Characterization of a family of structurally related glycoproteins expressing β 1-6-branched asparagine-linked oligosaccharides in human colon carcinoma cells

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Previous studies have established that metastatic tumour cells express high levels of β 1-6-branched Asn-linked oligosaccharides which can be detected with the lectin leucoagglutinin (L-PHA) [Dennis, Laferté, Waghorne, Breitman & Kerbel (1987) *Science* **236**, 582–585]. In order to identify L-PHA-binding glycoproteins which may play a role specifically in colon cancer, we have prepared monoclonal antibodies (MAbs) to the moderately well-differentiated human colon carcinoma cell line HT29. In this paper we present the initial characterization of a family of structurally related L-PHA-binding glycoproteins detected by MAb 1H9 which are differentially expressed and processed by HT29 cells and by two other human colon carcinoma cell lines, SW480 and SW620. In contrast to HT29, the SW480 and SW620 cell lines were established from a poorly differentiated grade III/IV primary tumour and one of its lymph node metastases respectively. MAb 1H9 detects in HT29 cells a conformational determinant present on three L-PHA-binding glycoproteins of 100, 70 and 25 kDa, as well as a 74 kDa glycoprotein with high-mannose-type Asn-linked oligosaccharides. Pulse-chase experiments and peptide mapping analyses revealed that the 74 kDa and 100 kDa species are related by carbohydrate processing and are probably derived from a common 76 kDa precursor. On the other hand, the 70 kDa glycoprotein is synthesized from an endoglycosidase H-sensitive precursor of 56 kDa which is structurally related to, but distinct from, the aforementioned 76 kDa precursor. In addition, the 100 kDa species is secreted into the culture medium, whereas the 70 kDa glycoprotein is retained intracellularly. SW480 and SW620 cells showed qualitative and quantitative differences from HT29 cells, including increased secretion of a smaller L-PHA-binding glycoprotein of 92 kDa into the culture medium, as well as apparent differences in glycosylation of the intracellular 66 kDa glycoprotein. These results suggested that the expression, glycosylation and subcellular localization of this family of L-PHA-binding glycoproteins may correlate with the differentiation status of colon cancer cells and/or reflect biochemical changes characteristic of more progressive metastatic tumours.

INTRODUCTION

Colorectal cancer remains one of the most common epithelially derived malignancies affecting both men and women [1]. Although significant progress has been achieved in elucidating some of the genetic mechanisms implicated in colon tumorigenesis [2], eradication of metastatic disease remains the single greatest obstacle to curing cancer [3].

Monoclonal antibodies (MAbs) have proven useful in detecting phenotypic and genotypic changes in pre-neoplastic and neoplastic colonic mucosa [4]. As a result, it has become apparent that alterations in glycoconjugate expression and/or structure are associated with both early and late stages of colon cancer [4–9]. Although the biological significance of these changes is not clear, various reports have suggested that carbohydrate changes on tumour cells can modulate cell adhesion [10,11] and cell motility [11], and influence anti-tumour immunity [12–15].

There is increasing evidence that in both rodent [16–18] and human [19–21] tumours, progression of cancer from a tumorigenic to a metastatic phenotype is directly associated with increased levels of β 1-6-branched Asn-linked oligosaccharides. These oligosaccharides, which have the structure GlcNAc(β 1-6)Man α 1-

6Man-R, can be characterized by their binding to the lectin leucoagglutinin (L-PHA) [22]. The functional significance of increased β 1-6 branching of Asn-linked oligosaccharides and the identity of glycoproteins bearing these structures have not been well established. However, there is evidence that the presence of such oligosaccharides may influence tumour cell adhesion [23,24] and invasion [24]. For example, two membrane glycoproteins of 110 kDa (called P2A) and 130 kDa (LAMP-1; lysosomal-associated membrane protein-1) bearing β 1-6-branched Asn-linked oligosaccharides have been purified from the highly metastatic lymphoid tumour cell line MDAY-D2 [25]. Both glycoproteins bound to immobilized type I collagen with affinities which were glycosylation-dependent. Enzymic removal of sialic acid and polylactosamine structures from LAMP-1 enhanced its binding to type I collagen, laminin and fibronectin. In contrast, the binding of P2A to type I collagen was dependent on sialic acid [23]. These results suggested that the expression of β 1-6-branched oligosaccharides on glycoproteins such as P2A and LAMP-1 may contribute to the metastatic process by modulating tumour cell adhesion. In additional studies, treatment of metastatic B16F10 melanoma and MDAY-D2 cells with the glycosylation inhibitor swainsonine led to decreased expression of β 1-6-

Abbreviations used: L-PHA, leucoagglutinin; D-PBS, Dulbecco's phosphate-buffered saline (8 mM- Na_2HPO_4 /1.5 mM- KH_2PO_4 /5.4 mM-KCl/137 mM-NaCl, pH 7.4); FCS, fetal calf serum; PMSF, phenylmethanesulphonyl fluoride; DMEM, Dulbecco's minimum essential medium; PBS, phosphate-buffered saline (2.5 mM- NaH_2PO_4 /7.3 mM- Na_2HPO_4 /144 mM-NaCl, pH 7.2); FITC, fluorescein isothiocyanate; TBS, Tris-buffered saline (50 mM-Tris/HCl, pH 8.0); TBT, 50 mM-Tris/HCl (pH 8.0)/0.1% BSA/0.25% Tween-20; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, NitroBlue tetrazolium; endo H, endoglycosidase H; LAMP-1, lysosomal-associated membrane protein-1; CEA, carcinoembryonic antigen; PM, plasma membrane fraction; MAb, monoclonal antibody.

