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Purification and Characterization of a Human Pancreatic Adenocarcinoma Mucin[1]

Ali M. Khorrami,[*] Amit Choudhury,[†] Mahefatiana Andrianifahanana,[†] Grish C. Varshney,[†] Sambhu N. Bhattacharyya,[‡] Michael A. Hollingsworth,[§] Bernard Kaufman,[‖] and Surinder K. Batra[†,§,2]

[*]S.U.N.Y., Upstate Medical University, Syracuse, NY 13202, USA; [†]Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE 68198, USA; [§]Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, NE 68198, USA; [‡]Department of Clinical Investigation, William Beaumont Army Medical Center, El Paso, TX 79920, USA; and [‖]Department of Biochemistry, Duke University Medical Center, Durham, NC 27710, USA

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Pancreatic mucins consist of core proteins that are decorated with carbohydrate structures. Previous studies have identified at least two physically distinct populations of mucins produced by a pancreatic adenocarcinoma cell line (HPAF); one is the MUC1 core protein, which includes an oligosaccharide structure identified by a monoclonal antibody (MAb) recognizing the DU-PAN-2 epitope. In this study, we purified and characterized a second mucin fraction, which also shows reactivity with the DU-PAN-2 antibody, but which has an amino acid composition that is not consistent with the MUC1 core protein. This new mucin was purified by ammonium sulfate precipitation, molecular sieve chromatography, and density gradient centrifugation. It eluted in the void volume of a Sepharose 4B column together with an associated low molecular weight protein, which could be further resolved. The mucin is highly polyanionic due to numerous sulfated and sialylated saccharide chains. Carbohydrate analyses of the purified mucin showed the presence of galactose, glucosamine, galactosamine, and sialic acid, but no mannose, glucose, or uronic acid. The purified and deglycosylated mucin shows no reactivity with anti-MUC1 apomucin antibody, but reacts with antiserum against deglycosylated tracheal mucins and antiserum against the MUC4 tandem repeat peptide. Analysis of mucin expression in HPAF cells revealed high levels of *MUC1* and *MUC4* mRNA, and moderate levels of *MUC5AC* and *MUC5B* mRNA. The amino acid composition of the purified mucin shows a high degree of similarity to the MUC4 core protein.

Key words: adenocarcinoma, DU-PAN-2, glycoproteins, MUC4, mucins, pancreatic neoplasms.

Mucins are important molecules produced by secretory epithelial cells for the lubrication and protection of ducts and lumens within the human body. Mucins contain a high percentage of serine, threonine, proline, alanine, and glycine, and are heavily glycosylated (*O*-linked) (*1–3*). Mucins from different organs, however, usually have distinct physical and biochemical properties (including oligosaccharide structure and composition, sulfation, molecular weight, and viscoelastic properties) (*4*).

Currently, 12 distinct genes encoding human mucins have been identified, designated *MUC1-4*, *MUC5B*, *MUC5AC*, *MUC6-8*, *MUC9*, *MUC11*, and *MUC12* (*5–13*). The complete primary amino acid sequences of *MUC1*, *MUC2*, *MUC4*, *MUC5B*, and *MUC7* are known. Mucins have been classified into two main categories: secreted (soluble non-gel-forming and gel-forming) and membrane-bound. The membrane-bound mucin family comprises *MUC1*, *MUC3*, *MUC4*, and *MUC12*. In pancreatic cancer, dysregulation of mucin genes leads to the overexpression of *MUC1* and the ectopic expression of *MUC3*, *MUC4*, and *MUC5AC*.

Pancreatic mucins are expressed at high levels in well-differentiated tumors; however, undifferentiated tumors show low or undetectable levels of mucin expression (*14*). There is a complex spatio-temporal regulation of the mucin genes during development in the pancreas that suggests a possible regulatory role for mucin gene products in gastroduodenal epithelial cell differentiation (*15*). Pancreatic tumor-associated mucins are also aberrantly glycosylated, which results in the expression of a variety of tumor-associated carbohydrate structures, some of which can be identi-

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[2]To whom correspondence should be addressed at: Department of Biochemistry and Molecular Biology, Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, 984525 Nebraska Medical Center, Omaha, NE 68198-4525, USA. Phone: +1-402-559-5455, Fax: +1-402-559-6650, E-mail: sbatra@unmc.edu

Abbreviations: SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; 2-ME, 2-mercaptoethanol.

fied by monoclonal antibodies. Mucin-associated antigens, including sialyl-Lewis (a), Lewis (x), and DU-PAN-2, are implicated in tumor cell metastasis and are used in diagnostic tests for cancers. The DU-PAN-2 antibody, originally raised against a human pancreatic adenocarcinoma, identifies a molecular component that is present in high concentrations in the serum of 70% of patients with pancreatic adenocarcinoma (*16, 17*). The DU-PAN-2 antibody has been shown to recognize the oligosaccharide structure of sialyl lewis C (*18*). The DU-PAN-2 epitope is expressed on a limited subset of normal secretory epithelia mostly derived from the embyonic foregut (*17*).

We previously showed that one core protein on which the DU-PAN-2 epitope is expressed in pancreatic adenocarcinomas is MUC1, a cell surface-associated mucin-type glycoprotein (*19*). Previously, agarose gel electrophoretic analysis of DU-PAN-2 antigen purified from a pancreatic tumor cell line led to the detection of two primary components, designated FI and FII, and a trace component designated FIII (*20, 21*). Although FII was initially thought to be a proteolytic product of FI, $^{35}SO_4$-labeling experiments indicated that these might be distinct glycoproteins. Subsequent molecular analysis led to the determination that the *MUC1* gene encodes the protein core of FII (*5, 21*). In the present report, we describe the characterization of a polyanionic mucin, the protein core of which bears the DU-PAN-2 antigen, but which is distinct from MUC1. A comparison of the amino-acid composition of this mucin with those of known mucin genes expressed in HPAF cells suggests that the purified mucin may be the protein product of the *MUC4* gene.

