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Integrin α3β1 Expressed by Human Colon Cancer Cells Is A Major Carrier of Oncodevelopmental Carbohydrate Epitopes

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Abstract Oncodevelopmental carbohydrate epitopes are a common feature of human colorectal carcinoma, yet their biological significance remains unclear. We have shown previously that monoclonal antibody (MAb) 3A7, which recognizes a determinant on type 2 chain blood group A and B oligosaccharides, detects oncodevelopmental changes in azoxymethane-induced rat colon tumors and some human colon cancer cell lines. (Laferté S et al. [1995] J. Cell. Biochem. 57:101–119). In this study, we set out to purify gp140, the major glycoprotein carrier of the 3A7 epitope expressed by human colon cancer cells, as a first step towards elucidating the contribution of the 3A7 epitope and its major glycoprotein carrier to colon cancer progression. To this end, gp140 was purified from HT29 cells and used for the preparation of polypeptide-specific monoclonal antibodies. Five monoclonal antibodies (7A8, 7B11, 8C7, 8H7, and 11D4) immunoprecipitated a 3A7-immunoreactive glycoprotein complex of 140 kDa which was subsequently identified by partial protein sequencing as α3β1 integrin. Flow cytometric analysis of Chinese hamster ovary (CHO) cells expressing different human integrin chains revealed that MAbs 7A8 and 7B11 detect the α3 integrin subunit whereas MAbs 8C7 and 8H7 detect the β1 integrin subunit. MAb 11D4, which did not bind to any of the CHO transfectants, detected type 2 chain blood group A determinant. Flow cytometric analysis of a panel of human colon carcinoma cell lines obtained from blood group A, AB, or B individuals revealed a direct correlation between cell-surface expression of the 3A7 epitope and α3 integrin subunit, suggesting that α3β1 integrin is a preferred target of the 3A7 epitope in colon cancer cells. Using lectins and glycosidases to examine further the carbohydrate structure of α3β1 integrin, we demonstrated that it is a sialoglycoprotein containing both N- and O-linked oligosaccharides. In addition, both α3 and β1 integrin subunits express β1–6 branched Asn-linked oligosaccharides and short poly-N-acetyllactosamine units (Galβ1–4GlcNAc-R; $n \leq 3$), glycans previously implicated in cancer metastasis.Thus, α3β1 integrin expressed by human colon carcinoma cells is a major carrier of oncodevelopmental carbohydrate epitopes whose presence may modulate tumor cell adhesion, migration, and/or invasion. J. Cell. Biochem. 72:189–209, 1999.

Key words: colon; cancer; carbohydrate epitopes; colorectal carcinoma

Abbreviations: APC, adenomatous polyposis coli; DCC, deleted in colon carcinoma; ABH, serologically-defined carbohydrate epitopes having the following structures-A = GalNAcα1–3[Fucα1–2]Gal-R, B = Galα1–3[Fucα1–2]Gal-R and H = Fucα1–2Gal-R; Asn, asparagine; GlcNAc, N-acetyl-D-glucosamine; GalNAc, N-acetyl-D-galactosamine; Le, Lewis blood group determinant; Man, mannose; Fuc, fucose; Gal, galactose; GlcNAc-TV, β-N-acetylglucosaminyltransferase V; MAb, monoclonal antibody; CHO, Chinese hamster ovary; DMEM, Dulbecco's minimum Essential medium; FBS, fetal bovine serum; PBS, phosphate buffered saline; IF, immunofluorescence; IP, immunoprecipitation; EIA, enzyme-linked immunoassay; BSA, bovine serum albumin; NMS, normal mouse serum; NRS, normal rabbit serum; TBS, Tris-buffered saline (50 mM Tris HCl pH 8.0, 0.02% sodium azide; TBT, 50 mM Tris HCl pH 8.0, 0.1% bovine serum albumin, 0.025% Tween-20, 0.02% sodium azide; PBS, phosphate buffered saline (2.5 mM NaH_2PO_4, 7.3 mM Na_2HPO_4, 144 mM NaCl, pH 7.2); FITC, fluoresceine isothiocyanate; BCIP, 5-bromo-4-chloro-3-indolyl phosphate;NBT, nitro blue tetrazolium; PMSF, phenylmethanesulfonyl fluoride; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; L-PHA, leucophytohemagglutinin; WGA, wheat germ agglutinin; DBA, *Dolichos biflorus*; HPA, *Helix pomatia*; DSA, *Datura stramonium*; LEA, *Lycopersicon esculaentum*; lamp-1, lysosome-associated membrane protein-1; lamp-2, lysosome-associated membrane protein-2; CEA, carcinoembryonic antigen; TAA90, tumor-associated antigen 90 K.

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Colorectal cancer is the second leading cause of cancer death, resulting in nearly 70,000 deaths annually in North America [Statistics Canada, 1997; Parker et al., 1997]. A decade of intense research has revealed that colorectal cancer results from the accumulation of mutations which can lead to the activation of dominant acting oncogenes (e.g. *ras*), loss of tumor suppressor genes, e.g., *APC, p53, DCC,* and inactivation of DNA mismatch repair genes [Fearon and Vogelstein, 1990; Jessup and Gallick, 1992, Kinzler and Vogelstein, 1996]. However, it is not yet clear how these genetic events lead to the numerous biochemical changes detected in colon cancer cells or influence their invasive and metastatic phenotype in vivo.

One of the most frequent biochemical alterations associated with colon tumorigenesis and metastasis is altered expression and/or structure of cell-associated complex carbohydrates [Hakomori and Kannagi, 1983; Nicolson, 1984; Rademacher et al., 1988, Dennis, 1992]. Although the biological significance of altered glycosylation on colon cancer cells is not completely understood, complex carbohydrates play a vital role in normal growth and development of living organisms [Varki, 1993]. For example, they can influence the three-dimensional structure and function of glycoproteins [Sairam and Jiang, 1992], decrease the protease susceptibility of some glycoproteins [Varki, 1993], serve as ligands for mammalian lectins [Zhou and Cummings, 1992] and contribute to cell-cell [Lowe, 1994] and cell-extracellular matrix interactions [Carson, 1992]. When expressed aberrantly on cancer cells, complex carbohydrates have been shown to modulate tumor cell adhesion [Olden, 1993], influence host anti-tumor immunity [Dennis and Laferté, 1985; Castronovo et al., 1989], and contribute to the malignant behavior of these cells [Dennis, 1992]. Specific examples of colon cancer-associated glycosylation changes include aberrant expression of ABH and Lewis (Le) blood group antigens [Hakomori and Kannagi, 1983; Coon and Weinstein, 1986; Hoff et al., 1989; Bloom et al., 1990], increased sialylation and fucosylation of type 1 (Galβ1–3GlcNAcβ-R) and type 2 (Galβ1–4GlcNAcβ-R) chains [Hakomori and Kannagi, 1983], increased expression of poly-N-acetyllactosamine structures (i.e., repeating type 2 chains) [Saitoh et al., 1992], alterations in O-linked oligosaccharides of colonic mucins [Kannagi et al., 1986; Baeckstrom et al., 1991; Fernandes et al., 1991], and increased branching of N-linked oligosaccharides [Fernandes et al., 1991; Saitoh et al., 1992], in particular initiation of the β1–6 antenna by GlcNAc T-V [Dennis et al., 1987; Dennis, 1992]. Notably, several of these cancer-associated carbohydrate determinants, including sialyl-Le^a, sialyl Le^x, difucosyl-Le^x, and ABH determinants, are also expressed in fetal colon and as such represent oncodevelopmental antigens [Hakomori and Kannagi, 1983; Bloom et al., 1990].

Although expression of oncodevelopmental carbohydrate epitopes, including Lewis and ABH determinants, on colon cancer cells has been well-documented [Denk et al., 1974; Atkinson et al., 1982; Ernst et al., 1984; Fukushi et al., 1984; Yuan et al., 1985; Bloom et al., 1990; Matsumoto et al., 1993], their functional relevance to colon tumorigenesis and metastasis is still under investigation. The demonstration that Le^x and sialylated Le^x constitute the ligands for selectins, a family of receptors involved in cell-cell adhesion [Lowe et al., 1990; Phillips et al., 1990; Walz et al., 1990], has led to the suggestion that accumulation of these structures on metastatic colon cancer cells may facilitate their arrest on vascular endothelium, an important step in the metastatic process [Nicolson, 1988, 1994; Sawada et al., 1994]. On the other hand, loss of expression of ABH blood group determinants on lung and kidney tumors, which was shown to result from decreased transcription of mRNA encoding A and B transferases, correlated with poor prognosis [Matsumoto, 1993; Orntoft, 1996]. This suggested that persistent expression of A and B blood group determinants on colon cancer cells may play an active role in preserving tumor cell-tumor cell and tumor cell-extracellular matrix interactions characteristic of benign tumors but not of invasive tumors. In this regard, recent studies by Ichikawa et al. [1997] have shown that transfection of cDNAs encoding A and B transferase into blood group H-expressing colon carcinoma cells resulted in decreased tumor cell motility, which appears to be mediated by integrins composed of α3, α6, and β1 subunits.

One approach to understanding the biological role of cell surface glycosylation changes in colon cancer progression is to identify and characterize the major glycoproteins carrying these structures and examine the contribution of cancer-associated carbohydrate structures to their

function. In a previous study [Laferté et al., 1995], we isolated a monoclonal antibody, 3A7, which detects an epitope on type 2 chain (Galβ1–4GlcNAc-R) bearing blood group A (GalNAcα1–3[Fucα1–2]Gal-R) or B (Galα1–3[Fucα1–2]Gal-R) determinant that is developmentally-regulated in rat colon and expressed at elevated level in azoxymethane-induced rat colon tumors and human colon cancer cell lines established from blood group A, B, or AB patients. Analysis of a panel of azoxymethane-induced rat colon neoplasms of varying histopathological grades, which provided a useful model system to examine tumor progression in vivo [Bird et al., 1985], revealed that increased expression of the 3A7 epitope in colonic neoplasms is detectable as early as the adenoma stage, with maximal expression in invasive carcinomas (i.e., Duke's B1 and B2). In Western blotting analyses of rat and human colon tumors, MAb 3A7 detected a number of glycoproteins ranging in size between 60 and 200 kDa, including a major species of 140 kDa (gp140) [Laferté et al., 1995]. As a first step towards elucidating the role of the 3A7 epitope in colon cancer progression and determining the importance of its glycoprotein carrier in this process, we undertook to purify and characterize gp140, the major glycoprotein carrying this epitope in human colon carcinoma cells. In this study, we describe the purification of gp140 from the human colon carcinoma cell line HT29, the preparation of gp140-specific monoclonal antibodies and their subsequent use in the identification of gp140 as α3β1 integrin, a heterodimeric cell surface receptor mediating cell-cell [Sriramarao et al., 1993; Symington et al., 1993] and cell-extracellular matrix interactions [Takada et al., 1988; Carter et al., 1991; Dedhar et al., 1992]. We also show using a panel of blood group A, B, or AB-positive human cancer cell lines that cell-surface expression of the 3A7 epitope correlates directly with the level of α3 integrin subunit on the surface, suggesting that α3β1 integrin is a major carrier of the 3A7 epitope. Lastly, we demonstrate that α3β1 integrin is a sialoglycoprotein containing β1–6 branched Asn-linked oligosaccharides and short poly-N-acetyllactosamine structures $[(GlcNAc\beta1–4Gal)_n$ where $n \leq 3]$, oligosaccharides previously implicated in cancer metastasis [Dennis et al., 1987]. These data indicate that α3β1 is a major carrier of oncodevelopmental and cancer-associated carbohydrate epitopes which may contribute to colon cancer progression by modulating tumor cell adhesion and invasion.

