Purification of two glycoproteins expressing β 1-6 branched Asn-linked oligosaccharides from metastatic tumour cells

Suzanne LAFERTE* and James W. DENNIS†‡

*Department of Biochemistry, A3 Health Sciences Building, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0, Canada, and †Division of Cancer and Cell Biology, Mount Sinai Hospital Research Institute, 600 University Avenue, Toronto, Ontario M5G 1X5, Canada/Department of Medical Genetics, University of Toronto, Toronto, Ontario, Canada

Increased branching at the trimannosyl core of 'complex-type' Asn-linked oligosaccharides has been observed in both human and murine tumour cells, and appears to be associated with enhanced metastatic potential in several murine tumour models [Dennis, Laferte, Waghorne, Breitman & Kerbel (1987), *Science* **236**, 582–585]. The lectin leucoagglutinin (L-PHA) requires the-GlcNAc β 1-6Man α 1-6Man-linked lactosamine antenna in complex-type oligosaccharides for high-affinity binding and can be used to detect these structures in glycoproteins separated on SDS/polyacrylamide-gel electrophoresis. The major L-PHA-binding glycoproteins in the highly metastatic lymphoid tumour cell line called MDAY-D2 were purified and resolved into two major species, termed P2A (110 kDa) and P2B (130 kDa). P2A had L-PHA-reactive Asn-linked oligosaccharides with polylactosamine sequences as well as a large component of sialylated *O*-linked carbohydrates. The glycoprotein showed structural characteristics similar to those of leukosialin (i.e. CD43), a glycoprotein previously identified on the surface of leukocytes. Based on monosaccharide compositional analysis and glycosidase digestions, P2B was found to be 50–60 % Asn-linked oligosaccharide containing polylactosamine sequences and sialic acid. The *N*-terminal peptide sequence of P2B was determined to be very similar to that of murine lysosomal membrane glycoprotein (LAMP-1), a ubiquitous glycoprotein found largely in the lysosomal membranes but also in the plasma membrane of several murine and human tumour cell lines.

INTRODUCTION

Neoplastic transformation and tumour progression are often accompanied by structural alterations in the carbohydrate portion of glycoproteins and glycolipids [1–3]. Some of the commonly observed changes include expression of embryonic carbohydrate antigens [1–4], aberrant expression of blood group determinants [5] and incomplete processing of Asn-linked oligosaccharides [6], as well as increased sialylation [7], increased polylactosamine content [8] and increased branching of the trimannosyl core of 'complex-type' *N*-linked structures [9–11]. We have recently shown that increased-GlcNAc β 1-6Man α 1-6Man/ β 1-branching of complex-type oligosaccharides is required for expression of the metastatic phenotype in two experimental models [12]. First, loss of β 1-6 branched oligosaccharides due to decreased GlcNAc-transferase V activity in leucoagglutinin (L-PHA)-resistant mutants (i.e. class 3) of the highly metastatic tumour line MDAY-D2 was associated with a dramatic decrease in metastatic potential. Secondly, a strong correlation between the acquisition of β 1-6 branched oligosaccharides and enhanced metastatic potential was observed in a series of oncogene-transfected SP1 murine mammary carcinoma cell lines [12]. Finally, the plant alkaloid swainsonine, which inhibits α -mannosidase II, a processing step prior to the addition of the β 1-6 antenna, also inhibits tumour cell metastasis [13,14].

In the metastatic tumour cell line MDAY-D2, the majority of the β 1-6 branched oligosaccharides appeared to be associated with only a few glycoproteins in the 80–160 kDa size range as indicated by ¹²⁵I-labelled L-PHA staining of membrane glycoproteins separated by SDS/polyacrylamide-gel electrophoresis (SDS/PAGE) [15]. The majority of complex-type oligosaccharides isolated from total cellular glycoprotein of MDAY-D2 cells were determined to be tetra-antennary structures containing polylactosamine sequences with both Gal α 1-3- and NeuNAc α 2-3-linked terminal sugars [16]. Similarly, transformation and progression-related increase in β 1-6 branching (i.e. L-PHA binding) observed in rat 1 fibroblasts and SP1, a murine mammary carcinoma cell line, appeared to be associated mainly with glycoprotein(s) of a similar size [12].

As a first step towards understanding how increased β 1-6 branching of the trimannosyl core may enhance the malignant potential of tumour cells, the major L-PHA-binding glycoproteins were isolated from the highly metastatic tumour line MDAY-D2. Structural characterization of the glycoproteins termed P2A and P2B suggested that they are related to the previously described leukosialin isolated from rat and human lymphocytes [17–19] and lysosomal-associated membrane glycoprotein 1 (LAMP-1) from murine cells respectively [20]. Recent studies on the binding of purified P2A and P2B to extracellular matrix (ECM) proteins suggest that

Abbreviations used: L-PHA, leucoagglutinin; LAMP-1, lysosomal-associated membrane glycoprotein 1; PAGE, polyacrylamide-gel electrophoresis; PNA, peanut agglutinin; WGA, wheatgerm agglutinin; ECM, extracellular matrix; PBS, phosphate-buffered saline (10 mM-sodium phosphate, pH 7.5/0.15 M-NaCl).