MATERIALS AND METHODS

HPAF Cell Line—The cell line was established from the ascites of a patient with pancreatic adenocarcinoma (*17*). Cells were cultured in Dulbecco's modified Eagle's medium (GIBCO-BRL, MD) supplemented with 10% fetal calf serum.

Purification of Human Pancreatic Mucin—HPAF culture fluid was used as the starting material. The purification of DU-PAN-2–positive material was monitored for total carbohydrate, protein, and units of DU-PAN-2 binding activity. The quantity of DU-PAN-2 antigen in a sample was designated as arbitrary units per milliliter and calculated by a competitive inhibition radioimmmunoassay (*22*). A 50–75% saturated ammonium sulfate fraction was prepared from HPAF culture fluid at 4°C. The precipitate was dissolved in 0.1% (w/v) sodium azide, dialyzed against 0.01 M ammonium acetate, and concentrated 10-fold by pervaporation. This sample was chromatographed on a Sepharose 4B column (Pharmacia Fine Chemicals, Piscataway, NJ) (2 cm × 95 cm) using 0.1% SDS (w/v) in 0.1 M ammonium acetate. The void volume eluate was concentrated, 2-ME was added (0.5 ml per 6 ml of concentrate), and the mixture was rechromatographed on a Sepharose 4B column using 0.1% SDS/0.1 M ammonium acetate/1 mM DTT as the elution buffer. The void volume fraction was dialyzed against two changes of 4 liters of 1 M urea (deionized with AG 501-X8 prior to use), 0.01 M NaCl at 25°C for 24 h, and then for 3 days at 4°C against 4 liters of distilled water, with the water changed every 12–18 h. Following dialysis, no SDS was detected when measured by the methylene blue procedure (*23*). The mucin components were purified further by CsCl density gradient centrifugation at a loading density of 1.42 g/ml as described previously (*21, 24*). Gradients were formed by centrifugation in a Beckman SW 41 Ti rotor at 28,000 rpm for 96 h at 10°C. One-milliliter fractions were collected, and the density of each was determined by weighing a 100-μl aliquot.

Preparation of Radiolabeled Mucin—Mucin was labeled using a method previously described (*21*). Briefly, five 150-cm^2 flasks of 50% confluent HPAF cells were labeled with 1 mCi D-[6-^{3}H]Glucosamine-HCl (E.I. Dupont DeNemours, Wilmington, DE) for 3 days. Another five flasks were labeled with both 1 mCi of D-[6-^{3}H]Glucosamine-HCl and 5 mCi of ^{35}S Na_2SO_4 (E.I. Dupont DeNemours). The culture medium was separated, and the radioactive mucin was purified according to the procedure described above.

Gel Electrophoresis—SDS-PAGE was done under denaturing and reducing conditions in 7% running and 4.0% stacking gels (*25*). Proteins and glycoproteins were visualized either by silver staining (*26*) or by periodate SchiffAfs followed by Coomassie Blue staining (*27*).

Chemical Analyses—Amino acid composition was determined on a Glenco amino acid analyzer using an AA911 amino acid analysis column (Interaction Chemicals, Mountain View, CA). Samples were hydrolyzed in 6 N HCl at 105°C for 20 h *in vacuo*. Cysteine/cystine residues were measured by conversion to cystic acid using a mixture of 5 volumes of 30% hydrogen peroxide and 95 volumes of 99% formic acid (*28*).

Total neutral sugars were estimated by the phenol-sulfuric acid method with galactose as a standard (*29*). To 1 ml of the sample, 30 μl of 80% (w/v) phenol solution was added followed by 2.5 ml of concentrated H_2SO_4. The color of the solution was measured at 490 nm in a spectrophotometer.

The mole percent and identity of each saccharide were determined by gas-liquid chromatography of trimethylsilyl derivatives of the methyl glycosides on a 3% SE-30 column (6′ × 1/8″) (*30*). Methanolysis was performed in 0.5 N methanolic-HCl (anhydrous) (Supelco, Bellefonte, PA) at a 65°C for 24 h followed by removal of the methanol and HCl by evaporation *in vacuo*. The amino sugars were *N*-acetylated by reaction at 25°C for 30 min in a reaction mix containing 0.25 ml methanol, 0.05 ml pyridine, and 0.025 ml acetic anhydride (*31*). The samples were evaporated to dryness and derivatized by incubation in TRI-SIL′Z′ (Pierce, Rockford, IL) reagent for 15 min at 25°C. The sialic acid content of the samples was determined by the thiobarbituric acid (Eastman Organic Chemicals, Rochester, NY) method using *N*-acetylneuraminic acid as the standard (*32*).

Hexosamines were measured as previously described (*33*). Samples were hydrolyzed in 4 N HCl at 105°C for 6 h in a N_2 atmosphere and dried *in vacuo*. The sulfate content was measured by the sodium rhodizonate (Fisher Scientific, Fairlawn, NJ) method using H_2SO_4 as the standard (*34*). The sulfate content was measured before and after hydrolysis to correct for any inorganic sulfate in the preparation.