MATERIALS AND METHODS

Cell Lines

The human colon carcinoma cell lines HT29 (HTB 38), LoVo (CCL 229), SW48 (CCL 231), SW480 (CCL 228), SW620 (CCL 227), SW1417 (CCL 238), as well as the nonsecreting mouse myeloma cell line FO (CRL 1646) were obtained from the American Type Culture Collection (A.T.C.C., Rockville, MD) and cultured in Dulbecco's minimum Essential medium (DMEM, high glucose) supplemented with 10% FBS, 2 mM L-glutamine (GIBCO BRL, Gaithersburg, MD), 1 mM sodium pyruvate (GIBCO BRL), 1% nonessential amino acids (GIBCO BRL), and 2% MEM vitamine solution (GIBCO BRL). The cell line KM20C, which was established from a liver metastasis derived from a human colon carcinoma [Morikawa et al., 1988], was obtained from Dr. I.J. Fidler at MD Anderson Cancer Center (Houston, TX). In routine testing, the cell lines were found to be free of mycoplasma contamination.

Preparation of Detergent Lysates of HT29 Cells

HT29 cells (5 ml packed cells) were homogenized using a Polytron (Brinkmann Instruments, Edmonton, Alberta, Canada) in four volumes of 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 1 mM PMSF, 1 mM benzamidine, 10 μg/ml aprotinin, 0.5 μM leupeptin, 0.7 μM peptstatin. The sample was adjusted to 1% Triton X-100, incubated on ice for 1 h and centrifuged at 20,000*g* for 30 min. The supernatant was assayed for protein using the Bio-Rad DC protein assay kit.

Isolation of gp140

HT29 cells (60 ml of pelleted cells) were lysed by repeated freeze/thaw of the cell pellet in four volumes of 50 mM Tris-HCl pH 7.4, 0.25 M sucrose, 1 mM EDTA, 1 mM PMSF, 1 mM benzamidine, 0.02% sodium azide, and centrifuged at 8,000*g* for 6 min to remove cellular debris. The microsomal pellet was collected after centrifugation at 100,000*g* for 1 h at 4°C. HT29 microsomes were solubilized on ice for 1 h in four volumes of buffer consisting of 50 mM Tris-HCl pH 7.4, 1 mM PMSF, 1 mM benzamidine, 0.5 μM leupeptin, 0.7 μM pepstatin, 10 μg/ml aprotinin, and 0.5% CHAPS, and cleared by centrifugation at 20,000*g* for 45 min. The

cell lysate was applied to a column (1 × 30 cm) of wheat-germ agglutinin (WGA)-Sepharose (1 mg lectin/ml gel) equilibrated in TBS pH 7.4, 0.5% CHAPS, 0.02% sodium azide. The column was washed with 20 column volumes of WGA buffer (50 mM Tris-HCl pH 7.4, 0.5 M NaCl, 0.1% CHAPS, 0.02% sodium azide) and eluted with five volumes of WGA buffer containing 5% N-acetyl-D-glucosamine (Sigma, St. Louis, MO). Fractions were tested for the presence of 3A7-immunoreactive gp140 by EIA and Western blotting. Immunoreactive fractions were pooled and concentrated to 1 ml using a Centricon 10 microconcentrator (Amicon, Beverly, MA). The WGA-unbound material was reapplied to the WGA-Sepharose column until the unbound fractions were devoid of 3A7-immunoreactive material. The pooled 3A7-immunoreactive material in the WGA-Sepharose eluted fractions was applied to a 5 ml column of *Dolichos biflorus* (DBA)-agarose (4 mg lectin/ml gel; Intermedico, Markham, Ontario, Canada) equilibrated in TBS pH 7.4, 0.1% CHAPS, 0.02% sodium azide (DBA/HPA buffer), and allowed to bind overnight at 4°C. The column was washed with 10 volumes of DBA/HPA buffer adjusted to 0.5 M NaCl and then eluted with five column volumes DBA/HPA buffer containing 100 mM N-acetyl-D-galactosamine (Sigma). DBA-unbound and eluted fractions (1 ml) were collected and assayed by EIA and Western blotting using MAb 3A7. The eluted fraction enriched with gp140 was concentrated to 1 ml and applied to a 5 ml column of *Helix pomatia* (HPA)-agarose (1.9 mg lectin/ml gel; Sigma) pre-equilibrated in DBA/HPA buffer. After extensive washing of the column, fractions were eluted in DBA/HPA buffer containing 100 mM N-acetyl-D-galactosamine. Unbound and eluted fractions were assayed as described for DBA-agarose affinity chromatography. All of the gp140 was recovered in the HPA-eluted fractions. Immunoreactive fractions were pooled, concentrated to 0.5 ml and applied to a Biogel A5m column (1.5 × 120 cm; Bio-Rad, Mississauga, Ontario, Canada) equilibrated in DBA/HPA buffer. Fractions of 1.6 ml were collected and assayed by EIA and Western blotting. Immunoreactive fractions were pooled, concentrated and assayed for protein content using the BioRad DC protein assay kit.

Monoclonal Antibodies

The isolation and characterization of MAb 3A7 has been described elsewhere [Laferté et al., 1995]. Monoclonal antibodies 7A8, 7B11, 8C7, 8H7, and 11D4 were isolated as described previously [Laferté et al., 1995] following immunization of mice with purified gp140 or partially purified gp140 obtained following lectin affinity chromatography of a detergent solubilized HT29 cell lysate on HPA-agarose. Hybridoma subclones were injected intraperitoneally into Balb/c mice for the production of ascites which were subsequently used for indirect immunofluorescence, flow cytometry, EIA, immunoprecipitation, and Western blotting analyses. Isotyping of hybridomas was carried out using the Isotyping Kit (Bio-Rad Laboratories) according to manufacturer's instructions. MAbs 7B11 and 11D4 were of the IgG_1 subclass, 7A8 and 8H7 were of the IgG_{2a} subclass while 8C7 was of the IgG_{2b} subclass (see Table I).

Immunofluorescence

Immunofluorescence analysis was carried out as described previously [Laferté and Loh, 1992]. Subconfluent cultures of human colon carcinoma cells grown in 100 mm culture dishes were washed twice with PBS and incubated with 2 ml of PBS containing 5 mm EDTA (PBS-EDTA) in a 37°C incubator until the cells appeared rounded. The cells were harvested from the plates with DMEM containing 10% FBS, washed twice with PBS and counted. For analysis of live cells, 2×10^5 cells were pelleted in borosilicate glass tubes (6 × 50 mm) and incubated for 1 h at 4°C with a 1:500 dilution of NMS or monoclonal antibody in immunofluorescence (IF) buffer (PBS containing 3% FBS). Following four washes with 400 µl IF buffer, the cells were incubated for 30 min with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse Ig (Jackson, Dimension Laboratories, Mississauga, Ontario, Canada), diluted 1:50 in IF buffer. Cells were resuspended in 50 µl PBS: glycerol (1:1), dropped onto glass slides, mounted with a cover slip, and examined under a fluorescence microscope (Nikon). For analysis of fixed and permeabilized cells, cells harvested with PBS-EDTA were resuspended in PBS at a concentration of 2×10^6 cells per ml and 10 µl spotted per well of eight-well toxoplasmosis slides (Bellco Glass Inc., Vineland, NJ). The slides were air-dried and fixed by incubation at −20°C for 4 min with methanol and 2 min with acetone. Slides were air-dried and stored at −20°C. Prior to use, slides were washed in PBS for 5 min. Slides were incubated with primary antibody and FITC-conjugate as described above. Slides were washed three times by re-

TABLE I. Summary of Properties of gp140-Specific Monoclonal Antibodies

MAb	Isotype	Immunofluorescence pattern	Immunoprecipitation	Western blotting[a]	Specificity
7A8	IgG2a	Membrane	135 kDa 145 kDa	−	α3 chain[b]
7B11	IgG1	Membrane	135 kDa 145 kDa	−	α chain[b]
8C7	IgG2b	Membrane	135 kDa 145 kDa 160 kDa	−	β1 chain[b]
8H7	IgG2a	Membrane	135 kDa 145 kDa 160 kDa	−	β1 chain[b]
11D4	IgG1	Membrane	55 kDa 135 kDa 145 kDa 160 kDa	+	A type 2[c]

[a]Microsomes prepared from HT29 cells (see Materials and Methods) were separated by SDS-PAGE, transferred to nitrocellulose, and blotted with normal mouse serum, hybridoma supernatants, or a 1:500 dilution of MAb 3A7 (positive control). Antibodies which produced a signal above the normal serum control were scored as '+.' Antibodies which failed to produce a signal above the normal serum control were scored as '−.'
[b]Antibody specificity was determined by flow cytometric analysis of CHO cells transfected with cDNA encoding different human integrin chains (see Table 2).
[c]Antibody specificity was assessed by EIA using BSA-glycan conjugates.

peated dipping in PBS and processed for analysis as described above.

Flow Cytometry

Human colon carcinoma cell lines grown to 70–80% confluency were washed with PBS and trypsizined using standard procedures. The cells were washed three times with PBS, resuspended in PBS containing 0.1% BSA, 0.2% sodium azide (FC buffer), and counted. One milliliter containing 1×10^6 cells was supplemented with normal mouse serum or ascites to a final concentration of 1:300 and incubated on ice for 1 h. The cells were washed three times with FC buffer and incubated for 30 min with 100 µl of a 1:50 dilution of FITC-labeled goat anti-mouse Ig in FC buffer (Zymed, Dimension Laboratories). After washing three times with FC buffer, cells were resuspended in 1 ml of FC buffer, filtered through 100 µm nylon mesh, and kept on ice until analysis. Flow cytometry was carried out using an EPICS XL flow cytometer (Coulter, Miami Lakes, FL). Analysis of Chinese hamster ovary cells (CHO) or CHO cells expressing recombinant human integrin α2 [Kamata et al., 1994], α3 [Takada et al., 1991], α4 [Takada et al., 1995], α5 [Sechler et al., 1996], β1 [Takada et al., 1992], or β3 [Takada et al., manuscript in preparation] chain was carried out using similar procedures except that normal mouse immunoglobulin and anti-integrin antibodies were used at dilutions of 1:500 and the analysis was carried out using a FACS IV fluorescence-activated cell sorter (Becton Dickinson col, Oxnard, CA).

