

| Treatment                  | Antigen binding activity<sup>a</sup> (A492)  |
|----------------------------|----------------------------------|
| None                      | 0.24 ± 0.031                   |
| Trypsin                   | 0.17 ± 0.021                   |
| Chymotrypsin              | 0.21 ± 0.006                   |
| Neuraminidase             | 0.29 ± 0.017                   |
| O-Glycosidase             | 0.11 ± 0.016                   |
| PNGase F                  | 0.16 ± 0.014                   |
| Keratanase                | 0.26 ± 0.003                   |
| Sulfatase                 | 0.11 ± 0.02                    |
| Chondroitinase AC         | 0.17 ± 0.02                    |
| Chondroitinase ABC        | 0.01 ± 0.01                    |
| Alkaline phosphatase      | 0.13 ± 0.01                    |

**Fig. 1.** A, elution profile of radioactivity bound to an affinity column after biosynthetic labeling of MAC16 cells with [35S]sulfate (●) and [6-<sup>3</sup>H]GlcN (○). The details of the procedure are given under “Experimental Procedures.” B, autoradiograph of material shown in A after SDS-PAGE electrophoresis. Lane 1, from tissue culture supernatant; lane 2, from MAC16 cells.
containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM EGTA, and 1 mM dithiothreitol and dissociated using an ultrasonic oscillator. Debris was removed by centrifugation (15,000 rpm for 20 min), and solid ammonium sulfate (80% w/v) was added to the supernatant, and the mixture was stored overnight at 4 °C. The precipitated proteins were collected by centrifugation, and the pellet was resuspended in 10 mM Tris HCl, pH 8.0, containing 0.5 mM PMSF, 0.5 mM EGTA, and 1 mM dithiothreitol, and salt was removed by ultrafiltration with an Amicon filtration cell containing a membrane filter with a molecular weight cut-off of 10,000 against the same solution. The concentrated sample was loaded onto an affinity column containing mouse monoclonal antibody (6) coupled to Affi-Gel HZ (Bio-Rad, Hemel Hempstead, United Kingdom) equilibrated with 10 mM Tris HCl, pH 8.0.

After overnight circulation at a flow rate of 5 ml/h, the column was washed with 10 mM Tris HCl, pH 8.0, and the retained material was eluted with 100 mM glycine HCl, pH 2.5, into tubes containing 1 M Tris HCl, pH 8.0, for neutralization. The fractions were counted for radioactivity using a Packard 2000 CA liquid scintillation analyzer.

Enzymatic and Chemical Deglycosylation—Enzyme treatments were performed as follows: PNGase F (1 unit/20 µl) for 24 h at 37 °C in 20 mM phosphate, pH 7.5, containing 50 mM EDTA and 0.2 mM PMSF; O-glycosidase (1 milliunit/20 µl) for 20 h at 37 °C in 100 mM phosphate/citrate, pH 6.0; neuraminidase (1.2 milliunits/ml) for 16 h at 37 °C in 50 mM sodium acetate, pH 5.0, containing 2 mM CaCl2; sulfatase (type H-1 from Helix pomatia), (16 units/ml) for 24 h at 37 °C in 200 mM sodium acetate, pH 5.0; endo-β-galactosidase for 20 min at 37 °C in 50 mM sodium acetate, pH 5.8; alkaline phosphatase (5 units) for 18 h at 37 °C in 0.5M NH4HCO3, pH 8.5; chondroitinase AC (1 unit/ml) for 18 h at 37 °C in 50 mM Tris HCl, pH 8.0, containing 50 mM NaCl; chondroitinase ABC (2 units/ml) for 18 h at 37 °C in 0.25M Tris HCl, pH 8.0. In all cases buffers contained 0.5 mM PMSF and 1 mM dithiothreitol to inhibit proteolytic degradation. Trypsin (300 µg/ml), chymotrypsin (300 µg/ml), and pepsin (200 µg/ml) were incubated for 1 h at 37 °C in 10 mM Tris-HCl, pH 8.0, containing 2 mM CaCl2 and Pronase (predigested for 10 min at 37 °C) for 1 h at 37 °C (4.3 units/ml) in 100 mM Tris-HCl, pH 8.0. Both chondroitinase AC and ABC were assayed before use using chondroitin A as substrate. The reaction rate was monitored by measuring the increase in absorbance at 232 nm. Enzyme activity calculated...
from the initial rate and using $E = 3800 \text{ m}^{-1}$ for reaction products at pH 8 was 2.85 and 2.70 m mol/min for 1 unit of chondroitinase ABC and AC, respectively. Chemical deglycosylation by anhydrous trifluoromethane sulfonic acid was achieved with a Glycofree deglycosylation kit according to the manufacturer's instructions. Nitrous acid deamination was carried out on the sample (in 50 ml of water) kept at room temperature for 10 min. The products of the reaction were then analyzed on a column of Sephadex G-50.