Alkaline Reductive β-Elimination—The alkaline borohydride reaction conditions were the same as previously reported, except that the temperature was raised to 50°C and the time was extended to 24 h (*35*). Excess borohydride was destroyed by the careful addition of acetic acid, and the reaction mix was dialyzed against distilled water. The sample was then hydrolyzed for hexosamine and hexosaminitol

analyses or passed through a DEAE-Sephadex A-25 column to separate oligosaccharide chains based on charge.

Deglycosylation of Mucins—Deglycosylation of mucin glycoprotein was performed by a modified procedure (*36–38*). Briefly, one part of anisol and two parts of TFMS (Aldrich Chemical Company, Milwaukee, WI) were mixed and cooled to 0°C, and then purified the mucins were dissolved in 1 ml of this mixture in a reaction vial under nitrogen. The reaction was performed at 25°C for 3 h. The reaction mixture was neutralized with an equal volume of ice-cold 50% (v/v) aqueous pyridine. Ether extraction of the aqueous phase was repeated several times, and the final products in the aqueous phase were dialyzed against several changes of 0.1 M ammonium acetate buffer and further purified by HPLC (*38*).

Binding of Antibodies to Apomucin and Synthetic Peptides—Purified pancreatic apomucin (200 ng), tracheal apomucin (200 ng), synthetic peptides (400 ng) corresponding to the MUC1 tandem repeat sequence (*5*) and the MUC4 tandem repeat sequence (*39, 40*) were dried in the wells of a micotiter plate. After blocking non-specific binding with 5% bovine serum albumin, rabbit antiserum (1:200) or HMFG2 (0.5 μg/ml) was added to the wells for 2 h at room temperature. Bound antibodies were detected by adding either ^{125}I-Protein A or goat anti-rabbit antibodies conjugated to alkaline phosphatase (Sigma) as a secondary ligand. In the latter case, the color was developed using *p*-nitrophenyl phosphate as the substrate, and the absorbance was read at 410 nm using a Dynatech MR 5000 automatic 96-well microtiter reader (Chantilly, VA).

Isolation of RNA—Total cellular RNA was isolated from the tumor cells by guanidine isothiocyanate cesium chloride cushion ultracentrifugation (*41, 42*). The cells were washed twice with ice-cold phosphate buffered saline (pH 7.4) and lysed with a solution containing 4 M guanidine isothiocyanate, 0.05 M sodium acetate, and 250 mM 2-mercaptoethanol. Total RNA was recovered via sedimentation through a 5.7 M CsCl in 0.025 M sodium acetate cushion in a Beckman SW40Ti rotor centrifuged at 32,000 rpm for 18 h. RNA pellets were resuspended in 0.3 M sodium acetate and precipitated with ethanol.

Semi-Quantitative Reverse Transcription-PCR (RT-PCR)—The total RNA (0.5 μg) from the tumor tissue or cell lines was reverse transcribed using a first-strand cDNA synthesis kit (Perkin Elmer, Branchburg, NJ) and oligo d(T) primers according to the manufacturer's instructions. Oligonucleotide primers to the nontandem repeat region of the *MUC 1, 2, 3, 4, 5AC, 5B, 6, 7, 11,* and *12* (*43*) genes were designed from the sequences published in GenBank. Amplifications were performed in a programmable thermal controller (PTC-100, MJ Research, Watertown, MA). The PCR amplification reactions were conducted in 50 μl reaction volume containing 5 μl of 10× Perkin Elmer buffer, 5 μl of 10 mM deoxynucleoside triphosphates, 2 μl of first-strand pancreatic cell line cDNA, 5 μl of 25 mM $MgCl_2$, 10 pmol of each primer, and 2 units of Taq DNA polymerase (ampli Taq Gold, Perkin Elmer). The mixture was denatured at 96°C for 10 min followed by 30 cycles at 96°C for 30 s, 60°C for 30 s, and 72°C for 1 min. The final elongation step was extended for an additional 15 min. The sequence of the PCR product was confirmed by the dideoxy-mediated chain termination method. The positive controls for *MUC1* and *MUC4* were HPAF/CD18, for *MUC6*, normal pancreas; *MUC2* and *MUC3*, small intestine; *MUC5AC* and *5B*, trachea; *MUC7*, salivary gland; and *MUC11* and *MUC12*, colon (*5–13*).

PCR products were run in 1% agarose gels, stained with ethidium bromide, and scanned on a Nucleo Vision™ 760 Imaging Workstation. The amplified products were quantified for each sample using the gel expert™ 3.5 software suite (Nucleotech Corporation, San Mateo, CA). The densitometric values were calculated for the specific gene product and *GAPDH* for each reaction. The value for mucin gene-specific products are expressed per unit of *GAPDH* to account for any differences in starting amounts of RNA.

Northern Blot Analysis—Total RNA (20 μg) was fractionated by electrophoresis in 1.0 % agarose gels containing 0.66 M formaldehyde and transferred to nylon membranes