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branched Asn-linked oligosaccharides, resulting in enhanced adhesion to collagenous basement membranes but decreased invasion into these membranes [24]. These results suggested that β 1-6-branched oligosaccharides may also play an important role in modulating the activities of specific proteolytic enzymes.

In order to identify glycoproteins expressing β 1-6-branched Asn-linked oligosaccharides which may contribute to progression of human colonic neoplasms and to elucidate their mechanisms of action, we have generated MABs to the moderately well-differentiated human colon carcinoma cell line HT29. MABs were selected based on their ability to recognize L-PHA-binding glycoproteins as well as to discriminate between colon tumours of different histopathological types. In the present study we report the initial characterization of a family of structurally related L-PHA-binding glycoproteins detected with MAB 1H9. These glycoproteins are expressed by HT29 cells and by two other human colon cancer cell lines established from more poorly differentiated, more progressive tumours. In addition, we show that the biosynthesis, processing and subcellular distribution of these glycoproteins may correlate with the differentiation status and/or metastatic potential of colon cancer cell lines.

EXPERIMENTAL

Cell lines

The human colon carcinoma cell lines HT29 (HTB 38), SW480 (CCL 228) and SW620 (CCL 227) were obtained from the American Type Culture Collection (A.T.C.C., Rockville, MD, U.S.A.) and were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum.

Preparation of HT29 cell plasma membranes

Confluent cultures of HT29 cells (10×150 mm dishes; approx. 5×10^8 cells) were washed three times with Dulbecco's phosphate-buffered saline (D-PBS), harvested with a rubber policeman and pelleted by centrifugation (500 *g*, 5 min). Plasma membranes were prepared as described [26]. Briefly, the pellet was homogenized in 4 vol. of 10 mM-Hepes, pH 7.6, 8.55% sucrose, 1 mM-EDTA, 0.5 mM-dithiothreitol, 2 mM phenylmethanesulphonyl fluoride (PMSF) and 10 μ g of aprotinin/ml using a Polytron homogenizer (Brinkman Instruments, Calgary, Alberta, Canada) and centrifuged at 300 *g* for 10 min, and the supernatant was recentrifuged at 20000 *g* for 60 min. The resultant microsomal pellet was resuspended in 10 mM-Hepes, pH 7.6, 8.55% sucrose and 1 mM-EDTA, layered on a discontinuous sucrose gradient of 8.55%, 21%, 40 and 54% sucrose, and centrifuged at 100000 *g* for 2 h. The fraction enriched in plasma membranes (PM) was collected from the interface between the 21% and 40% sucrose layers, pelleted at 23500 *g* for 60 min, and resuspended in phosphate-buffered saline (PBS). This preparation was used for immunizing mice and coating 96-well e.l.i.s.a. plates, as described below. For immunoprecipitation studies, the plasma membrane fraction was solubilized by adding Triton X-100 to a final concentration of 1%. Protein content was determined with the Bio-Rad protein assay kit using γ -globulin as standard, according to manufacturer's instructions.

Monoclonal antibodies

Balb/c mice were injected intraperitoneally with 100 μ g of HT29 PM emulsified with Freund's complete adjuvant. At 3, 6 and 9 weeks later, mice were boosted intraperitoneally with 50 μ g of HT29 PM. Serum was taken from each mouse and tested by e.l.i.s.a. with HT29 PM, as described below. Then, 1 month after the final boost, the mouse producing the serum with the highest titre against HT29 PM was given intraperitoneal injections of 25 μ g of HT29 PM on 3 successive days. On the fourth day,

spleen cells and F0 myeloma cells (A.T.C.C.) were fused in the presence of 40% poly(ethylene glycol) 1540 by standard protocols [27] and 2–3 weeks later supernatants were tested by standard e.l.i.s.a. protocols using 10 μ g of HT29 PM/well [27]. Positive hybridoma lines were subcloned by limiting dilution and stored frozen in liquid nitrogen. Hybridoma subclones were injected intraperitoneally into Balb/c mice for the production of ascitic fluid which was subsequently used for immunofluorescence and immunoprecipitation studies. Isotyping of MABs was carried out using an Isotyping Kit (Bio-Rad Laboratories) according to manufacturer's instructions. MAB 1H9 was found to be of the IgG₁ subclass.

Immunofluorescence

For immunofluorescence analysis of live cells, subconfluent cultures were washed three times with PBS, and cells were harvested by incubating plates for 2 min with PBS containing 5 mM-EDTA. Cells were washed three times with immunofluorescence buffer [IF buffer; PBS containing 3% fetal calf serum (FCS) and 5.5 mM-glucose] and counted. A total of 2×10^5 cells was pelleted in six 50 mm borosilicate glass tubes and incubated with the appropriate antibody diluted 1:200 with IF buffer. Following three washes, cells were incubated for 30 min with fluorescein isothiocyanate (FITC)-labelled goat anti-(mouse Ig), diluted 1:50 in IF buffer (Dimension Laboratories, Mississauga, Ontario, Canada). Cells were dropped on to glass slides and mounted in PBS/glycerol (1:1, v/v) and examined using a Nikon fluorescence microscope. For similar analysis of permeabilized cells, cells grown on glass coverslips were washed three times with D-PBS, and incubated at -20°C for 4 min with methanol and 2 min with acetone. Coverslips were air-dried, affixed to microscope slides and stored at -20°C until ready to use. At the start of the experiment, slides were washed for 5 min in PBS and incubated with the first and second antibodies, as described above. Slides were washed three times, mounted and examined using a Nikon fluorescence microscope.