**Enzyme-linked Immunosorbent Plate Assay**—Samples were divided into two and immobilized on a 96-well polyvinylchloride assay plate overnight at 4 °C. The liquid was removed by aspiration, and the wells were washed three times with PBS + 0.1% Tween 20 (200 μl). Blocking solution (200 μl of PBS containing 0.1% Tween 20 and 3% bovine serum albumin) was added to the wells, and the plate was incubated for 2 h at 37 °C. One-half of the sample was incubated with the monoclonal antibody (10 μg/ml) in blocking solution (100 μl) for 1 h at room temperature, while the other half was incubated in the same solution but in the absence of the antibody. After removal of the antibody solution the wells were washed six times before the addition of a peroxidase-conjugated anti-mouse immunoglobulin, diluted 1 in 500 in blocking solution (100 μl/well), and the plates were incubated for a further 1 h at 37 °C. The wells were washed six times, and then the substrate solution, o-phenylenediamine dihydrochloride (0.04%), hydrogen peroxide (0.012%) in 0.15 m phosphate citrate buffer, pH 5.0 (100 μl/well), was added for 30 min. The reaction was terminated by the addition of 0.2 m H$_2$SO$_4$ (50 μl/well), and the absorbance was determined at 492 nm using a microplate reader (Anthos Labtec Instruments).

**RESULTS**

When isolated mouse gastrocnemius muscle was incubated with serum from mice bearing the MAC16 tumor and with a weight loss between 2 and 4.4 g an increased protein degradation was observed as measured by tyrosine release (Table I). This effect was attenuated by incubation of the serum with monoclonal antibody prior to addition to the muscle preparation. An increased tyrosine release could also be produced by addition of affinity purified antigen to serum from non-tumor-bearing mice (Table I), thus confirming that this material was the serum component responsible for the degradation of skeletal muscle proteins. The increased tyrosine release produced by the affinity purified antigen was abolished by preincubation with PNGase F, O-glycosidase, and sulfatase but unaffected by treatment with neuraminidase or trypsin. These results suggest that protein degradation is mediated by N- and O-linked carbohydrate chains in the molecule.

A similar relationship was obtained for antigen binding activity (Table II). Immunological activity was completely destroyed by treatment with periodate, indicating that the carbohydrate moieties are involved in the epitope. Antibody binding activity was inhibited by PNGase F, O-glycosidase, and sulfatase (Table II) but unaffected by treatment with neuraminidase or chymotrypsin, indicating that N- and O-linked