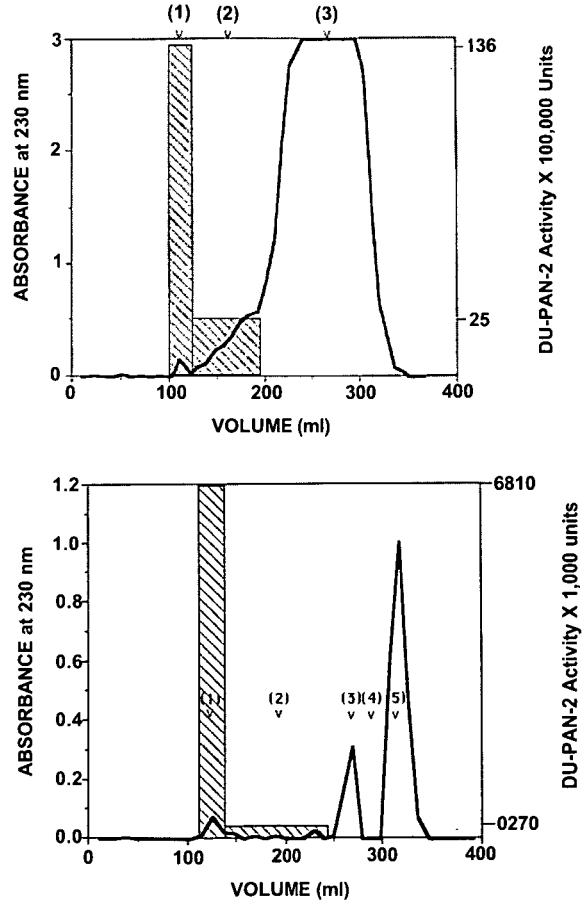

Fig. 1. **Molecular sieve analysis on Sepharose 4B.** A) The ammonium sulfate fraction (50–75%) was chromatographed on a Sepharose 4B column as described in "MATERIALS AND METHODS." The bar graphs indicate the pooled fractions (width) and the total units of DU-PAN-2 activity (height). The solid bold line indicates the OD profile. Numbers in parentheses indicate the fraction number. Fraction 1 (void volume) was used for further purification. B) The void volume fraction from the first Sepharose 4B column was rechromatographed. Fraction 1 from the previous Sepharose 4B chromatogram was concentrated, 2-ME was added, and the solution was then applied to a second Sepharose 4B column. The bar graphs indicate the pooled fractions (width) and the total units of DU-PAN-2 activity (height). The solid line indicates the OD profile. Numbers in parentheses indicate the fraction number. Fraction 1 (void volume) was used for further purification.

via capillary blotting. The cDNA probes were labeled with ^{32}P-dCTP using a random primed labeling kit, and separated from the free label by Sephadex G-50 column chromatography. Prehybridization and hybridization of blots were carried out in a solution of 5× SSPE, 50% formamide, 5× Denhardt's reagent, 200 μg/ml of sheared salmon sperm DNA, and a minimum of 10^6 cpm/ml of probe at 42°C for 18 h. Blots were washed twice with 2× SSC containing 0.1% SDS at room temperature for 15 min, followed by four washes with 0.2× SSC, 0.1% SDS at 60°C.

The human cDNA probes used in this analysis were: *MUC1* (*5*), *MUC4* (*40*), *MUC5AC* (*44*), and a 780-bp human glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). The *GAPDH* probe has 31 bp of the 5′ untranslated region and the region coding the first 250 amino acid residues of the protein.

Analysis of Deduced Amino Acid Composition—The mole percent of the amino acid residues in various mucin genes were calculated using the peptidesort program of GCG software (Madison, WI).

RESULTS

Purification of Human Pancreatic Mucin—Mucin purification was monitored for total protein and units of DU-PAN-2 binding activity. Ninety percent of the activity was present in the extracellular fraction, which was used as the starting material in the purification protocol. To test for the presence of mucin and mucin-type glycoproteins in fetal calf serum that would co-fractionate with the adenocarcinoma mucin, 100 ml of serum was mock-processed according to the same purification protocol, and CsCl density gradient fractions with densities higher than 1.427 g/ml were combined. The hydrolysate of the mock sample contained no detectable amino acid or hexosamine.

Fractional ammonium sulfate precipitation was used as the first step in the purification to reduce the working volume. The 50–75% $(NH_4)_2SO_4$ fraction was passed through a Sepharose 4B column (Fig. 1A). Eluates constituting the void volume fraction (fraction 1, Fig. 1A) contained 76% of the total DU-PAN-2 units (Table I). The remainder of the activity was present in fraction 2, which corresponded to the elution position of the previously described MUC1 gene product. This fraction also contained higher molecular weight components in the leading edge of the principal protein peak (fraction 3, Fig. 1A). The SDS-PAGE analysis of fraction 1 showed only intense Periodate-Schiff's staining with lightly stained Coomassie Blue component(s) in the spacer and at the top of the running gel. Silver stained gels, however, showed two additional components (molecular masses approximately 68 and 70 kDa) that were predicted to be the fraction 2 or 3 eluates. Re-chromatography of fraction 1 on a bed of Sepharose 4B in SDS/1 mM DTT/0.1 M NH_4Ac buffer yielded only the V_0 components, and SDS-PAGE analysis gave the same results as described above. Mercaptoethanol, a constituent of the sample buffer used in the SDS-PAGE analyses, was added to the chromatography sample buffer, and the V_0 was again subjected to chromatography on Sepharose 4B with elution buffer containing SDS/1 mM DTT/0.1 M NH_4Ac. The elution profile now

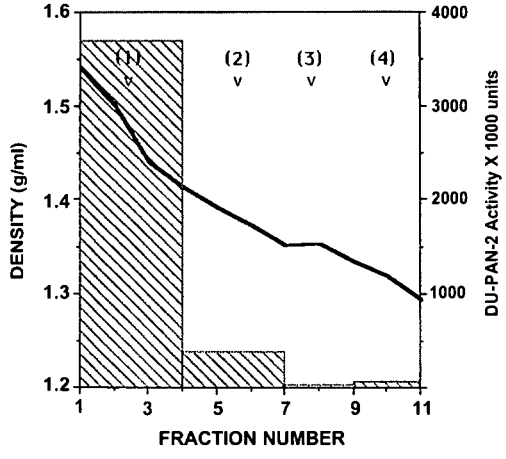

Fig. 2. **Buoyant density gradient ultracentrifugation.** The void volume eluates from the second Sepharose 4B chromatography were combined and subjected to buoyant density gradient centrifugation. The bar graph indicates the pooled fractions (width) and the total units of DU-PAN-2 activity (height). Numbers in parentheses indicate the fraction number. The solid line represents the buoyant density gradient as determined by weighing 100-μl aliquots. Fraction 1 was the purified mucin.