Preparation of BSA-Glycan Conjugates

BSA-glycan conjugates were prepared and used as described previously [Laferté et al., 1995]. The incorporation of carbohydrate on each BSA-glycan conjugate was as follows (where n = number of oligosaccharide coupled per mole of BSA): H type 2-BSA (n = 19); A disaccharide-BSA (n = 17); A type 2-BSA (n = 57); A type 1-BSA (n = 13); A type 4-BSA (n = 17); A type 6-BSA (n = 13); B disaccharide-BSA (n = 19); B type 2-BSA (n = 18); B type 4-BSA (n = 14); B type 5-BSA (n = 15); B type 6-BSA (n = 16). Because of the different level of incorporation of oligosaccharide chains per BSA molecule, all BSA-glycan conjugates were diluted to a standard concentration of 50 nmol oligosaccharide/ml. Suitable dilutions of this stock solution were used in enzyme-linked immunosorbent assay, as described in the Results section.

Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assays (EIA) were carried out as described previously [Laferté and Loh, 1992; Laferté et al., 1995] using 100 µl aliquots of BSA-glycan conjugates or column fractions.

Metabolic Labeling of Cells

Cell monolayers grown to 70–80% confluency were washed twice with PBS and radiolabeled for 24 h in methionine-free DMEM supplemented with 1/10 the normal content of methionine, 10% FBS, and 50 µCi/ml of [^{35}S]-methionine (800 Ci/mmole; NEN, Calgary, Alberta, Canada). Following radiolabeling, the plates were washed three times with ice-cold PBS, the cells harvested with a rubber policeman, and pelleted. Cell pellets were solubilized on ice for 1 h in extraction buffer consisting of 50 mM Tris-HCl pH 7.4, 1 mM PMSF, 1 mM benzamidine, 0.5 µM leupeptin, 0.7 µM pepstatin, 10 µg/ml aprotinin, and 1% Triton X-100, and cleared by centrifugation at 22,500*g*.

Immunoprecipitation

Unlabeled or radiolabeled cell lysates (2×10^6 cpm) were incubated overnight at 4°C with 3 µl of normal mouse serum or ascitic fluid. Protein A Sepharose-4B beads (100 µl of a 1:1 suspension; Pharmacia, Montreal, Canada) were added and the suspension rocked gently at 4°C for 1 h. After washing the beads once with immunoprecipitation (IP) buffer (50 mM Tris-HCl pH 8.0, 1 mM PMSF, 1 mM benzamidine, 1% deoxycholate, 1% Triton X-100, 0.02% sodium azide), twice with IP buffer containing 0.5 M NaCl, twice with IP buffer containing 0.1% SDS and once with IP buffer, the immunoprecipitated proteins were eluted by boiling for 5 min in SDS-PAGE sample buffer (0.125 M Tris-HCl pH 6.8, 2% SDS, 2% 2-mercaptoethanol, 10% glycerol) and analyzed by SDS-PAGE, as described below.

SDS-PAGE

SDS-PAGE was performed as described by Laemmli et al. [1970] using 6.0, 6.5, 7.5, or 15% polyacrylamide gels. [^{35}S]-methionine labeled proteins separated by SDS-PAGE were visualized by fluorography [Bonner and Laskey, 1974] using Enlightning (NEN DuPont, Mississauga, Ontario, Canada).

Western Blotting Analysis

Proteins separated by SDS-PAGE were transferred electrophoretically onto nitrocellulose for 1 h at 100 V. Nitrocellulose blots were blocked for 1 h at room temperature or overnight at 4°C in TBS/4% BSA. Following three 5-min washes in TBT buffer (50 mM Tris-HCl, pH 8.0, 0.15 M sodium chloride, 0.1% BSA, 0.025% Tween-20, 0.02% NaN_3), blots were incubated for 2 h with either NMS or monoclonal antibody, diluted 1:3,000 in TBT, or lectin diluted to 0.4 µg/ml in TBT. Following three 5-min washes in TBT, antibody blots were incubated for 1 h with alkaline phosphatase-coupled affinity purified goat antimouse Ig (Bio-Rad Laboratories). Following three 5-min washes in TBT, one wash in TBS containing 0.5% Tween-20 and one wash in TBS, blots were developed colorimetrically by incubation with the substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and Nitro Blue Tetrazolium (NBT). Blots incubated with biotinylated lectins were washed three times with TBT, incubated for 20 min with streptavidin-coupled alkaline phosphatase diluted 1:4,000 in TBT and processed as described for antibody blots. Blots incubated with L-PHA or DSA were washed and incubated for 1 h with rabbit anti-L-PHA or rabbit anti-DSA antiserum, respectively, diluted 1:3,000 in TBT. Following three 5-min washes in TBT, blots were incubated for 1 h with alkaline phosphatase coupled goat anti-rabbit Ig diluted 1:3,000 in TBT, washed, and incubated with colorimetric substrates.

Peptide Mapping

Limited proteolytic peptide mapping was carried out using a protocol originally developed by Cleveland et al. [1977] and modified by Loh [1991]. Briefly, gel slices corresponding to the desired bands on the autoradiogram were excised from unfixed dried SDS-polyacrylamide gels, rehydrated in overlay buffer (0.125 M Tris-HCl, pH 6.8, 1 mM EDTA, 0.1% SDS, 30% glycerol) and transferred to wells of a 1.5 mm thick SDS-polyacrylamide gel consisting of a 3% stacking gel and a 15% separating gel. The gel slice was covered with staphyloccoccal V8 protease (0.1 µg/well, ICN Biomedicals Canada Ltd., Montreal, Canada) in enzyme buffer (0.125 M Tris-HCl, pH 6.8, 1 mM EDTA, 0.1% SDS, 10% glycerol). The proteins were electrophoresed through the stacking gel for 4 h at 12 mA/gel to allow proteolysis to occur, followed by a 30 min period without current. The remainder of the electrophoresis was carried out at 25 mA/gel. The gel was stained with Coomassie Blue R-250, destained, dried, and bands were visualized by fluorography, as described above.

Protein Sequencing

HT29 cells (10 ml of packed cells) were extracted in 4 volumes of IP buffer containing 1% Triton-X100 for 1 h on ice and then clarified by centrifugation at 20,000*g* for 45 min at 4°C. The detergent lysate was immunoprecipitated overnight at 4°C with 3 µl MAb 7A8 per ml of extract. One hundred µl of a 1:1 suspension of protein A-Sepharose beads per ml of sample was added and incubated for 1 h at 4°C. The beads were washed and eluted with SDS-PAGE sample buffer, as described above for immunoprecipitation analyses. Eluted proteins were separated on a 6% preparative SDS-polyacrylamide gel and transferred electrophoretically for 1 h at 100 V to an Immobilon-P membrane (Millipore, Mississauga, Ontario, Canada) prewashed in methanol. Following transfer, the membrane was washed with distilled water and stained with 0.1% Ponceau S (Sigma) in 0.1% acetic acid for 2 min [Aebersold et al., 1987]. The membrane was washed with 0.1% acetic acid until protein bands were visible. The two protein bands migrating with apparent sizes of 135 and 145 kDa were excised and the membrane slices were washed repeatedly with distilled water. The excised bands were stored at −20°C until use. Samples were sent to Dr. William Lane at Harvard Microchem (Harvard University, Boston, MA) where they were digested with trypsin and subjected to partial protein sequence analysis.

RESULTS

Purification of the Major 140 kDa Glycoprotein Detected by MAb 3A7 (gp140) From the Human Colon Carcinoma Cell Line HT29

In a previous study [Laferté et al., 1995], we demonstrated that MAb 3A7 detects several glycoproteins in human and rat colon cancer cells ranging in size between 60 and 200 kDa, including a major species of 140 kDa (gp140). We chose to purify gp140 from the human colon carcinoma cell line HT29 because it expresses relatively high levels of gp140 and grows rapidly in culture, thereby facilitating the purification process. Moreover, analysis of unfixed HT29 cells by indirect immunofluorescence using MAb 3A7 revealed a ring-like staining pattern consistent with localization of the 3A7 epitope on the cell surface (Fig. 1). For these reasons, gp140 was purified from a detergent-solubilized microsome fraction prepared from HT29 cells using serial lectin affinity chromatography and gel filtration chromatography. Three lectin affinity resins were used including WGA-Sepharose, which binds glycan chains terminating in N-acetylglucosamine, and/or N-acetylneuraminic acid [Osawa and Tsuji, 1987], and two blood group A-specific lectins, *Dolichos biflorus*-agarose and *Helix pomatia*-agarose. In contrast to *Helix pomatia*, which recognizes blood group A determinants irrespective of the core oligosaccharide [Hammarstrom et al., 1977], *Dolichos biflorus* lectin exhibits more restricted specificity [Etzler and Kabat, 1970], making the latter potentially useful for the separation of glycoproteins bearing blood group A determinants on different oligosaccharides. Lectin affinity chromatography was followed by gel filtration chromatography on Biogel A5m. As shown in Figure 2A, most of the proteins in the detergent cell lysate were recovered in the WGA-unbound fraction, as detected by optical density at 280 nm. In contrast, nearly all of the immunoreactive material detected by EIA with MAb 3A7 was recovered in the WGA-eluted fraction, which was subsequently applied to DBA-agarose. Consistent with our previous studies showing that rat colon tumor extracts, unlike normal rat colon extracts, bind poorly to DBA-agarose [Laferté et al., 1995], nearly all of the 3A7-immunoreactive material from HT29 cells was recovered in the unbound fraction of DBA-agarose (Fig. 2B). Analysis of DBA-unbound and eluted fractions by Western blotting with MAb 3A7 revealed that each fraction differs with respect to the relative amount of three species having apparent molecular weights of 140, 120, and 90 kDa (Fig. 2E). The major species detected in the DBA-eluted fraction was the 120 kDa glycoprotein whereas the major species detected in the DBA-unbound fraction was the 140 kDa glycoprotein (gp140). The DBA-unbound fraction was subjected to affinity chromatography on HPA-agarose and fractions were assayed by EIA (Fig. 2C) and Western blotting (Fig. 2F) using MAb 3A7. The results indicated that the 140 kDa glycoprotein is specifically eluted from the column using N-acetylgalactosamine. Subsequent gel filtration chromatography of the HPA-eluted material on Biogel A5m (Fig. 2D) yielded a major immunoreactive species eluting near the void volume which migrated as a doublet of 135 and 145 kDa in Western blotting analysis carried out with MAb 3A7 (Fig. 2G). Analysis of pooled immunoreactive fractions by SDS-PAGE and silver staining revealed that the major species consists of gp140 (data not shown).