Metabolic labelling of cells

Cell monolayers grown to 70–80% confluence were washed twice with D-PBS and radiolabelled for 24 h in methionine-free DMEM supplemented with one-tenth the normal content of methionine, 10% FCS and 50 μ Ci of [^{35}S]methionine/ml (800 Ci/mmol; NEN, Calgary, Alberta, Canada). In experiments where cells were radiolabelled in the presence of the glycosylation inhibitor tunicamycin, the drug was added to a final concentration of 5 μ g/ml, 24 h before addition of [^{35}S]methionine, and was maintained in the culture medium during the entire radiolabelling period. For pulse-chase labelling experiments, cell monolayers grown in 60 mm dishes were washed in D-PBS and incubated for 1 h in methionine-free medium prior to the addition of [^{35}S]methionine (0.5 ml; 500 μ Ci/ml). Cells were pulse-labelled for 10 min and chased for 0.5, 1.5, 5 and 24 h in DMEM containing 10% FCS and $50\times$ the normal concentration of unlabelled methionine. At the indicated times, the medium was removed, centrifuged to remove cells and saved for analysis of secreted antigens. The plates were then washed three times with ice-cold PBS, and the cells were 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 μ M-leupeptin, 0.7 μ M-pepstatin, 10 μ g of aprotinin/ml and 1% Triton X-100, and cleared by centrifugation at 22500 *g* for 30 min.

Immunoprecipitation

[^{35}S]Methionine-labelled cell lysates [(0.5–2) $\times 10^6$ c.p.m.], [^{35}S]methionine-labelled culture media (0.5 ml) or unlabelled cell

lysates (1 mg) were incubated overnight at 4 °C with 3 μ l of ascitic fluid. Then, 100 μ l of a 1:1 suspension of Protein A-Sepharose CL-4B beads (Pharmacia, Montreal, Canada) was then added, and the suspension was gently rocked 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 and 0.02 % NaN₃), 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 and 10 % glycerol). Samples to be digested with glycosidases were immunoprecipitated with Protein A-Sepharose and washed as described above. Following the last wash, beads were resuspended in glycosidase buffer, as specified by the manufacturer, boiled for 1–2 min, cooled and incubated overnight at 37 °C with enzyme. Prior to gel electrophoresis, samples were adjusted to 1 $\times$ SDS sample buffer and boiled for 5 min. The glycosidases used in individual samples were as follows: (a) endoglycosidase H (endo H), 1 unit (Boehringer-Mannheim, Montreal, Canada), (b) neuraminidase Type X from *Clostridium perfringens*, 0.1 unit (Sigma), (c) *N*-glycanase, 0.3 unit (Genzyme), (d) *O*-glycanase, 0.3 unit (Genzyme) and (e) endo- β -galactosidase, 0.01 unit (ICN Biomedicals Ltd., Montreal, Canada).

SDS/polyacrylamide-gel electrophoresis

SDS/PAGE was performed as described by Laemmli [28], using 7.5 % acrylamide. Gels of ³⁵S-labelled samples were visualized by fluorography [29], using Enlightening (NEN).

Western blotting

Proteins separated by SDS/PAGE were transferred electrophoretically on to nitrocellulose (Schleicher and Schull) for 1 h at 100 V. Nitrocellulose blots were blocked for 1 h at room temperature or overnight at 4 °C in Tris-buffered saline (TBS)/4 % BSA. Following three 5 min washes in TBT buffer, blots were incubated for 2 h with the lectin L-PHA (0.2 μ g/ml), washed three times with TBT and incubated for 1 h with rabbit anti-L-PHA antibody. Following three washes in TBT, lectin blots were incubated for 1 h with alkaline phosphatase-coupled affinity-purified goat anti-(rabbit Ig) (Bio-Rad Laboratories). 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) and Nitro Blue Tetrazolium (NBT) (Bio-Rad) were the substrates for colour development.

Peptide mapping

Limited proteolytic peptide mapping was carried out by a protocol originally developed by Cleveland *et al.* [30] and modified as previously described [31].

RESULTS

Characterization of the antigen(s) detected in HT29 cells by Mab 1H9

Preliminary studies in our laboratory indicated that the profile of L-PHA-binding glycoproteins in human and rodent colon tumours was more complex than previously detected in the metastatic tumour cell line MDAY-D2 [16,25]. Species which migrated on SDS/polyacrylamide gels with apparent mobilities ranging from 60 kDa to 200 kDa were detected. In order to characterize glycoproteins expressing β 1-6-branched Asn-linked oligosaccharides, which may contribute to progression of colon cancer, we generated MAbs against the moderately well-differentiated colon cancer cell line HT29 and screened for antibodies which detected L-PHA-binding glycoproteins. The

approach used consisted of immunoprecipitating HT29 cell lysates with MAbs followed by Western blotting and detection of glycoproteins bearing β 1-6-branched oligosaccharides with L-PHA and anti-L-PHA antiserum (see the Experimental section). As shown in Fig. 1, Mab 1H9 immunoprecipitated three L-PHA-binding glycoproteins which migrated on SDS/PAGE with apparent mobilities of 100, 70, and 25 kDa, suggesting that each glycoprotein contained β 1-6-branched Asn-linked oligosaccharides [22]. When [³⁵S]methionine-labelled HT29 cell lysates were immunoprecipitated with the same antibody, an additional weakly labelled endo H-sensitive species of 74 kDa was apparent, suggesting that this glycoprotein differed from the other species in its carbohydrate structure (Fig. 2, lanes b and c).

In order to characterize the carbohydrate structure of these glycoproteins in more detail, immunoprecipitates of [³⁵S]methionine-labelled HT29 cells were treated with glycosidases of different specificities. Treatment of immunoprecipitates with *N*-glycanase altered the mobility of each of the species recognized by Mab 1H9, resulting in bands which migrated on an SDS/polyacrylamide gel with apparent mobilities of 62, 60, 58, 46 and 43 kDa after enzyme digestion (Fig. 2, lane e). Longer exposure of the autoradiogram revealed an additional species of 20 kDa. This indicated that each glycoprotein species detected with Mab 1H9 contained Asn-linked oligosaccharides.