TABLE I. **Summary of the purification steps for DU-PAN-2 reactive mucin from HPAF culture.**

Fraction	Volume (ml)	DU-PAN-2 activity[a]	Protein concentration[b]	Specific activity DU-PAN-2[c]
Spent culture supernatant	10,500	5,242	3,864	1.36
0–50% $(NH_4)_2SO_4$ precipitate	770	560	5,300	0.11
50–75% $(NH_4)_2SO_4$ precipitate	1,085	21,658	15,600	1.39
50–75% $(NH_4)_2SO_4$ supernatant	26,565	127	2,500	0.05
First sepharose-4B column				
Fraction 1	185	55,132	NA	–
Fraction 2	567	5,733	18.1	317
Fraction 3	1,335	0	3,688	0
Second sepharose-4B column				
Fraction 1	187	42,042	0.88	47,775
Fraction 2	514	1,747	1.00	1,747
Fraction 3	178	0	7.38	0
CsCl density gradient				
Fraction 1	142	19,600	0.38	51,579
Fraction 2	99	3,880	NA	–

[a]Expressed in units/ml. [b]Expressed in mg/ml. [c]Expressed in units/mg protein

showed a protein component in the 70 kDa range in addition to the V_0 component (Fig. 1B). SDS-PAGE analysis of the V_0 component no longer showed the presence of the 68 and 70 kDa components, the latter component now being found in fraction 3 (Fig. 1B). Once separated from the V_0 component, the 68 and 70 kDa components eluted in the same fractions as before mercaptoethanol was added to the sample buffer, and all attempts to reconstitute binding to the V_0 component were unsuccessful.

The mucin (V_0) fraction was extensively dialyzed after the second Sepharose 4B column chromatography to remove SDS, and then further purified by density gradient centrifugation. Fractions with densities greater than 1.44 g/ml contained the highest levels of DU-PAN-2 activity and were pooled (Fig. 2). Table I shows the DU-PAN-2 activity and protein content at the various purification steps. The specific activity of the purified component was defined as DU-PAN-2 units per μg of protein, and ranged from 1.36 for HPAF culture fluid (starting material) to 51,579 for the purified material. SDS-PAGE gels were used to evaluate the purity of the fractions and were developed with Periodate-Schiff's, Coomassie Blue, and silver stain reagents. No protein components from the crude starting material were detected in the purified fractions, as shown in Fig. 3.

Chemical Composition—Amino acid analysis of the purified material (Table II) showed components typical of mucin-type glycoproteins with a predominance of serine, threonine, glycine, and alanine residues.

Carbohydrate accounted for 89% of the weight calculated by summing the carbohydrate, amino acid, and sulfate contents. Mole percent of saccharide is shown in Table III. Neither mannose nor uronic acid was detected in the purified sample, with the detection limit being approximately 10 nmol per 1,000 nmol of galactose. Under alkaline reductive β-elimination, 84% of the galactosamine was converted to galactosaminitol, which is within the range of yields commonly observed for complex oligosachharides (*45*). The production of α-amino butyrate was evident in the amino acid analyses. A balance sheet comparing serine and threonine destruction with the formation of alanine and α-amino butyrate and galactosaminitol could not be obtained because of extensive fragmentation of the peptide chain. When the reaction products were dialyzed, all hexosamine and hexosaminitol was recovered within the dialysis bag, whereas a considerable portion of the amino acids was dialyzed out with the salts. It was not possible to recover the dialyzable peptides from the salts in order to obtain quantitative numbers needed to generate a balance sheet. Furthermore, size exclusion chromatography was not useful for separating peptides from the salts.

Sulfate analysis together with the incorporation of ^{35}S sulfate showed that the purified mucin was sulfated. The quantitative value for sulfate is shown in Table III.

Ion-Exchange and Bio-gel P-4 Chromatography—The products of reductive β-elimination of HPAF pancreatic tumor mucin were passed through a DEAE Sephadex A-25

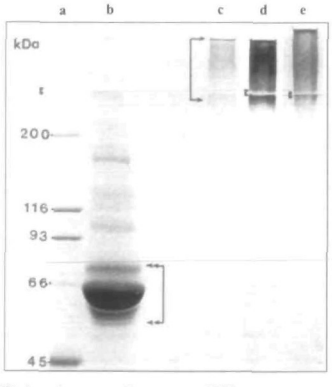

Fig. 3. **SDS-PAGE of samples at different steps of purification.** Lanes a, b, and c were stained with periodate Schiff's reagent followed by Coomassie Blue. Bands that stained with periodate SchiffAfs reagent are indicated by single arrows, while bands that stained with Coommasie Blue are indicated by double arrows. Lanes d and e were developed by a silver staining protocol. Lane a, protein standard; Lane b, crude HPAF culture medium; Lanes c and d, mucin sample the after second Sepharose 4B chromatography; Lane e, purified mucin. The bracket denotes the space between the 4% stacking and 7% running gels.