**FIG. 3.** Elution profile of radioactivity determined as $^35$S (●) and $^3$H (□) in the oligosaccharide chains released after enzymatic deglycosylation with PNGase F (A) and fractionation by high performance liquid chromatography on a DEAE-cellulose column (Applied Biosystems Inc.) under the influence of a NaCl gradient from 0 to 0.3 m NaCl. Affinity purified biosynthetically labeled material (Fig. 2) was subjected to enzymatic deglycosylation as described under “Experimental Procedures,” and the products were desalted using a microcon microconcentrator containing a filter with a molecular size cut-off of 10,000 (Amicon Corp.) and fractionated on a DEAE-cellulose column. The flow rate was 0.2 ml/min with solvent system A, 10 mm sodium phosphate buffer, pH 5.3, and solvent system B, 10 mm sodium phosphate containing 0.3 m NaCl. The gradient was for 10 min at 0% B, 40 min at 100% B, 50 min at 100% B, and 60 min at 0% B. Absorbance was monitored at 214 nm, and the radioactivity of the individual fractions was determined using a dual counting procedure. The bands eluting at 0.28 m NaCl (B) and 0.20 m NaCl (C) were further fractionated on a Sephadex G-50 column.
carbohydrate chains are the antigenic determinants. In addition antigen reactivity was reduced by treatment with chondroitinase AC and completely destroyed by treatment with chondroitinase ABC but was unaffected by treatment with endo-β-galactosidase. Since the latter enzyme only hydrolyzes internal β-galactoside linkages of oligosaccharides having non-sulfated β-galactose residues, these results suggest that the principal immunological determinants reside in sulfated oligosaccharide chains and that the material is a glycoprotein or proteoglycan.

To obtain information on the nature of the linkage and the number and types of glycan chains, MAC16 cells were doubly labeled with [35S]sulfate and [3H]GlcN, and the antigen was purified by ammonium sulfate fractionation and affinity chromatography. The elution profile from the affinity column showed a single band of radioactivity containing both radiolabels (Fig. 1A), which represented 13% of the [35S] and 15% of the [3H] radiolabel from the ammonium sulfate precipitate. This material showed a single band of radioactivity corresponding to a Mr of 24,000 on SDS-PAGE (Fig. 1B). Similar material could not be isolated from a cell line (MAC13) originating from a tumor that does not produce cachexia in vivo. Fractionation of the MAC16 material on a Sephadex G-50 column under dissociating conditions confirmed a single band of Mr 24,000 that contained both 35S and 3H (Fig. 2A). The Mr of this material was the same as that previously isolated from the MAC16 tumor using a combination of affinity and reverse phase hydrophobic chromatography (7). Identical sham incubations (with-
FIG. 5. A, autoradiograph of material, obtained by biosynthetic labeling of MAC16 cells with $^{32}$P orthophosphate and purification by affinity chromatography, on a 16.5% Tricine/SDS-PAGE gel. *Lane 1*, without treatment; *lane 2*, with chondroitinase AC; *lane 3*, with PNGase F; *lane 4*,
of PNGase F) showed no radioactivity eluting at positions corresponding to the released material. Re-chromatography of this material after overnight digestion with PNGase F gave two bands of radioactivity eluting at positions corresponding to Mr of 14,000 and 10,000 (Fig. 2B). Both fragments contained $^3$H and $^{35}$S. Digestion of the Mr 24,000 material with O-glycosidase and fractionation on Sephadex G-50 showed conversion to three bands of radioactivity corresponding to Mr of 14,000, 6,000, and 4,000 (Fig. 2C). Incubation with buffer in the absence of O-glycosidase showed no degradation of the Mr 24,000 material. The first two bands contained both $^3$H and $^{35}$S, and the band of Mr 4,000 contained only $^3$H. Treatment of the material of Mr 14,000 produced from PNGase F digestion with O-glycosidase converted it into two fractions corresponding to Mr of 6,000 and 4,000 (Fig. 2D).

To determine the acidity of the charged groups on each of the oligosaccharide chains, the Mr 24,000 material was subjected to enzymatic digestion as above, and the products were fractionated on a DEAE-cellulose column under the influence of a linear gradient from 0 to 0.3 M NaCl. The identity of the eluted peaks was determined by exclusion chromatography on Sephadex G-50. Anion exchange chromatography after digestion with PNGase F gave two bands eluting at 0.20 M NaCl (Mr 10,000) and 0.28 M NaCl (Mr 14,000) (Fig. 3). Digestion with O-glycosidase gave three bands that all adhered to the DEAE-cellulose column in order of elution 0.20 M NaCl (Mr 6,000), 0.24 M NaCl (Mr 4,000), and 0.29 M NaCl (Mr 14,000) (Fig. 4). In both cases there was no further fractionation between the $^3$H- and $^{35}$S-labeled oligosaccharide chains. Although the O-linked oligosaccharide chain of Mr 4,000 did not contain sulfate residues as determined by biosynthetic labeling, it was more acidic than the sulfated chains of Mr 6,000 and 10,000, as determined by the concentration of NaCl required to elute it from the DEAE-cellulose column.