The 100, 70 and 25 kDa species were also sensitive to neuraminidase, yielding species of 87, 61 and 22 kDa respectively after digestion with the enzyme (Fig. 2, lane d). This indicated that these species were sialoglycoproteins. In contrast, the 74 kDa species, which had no detectable terminal sialic acid residues, was found to retain Asn-linked high-mannose-type oligosaccharides, since digestion with endo H produced two species, of 60 and 58 kDa (Fig. 2, lane c). The presence of two bands after endo H treatment may result from incomplete digestion or from heterogeneity in the oligosaccharide structure of the polypeptide chain.

Treatment of immunoprecipitates of [³⁵S]methionine-labelled HT29 cells with *O*-glycanase alone had no effect, indicating that these glycoproteins lack *O*-linked oligosaccharides with the sequence Gal β 1-3GalNAc (results not shown). In addition, digestion with *O*-glycanase after neuraminidase treatment did not further alter the mobilities of the digestion products produced by neuraminidase alone, indicating that resistance to *O*-glycanase

Fig. 1. Detection of L-PHA-binding glycoproteins in HT29 cells with Mab 1H9

Lysates from HT29 cells containing 1 mg of protein were immunoprecipitated with Mab 1H9 (lanes a) or normal mouse serum (lanes b) and analysed by Western blotting with (a) L-PHA and anti-L-PHA antiserum or (b) L-PHA and normal rabbit serum. Bands were visualized with alkaline phosphatase-coupled goat anti-(rabbit Ig), as described in the Experimental section.

Fig. 2. Analysis of glycoproteins defined by MAb 1H9 in [35 S]methionine-labelled HT29 cells

[35 S]Methionine-labelled HT29 cell lysates were immunoprecipitated with normal mouse serum (lane a) or MAb 1H9 (lanes b–e). Samples immobilized to Protein A–Sepharose were left untreated (lanes a and b) or digested with endo H (lane c), neuraminidase type X from *Clostridium perfringens* (lane d) or *N*-glycanase (lane e). Samples were analysed by SDS/PAGE (7.5% gels). Bands were visualized after fluorography and autoradiography.

Fig. 3. Effect of SDS and 2-mercaptoethanol on the conformation of antigenic determinants detected by MAb 1H9

[35 S]Methionine-labelled HT29 cell lysates were immunoprecipitated with normal mouse serum (lane a) or MAb 1H9 (lanes b–e). In lane c, the immunoprecipitation was carried out in the presence of 1% 2-mercaptoethanol. In lane d, samples were adjusted to 1% SDS, heated to 100 °C for 5 min and diluted to 0.1% SDS prior to the addition of MAb 1H9. In lane e, HT29 cell lysates were immunoprecipitated with MAb 1H9 and analysed by SDS/PAGE (7.5% gels) under non-reducing conditions.

digestion was not simply due to sialylation of *O*-linked oligosaccharides (results not shown). However, it is possible that these glycoproteins contain more complex, substituted, *O*-linked oligosaccharide structures not sensitive to cleavage by *O*-glycanase.

The epitope detected in HT29 cells by MAb 1H9 appeared to be on each of the four glycoprotein species (ie. 100, 75, 70 and 25 kDa), since pre-treatment of cell extracts with 1% 2-mercaptoethanol, to disrupt possible disulphide-linked complexes, followed by immunoprecipitation yielded the same species as in the

Fig. 4. Effect of tunicamycin on the epitope recognized by MAb 1H9

HT29 cells were radiolabelled with [35 S]methionine in the absence (a, lanes a, b and c) or presence (a, lanes d and e; b, lanes a–e) of tunicamycin (5 μ g/ml). Cell lysates were immunoprecipitated with normal mouse serum (lanes a) or MAb 1H9 (lanes b–e). Samples immobilized to Protein A–Sepharose were left untreated (a, lanes a, b and d; b, lanes a and b) or were digested with endo H (a, lanes c and e), *O*-glycanase (b, lane c), neuraminidase (b, lane d) or a combination of neuraminidase and *O*-glycanase (b, lane e). Samples were analysed by SDS/PAGE (7.5% gels) and bands were visualized by fluorography and autoradiography.

control samples (Fig. 3, lane c). The 43 kDa species immunoprecipitated under these conditions (Fig. 3, lane c) was considered to be non-specific, because it was only detectable when 2-mercaptoethanol was added to samples prior to immunoprecipitation (results not shown). The 100, 75 and 70 kDa glycoproteins appeared to contain intramolecular disulphide bonds, since elution of immunoprecipitates with sample buffer devoid of 2-mercaptoethanol resulted in increased mobility of these glycoproteins on SDS/PAGE (Fig. 3, lane e). This may be due to a more compact conformation resulting from intramolecular interactions [32].

MAb 1H9 detected a conformational determinant, since no glycoprotein could be immunoprecipitated when the cell lysate was boiled in the presence of 1% SDS and diluted to 0.1% SDS prior to immunoprecipitation (Fig. 3, lane d). Furthermore, the antibody failed to react in Western blots (results not shown). In order to determine whether the epitope recognized by MAb 1H9 was carbohydrate or protein in nature, HT29 cells were radiolabelled with [35 S]methionine in the presence of the glycosylation inhibitor tunicamycin. As shown in Fig. 4, MAb 1H9 immunoprecipitated a species of 55 kDa which was resistant to further digestion with endo H, neuraminidase, *O*-glycanase or a combination of neuraminidase and *O*-glycanase. These results indicated that the determinant recognized by MAb 1H9 is not dependent on the presence of Asn-linked oligosaccharides, and suggested that the epitope is present on the polypeptide backbone. However, we cannot exclude the possibility that complex *O*-linked oligosaccharides, not susceptible to *O*-glycanase digestion, may be part of the epitope recognized by MAb 1H9.