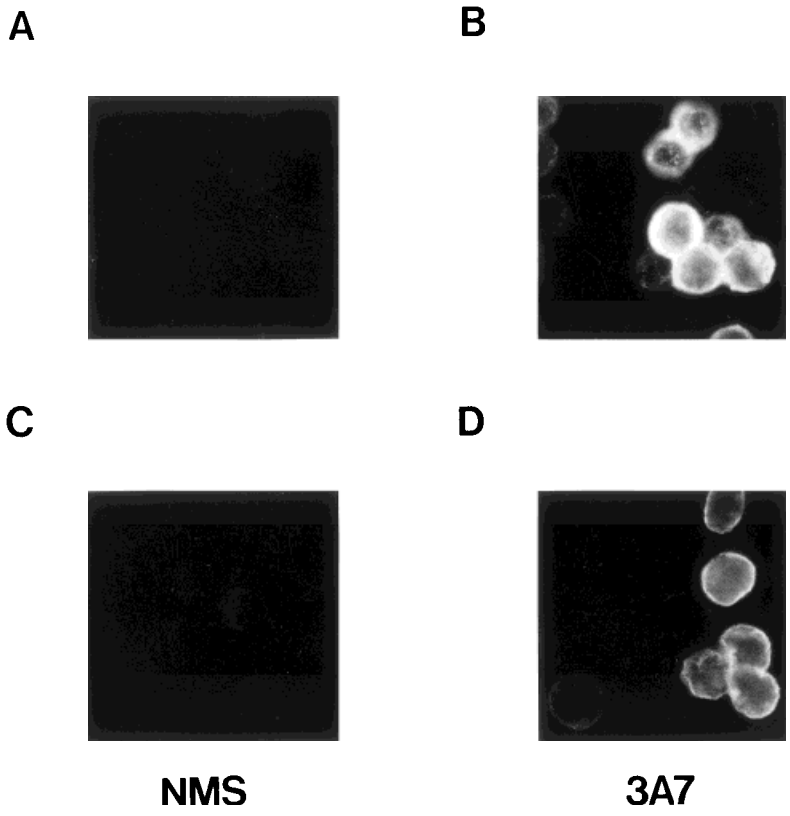

Fig. 1. Indirect immunofluorescence staining of HT29 cells with MAb 3A7. Methanol/acetone-fixed HT29 cells (**A,B**) and unfixed cells (**C,D**) were incubated with normal mouse serum (A,C) or MAb 3A7 (B,D) followed by FITC-labeled goat anti-(mouse Ig). Magnification ×400.

Isolation of gp140-Specific Monoclonal Antibodies

Although MAb 3A7 proved useful in monitoring purification of gp140, the specificity of this antibody for a carbohydrate determinant made it difficult to assess whether the purified gp140 fraction contained additional co-migrating glycoproteins. In order to identify gp140 as well as gain a better understanding of its biological role and the contribution of the 3A7 epitope to its function, we prepared monoclonal antibodies specific for the polypeptide moiety of gp140. Purified gp140, or partially purified gp140 isolated from HT29 cells following a single lectin affinity chromatography step on HPA-agarose, were used for the preparation of monoclonal antibodies. Hybridoma culture supernatants were screened initially for their ability to produce the same ring-like membrane immunofluorescence pattern with HT29 cells as was seen with MAb 3A7 (Fig. 1). Positive hybridomas were subsequently tested for their ability to immunoprecipitate a 140 kDa glycoprotein from detergent-solubilized HT29 cell lysates which could be detected in Western blots with MAb 3A7. As shown in Figure 3 and summarized in Table I, five monoclonal antibodies, namely 7A8, 7B11, 8C7, 8H7, and 11D4, met this criterion. Moreover, the 3A7-immunoreactive 140 kDa species immunoprecipitated by these antibodies co-migrated with the major glycoprotein species immunoprecipitated by MAb 3A7.

The specificity of the gp140-specific antibodies was examined further by analyzing immunoprecipitates of [^{35}S]-labeled HT29 cell lysates using 6.5% SDS-polyacrylamide gels (Fig. 4). By using the lower percentage polyacrylamide gels for improved band resolution, it was now apparent that MAbs 7A8 and 7B11 immunoprecipitate two closely-migrating species of 135 kDa and 145 kDa (Fig. 4). Under the same conditions, MAbs 8C7 and 8H7 also immunoprecipitated species of 135 and 145 kDa as well as a third species of 160 kDa (Fig. 4). While MAb 11D4 also immunoprecipitated species of 135, 145, and 160 kDa, an additional species of 55 kDa was also detected (data not shown). Similar analysis carried out under nonreducing con-

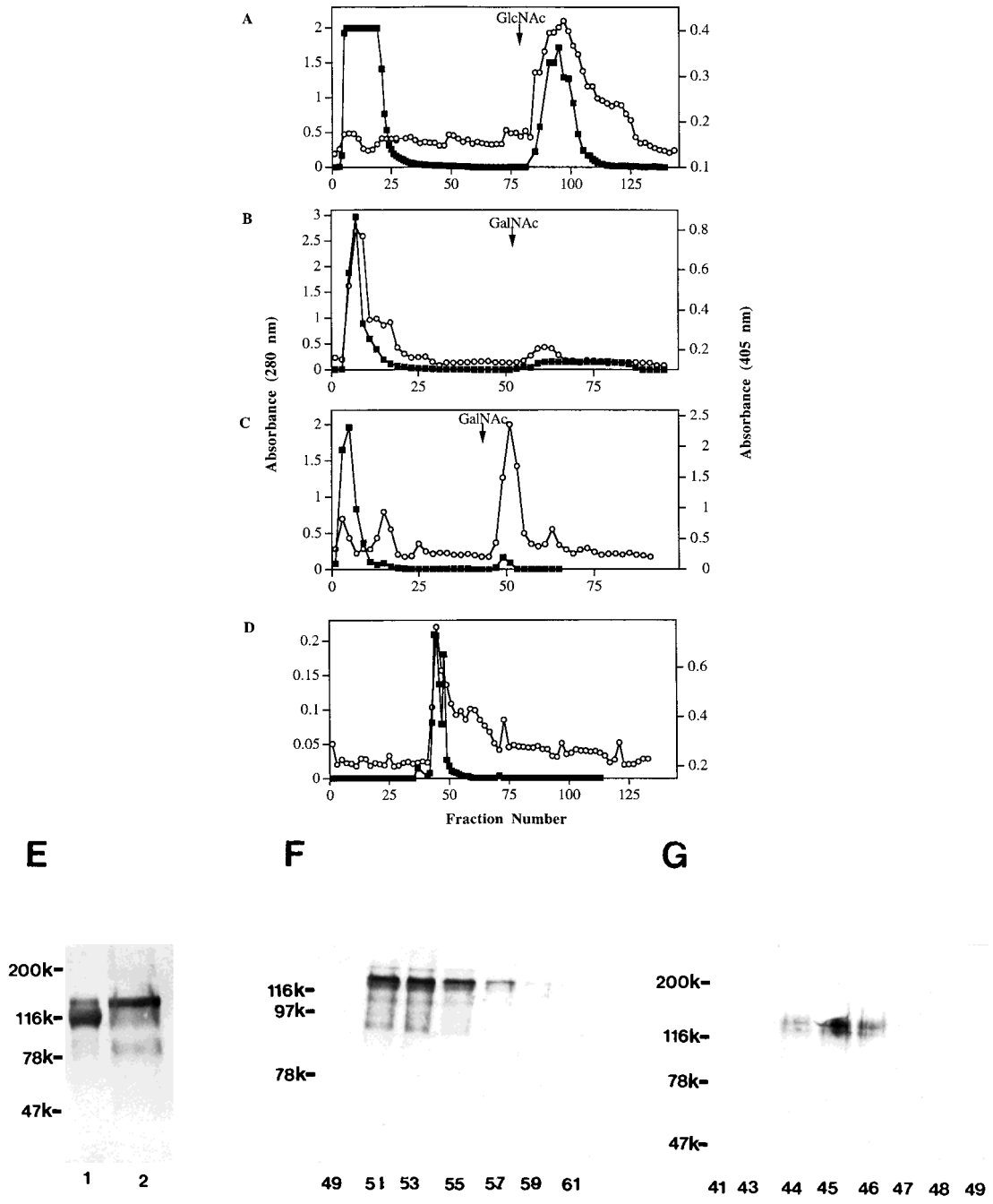

Fig. 2. Purification of gp140. A detergent-solubilized HT29 cell lysate prepared from 60 ml of pelleted cells was subjected to lectin affinity chromatography on WGA-Sepharose, DBA-agarose, and HPA-agarose, and gel filtration chromatography on Biogel A5m, as described below. Run-through and eluted fractions from each column were monitored by optical density at 280 nm (closed squares) and EIA at 405 nm using MAb 3A7 (open circles). **A:** The HT29 cell lysate was applied to a column of WGA-Sepharose (1 × 30 cm) and eluted with 5% N-acetyl-D-glucosamine (GlcNAc). **B:** MAb 3A7-immunoreactive glycoproteins eluted from WGA-Sepharose were applied to a column of DBA-agarose and eluted with buffer containing 100 mM N-acetyl-D-galactosamine (GalNAc). **C:** DBA-agarose unbound fractions enriched in gp140 were pooled and applied to a column of *Helix pomatia* (HPA)-agarose and eluted with buffer containing 100 mM N-acetyl-D-galactosamine (GalNAc). **D:** Fractions eluted from HPA-agarose were pooled, concentrated to 0.5 ml and applied to a column of Biogel A5m. **E:** The DBA-eluted (**lane 1**) and unbound (**lane 2**) fractions were pooled, concentrated to 0.5 ml and analyzed by Western blotting with MAb 3A7 (1:3,000 dilution). **F:** Aliquots (20 µl) of HPA-eluted fractions (the fraction number is shown below each lane) were analyzed by Western blotting with MAb 3A7 (1:3,000 dilution). **G:** MAb 3A7-immunoreactive HPA-eluted fractions shown in F were pooled, concentrated to 0.5 ml and separated by gel filtration using a Biogel A5m column. Aliquots (20 µl) of eluted fractions (fraction number shown below each lane) were analyzed by Western blotting with MAb 3A7 (1:3,000 dilution).