TABLE II. **Amino acid composition of pancreatic tumor mucin (mole percent).**

Amino acid	Purified mucin[a]	Mucin cDNAs			
		MUC1[b]	*MUC4*[c]	*MUC5AC*[d]	*MUC5B*[e]
Asp	4.5	4.5	5.7	4.1	1.0
Thr	**20.4**	**13.5**	**20.6**	**10.9**	**28.3**
Ser	**20.7**	**12.2**	**21.8**	**9.9**	**13.5**
Glu	8.0	1.4	1.1	4.4	2.7
Pro	**10.1**	**19.0**	**8.3**	**7.9**	**11.2**
Gly	**7.2**	**8.9**	**5.7**	**7.2**	**6.3**
Ala	**6.4**	**16.1**	**8.7**	**5.7**	**8.8**
Cys*	trace	0.2	0.5	7.6	2.0
Val	6.6	6.0	7.9	7.3	4.4
Met	ND	0.3	0.4	1.3	1.2
Ile	5.3	0.9	0.8	3.0	2.0
Leu	4.8	2.7	8.0	5.8	5.1
Tyr	0.4	1.0	0.5	2.4	0.8
Phe	0.7	1.3	0.9	3.0	1.4
Lys	1.1	0.8	0.4	3.0	1.3
His	1.6	4.5	5.0	2.7	1.9
Arg	2.4	4.2	1.1	4.5	2.9

*Measured as cysteic acid; ND, not detected. [a]For amino acid composition, samples (in duplicates) were hydrolysed for 20 h and analyzed to give the average values shown above. The variation from the average of any single amino acid did not exceed 10%. Trace is considered between 1–3 residues/100 residues. [b]The mole percents of residues were calculated from J05582. [c]The mole percents of residues were calculated from AJ276359. [d]The mole percents of residues were calculated from AF015521, Z34277, and AJ001402. [e]The mole percent of residues were calculated from AJ004862, Z72496, and Y09788. Note: The major amino acid residues and their compositions are shown in bold.

TABLE III. **Carbohydrate and sulfate composition of purified pancreatic mucin.**

Component	Ratio[a]	Theory[b]
$GlcNH_2$	3.3	3.0
$GalNH_2$	1.0	1.0
NeuAc	1.7	2.0
Gal	2.8	3.0
Sulphate	0.8	1.0

[a]Galactosamine is assumed to be the linkage saccharide, and the molar ratios are calculated on the basis of one galactosamine per chain. [b]Given the differences in recoveries for the various hydrolytic procedures together with the differing precision and accuracy of the four analytical techniques, the variation between the observed molar ratios and those for a nine unit oligosachhride chain (theory) are within the range of experimental error.

column as shown in Fig. 4A. Eighty-five percent of the radioactivity loaded onto the column was recovered in the eluates, and 95% of that was recovered in the 0.5 M NaCl fraction. When the starting material was treated with neuraminidase, the elution profile remained unchanged (Fig. 4B), indicating that all the chains were sulfated. The fraction eluted by 0.5 M NaCl was next passed through a Bio-Gel P-4 column to verify different sizes of carbohydrate chains; however, only a single peak eluted from the column, which contained both ^{3}H and ^{35}S labels (Fig. 4C).

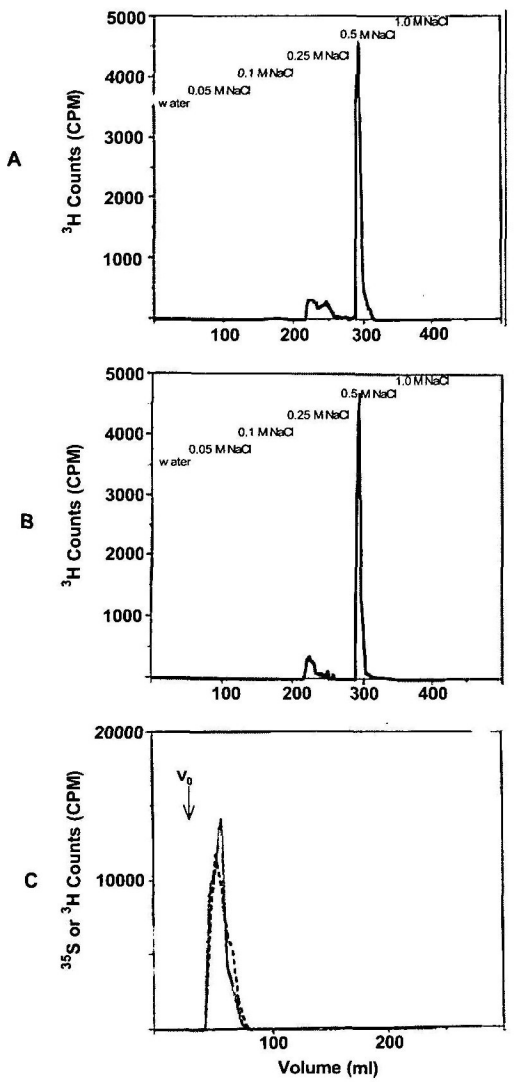

Fig. 4. **Ion-exchange and Bio-Gel P4 chromatography of the β-elimination product.** Glucosamine-labeled mucin treated with alkaline borohydride and dialyzed against distilled water. a) the β-elimination product (81,000 cpm) was passed through a 4 ml column of DEAE Sephadex A-25 using water and stepwise elution with different concentrations of NaCl, as indicated above the solid lines. b) the β-elimination product (81,000 cpm) was first treated with neuraminidase, dialyzed against distilled water, which removed only free sialic acid, and passed through a DEAE Sephadex A-25 column using the same elution profile. c) HPAF mucin labeled with both glucosamine and sulfate was passed through a DEAE Sephadex A-25 column (1,030,000 cpm ^{3}H), and the major peak, which eluted at 0.5 M NaCl, as passed through a Bio-Gel P-4 column (1.5 cm × 75 cm) using 0.1 M ammoniuum acetate. Fractions were collected and ^{3}H (solid line) and ^{35}S (broken line) levels new counted. V_0 indicates the void volume.