Immunofluorescence staining of HT29 cells using MAb 1H9

To evaluate the intracellular distribution of the glycoproteins detected by MAb 1H9, immunofluorescence studies on intact and permeabilized HT29 cells were carried out. Experiments performed with intact cells failed to show any staining with the antibody (Fig. 5d). However, when control experiments were carried out with another MAb which recognizes cell-surface

Fig. 5. Immunofluorescence staining of HT29 cells using MAb 1H9

This Figure shows the immunofluorescence staining patterns of methanol/acetone-fixed (*a* and *b*) and unfixed (*c* and *d*) HT29 cells following incubation with normal mouse serum (*a* and *c*) or MAb 1H9 (*c* and *d*) followed by FITC-labelled goat anti-(mouse Ig).

carbohydrate antigens (S. Laferté, unpublished work), bright membrane fluorescence was detected (results not shown), indicating that the epitope recognized by MAb 1H9 is not exposed on the cell surface. On the other hand, when similar studies with MAb 1H9 were carried out with HT29 cells permeabilized with methanol/acetone, intense cytoplasmic fluorescence was observed. HT29 cells were homogeneously stained with the antibody, producing diffuse as well as punctate cytoplasmic staining (Fig. 5*b*). In addition, there was staining of cellular processes extending away from the perinuclear area. Thus our results indicated that the antigens recognized by MAb 1H9 are located primarily in the cytoplasm.

Biosynthesis and processing of the HT29 antigens detected by MAb 1H9

Pulse-chase experiments with HT29 cells were carried out to investigate the precursor-product relationship of the four glyco-

Table 1. Properties of pulse-chase products immunoprecipitated from [³⁵S]methionine-labelled HT29 cells by MAb 1H9

Letters in parentheses refer to the lanes shown in Fig. 6. The differences in size of the various pulse-chase products can be more clearly seen in Fig. 7(*a*).

Sample time (h)	Size of products (kDa)	Endo H sensitivity	Neuraminidase sensitivity
0 (pulse)	76 (a)	+ (b)	– (d)
0.5	76 (e)	+ (f)	– (h)
	56 (e)	+ (f)	– (h)
1.5	100 (i)	– (j)	+ (l)
	75 (i)	+ (j)	– (l)
	56 (i)	+ (j)	– (l)
5	100 (m)	– (n)	+ (p)
	74 (m)	+ (n)	– (p)
	70 (m)	– (n)	+ (p)
24	100 (q)	– (r)	+ (t)
	70 (q)	– (r)	+ (t)

proteins detected by MAb 1H9. As shown in Fig. 6 and summarized in Table 1, the pulse-labelled species consisted of an endo H-sensitive, neuraminidase-resistant precursor of 76 kDa, consistent with the cotranslational addition of high-mannose-type Asn-linked oligosaccharide chains (Fig. 6, lanes a–d) [33]. Digestion of the 76 kDa species with endo H (lane b) or *N*-glycanase (lane c) yielded a species of 60 kDa and a second, fainter species of 58 kDa. Immunoprecipitates of the 30 min chase sample revealed the persistence of the 76 kDa species, which produced products of 62, 60 and 58 kDa following endo H treatment, as well as the appearance of an additional band of 56 kDa which was also sensitive to endo H (Fig. 6, lanes e and f; more detectable in lanes i and j). After a 1.5 h chase period, the 76 kDa band was further processed to a species of 75 kDa (Fig. 6, lane i; more clearly depicted in Fig. 7*a*, lane c), consistent with trimming of the precursor high-mannose-type oligosaccharide [33]. In addition, a third band of 100 kDa appeared (Fig. 6, lane i) which was resistant to endo H (lane j) but susceptible to neuraminidase, giving rise to a band of 87 kDa upon digestion with the latter (lane l). This indicated that the 100 kDa glycoprotein contained complex-type Asn-linked oligosaccharides.

Fig. 6. Pulse-chase analysis of [³⁵S]methionine-labelled HT29 cells

HT29 cells were pulse-labelled with [³⁵S]methionine (500 μ Ci/ml) for 10 min and chased for 0.5, 1.5, 5 and 24 h with complete medium containing 50 times the normal concentration of unlabelled methionine. At the indicated times, cell lysates were prepared and immunoprecipitated with MAb 1H9. Immunoprecipitated samples were either left untreated (lanes a, e, i, m and q) or digested with endo H (lanes b, f, j, n and r), *N*-glycanase (lanes c, g, k, o and s) or neuraminidase (lanes d, h, l, p and t). Samples were analysed by SDS/PAGE (7.5% gels) and bands were visualized following fluorography and autoradiography.

Fig. 7. Limited proteolytic peptide mapping of glycoproteins detected in HT29 cells and culture medium with MAb 1H9

HT29 cells were pulse-labelled for 10 min with [35 S]methionine and chased for 0.5, 1.5, 5 and 24 h. At the indicated times, cells were harvested and solubilized, and the lysates were immunoprecipitated with MAb 1H9. Samples were separated by SDS/PAGE (7.5% gels), as shown in (a). Seven glycoprotein species were excised from the dried, unfixed gel (labelled a–g) and subjected to limited proteinase V8 proteolytic peptide mapping, as described in the Experimental section (b). These glycoproteins consisted of the pulse-labelled 76 kDa glycoprotein (a), the 76 kDa species detected in the 30 min chase sample (b), the 75 kDa species (c) and the 56 kDa species (d) detected in the 1.5 h chase sample, the 100 kDa (e) and the 70 kDa (f) species detected in the 5 h chase sample, and the 25 kDa species (g) detected in the 24 h chase sample. Open triangles (∇) represent peptides shared by the 76/75 kDa and 56 kDa glycoproteins; closed triangles ($\blacktriangledown$) represent peptides unique to the 56 kDa glycoprotein. (c) Analysis of glycoproteins detected by MAb 1H9 in the culture medium of HT29 cells. HT29 cells were pulse-labelled for 10 min with [35 S]methionine and chased for 1.5, 3, 5 and 24 h. At each of the indicated times, culture medium was collected, immunoprecipitated with MAb 1H9 and analysed by SDS/PAGE (7.5% gels). Bands were visualized by fluorography and autoradiography.