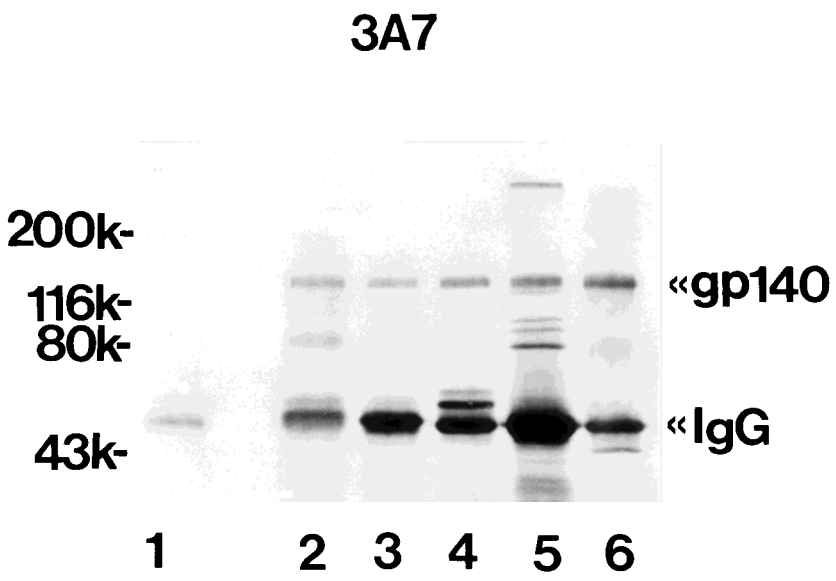

Fig. 3. Identification of monoclonal antibodies specific for gp140. A glycoprotein fraction purified from detergent-solubilized HT29 cells by HPA-agarose which contained 30 µg total protein was immunoprecipitated with normal mouse serum (**lane 1**), MAb 3A7 (**lane 2**), MAb 7A8 (**lane 3**), MAb 8C7 (**lane 4**), MAb 8H7 (**lane 5**), and MAb 11D4 (**lane 6**), and analyzed by Western blotting with MAb 3A7 (1:3,000 dilution). The band migrating at approximately 58 kDa represents the immunoglobulin heavy chain (IgG) detected by the alkaline phosphatase-coupled goat anti-mouse Ig.

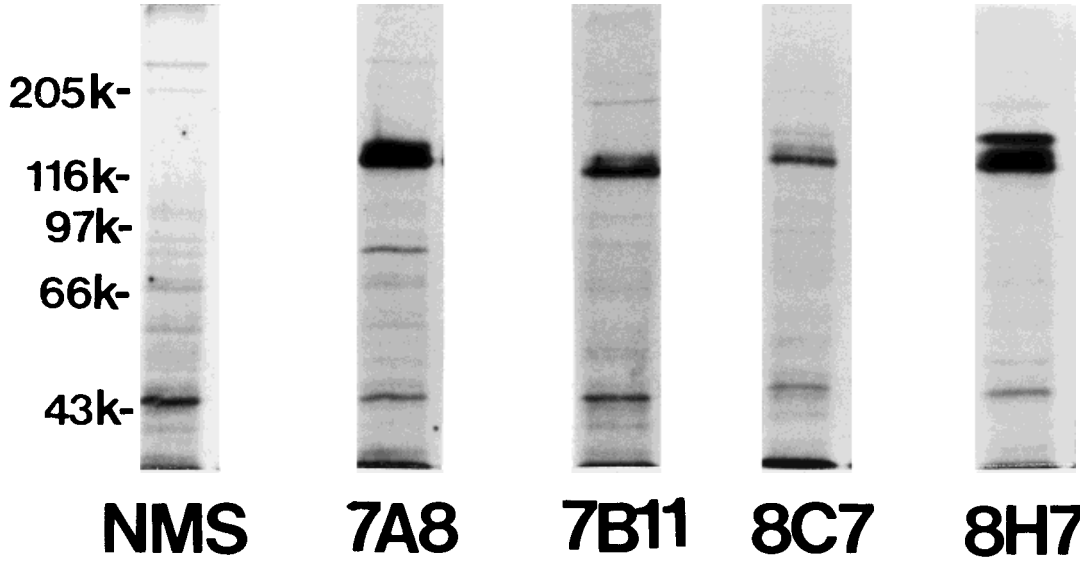

Fig. 4. SDS-PAGE analysis of gp140 immunoprecipitates. [^{35}S]methionine-labeled HT29 cell lysates were immunoprecipitated with normal mouse serum (NMS) or gp140 specific monoclonal antibodies, as indicated below each lane, and separated by SDS-PAGE (6.5%) under reducing conditions. Bands were visualized following fluorography and autoradiography.

ditions revealed that MAbs 7A8, 7B11, 8C7, and 8H7 detect a nondisulfide-linked complex of two proteins/glycoproteins having apparent sizes of 125 and 155 kDa (data not shown). Peptide mapping analysis of the 135 kDa species immunoprecipitated by each of the five antibodies revealed nearly identical peptides (Fig. 5A), which differed significantly from peptides generated from the 145 kDa species (Fig. 5B). Thus, the new panel of monoclonal antibodies appeared to detect a similar complex of structurally distinct proteins/glycoproteins of 135 and 145 kDa.

To identify the glycoproteins detected by these antibodies, the 135 and 145 kDa species were immunoprecipitated with MAb 7A8, separated

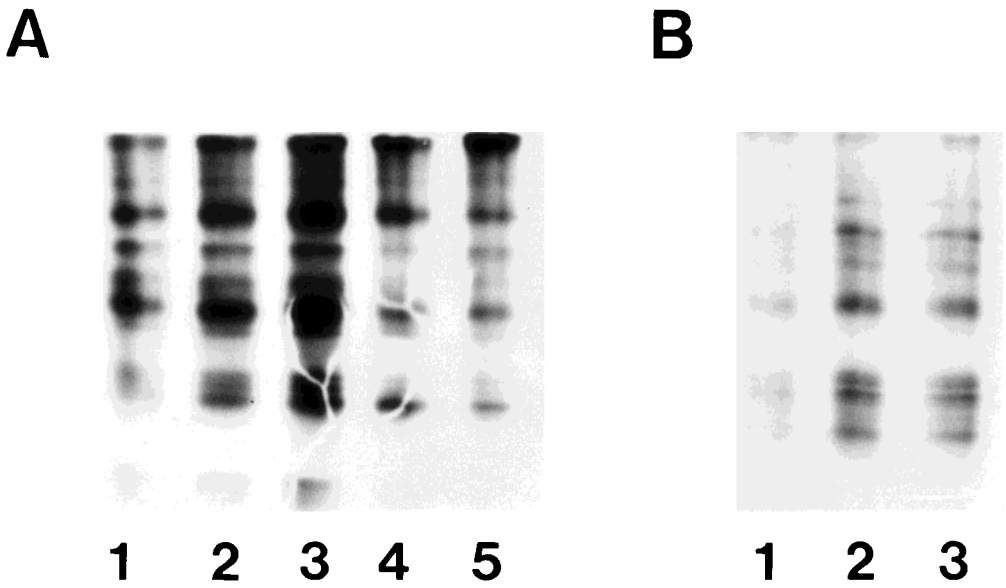

Fig. 5. Limited V8 protease peptide mapping of species immunoprecipitated by gp140-specific monoclonal antibodies. [^{35}S]-methionine HT29 lysates were immunoprecipitated with MAbs 3A7 (**lane 1**), 7A8 (**A, lane 2; B, lane 1**), 8C7 (A, **lane 3;** B, **lane 2**), 8H7 (A, **lane 4;** B, **lane 3**), or 11D4 (A, **lane 5**), and separated by 6.0% SDS-PAGE. The 135 kDa (A) and 145 kDa (B) species were excised from the dried, unfixed gel, and subjected to limited protease V8 peptide mapping, as described in Materials and Methods.

TABLE II. Analysis of Antibody Specificity by Flow Cytometry Using CHO Transfectants Expressing Human Integrin Chains

MAb	CHO	CHO-α2	CHO-α3	CHO-α4	CHO-α5	CHO-β1	CHO-β3
mIg[a]	4.96[b]	7.59	2.35	2.30	5.79	4.33	3.62
	(5.8)[c]	(6.8)	(0.3)	(0.5)	(5.2)	(0.3)	(1.5)
7A8	4.77	7.38	130.18	2.19	5.35	3.67	3.68
	(5.4)	(6.1)	(94.6)	(0.4)	(4.0)	(0.6)	(1.7)
7B11	5.07	7.21	118.78	2.41	5.87	5.65	3.74
	(5.8)	(5.5)	(95.0)	(0.4)	(4.1)	(2.2)	(1.8)
8C7	5.06	7.01	2.91	2.33	5.75	43.33	3.65
	(5.8)	(5.1)	(0.7)	(0.7)	(4.4)	(99.8)	(1.8)
8H7	5.10	7.36	2.54	2.33	5.88	186.82	3.80
	(6.0)	(6.8)	(0.7)	(0.5)	(4.7)	(99.8)	(1.4)
11D4	5.41	7.57	2.33	2.29	6.27	3.91	4.56
	(6.4)	(7.1)	(0.2)	(0.6)	(7.1)	(0.9)	(1.6)

[a]Normal mouse immunoglobulin.
[b]Antibody specificity was determined by flow cytometry using control CHO cells or CHO cells transfected with different human alpha or beta chains, as indicated at the top of each column. The numbers represent the mean fluorescence intensity obtained following incubation of each transfectant with monoclonal antibody followed by FITC-labeled goat anti-mouse Ig.
[c]The numbers in parentheses represent the percentage of positive cells.

by 6.0% SDS-PAGE, transferred to an Immobilon-P membrane and the excised bands subjected to partial protein sequencing following trypsin digestion. A tryptic peptide having the sequence AGNPGSLFGYSVALHR was generated from the 135 kDa species while a peptide with the sequence LKPEDITQIQPQ(Q)L was obtained from the 145 kDa species. A search of these sequences in the Genebank protein sequence database using the BLAST search system revealed that the former peptide was identical to a sequence found within the human α3 integrin chain while the latter peptide was nearly identical (13 out of 14 residues) to human β1 integrin, with a threonine residue instead of histidine at position 7.

The integrin chain specificity of MAbs 7A8, 7B11, 8C7, 8H7, and 11D4 was examined further by testing the ability of each antibody to detect CHO transfectants expressing different human α and β integrin chains. As shown in Table II, MAbs 7A8 and 7B11 stained CHO

transfectants expressing α3 integrin subunit while 8C7 and 8H7 were specific for CHO transfectants expressing β1 integrin subunit. In contrast, 11D4 failed to bind to any of the transfectants tested, including those expressing integrin α2, α3, α4, α5, β1, or β3 chains. In light of these data, we investigated the possibility that this antibody detected a carbohydrate epitope. Using a panel of BSA-glycan conjugates expressing blood group A or B determinants (Table III), we were able to show by EIA that 11D4 detects specifically type 2 chain blood group A determinant but not type 2 chain blood group B (Fig. 6), indicating that the specificity of MAb 11D4 differs significantly from that of MAb 3A7. However, as we have shown previously for MAb 3A7 [Laferté et al., 1995], treatment of BSA-type 2 blood group A with N-acetylglucosaminidase, which removes the terminal α1–3 linked N-acetylgalactosamine [Izumi et al., 1992], destroyed the epitope detected by MAb 11D4 (data not shown).