The nature of the charged groups on this non-sulfated oligosaccharide chain was investigated by digestion with either neuraminidase or phosphatase followed by fractionation of the products by anion exchange chromatography. After treatment of the oligosaccharide with neuraminidase, the product still adhered to the DEAE-cellulose column and eluted at 0.24 M NaCl, suggesting the absence of sialic acid residues. However, after incubation with alkaline phosphatase the acid group was removed and the product no longer attached to the anion exchange column, although the apparent Mr was not affected (results not shown). The Mr of the intact molecule was also unaffected by alkaline phosphatase as determined by exclusion chromatography on Sephadex G-50. This suggests that phosphate residues are responsible for the negative charge on the O-glycosidase digestion product of Mr 4,000. Antibody binding activity of the Mr 24,000 material was reduced by 53% after treatment with alkaline phosphatase (Table II), and immunoreactivity on Western blotting was completely destroyed, suggesting that the phosphate residues are also important antigenic determinants. To confirm the presence of phosphate groups on the chains of Mr 4,000, MAC16 cells were biosynthetically labeled with $^{32}$P orthophosphate, and the antigen was purified by ammonium sulfate precipitation and affinity chromatography as before. The $^{32}$P was confined to a single band of Mr 24,000 as determined by SDS-PAGE (Fig. 5A) and exclusion chromatography on Sephadex G-50 (Fig. 5B). Treatment with PNGase F (Fig. 5, A and C) or chondroitinase AC (Fig. 5A) yielded a single fraction containing $^{32}$P of Mr 14,000. Digestion of the material of Mr 24,000 with either O-glycosidase (Fig. 5, A and D) or chondroitinase ABC gave a single band containing $^{32}$P of Mr 4,000. This material adhered to a DEAE-cellulose column and was eluted with 0.24 M NaCl (Fig. 5E) as did the fragment from material labeled with $^3$H(GlcN) (Fig. 3).

To determine the size of the polypeptide core, MAC16 cells were labeled with 1-[2,5-$^3$H]His, which is the amino acid at residue 16 of the core (6, 7). The radiolabel was incorporated into a single component of Mr 24,000 after fractionation of the cell extract by affinity chromatography (Fig. 5B; Fig. 6). Treatment with PNGase F showed $^3$H labeling of the fragment of Mr 14,000 (Fig. 5C; Fig. 6, the lower band in the autoradiograph may be an artifact). Treatment with O-glycosidase showed $^3$H labeling of the Mr 4,000 fragment (Fig. 5D; Fig. 6). This fragment was the same as that found after biosynthetic labeling with $^{32}$P since both adhered to a DEAE-cellulose column and were eluted with 0.24 M NaCl (Fig. 5E). Chemical deglycosylation with anhydrous trifluoromethanesulfonic acid showed a major band with a Mr near 4,000 but lower than that formed by treatment with O-glycosidase (Fig. 6) and a minor band at Mr 2,000. This suggests that the molecular weight of the polypeptide core is 4,000 and that there may be a short oligosaccharide chain (labeled with $^3$H(GlcN) which is phosphorylated or the peptide chain itself may be phosphorylated.