The 75 and 56 kDa bands detected remained endo H-sensitive (Fig. 6, lane j), the latter giving rise to two species of 45 and 43 kDa upon digestion. The increase in intensity of the 100 kDa species in the 5 h chase sample (lane m), concomitant with a comparable decrease in intensity of the 75 kDa species, suggested that it was the 75 kDa species which was processed to the 100 kDa glycoprotein. Furthermore, the disappearance of the 56 kDa species after the 5 h chase period and the appearance of a 70 kDa species (lane m) which was resistant to endo H (lane n) but sensitive to neuraminidase (lane p), giving rise to a species of 62 kDa, suggested that these two glycoproteins may be related by carbohydrate processing. A residual endo H-sensitive species of 74 kDa remained in the 5 h chase sample (Fig. 6, lanes m and n; more clearly depicted in Fig. 7a, band f), which probably corresponds to the endo H-sensitive species detected during long-term labelling of HT29 cells (see Fig. 2, lanes b and c). By 24 h post-chase, three neuraminidase-sensitive species with apparent

molecular masses of 100, 70 and 25 kDa were detected (Fig. 6, lanes q and t). The intensity of the pulse-labelled 100 kDa species had substantially decreased by 24 h, while that of the 70 kDa species had increased significantly. This suggested either that the 100 kDa glycoprotein was unstable within the cell or that it was secreted into the culture medium. The latter possibility is dealt with in a later section. Treatment of the 24 h chase sample with *N*-glycanase produced five bands, of 62, 58, 46, 43 and 20 kDa (Fig. 6, lane s).

Structural relationship between the pulse-labelled glycoproteins detected by MAb 1H9

In order to examine the structural relatedness of the glycoproteins detected by MAb 1H9, we carried out peptide mapping analyses on most of the species detected during the pulse-chase experiment described in Fig. 6. Each of the species analysed is identified in Fig. 7(a). As shown in the peptide maps in Fig. 7(b), the 76 kDa species detected in the pulse sample (lane a) and 30 min chase sample (lane b), as well as the 75 kDa species detected 1.5 h post-chase (lane c), produced identical peptide maps, confirming the precursor-product relationship of these glycoprotein species. The 56 kDa species detected in the 1.5 h chase sample (Fig. 7b, lane d) was found to be structurally related to the 76/75 kDa species (lanes a–c), as indicated by the similarity in the four smallest peptides (∇). However, the 56 kDa species also contained unique peptides not found in the 76/75 kDa species ($\blacktriangledown$). This result made it unlikely that the 56 kDa species is derived from the 76/75 kDa species by proteolytic cleavage. Similarly, comparison of the 100 kDa species and the 70 kDa species from the 5 h chase sample (Fig. 7b, lanes e and f respectively) revealed that striking similarity in the smallest peptides was conserved; however, most of the remaining peptides were distinct. These results, together with the *N*-terminal sequence analysis of the 100 and 70 kDa glycoproteins which revealed sequence identity of the first 25 amino acid residues examined (S. Laferté, unpublished work), indicated that the 100 kDa and 70 kDa glycoproteins detected by MAb 1H9 are structurally related, but not identical. This is also consistent with our previous predictions that the 70 kDa glycoprotein may be derived from the 56 kDa glycoprotein by carbohydrate processing, while the 76/75 kDa glycoproteins may be precursors of the 100 kDa glycoprotein. The 25 kDa species detected in the 24 h chase sample (Fig. 7b, lane g) produced one major peptide and a second, fainter peptide, a pattern distinct from those of the 100 kDa and 70 kDa species (lanes e and f respectively). Because of the limited number of proteolytic fragments generated from the 25 kDa glycoprotein, it is not yet possible to establish conclusively its structural relationship to the 100 and 70 kDa glycoproteins.

Analysis of antigen(s) detected in the culture medium of HT29 cells by MAb 1H9

In order to examine whether the antigens detected by MAb 1H9 are secreted by HT29 cells, the culture medium of [35 S]methionine-labelled cells was collected at the end of the 10 min pulse-labelling period and at each time a chase sample was harvested. The results of the immunoprecipitation studies, which are shown in Fig. 7(c), demonstrated that the 100 kDa species is detectable in the medium by 5 h of chase and persists at 24 h. A second species of 75 kDa was sometimes observed in the culture medium, which appeared to be distinct from the cell-associated endo H-sensitive 76/75 kDa precursor. This conclusion is based on the fact that the secreted 75 kDa glycoprotein is neuraminidase-sensitive and binds L-PHA (results not shown). In light of the pulse-chase studies shown in Fig. 7(a), which depict decreased levels of the 100 kDa species in HT29 cells by

Fig. 8. Expression of glycoproteins detected by MAb 1H9 in HT29, SW480 and SW620 cells

Lysates of [35 S]methionine-labelled HT29 (lanes a and b), SW480 (lanes c and d) and SW620 (lanes e and f) cells were immunoprecipitated with normal mouse serum (lanes a, c and e) or MAb 1H9 (lanes b, d and f) and analysed by SDS/PAGE. Bands were visualized following fluorography and autoradiography.