Expression of Mucin Genes in HPAF Cell Line—To investigate putative mucin gene products that could be expressed by the HPAF cell line, we analyzed the expression of the mRNAs for 10 mucin genes. An RT-PCR amplification was performed using mucin gene-specific and *GAPDH* primers designed from the sequences published in Genbank as described earlier (*43*). The RT-PCR products for *MUC1* and *MUC4* were found at high levels, those for *MUC5AC* and *MUC5B* at moderate levels, and those for *MUC2* at low levels (Table IV). Only trace amounts of the *MUC11* and *MUC12* products were found. There was no detectable product for *MUC3*, *MUC6*, or *MUC7*. All positive controls (as listed in the experimental procedure section) showed the appropriate amplification.

The expression of *MUC* mRNA (*MUC1*, *MUC4*, and *MUC5AC*) was confirmed by northern blot analysis. As shown in Fig. 5, MUC specific probes hybridized to mRNAs

TABLE IV. **RT-PCR analysis of mucin gene expression in an HPAF tumor cell line.**

Mucin genes	Amount of RT-PCR product
MUC1	+++
MUC2	+
MUC3	–
MUC4	+++
MUC5AC	++
MUC5B	+
MUC6	–
MUC7	–
MUC11	+/–
MUC12	+/–

PCR amplified products were quantified for each sample using the gel expert™ 3.5 software suite. The densitometric values were calculated for the specific gene product and GAPDH for each reaction. The value for the mucin gene–specific product is expressed per unit of *GAPDH* to account for any differences in starting amounts of RNA. The lowest densitometric values (weakest band) for *GAPDH* are taken as an arbitrary unit. For convenience, the corrected densitometric values for different products were categorized in 3 different ranges: high (+++), moderate (++), and weak values (+). Each value was determined as the mean of four densitometric readings.

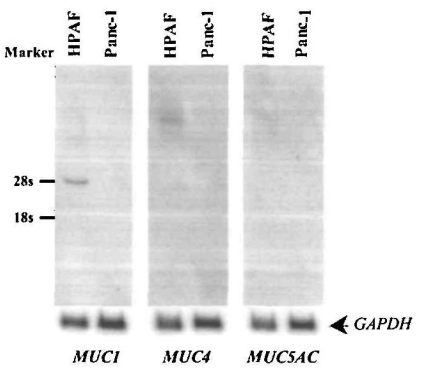

Fig. 5. **Northern blot analysis for mucin expression in HPAF and Panc-1 cells.** (A) Twenty micrograms of total RNA from HPAF and Panc-1 cells were fractionated electrophoretically in 1% formaldehyde-agarose gels and transferred to nylon membranes by capillary blotting. Blots were reacted with ^{32}P-labeled cDNA probes specific for human *MUC1*, *MUC4*, and *MUC5AC*. A *GAPDH*-specific probe was used as an internal control.

TABLE V. **Immunoreactivity of antibodies against tracheobronchial mucins with TFMS-treated pancreatic mucin, TFMS-treated respiratory mucin, and synthetic MUC1 peptide.**

Antibodies	Pancreatic apomucin	Respiratory apomucin	MUC1 peptide
Pre-RS	356 ± 33	991 ± 79	309 ± 27
RS	38,014 ± 2001	63,559 ± 3,889	998 ± 90
HMFG2	164 ± 20	3,284 ± 289	6695 ± 560

Direct binding assay counts per minute (cpm ± SD) were measured using ^{125}I-Protein A. RS, rabbit serum; Pre-RS, pre-immune rabbit serum.

TABLE VI. **Immunoreactivity of anti-MUC4 rabbit antibodies to TFMS-treated pancreatic mucin.**

	Absorbance
Blank	0.249 ± 0.023
Pre-RS	0.313 ± 0.003
RS	1.085 ± 0.023

Direct binding ELISA (mean ± SD) values measured using goat anti-rabbit antibodies conjugated to alkaline phosphatase. RS, rabbit serum; Pre-RS, pre-immune rabbit serum. The optical density was measured using a Dynatech MR 5000 automatic 96-well microtiter reader at 410 nm.

of specific sizes. *MUC1* and *MUC4* mRNA were expressed at high levels whereas *MUC5AC* was expressed at low levels in HPAF cells. The poorly differentiated cell line, Panc1, showed no expression of *MUC1*, *MUC4*, or *MUC5AC*.

Characterization of Pancreatic Mucin—The nature of the polypeptide core of the purified pancreatic mucin was further investigated. From the expression studies, we learned that MUC1, MUC4, MUC5AC, and MUC5B are the major mucin gene products expressed in HPAF cells. Based on the sequence information of these mucin genes, the amino acid compositions were deduced as described in Table II. Amino acid analysis of the purified mucin (Table II) revealed a composition consistent with that of a typical mucin. Comparison with the predicted amino-acid compositions of MUC1, MUC5AC, and MUC5B revealed a disparity, raising the possibility that the purified material contained a core protein other than these mucins. Previous analysis of mucin expression by HPAF showed the expression of MUC4 (*14*), a mucin associated with tracheo-bronchial secretions (*46*). The deduced amino acid composition of MUC4 (*40*) was found to be consistent with the amino acid composition of the purified DU-PAN-2–reactive fraction (Table II).