To determine the nature of the oligosaccharide chains, material of Mr 10,000, released by treatment of $^{32}$Sulfate- and $[^3]$H(GlcN-labeled Mr 24,000 with PNGase F, was treated with chondroitinase ABC followed by re-chromatography on a Sephadex G-50 column. Two bands of radioactivity corresponding to Mr of 8,000 and 2,000 were obtained (Fig. 7A) but no low molecular weight material corresponding to disaccharides. Treatment of the oligosaccharide chain of Mr 6,000 produced by cleavage with O-glycosidase with either chondroitinase AC or ABC (Fig. 7B) had no effect on the molecular weight. These results suggest that material of Mr 24,000 does not contain glycosaminoglycan chains and that it is a sulfated glycoprotein rather than a proteoglycan. This conclusion was substantiated by analysis of the carbohydrate chains released after treatment with 0.1 M NaOH and 2 M NaBH₄ for 16 h. Material of Mr 24,000 with O-glycosidase; lane 5, with chondroitinase ABC. B, elution profile of material from MAC16 cells on a Sephadex G-50 column after biosynthetically labeling with either $^{32}$P (●) or $[^3]$HHis (○) and purified by affinity chromatography; C, after treatment with PNGase F; D, with O-glycosidase. E, elution profile of radioactivity, determined either as $^{32}$P (●) or $^3$H (○) on a DEAE-cellulose column, as described in the legend to Fig. 4, of the fragment released by treatment with O-glycosidase as shown in D.
doubly labeled with [35S]sulfate and [3H]His released fragments of \( M_r \) 14,000, 6,000, and 4,000 (Fig. 7D). The latter fragment was labeled only with \(^3\)H, suggesting that it represented the polypeptide core. The elution positions of both the fragments of \( M_r \) 14,000 and 6,000 were not affected by further treatment with chondroitinase ABC, AC, or nitrous acid (data not shown) again confirming that the oligosaccharide chains were not of the chondroitin, dermatan, or heparan sulfate type.

**DISCUSSION**

The data in the present study provide strong support that protein degradation in skeletal muscle during the process of cancer cachexia is mediated by a tumor-produced sulfated glycoprotein of \( M_r \) 24,000. This material could be responsible for the accelerated breakdown of isolated rat diaphragm muscle observed when incubated with plasma from cancer patients with weight loss greater than 10% (12), since we have shown similar material in both human colonic carcinomas and in the urine of patients with pancreatic carcinoma and established cachexia (7). Although interleukin-1\( \alpha \) and interleukin-1\( \beta \) were shown to stimulate protein catabolism in the rat diaphragm muscle bioassay, in the study of Belizario et al. (12) antibodies to the recombinant human cytokines gave only partial neutralization of bioactivity in less than half of the patients investigated, suggesting other active factors that could not be defined.

The structure of the \( M_r \) 24,000 material is novel, and the peptide sequence (6, 7) is distinct from the recognized cytokines. Antisera to this material was not cross-reactive with the cytokines' tumor necrosis factor-\( \alpha \) and interleukin-6. In addition, the ability of MAC16 cells to produce this material \textit{in vitro}, as demonstrated by biosynthetic labeling studies, confirms that it arises from tumor rather than host cells. A histologically related cell line, MAC13, derived from a non-cachexia-inducing tumor was found not to be capable of producing radioactively labeled \( M_r \) 24,000 material using either [6-\(^3\)H]GlcN or \( \text{Na}_2\text{SO}_4 \) as the precursors. This suggests that it is only produced by some tumor cell lines which are capable of inducing cachexia.

Both functional and immunological studies provide evidence that biological activity is mediated through \( N \)- and \( O \)-linked oligosaccharide chains in the molecule. To identify the size and position of attachment of the oligosaccharide chains as well as the size of the polypeptide core, MAC16 cells have been biosynthetically labeled with \(^{35}\text{SO}_4 \), [\(^3\)H]GlcN, [\(^3\)H]His, and \(^{32}\text{P}_i \) followed by affinity purification to isolate the labeled product. In all cases a single component of \( M_r \) 24,000 was obtained as determined by SDS-PAGE and exclusion chromatography. Similar results have previously been obtained with high performance liquid chromatography-purified material labeled with \( \text{Na}^{125}\text{I} \) (7).