24 h of chase but persistence of the 70 kDa species, the results shown in Fig. 7(c) suggest that there is preferential secretion of the 100 kDa species into the culture medium. In this regard, excision of the secreted 100 kDa glycoprotein band from SDS/acrylamide gels followed by elution and digestion with *N*-glycanase produced two products of 62 and 58 kDa (compare with Fig. 6, lane s), supporting the structural identity of the cell-associated and secreted 100 kDa glycoproteins (results not shown).

Expression of glycoproteins detected by MAb 1H9 in other human colon cancer cell lines

HT29 is a moderately well differentiated human colon cancer cell line which was established from a grade II tumour [34]. When injected into nude mice, it produces well-differentiated tumours consistent with grade I tumours. In order to determine whether MAb 1H9 detects glycoproteins unique to HT29 and whether there is any difference in glycoprotein expression in cell lines established from more progressive tumours, we compared the glycoproteins detected in HT29 cells with those expressed by two other human colon carcinoma cell lines, namely SW480 and SW620. In contrast to HT29, SW480 is a poorly differentiated cell line which was established from a grade III/IV primary tumour [34,35]. A lymph node metastasis arising from this tumour was isolated and used to generate the SW620 cell line. The availability of the paired cell lines SW480 and SW620 therefore provided an opportunity to examine a primary colonic tumour and lymph node metastasis from the same patient.

Immunoprecipitation studies carried out with [35 S]methionine-labelled SW480 and SW620 cells demonstrated that the glycoproteins detected by MAb 1H9 were present in lower amounts compared with in HT29 cells, and migrated on SDS/polyacrylamide gels as a diffuse band with apparent molecular mass in the 66–75 kDa range (Fig. 8, lanes d and f respectively).

In order to investigate the reasons for the decreased expression of antigens detected by MAb 1H9 in SW480 and SW620 cells, we carried out pulse-chase labelling studies on [35 S]methionine-labelled cells. As shown in Figs. 9(a) and 9(b), immunoprecipitation studies combined with glycosidase digestions revealed that during the pulse and early chase periods (i.e. 0.5 and 1.5 h) (lanes a–m) there were few differences in the

Fig. 9. Pulse-chase analysis of [35 S]methionine-labelled SW480 and SW620 cells

SW480 (a) and SW620 (b) cells were pulse-labelled for 10 min with [35 S]methionine (lanes a–d) and chased for 0.5 h (lanes e–i), 1.5 h (lanes j–m), 5 h (lanes n–r) and 24 h (lanes s–y). Cell lysates were immunoprecipitated with normal mouse serum (lanes a, e, j, n and s) or MAb 1H9 (all other lanes). Samples immunoprecipitated with MAb 1H9 were either left untreated (lanes b, f, k, o and t) or digested with endo H (lanes c, g, l, p and u), neuraminidase (lanes d, h, m, q and v), *N*-glycanase (lanes i, r and w), endo- β -galactosidase (lane x) or neuraminidase plus endo- β -galactosidase (lane y).

synthesis and processing of the 76/75 kDa glycoproteins in SW480 and SW620 cells as compared with HT29 cells. However, during the 5 h chase period, some of the 76/75 kDa precursor was processed to an endo H-resistant neuraminidase-sensitive species of 92 kDa (Fig. 9, lane o), in contrast to the 100 kDa species detected in HT29 cells (Fig. 6, lane m). Since treatment of the 92 kDa species with *N*-glycanase appeared to give products having the same size as those of the *N*-glycanase-treated 100 kDa species of HT29 cells (lane r), this difference in size appeared to be due to differences in carbohydrate content. Furthermore, the amount of cell-associated 92 kDa species appeared to be considerably less than that of the 100 kDa species of HT29 cells. This result suggested that there was more rapid turnover of the 76/75 kDa precursor or more rapid processing and secretion of the 92 kDa species into the culture medium. This would result in little or no 92 kDa species in SW480 and SW620 cells by the 24 h chase period. In addition, a 66 kDa species detected during the 5 h chase period (Fig. 9, lane o) was converted by 24 h to a very diffuse glycoprotein species which migrated in the 50–70 kDa range (lane t). The altered mobility of this glycoprotein, which was more apparent in SW480 cells, did not appear to result from

Fig. 10. SDS/PAGE analysis of glycoproteins detected in the culture medium of pulse-labelled SW480 and SW620 cells

SW480 (a) and SW620 (b) cells were pulse-labelled for 10 min with [35 S]methionine and chased for 1.5, 3, 5 and 24 h. At the indicated times, culture medium was collected, immunoprecipitated with MAb 1H9 and analysed by SDS/PAGE. Bands were visualized following fluorography and autoradiography. P, pulse.

proteolytic cleavage, since treatment of this species with *N*-glycanase produced the same two major species of 46 and 43 kDa (lane w) as were detected in the *N*-glycanase-treated 5 h chase sample (lane r). Treatment of immunoprecipitates with neuraminidase alone resulted in a reproducible decrease in the size and diffuseness of the 50–70 kDa glycoprotein (Fig. 9, lane v). Further digestion of glycoproteins immunoprecipitated from SW480 cell lysates with endo- β -galactosidase (lane y), which cleaves unsubstituted polylactosamine chains, produced a smaller and less heterogeneous glycoprotein species. These results indicated that the diffuse mobility of the glycoprotein was due in part to sialic acid and polylactosamine structures, and suggested that there may be additional substitutions of the oligosaccharide chains, such as branched polylactosamine chains (which are resistant to endo- β -galactosidase treatment [36]) or *O*-linked oligosaccharides, on these glycoproteins which may not be present in HT29 cells.