‡ To whom correspondence should be addressed, at Mount Sinai Hospital Research Institute.

oncodevelopmentally-regulated patterns of oligosaccharide processing on the glycoproteins may influence the malignant potential of tumour cells by modulating cellular adhesion [21].

MATERIALS AND METHODS

Cell lines

The origin of the highly metastatic DBA/2 mouse tumour called MDAY-D2 has been described previously [22,23]. The cell lines were routinely grown in antibiotic-free alpha-minimum essential medium containing 7% fetal calf serum. The cell lines were shown to be free of *Mycoplasma* as detected by staining with Hoeschst 33258.

Extraction and purification of P2A and P2B

MDAY-D2 cells were grown in 6-litre spinner flasks to a density of $(3-5) \times 10^5$ cells/ml, as described previously [16]. Packed cells (50 ml) were collected and pooled with 10^7 MDAY-D2 cells previously labelled for 24 h with 250 μ Ci of [3 H]mannose (10 Ci/mmol) and 50 μ Ci of [14 C]galactose (100 Ci/mmol). The pooled cell pellets were extracted at 4 °C for 2 h with constant stirring in 200 ml of 50 mM-Tris/HCl, pH 8.0/0.15 M-NaCl (buffer A) containing 1 mM-EDTA, 1% Triton X-100 and 2 mM-phenylmethanesulphonyl fluoride. The extract was centrifuged at 15000 *g* for 30 min and the supernatant was diluted 4-fold with buffer A, giving a final detergent concentration of 0.2%. The extract was applied to a 25 ml column of wheatgerm agglutinin (WGA)-Sephacrose (4 mg of lectin/ml of Sepharose), pre-equilibrated in buffer A containing 0.2% Triton X-100. The column was washed with 10 vol. of 10 mM-Tris/HCl, pH 8.0, containing 0.4% sodium deoxycholate and 1 mM-EDTA (buffer B) and eluted in 5 ml fractions with the same buffer containing 5% *N*-acetylglucosamine and 2 mM-phenylmethanesulphonyl fluoride. The WGA-affinity chromatography step was repeated until the counts eluted were down to background levels and L-PHA reactivity was negligible.

The L-PHA-binding glycoproteins were detected by dot-blot assay on nitrocellulose as follows. Sample aliquots (25 μ l) were precipitated at -20 °C for 3 h with 9 vol. of cold acetone (-20 °C) in order to remove detergent. Pellets were collected by centrifugation at 15000 *g*, dissolved in buffer A and spotted on to nitrocellulose. Dot blots were incubated for 1 h at 37 °C in buffer A containing 5% bovine serum albumin. Following a 2 h incubation at room temperature with 125 I-L-PHA (10^5 c.p.m./ml) in 50 mM-Tris/HCl, pH 8.0/0.15 M-NaCl/2% bovine serum albumin (buffer C), blots were subjected to three 15 min washes in the same buffer, dried and exposed overnight to X-ray film (Kodak XAR). In later experiments, L-PHA-binding glycoproteins were detected using L-PHA, a rabbit anti L-PHA antiserum and a goat anti-rabbit IgG conjugated with alkaline phosphatase in a Western blotting procedure (Bio Rad).

L-PHA-binding glycoproteins eluted from WGA-Sephacrose were pooled, concentrated to 5 ml by ultrafiltration using an Amicon P-10 membrane and applied to a column (2.5 cm $\times$ 120 cm) of Biogel A5m (100-200 mesh). The column separated the material into two fractions designated P2A and P2B which were then dialysed against 20 mM-NH₄HCO₃/0.01% SDS,

lyophilized, resuspended in 2 ml of SDS sample buffer (0.03 M-Tris/HCl, pH 6.8/5% 2-mercaptoethanol/5% glycerol/1.2% SDS) and subjected to preparative SDS/PAGE. A vertical strip of acrylamide was removed from each gel (0.35 $\times$ 16 cm), cut into 0.5 cm lengths and extracted overnight in buffer A containing 0.1% SDS. The extracted protein was counted in a liquid scintillation counter. Regions of the gel with counts and corresponding to the glycoproteins of interest were pooled, and purified P2A and P2B were isolated by electroelution into dialysis tubing at 100 V in buffer consisting of 50 mM-Tris/acetate, pH 7.4/0.5% SDS. The purified glycoprotein was separated from the acrylamide by centrifugation followed by filtration. The sample was dialysed against buffer B and concentrated to 3 ml by ultrafiltration. Protein determinations were carried out with the BCA (bicinchoninic acid) reagent (Pierce) using bovine serum albumin as standard.