With regard to the molecular weight of the material and the fragments generated by enzymatic deglycosylation, these are apparent rather than real, since glycosylated molecules have hydrodynamic volumes that differ per unit of molecular weight from those of globular proteins, which have been used as molecular weight standards.

With this caveat incubation with recombinant protease-free PNGase F, which specifically cleaves the GlcNAc-Asn bond of

\[ 37 \, ^{\circ}\text{C} \text{in 250 mM Tris-HCl, pH 8.0.} \]

\text{C, elution profile of radioactivity on Sephadex G-50 from affinity purified material of} \( M_r \) 24,000 biosynthetically labeled with [\(^{35}\text{SO}_4 \) (A) and O-glycosidase (B) after incubation with chondroitinase ABC (0.2 units in 0.1 ml) for 18 h at

\[ 37 \, ^{\circ}\text{C} \text{in 250 mM Tris-HCl, pH 8.0.} \]

\text{C, elution profile of radioactivity on Sephadex G-50 from affinity purified material of} \( M_r \) 24,000 biosynthetically labeled with [\(^{35}\text{SO}_4 \) (A) and [\(^3\)H]His (C) after incubation with 2 mM NaBH}_4 in 0.1 M NaOH for 16 h at 37 \, ^{\circ}\text{C}. Excess NaBH}_4 was destroyed with 0.25 M acetic acid in methanol, and the boric acid was removed by evaporation of the methanolic solution under a stream of nitrogen. The procedure was repeated twice with the same amount of acidified methanol and twice with methanol alone.
N-linked oligosaccharides (13), yielded two fragments one of M \(_r\) 14,000 and one of M \(_r\) 10,000. The former fragment contained the peptide chain since it contained \(^{3}H\)His (Fig. 6) or \(^{125}I\), when Na\(^{125}I\) was used (7). The latter fragment contained only \(^{35}S\) and \(^{1}H\) when \(^{35}SO_4\) and \(^{3}H\)GlcN were used in biosynthetic labeling studies (Fig. 2B), suggesting that it is an oligosaccharide chain. Retention of \(^{35}S\) and \(^{1}H\) in the M \(_r\) 14,000 fragment suggests that it also contains an oligosaccharide chain. Chondroitinase AC also cleaved material of M \(_r\) 24,000 into the fragment of M \(_r\) 14,000 (Fig. 5) and caused the same decrease in antibody binding activity as PNGase F. However, the fragment of M \(_r\) 14,000 was not cleaved into lower molecular weight material by chondroitinase AC or ABC and did not release any of the \(^{35}SO_4\) label with nitrous acid, whereas the oligosaccharide chain of M \(_r\) 10,000 formed two products with chondroitinase ABC M \(_r\) 8,000 and 2,000 (Fig. 7A). These results suggest the absence of N-sulfate residues and the lack of formation of disaccharides with chondroitinase AC or ABC suggest that the oligosaccharide chains were not of the chondroitin or dermatan sulfate type. The presence of sulfate in N-linked oligosaccharides has been reported in several proteins including the low density lipoprotein receptor (14), ovalbumin (15), and pituitary hormones (16, 17).

Treatment of material of M \(_r\) 24,000 with O-glycosidase yielded two fragments of M \(_r\) 6,000 and 4,000. Material biosynthetically labeled with \(^{35}SO_4\) and \(^{3}H\)GlcN showed the two labels to be incorporated into the fragment of M \(_r\) 6,000, suggesting that it was an O-linked glycan chain. O-Glycosidase has stringent specificity for the core structure Galβ1–3 GalNAc α1-Ser/Thr and will not cleave any other O-linked glycan. Thus the sulfoglycan of M \(_r\) 6,000 could not be a typical chondroitin sulfate chain with xylose O-glycosidically linked to serine. (18). This conclusion is substantiated by lack of cleavage of the oligosaccharide fragment by either chondroitinase ABC or AC. Thus the material of M \(_r\) 24,000 does not appear to contain glycosaminoglycan chains attached to either Asn or Ser residues and is therefore a sulfated glycoprotein rather than a proteoglycan.