In order to determine if the decreased expression of the 92 kDa species in these cells was a result of increased processing and secretion of this glycoprotein species into the culture medium, we carried out immunoprecipitation studies with cell lysates and the culture medium of SW480 and SW620 cells labelled with [35 S]methionine for 10 min and chased with unlabelled methionine for 1.5, 3, 5 and 24 h. Fig. 10 demonstrates that the decreased amount of the 92 kDa species in SW480 and SW620 cells was accompanied by a concomitant increase in the amount of 92 kDa species released into the medium, which appeared as early as 3 h. A second species of 66 kDa was sometimes detected in the culture medium of SW480 and SW620 cells, but its relationship to the 92 kDa glycoprotein is not known at present.

DISCUSSION

In this paper we report the initial characterization of a family of L-PHA-binding glycoproteins which are differentially expressed and processed by three human colon cancer cell lines, namely HT29, SW480 and SW620. MAb 1H9 detected in HT29 cells three L-PHA-binding glycoproteins of 100, 70 and 25 kDa. Pulse-chase experiments, peptide mapping analyses and *N*-

terminal amino acid sequencing (S. Laferté, unpublished work) revealed that the 100 kDa species is structurally related to, but distinct from, the 70 kDa species. In addition, the 100 kDa glycoprotein is secreted into the culture medium, whereas the 70 kDa glycoprotein is retained intracellularly. Together, these data suggested that the structural differences detected between these glycoproteins may account for their different subcellular localization, and may possibly affect their function. On the basis of the biochemical data demonstrating secretion of the 100 kDa glycoprotein into the culture medium, one may speculate that the punctate cytoplasmic immunofluorescence pattern detected in HT29 cells with MAb 1H9 represents secretory vesicles containing the 100 kDa glycoprotein.

In comparison to HT29 cells, SW480 and SW620 cells showed significant qualitative and quantitative differences in the expression of glycoproteins detected by MAb 1H9. These differences included: (i) increased mobility of 1H9-reactive glycoproteins in SDS/polyacrylamide gels, and (ii) an apparent increase in the level of 92 kDa glycoprotein in the culture medium (SW620 contained the highest level). The altered mobility of secreted and intracellular glycoproteins detected by MAb 1H9 appeared to be due to glycosylation differences, as treatment of immunoprecipitates with *N*-glycanase produced the same peptides as in HT29 cells (Fig. 8, and results not shown). Furthermore, the apparent differences in glycosylation of the cell-associated 66 kDa glycoprotein resulted in part from increased synthesis of polylactosaminoglycans. Because polylactosaminoglycans have been implicated in differentiation [37,38], cell adhesion [23,39,40] and metastasis [17,41], it will be important to determine what role, if any, the 1H9-reactive glycoproteins play in these processes.

In addition to glycosylation changes, MAb 1H9 detected significant differences in the amount of the 100/92 kDa glycoprotein in the culture medium of the cell lines under study. SW480 and SW620 secreted significantly higher levels of the 92 kDa glycoprotein into the culture medium, suggesting a possible correlation between the secretion of this glycoprotein and the degree of tumour progression, with invasive and metastatic tumours secreting increasing amounts of the glycoprotein. In order to assess the validity of this hypothesis, it is necessary to examine human serum samples from normal subjects and colon cancer patients. Preliminary results have indicated (S. Laferté, unpublished work) that the 100/92 kDa glycoprotein is present in normal human serum but that the amount is increased several-fold in the serum of certain colon cancer patients.

There exist several glycoproteins expressing β 1-6-branched Asn-linked oligosaccharides which have been implicated in tumour progression. Two of these glycoproteins consist of P2A and LAMP-1, which mediate carbohydrate-dependent adhesion to extracellular matrix proteins [23]. Another member of this group is carcinoembryonic antigen (CEA), an oncodevelopmentally expressed glycoprotein of 180 kDa which is secreted by colon tumours and is detectable in the serum of most colon cancer patients [42,43]. CEA isolated from a number of colon tumours and liver metastases has been shown to express β 1-6-branched Asn-linked oligosaccharides [44]. More recently, CEA has been shown to play a role in cell–cell adhesion, which may be important during organ colonization [45]. A large number of CEA-related glycoproteins ranging in size from 50 to 180 kDa have also been identified in normal and neoplastic tissues, but their functions have not yet been elucidated [43]. The 100 and 70 kDa glycoproteins detected by MAb 1H9 may constitute a distinct group of L-PHA-binding glycoproteins associated with tumour progression. These glycoproteins appear to differ from CEA and the previously characterized P2A and LAMP-1 [16,23,25]. This conclusion is based on a comparison of their size,

apparent polypeptide size after *N*-glycanase treatment, content of Asn-linked oligosaccharides and *N*-terminal amino acid sequence (S. Laferté, unpublished work). Furthermore, comparative analysis of the *N*-terminal sequence and two internal peptide sequences of the 70 kDa glycoprotein with the protein sequence data bank failed to reveal any homology with known proteins (S. Laferté, unpublished work), suggesting that MAb 1H9 defines a new family of L-PHA-binding glycoproteins. Although the function of these glycoproteins is not yet known, it is conceivable that, like other secreted glycoproteins, they may play a role in metastasis by influencing tumour cell proliferation [46], mediating cell-cell interactions [7] and/or degrading the extracellular matrix [48]. It is of interest that Yamagata *et al.* [49] have identified two gelatinases of 95 and 60 kDa that are secreted into the culture medium of various tumour cell lines, and have shown that the level of the 95 kDa gelatinase in the medium correlates with metastatic potential [49]. Isolation of cDNA clones encoding glycoproteins detected by MAb 1H9 should contribute to our understanding of the function of these glycoproteins and the contribution of their carbohydrate structure to that function.

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