Expression of α3β1 Integrin on Blood Group A, AB, and B-Positive Human Colon Carcinoma Cell Lines

The availability of monoclonal antibodies specific for the polypeptide portion of gp140, which define α3β1 integrin, made it possible to examine whether the 3A7 epitope detected on the surface of human colon carcinoma cells is present consistently on α3β1 integrin. In total, 10 cell lines originating from blood group A, AB, or B patients were assessed for expression of the 3A7 epitope, α3 integrin subunit, and β1 integrin subunit. As can be seen from Table IV, these cell lines were established from primary tumors of varying histological grade, lymph node metastases, or liver metastases. For example, the HT29 cell line was established from a moderately-well differentiated Duke's B2 tumor [Fogh and Trempe, 1975] while the SW480 and SW1417 cell lines were established from Duke's C tumors. The SW620 and KM20C cell lines were established from a lymph node metastasis [Leibovitz et al., 1976] and liver metastasis [Morikawa et al., 1988], respectively. It is noteworthy that the SW480 and SW620 cells were derived from the same individual. We also examined the two morphologically distinct populations present in the SW480 cell line, SW480-E, and SW480-R cells. In a recent study, Tomita et al. [1992] demonstrated that SW480-E cells, which constitute the majority of cells, form flat epithelial-like colonies, whereas R-type cells have a rounded shape and grow as dome-like structures. In addition, these authors showed that the R-type cells produce larger subcutaneous tumors in nude mice than the E-type cells. It was of interest, therefore, to include them in this analysis. Flow cytometric analysis of these cell lines was carried out using either MAb 3A7, 7B11 (anti-α3), or 8H7 (anti-β1). As shown in Table IV, the mean fluorescence intensity detected with each antibody varied among the panel of cell lines tested, with HT29 cells exhibiting the highest level of staining whereas SW480-E and SW620 cells exhibited the lowest level. This pattern of expression was identical for cells isolated using trypsin (Table IV) or PBS-EDTA (data not shown), consistent with the relatively protease-resistant nature of integrins [Hynes, 1992]. Moreover, the level of expression of epitopes detected by MAbs 3A7, 7B11, and 8H7 did not appear to correlate with the histopathological grading of the original tumor. This may be due to biochemical and phenotypic changes resulting from in vitro culture of the primary tumor. However, the relative level of expression of the 3A7 epitope and α3 integrin subunit was similar for

TABLE III. Structure of Oligosaccharide Moiety of BSA-Glycan Conjugates

Name	Structure
H type 2	Fucα1–2Galβ1–4GlcNAc-BSA
A disaccharide	GalNAcα1–3Gal-BSA
A type 1	GalNAcα1–3[Fucα1–2]Galβ1–3GlcNAc-BSA
A type 2	GalNAcα1–3[Fucα1–2]Galβ1–4GlcNAc-BSA
A type 4	GalNAcα1–3[Fucα1–2]Galβ1–3GalNAc-BSA
A type 6	GalNAcα1–3[Fucα1–2]Galβ1–4Glc-BSA
B disaccharide	Galα1–3Gal-BSA
B type 2	Galα1–3[Fucα1–2]Galβ1–4GlcNAc-BSA
B type 4	Galα1–3[Fucα1–2]Galβ1–3GalNAc-BSA
B type 5	Galα1–3[Fucα1–2]Galβ1–3Gal-BSA
B type 6	Galα1–3[Fucα1–2]Galβ1–4Glc-BSA

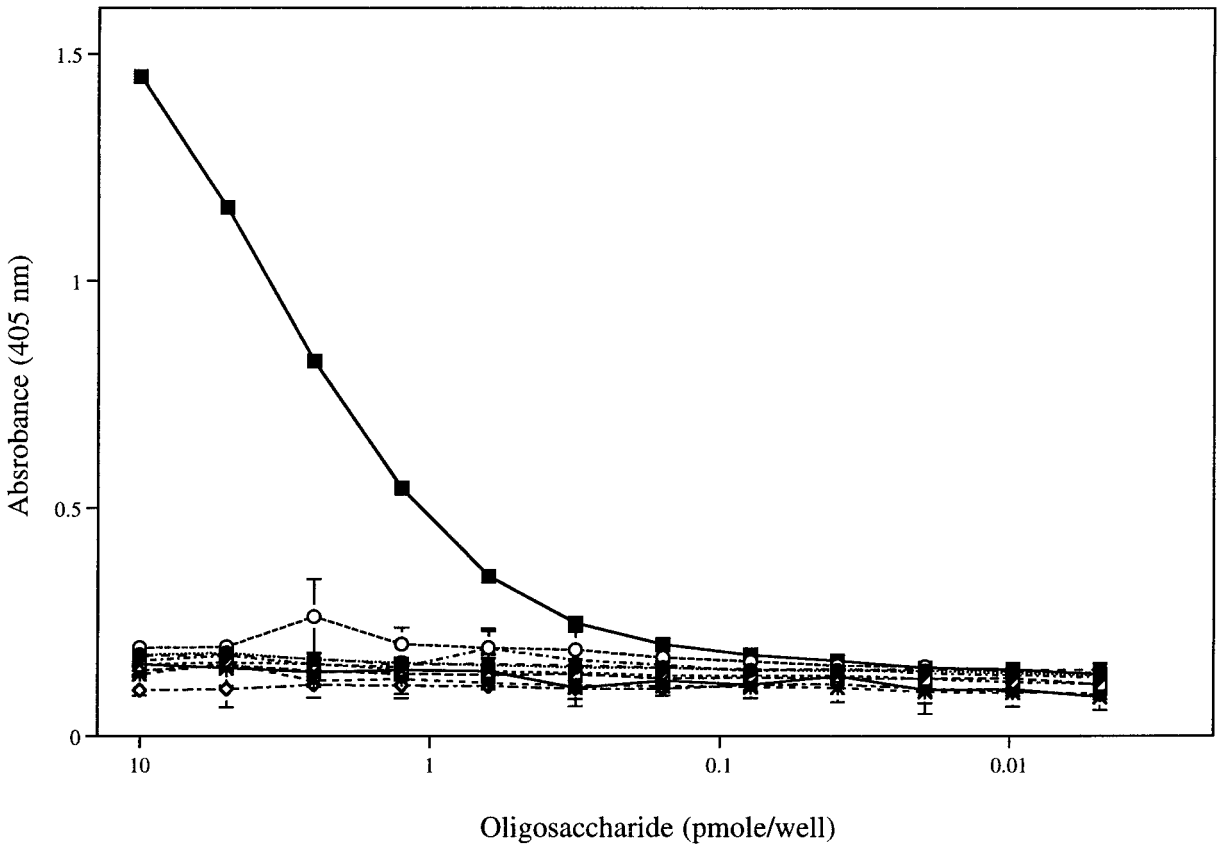

Fig. 6. Solid-phase immunosorbent assay of BSA-glycan conjugates with monoclonal antibodies 11D4. Serial dilutions of BSA-glycan conjugates (10 to 0.01 picomole oligosaccharide/well), depicted in Table III, were added in triplicate to an 96-well Immulon-2 plate and incubated overnight at 4°C. An enzyme-linked immunoassay was carried out using MAb 11D4 (1:3,000 dilution, 2 h) followed by alkaline phosphatase-coupled goat antimouse Ig (1,3,000, 1 h). Color development was monitored at 405 nm following addition of the substrate p-nitrophenyl phosphate. Each point on the graph represents the mean and standard deviation (note that for some of the data points, the standard deviation does not appear because values were lower than the graphic resolution). H type 2-BSA (◆); A disaccharide-BSA (☒); A type 1-BSA (○); A type 2-BSA (■); A type 4-BSA (◩); A type 6-BSA (●); B disaccharide (□); B type 2-BSA (▲); B type 4-BSA (△); B type 5-BSA (▽); B type 6-BSA (◇).

any given cell line (Table IV). In fact, the graphical representation of the fluorescence intensity of each cell line obtained with MAb 3A7 plotted against fluorescence intensity obtained with MAb 7B11 produced a straight line (Fig. 7). Linear regression analysis of this plot yielded a regression value (R) of 0.970. The proximity of this value to 1.00 provided evidence that expression of the 3A7 epitope on the tumor cell surface is dependent on cell surface expression of the α3 integrin subunit. Similar regression analysis of a plot of the mean fluorescence intensity obtained with 8H7 (anti-β1) against the intensity obtained with MAb 3A7 yielded a correlation coefficient of 0.852 (data not shown). This lower value suggested that the β1 integrin subunit is associated with other alpha integrin chains on the surface of human colon carcinoma cells which do not express the 3A7 epitope. Thus α3β1 is a major carrier of the 3A7 epitope in human colon carcinoma cell lines originating from blood group A, AB, and B individuals.

Carbohydrate Structure on Integrin α3β1

Glycosidase sensitivity and lectin blotting were used to examine further the carbohydrate moiety of α3β1 integrin. As shown in Figure 8A (lane 3), susceptibility of immunoprecipitated α3β1 to neuraminidase indicated the presence of N-acetylneuraminic acid (sialic acid) on this glycoprotein heterodimer. Treatment of α3β1 immunoprecipitates with glycopeptidase F produced a species of 100 kDa (lane 4), indicating that both α3 and β1 subunits contain N-linked oligosaccharides. The small decrease in apparent molecular weight of α3β1 following O-glycanase treatment (lane 5) was indicative of the presence of O-linked oligosaccharides. Since sialylated O-linked oligosaccharides are not susceptible to O-glycanase, pre-treatment of α3β1

TABLE IV. Expression of α3β1 Integrin and the 3A7 Epitope on Blood Group A, AB, or B-Positive Human Colon Carcinoma Cell Lines

Cell line	Duke's staging or origin	Blood group	Mean fluorescence intensity[a]		
			MAb 3A7	MAb 7B11	MAb 8H7
HT29	Duke's B2	A	34.2 ± 0.3	42.5 ± 3.6	70.3 ± 3.4
WiDr	Duke's B2	A	29.6 ± 2.8	30.5 ± 0.4	46.8 ± 2.4
SW1417	Duke's C1	B	10.8 ± 2.3	8.7 ± 1.9	17.5 ± 0.6
SW480	Duke's C2	A	8.4 ± 3.2	8.0 ± 1.0	25.4 ± 0.9
SW480-E	Variant of SW480; epithelial-like morphology	A	1.7 ± 0.4	4.27 ± 0.4	19.8 ± 1.1
SW480-R	Variant of SW480; round morphology	A	26.4 ± 0.2	35.1 ± 0.5	41.7 ± 0.1
SW620	Lymph node metastasis	A	1.9 ± 0.4	3.0 ± 0.3	14.1 ± 1.2
LoVo	Supraclavicular metastasis	B	12.9 ± 2.1	12.9 ± 0.5	22.7 ± 1.3
SW48	Duke's D	AB	19.8 ± 3.0	21.7 ± 1.3	31.4 ± 0.9
KM20C	Duke's D	A	29.9 ± 3.1	37.9 ± 5.2	33.4 ± 1.5

[a]The level of expression of the α3 integrin subunit, β1 integrin subunit and the 3A7 epitope was determined by flow cytometry. Human colon carcinoma cells were incubated with MAbs 3A7, 7B11 (anti-α3), or 8H7 (anti-β1), followed by FITC-coupled goat anti-mouse IgG, as described in Materials and Methods. The numbers represent the mean fluorescence intensity from two (SW480-E and SW480-R) or four independent experiments (all other cell lines).