The purified DU-PAN-2 component of pancreatic mucin was treated with trifluoromethane sulfonic acid to remove carbohydrate units. The nature of the purified deglycosylated material was investigated using a previously produced rabbit polyclonal antiserum against purified TFMS-treated tracheobronchial mucin (*47*) that recognizes MUC4 sequences (*40*), and rabbit serum raised specifically against the MUC4 tandem repeat peptide (*43*). Due to the high expression of MUC1 mucin in HPAF cell cultures, a murine monoclonal antibody (HMFG2) reactive with the MUC1 apomucin epitope was also tested for its reactivity with the pancreatic deglycosylated tumor mucin. The reactivity of HMFG2 was weak with this material, whereas there was strong reactivity with the rabbit antiserum. Direct binding data are shown in Table V. The identity of the newly purified mucin to MUC4 was further confirmed by its reactivity with antiserum raised against the MUC4 tandem repeat peptide (Table VI). The specificity of the anti-MUC4 antibody has been reported earlier (*43*).

DISCUSSION

Two distinct glycoproteins (molecular mass. > 250 kDa) possessing chromatographic and buoyant density characteristics of the mucin family of glycoproteins were previously identified as markers detected in the ascites and serum of pancreatic adenocarcinoma patients by the DU-PAN-2 monoclonal antibody (*21*). The protein core of one of these glycoproteins was identified as the product of the MUC1 gene (*5, 41*), characterized by a higher mole percent of proline relative to serine and threonine than is found in other members of the mucin family, and by a remarkably high mole percent of alanine. This mucin is anchored by a hydrophobic cytoplasmic tail in the membrane with the carbohydrate-rich domain projecting outside into the lumenal space. A series of identical 60-bp sequences is repeated in tandem and codes for a serine- and threonine-rich domain, the highly glycosylated domain, preceded and followed by unique amino- and carboxy-sequences. This repeat exhibits variable length polymorphism (20–125 units), each coded by a specific MUC1 allele.

The results presented in this report show that the protein core of the other DU-PAN-2–reactive protein fraction is distinct in both amino acid composition and immunoreactivity. The amino acid profile is typical of members of the mucin family with a predominance of serine and threonine and a lesser mole percent of proline and alanine than that of MUC1. Deglycosylation of the mucin by the TFMS protocol yielded a protein core that reacted with antiserum raised against human tracheobronchial mucins. Tracheobronchial mucins comprise mainly *MUC1*, *MUC4*, *MUC5AC*, and *MUC5B* transcripts as detected in northern blot analyses. These could be the source of the translation products reacting with the antiserum raised against the deglycosylated human tracheobronchial mucin core protein preparation (*14*). However, there was no reactivity with the HMFG-2 antibody directed against the MUC1-specified protein core. The carbohydrate composition of the HPAF cell line mucin and that of the ascitic fluid (*20*) were similar except for the absence of fucose and the higher sialic acid content of the cell line. These results support the presence of two different mucin core proteins containing the DU-PAN-2 structure. Furthermore, a rabbit polyclonal antibody raised against the MUC4 tandem repeat peptide reacted specifically with this newly purified mucin. To confirm the identity of this purified mucin further, we did attempt to determine the amino acid sequences. However, the N-terminal portion was blocked due to the TFMS-treatment (for deglycosylation) and no amino acid sequence could be obtained.

The RT-PCR analysis of mucin genes in the HPAF tumor

cell line revealed the expression of *MUC1*, *MUC4*, *MUC5AC*, and *MUC5B* transcripts. The data presented in Table II suggest that a molecule expressing the DU-PAN-2 epitope shows similarity to the *MUC4* cDNA sequence cloned from human tracheobronchial (*39, 49*) or pancreatic tumor cDNA libraries (*40*). The unglycosylated MUC4 gene product is predicted to contain 973 residues at the NH_2-terminus, followed by a polymorphic large tandem repeat domain varying in size from 3,285 to 7,285 residues. The COOH-terminus consists of 1,156 amino acid residues. Any amino acid composition of the MUC4 product, therefore, would have to be based in large measure on the consensus sequence, and there is appreciable variation among the non-conserved amino acids in the variable 16–amino acid repeat.

The pancreatic mucin described here, together with the MUC1 product, were found in extracellular fluids. MUC1 has been characterized as an integral membrane protein. Its presence in extracellular fluid results from the release of a proteolytic extracellular fragment of the carbohydrate-rich domain (*50*). Analysis of the MUC4 sequence also indicated the presence of a mucin-type subunit (MUC4α) and a transmembrane unit (MUC4β, 70 kDa) separated by a GDPH proteolytic cleavage site. The amino acid composition of MUC4β was found to be dominated by Asp/Asn, Glc/Glu, and Leu, which is similar to the 70 kDa protein that persisted in eluting in the V_0 fraction together with the mucin despite repeated chromatography in a denaturating/reducing solvent (0.1% SDS/1 mM DTT) (data not shown). Both are also rich in Cys residues with MUC4β containing 39 residues per 1,000 as compared to 38 residues/1,000 in the 70 kDa protein.

The data in this report suggest that the DU-PAN-2 epitope can also be expressed on the MUC4 core protein. Future studies should examine the relative presence of MUC1 and MUC4 in the serum of patients with pancreatic adenocarcinoma and other adenocarcinomas to determine whether analysis of multiple markers (DU-PAN-2, CA19-9, MUC1, MUC4) will improve the sensitivity or specificity of serum diagnostic assays. In addition, given that tumor-associated epitopes on MUC1 are under investigation for diagnostic use and for inclusion in tumor vaccines, there is merit in examining the expression and glycosylation of MUC4 by pancreatic adenocarcinomas to evaluate its utility as a target for immunodiagnostic and immunotherapeutic strategies for this disease.

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