Treatment of the fragment of M \(_r\) 14,000, obtained by digestion of material of M \(_r\) 24,000 with PNGase F, with O-glycosidase also yielded the two fragments of M \(_r\) 6,000 and 4,000. Although only material of M \(_r\) 6,000 was labeled with \(^{35}S\), both fragments were acidic as determined by the binding to a DEAE-cellulose column and subsequent elution with NaCl. The charge on the fragment of M \(_r\) 6,000 was removed by treatment with alkaline phosphatase, suggesting that the acidic group was phosphate. This was confirmed by biosynthetically labeling MAC16 cells with \(^{32}P\)orthophosphate, which led to \(^{32}P\) incorporation into the glycoprotein of M \(_r\) 24,000. The PNGase F cleavage fragment of M \(_r\) 14,000, and the O-glycosidase cleavage fragment of M \(_r\) 4,000 (Fig. 6). Biosynthetic labeling of the polypeptide core with \(^{3}H\)His also showed incorporation of the radiolabel into the same fragments (Fig. 6). This suggests that the phosphate residues are attached to the peptide core or a short oligosaccharide chain containing GlcN attached to the peptide core.

Amino acid sequence studies showed a short polypeptide core containing 18 (20) amino acids (6, 7). Attempts to determine the molecular weight of the polypeptide have yielded conflicting results. Thus chemical deglycosylation of the iodinated material using anhydrous trifluoromethanesulfonic acid gave a single band of M \(_r\) 2,500 (7). However, chemical deglycosylation using material that had been biosynthetically labeled with \(^{3}H\)His showed a strong band at M \(_r\) 4,000 and a weaker one at M \(_r\) 2,000. The latter result suggests that the polypeptide chain may be longer that that previously reported.

These results suggest a model for the M \(_r\) 24,000 glycoprotein consisting of a central polypeptide chain and a short oligosaccharide chain containing GlcN and with phosphate residues and of M \(_r\) 4,000, one O-linked sulfated oligosaccharide chain containing GlcN and of M \(_r\) 6,000, and one N-linked sulfated oligosaccharide chain of M \(_r\) 10,000 also containing GlcN. The apparent difference between the sum of the molecular weight of the subunits and that of the whole molecule may be due to differences in the true molecular weight of the carbohydrate fragments with that achieved using globular proteins as molecular weight standards. The high negative charge on all the fragments may also lead to disparities in calculating the exact molecular weight.

The sequence contained a single Asn residue, which must be the site for N-glycosylation (7). The sequence NXS is an N-glycosylation sequon, although the presence of proline in the middle of the sequon has been suggested to inhibit the attachment of sugars (19). Thus it is possible that the Asn that is glycosylated is downstream of that in the sequence reported.

Although only material of M \(_r\) 6,000 was recovered at all positions, and residue 15 often yielded a blank, strongly suggesting O-glycosylation at this site, since hydrophobic amino acid derivatives are not efficiently extracted in the non-polar solvent used after cleavage in the Edman degradation (20).

The biological significance of sulfate esters on O- and N-linked oligosaccharide chains is presently unknown. In thyroglobulin sulfated complex N-linked oligosaccharides may serve as recognition signals in directing the intracellular traffic, follicular secretion, or reabsorption (21). Sulfate residues would also provide a strong electrostatic linkage to cellular receptors leading to tight binding. It is not known why the M \(_r\) 24,000 glycoprotein should be expressed only in tumor cells capable of producing cachexia. There are quantitative and qualitative changes in the expression of acidic glycoconjugates in colon cancer, which are associated with progression and metastasis (22, 23). In addition to differential expression of the core polypeptide in tumors producing cachexia, there may be differences in expression of glycosyl and sulfotransferases. Considering the complexity of structure of this material it is not surprising that only certain tumor cells have the enzymatic machinery capable of production. However, the conservation of structure between mouse and man (7) suggests that this material may be important for tumor function.

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Penio T. Todorov, Melanie Deacon and Michael J. Tisdale

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