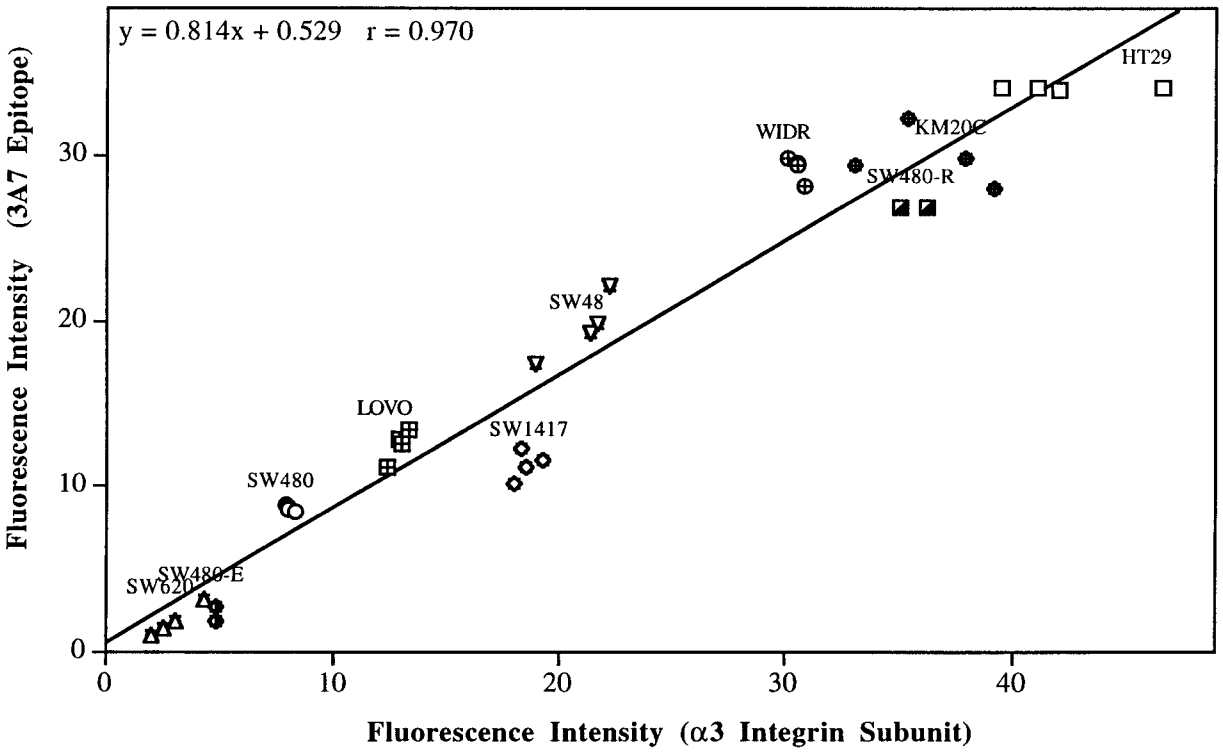

Fig. 7. Expression of α3 integrin subunit and the 3A7 epitope on blood group A, AB, or B human colon carcinoma cell lines. Human colon carcinoma cells listed in Table IV (1×10^6 cells in 1 ml FC buffer) were incubated on ice for 1 h with MAb 3A7 or 7B11 (anti-α3) diluted to a final concentration of 1:300. After washing, cells were pelleted, resuspended in 100 µl of FITC-coupled goat anti-mouse IgG and incubated on ice for 30 min, and analyzed by flow cytometry as described in Materials and Methods. The data from separate analyses are shown for each cell line.

immunoprecipitates with neuraminidase, which removes sialic acid, was carried out prior to O-glycanase digestion. This resulted in a further decrease in size of the deglycosylated product (lane 6) which exceeded that obtained with neuraminidase alone (lane 3), consistent with the presence of sialylated O-linked oligosaccharides. Susceptibility of α3β1 integrin to endo-β-galactosidase (lane 7) indicated the presence of poly-N-acetyllactosamine structures. This was

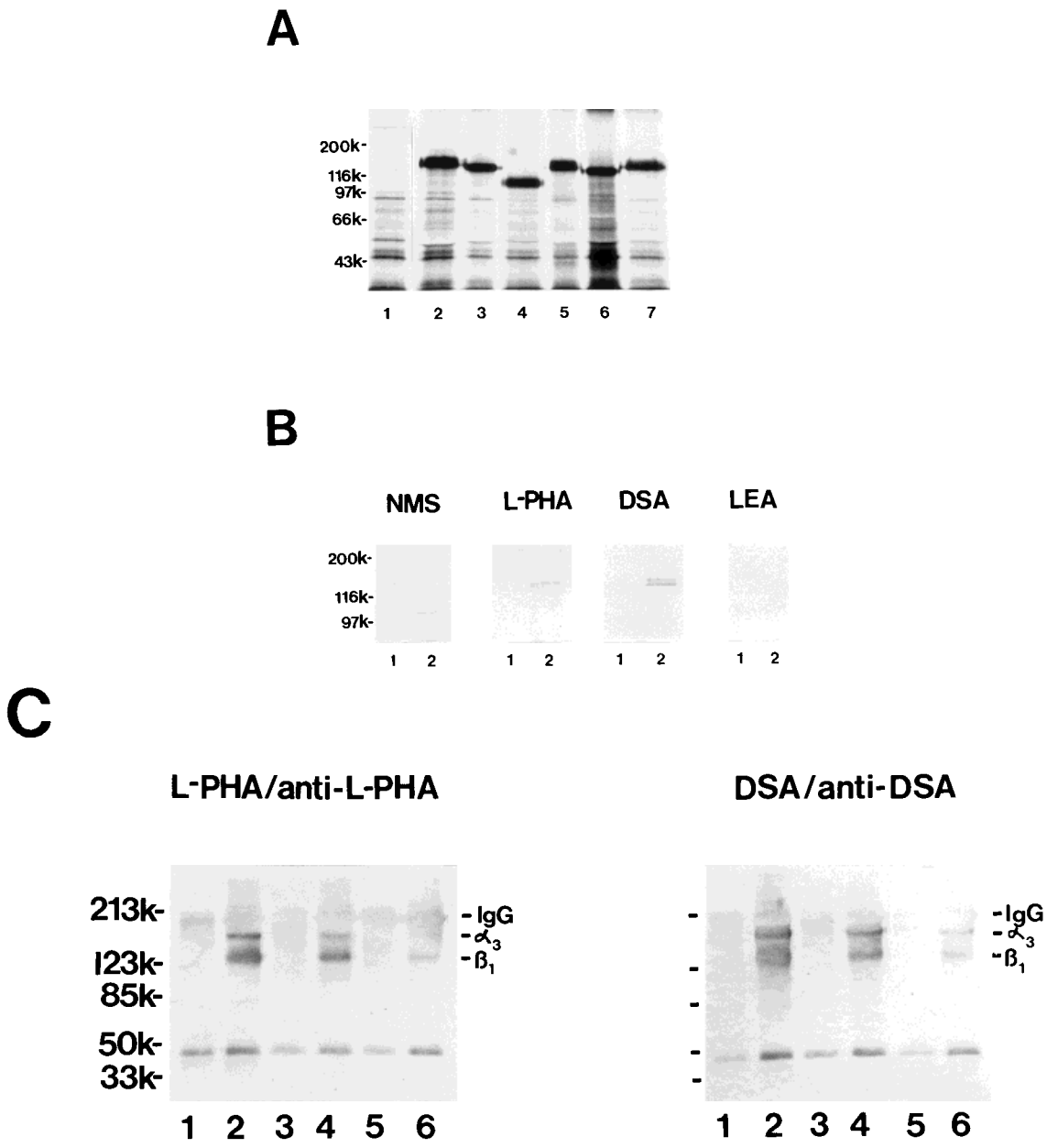

Fig. 8. Analysis of carbohydrate moiety of α3β1 integrin. **A:** α3β1 integrin was immunoprecipitated from [^{35}S]methionine-labeled HT29 cell lysates using MAb 7A8 (anti-α3 integrin subunit; lanes 2–7). Samples were left untreated (**lane 2**) or digested with neuraminidase (**lane 3**), glycopeptidase F (**lane 4**), O-glycanase (**lane 5**), neuraminidase and O-glycanase (**lane 6**), or endo-β-galactosidase (**lane 7**). **Lane 1** shows proteins immunoprecipitated with normal mouse serum. Bands were visualized following fluorography and autoradiography. **B:** A detergent-solubilized HT29 cell lysate was applied to HPA-agarose, as described in Materials and Methods. Eluted proteins were immunoprecipitated with either NMS (**lanes 1**) or MAb 7A8 (**lanes 2**), separated by 6.5% SDS-PAGE and Western blotted with biotinylated L-PHA, DSA, or LEA, as indicated above each figure, followed by alkaline phosphatase-coupled streptavidin. The numbers in the left margin refer to the sizes of pre-stained molecular weight markers. **C:** Detergent-solubilized HT29 cell lysates containing 200 μg (**lanes 1 and 2**), 40 μg (**lanes 3 and 4**), or 8 μg (**lanes 5 and 6**) total protein were immunoprecipitated with either normal mouse serum (lanes 1, 3, and 5) or MAb 7A8 (lanes 2, 4, and 6), and the immunoprecipitates separated by 6% SDS-PAGE under nonreducing conditions followed by Western blotting analysis with either L-PHA/anti-L-PHA or DSA/anti-DSA, as indicated above each figure.

confirmed by lectin blotting carried out with *Datura stramonium* agglutinin (DSA; Fig. 8B), which binds short ($n \leq 3$) poly-N-acetyllactosamine units [Merkle and Cummings, 1987]. Similar analysis carried out on α3β1 immunoprecipitates separated by SDS-PAGE under non-reducing conditions showed more clearly that both α3 and β1 integrin subunits stain with DSA, with more intense staining associated with the β1 subunit (Fig. 8C). However,

α3β1 integrin does not appear to contain long ($n \geq 3$) poly-N-acetyllactosamine units since this glycoprotein failed to stain with *Lycopersicum esculentum* agglutinin (LEA; Fig. 8B) [Merkle and Cummings, 1987]. Lastly, blotting of α3β1 immunoprecipitates with L-PHA, under both reducing (Fig. 8B) and nonreducing conditions (Fig. 8C), indicated that both α3 and β1 subunits contain β1–6 branched Asn-linked oligosaccharides, with more intense staining associated with the β1 subunit. The apparent increase in mobility of the α3 integrin subunit seen under nonreducing conditions has been attributed to the presence of a disulfide-linked peptide which is lost upon reduction [Sonnenberg, 1993]. Thus, each subunit of the α3β1 heterodimer expresses a number of cancer-associated oligosaccharide structures including β1–6 branched Asn-linked oligosaccharides, short ($n < 3$) poly-N-acetyllactosamine glycan chains and the oncodevelopmental carbohydrate epitope defined by MAb 3A7.

DISCUSSION

In this paper, we have shown that gp140, the major carrier of an oncodevelopmentally expressed carbohydrate epitope detected by MAb 3A7 in human colon cancer cells, corresponds to α3β1 integrin, a heterodimeric cell surface receptor involved in cell-extracellular matrix adhesion [Takada et al., 1988; Carter et al., 1991; Dedhar et al., 1992] as well as homotypic [Sriramarao et al., 1993], and heterotypic cell-cell interactions [Symington et al., 1993]. Our interest in identifying gp140 stemmed from our previous studies [Laferté et al., 1995] showing that it is the major species detected in azoxymethane-induced colon tumors and some human colon carcinoma cell lines by MAb 3A7, an antibody shown to detect a developmentally-regulated epitope on type 2 chain blood group A or B determinants. Our previous studies documenting increased expression of the 3A7 epitope in colon tumors, with the highest level detected in invasive rat colon tumors (Duke's B1 and B2) and human colon carcinoma cell lines established from invasive colon tumors, suggested that the 3A7 epitope contributes to colon tumor progression. In this paper, we have taken the first step towards elucidating the biological role of the 3A7 epitope by demonstrating using biochemical, immunological, and molecular approaches that its preferred glycoprotein carrier in human colon carcinoma cells is α3β1 integrin.

The fact that α3β1 integrin was eluted from WGA-sepharose, the first of three lectin affinity chromatography steps used for its purification, suggested that it is a sialoglycoprotein. This was confirmed by the susceptibility of α3β1 to neuramininase (Fig. 8A), an exoglycosidase specific for oligosaccharides terminating with N-acetylneuraminic acid. The affinity of α3β1 integrin for HPA-agarose was not surprising given the specificity of this lectin for terminal α-linked N-acetylgalactosamine, one of the sugars making up the blood group A determinant (GalNAcα1–3[Fucα1–2]Gal-R) and a necessary component of the 3A7 and 11D4 epitopes. Although the poor binding of α3β1 to DBA-agarose was expected in light of our previous studies [Laferté et al., 1995] and those of Yuan et al. [1985] showing that colon tumors, unlike normal colon, bind poorly to this lectin, the ability of DBA-agarose to separate α3β1 integrin from two other less abundant 3A7-immunoreactive glycoproteins of 90 and 120 kDa proved to be an effective purification step. In addition, this revealed the existence of additional glycoproteins bearing the 3A7 epitope in HT29 cells which may play a role in colon cancer progression.

Gel filtration chromatography of HPA-eluted glycoproteins on Biogel A5m yielded a single peak of 3A7-immunoreactive material (Fig. 2D) which consisted of two closely-migrating species of 135 and 145 kDa, as detected by Western blotting with MAb 3A7. This suggested that two 3A7-immunoreactive glycoproteins of similar size co-purified in the same column fraction or gp140 consisted of a complex of two glycoproteins bearing the 3A7 epitope. The evidence in support of the latter possibility included the fact that i) all five gp140-specific monoclonal antibodies (7A8, 7B11, 8C7, 8H7, and 11D4) immunoprecipitated two species of 135 and 145 kDa from [^{35}S]-labeled HT29 cells, ii) peptide mapping analyses revealed that the 135 kDa immunoprecipitated by each of these antibodies produced nearly identical peptides which were distinct from the 145 kDa species, iii) partial protein sequencing of the 135 and 145 kDa species immunoprecipitated by MAb 7A8 identified them as α3 and β1 integrin subunits, respectively, which form a heterodimer and iv) commercially-available anti-α3β1 (Chemicon), anti-α3 (Gibco/BRL), and anti-β1 (Gibco/BRL) monoclonal antibodies immunoprecipitated the same 135 and 145 kDa species as the five gp140-specific monoclonal antibodies described herein (data not shown).

Reactivity of α3β1 with the carbohydrate-specific monoclonal antibodies 3A7 and 11D4 confirmed that α3β1, like other integrins, is a glycoprotein [Sonnenberg, 1993]. In addition, the presence on α3β1 integrin of determinants defined by these antibodies, which both detect blood group A determinant specifically on type 2 chain, was consistent with studies by Dabelsteen et al. [1988] showing that human colon tumors and fetal intestine express blood group A determinants on type 2 chain whereas normal colon expresses this epitope exclusively on type 1 chain. The fact that α3β1 integrin was the major species immunoprecipitated from HT29 cells using these antibodies, which likely detect carbohydrate determinants at the terminal ends of oligosaccharide chains, is consistent with previous studies demonstrating the importance of glycoprotein acceptors in regulating terminal glycosylation of oligosaccharide chains [Yeh and Cummings, 1997].

In light of studies from several laboratories demonstrating the importance of N-linked oligosaccharides for integrin receptor assembly and ligand binding [Lampe et al., 1992; Chammas et al., 1993; Lindmark et al., 1993; Zheng et al., 1994], we examined further the oligosaccharide moiety of α3β1. Susceptibility of α3β1 to glycopeptidase F and O-glycanase indicated the presence of both N- and O-linked oligosaccharides, respectively (Fig. 8). Although the number and type of oligosaccharide chains present on α3β1 integrin expressed by human colon cancer cells is not yet known, the cDNA sequences of the α3 and β1 integrin subunits have revealed 13 and 12 potential N-linked glycosylation sites, respectively [Takada et al., 1991; Sonnenberg, 1993].

Reactivity of α3β1 with DSA and its susceptibility to endo-β-galactosidase were consistent with the presence of short poly-N-acetyllactosamine structures. Since poly-N-acetyllactosamine structures are often carriers of oncodevelopmental antigens, including ABH and Lewis blood groups [Fukuda, 1992], this suggested that the epitopes detected by MAb 3A7 and 11D4 may be present on these extended type 2 chains. It is noteworthy that poly-N-acetyllactosamine structures have been detected on a variety of cancer cells, including colon, and have been shown to reduce cell-cell and cell-extracellular matrix interactions [Dennis, 1992; Fukuda, 1992, 1994; Sawada et al., 1994]. Although lamp-1 and lamp-2 constitute major carriers of poly-N-acetyllactosamine structures in many cancer cells, including colon [Saitoh et al., 1992; Sawada et al., 1994; Fukuda, 1992, 1994], these glycan chains have also been found on the β1 integrin subunit expressed by transformed mammary epithelial cells where they have been shown to decrease tumor cell spreading on laminin-1 [Leppa et al., 1995]. Similarly, our data indicate that the β1 integrin subunit expressed by the human colon cancer cell line HT29 carries poly-N-acetyllactosamine structures, suggesting that these cancer-associated glycan chains may modulate tumor cell adhesion by affecting the ligand binding properties of α3β1 integrin. To our knowledge, this is the first report documenting the presence of poly-N-acetyllactosamine structures on the α3 integrin subunit. It is not yet clear, however, whether the presence of poly-N-acetyllactosamine structures and/or the 3A7 epitope on the α3 integrin subunit affects the ability of α3β1 to bind to its known ligands, namely, fibronectin, collagens I and IV [Takada et al., 1988], laminin 1 [Takada et al., 1988], and 5 [Carter et al., 1991], and entactin [Dedhar et al., 1992] or mediate cell-cell interactions [Sriramarao et al., 1993; Symington et al., 1993].

In addition to its role as major carrier of oncodevelopmental carbohydrate determinants, α3β1 was also shown to express β1–6 branched Asn-linked oligosaccharides, previously shown to contribute to cancer metastasis by influencing tumor cell adhesion and invasion [Dennis et al., 1987; Laferté and Dennis, 1989; Yagel et al., 1989; Demetriou et al., 1995]. This is consistent with studies demonstrating that cancer cells express elevated levels of GlcNAc T-V, the enzyme responsible for initiation of the β1–6 antenna [Pierce and Arango, 1986; Fernandes et al., 1991; Dennis, 1992]. Moreover, oligosaccharides containing β1–6 antenna are preferred substrates of β1–3 N-acetylglucosaminyl transferase (GlcNAc -Ti), one of the enzymes required for synthesis of poly-N-acetyllactosamine structures [van den Eijnden et al., 1988]. Thus, cancer-associated changes in the expression of GlcNAc T-V may play a key role in regulating the expression of a number of oncodevelopmental carbohydrate epitopes, including the 3A7 epitope. Although the mechanism responsible for the biological effects mediated by β1–6 branched Asn-linked oligosaccharides is not completely understood, the identification of glycoproteins bearing these structures has been the subject of intense research for several years. To date, only a limited number of glycoproteins have been shown to carry β1–6 branched Asn-linked oligosaccharides, most of which have been implicated in cell-cell or cell-extracellular

matrix interactions. These include lamp-1 [Carlsson et al., 1988; Laferté and Dennis, 1989], lamp-2 [Carlsson et al., 1988], CEA [Chandrasekaran et al., 1983], laminin [Jin et al., 1995], TAA90/Mac-2 binding protein/cyclophilin C-associated protein [Koths et al., 1993; Ullrich et al., 1994], and certain integrin subunits including α5, α6, αv, and β1 [Jasiulionis et al., 1996; Demetriou et al., 1995; Chammas et al., 1993]. Although it is not yet clear whether the presence of β1–6 branched Asn-linked oligosaccharides on α3β1 alters its adhesive functions, the presence of these structures on α6β1 expressed by ras-transformed fibroblasts [Jasiulionis et al., 1996] and the fibronectin receptor (α5β1) expressed by immortalized mink lung epithelial cells [Demetriou et al., 1995] has been shown to modulate tumor cell adhesion and spreading.

The presence of oncodevelopmental and cancer-associated carbohydrate determinants on α3β1 integrin suggests that it may play an important role in one or more steps of colon tumor progression. Moreover, our studies demonstrating that the amount of α3 integrin subunit expressed at the surface of blood group A, B, and AB colon carcinoma cell lines correlates directly with cell-surface expression of the 3A7 epitope suggest that expression of specific glycoproteins, such as α3β1 integrin, may play a key role in displaying functionally-relevant carbohydrate epitopes, provided that the glycosyltransferases responsible for their synthesis are not limiting. Thus, activation of oncogenes and loss of tumor suppressor genes, which characterize colon tumors, may not only affect the level of expression of α3β1 integrin but also influence cell-surface glycosylation patterns in a way which affects cell-extracellular matrix as well as cell-cell interactions. Although the role of α3β1 integrin in colon cancer progression is not well understood, recent studies by Lindmark et al. [1994] have shown that the discontinuous pattern of expression of the α3 subunit in the basolateral region of colon tumors correlates with poor prognosis. In light of recent studies of α3-null mice which implicate α3β1 integrin in assembly and maintenance of the extracellular matrix [DiPersio et al., 1997], it is possible that cancer-associated alterations in α3β1 integrin expression as well as glycosylation may lead to abnormal extracellular matrix structure, thereby facilitating tumor cell invasion and metastasis. Availability of α3 chain-specific monoclonal antibodies as well as monoclonal antibodies which detect oncodevelopmental carbohydrate epitopes should facilitate future studies on the contribution of complex carbohydrate to α3β1 integrin function.

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