Protein iodination

Purified P2A or P2B, 25 μ g in 0.5 ml of phosphate-buffered saline (PBS) (10 mM-sodium phosphate, pH 7.5/0.15 M-NaCl) and PBS containing 0.4% deoxycholate, respectively, were labelled with 500 μ Ci Na¹²⁵I and 3 Iodogen beads (Pierce) for 10 min at room temperature. L-PHA was iodinated with the Bolton-Hunter reagent as previously described [24]. The sample was separated from the free iodine on a column (1 cm $\times$ 50 cm) of Biogel P10 (Pharmacia).

Antibody production

P2B (60 μ g) dissolved in buffer B was emulsified with an equal volume of complete Freund's adjuvant and injected intramuscularly into 8-week-old rabbits. After 4 weeks, animals were boosted with 30 μ g of antigen emulsified in Freund's incomplete adjuvant and injected at several subcutaneous sites. Animals were boosted monthly and serum collected one week later. For production of antibodies to L-PHA, an identical protocol was followed except that an initial injection dose of 200 μ g was administered followed by monthly boosts with 100 μ g of the lectin.

Glycosidase treatment

Iodinated P2A and P2B in 10 μ l of 10 mM-Tris/HCl, pH 7.5/0.4% deoxycholate were digested with 2 munits of glycopeptidase F (Boehringer Mannheim). For digestions with neuraminidase from *Clostridium perfringens* (type X, Sigma) (100 munits) and endo- β -galactosidase (Miles) (10 munits), samples were diluted with an equal volume of 50 mM-sodium acetate (pH 5.0) buffer. Sequential digestions were done first with glycopeptidase for 18 h, followed by dilution of the sample with 2 vol. of 50 mM-sodium acetate buffer, pH 5.0, and neuraminidase digestion for 4 h.

Western blotting

Proteins separated by SDS/PAGE (10 or 12% gels) under reducing conditions were transferred electrophoretically on to nitrocellulose sheets in buffer consisting of 25 mM-Tris/0.2 M-glycine which contained 20% methanol. Blots were blocked for nonspecific binding by incubation at 37 °C for 1 h in buffer A containing 5% (w/v) milk powder (blotto) in the case of immunoblots,

and buffer A/5% bovine serum albumin for L-PHA and peanut agglutinin (PNA) blots. For PNA, blots were incubated in 10 ml of 50 mM-sodium acetate, pH 5.5, either alone or with 0.5 U of neuraminidase (Sigma, type x) overnight.

Immunoblots were incubated for 3 h with 1/200 dilution of pre-immune serum or anti-P2B serum in blotto. Lectin blots were incubated for 1 h with L-PHA and PNA (0.1 μ g/ml) in PBS/0.1% bovine serum albumin. Following three 5 min washes in the same buffer, lectin blots were incubated for 1 h with a 1/1000 dilution of rabbit anti-L-PHA antibody or anti-PNA in PBS/0.1% bovine serum albumin. Immunoblots and lectin blots were subjected to three 5 min washes in PBS/0.1% bovine serum albumin and incubated for 1 h in alkaline phosphatase-coupled goat anti-rabbit antibody diluted 1/3000 in blotto. Blots were washed 4 times (5 min each) in blotto followed by one wash in buffer A/0.05% Tween-20 and one wash in buffer A. The blots were developed according to manufacturer's instructions (Bio Rad). Pre-immune rabbit serum did not stain proteins under these conditions.

The specificity of L-PHA for -GlcNAc β 1-6Man α 1-6Man β -branched oligosaccharides [25,26] was confirmed by comparing reactivity with bovine thyroglobulin, fetuin and human transferrin immobilized on nitrocellulose. These glycoproteins contain predominantly tetra- [26], tri- (lacking the β 1-6 antenna) [27] and bi-antennary [28] complex-type oligosaccharides respectively. When L-PHA and anti L-PHA antiserum were diluted appropriately (i.e. 0.1 μ g/ml and 1/3000 respectively), only bovine thyroglobulin was detected (results not shown). Galactose is also required for L-PHA binding [26], and consequently glycoproteins from the class 1 mutants of MDAY-D2 which have an apparent defect in transport of UDP-Gal into the Golgi do not bind L-PHA [16].

Amino acid analysis and sequencing

Analyses were kindly performed by Dr. C. Hew (University of Toronto, Toronto, Canada) on a Waters pico-tag system. Glycoprotein samples were precipitated with ethanol and the lyophilized samples were hydrolysed using 6 M-HCl/0.1% phenol for 4 h at 110 °C. Following hydrolysis, samples were dried under N₂ and then treated with methanol/water/triethylamine/phenylisothiocyanate (1:1:1:1, by vol.) for 29 min at room temperature. The samples were redried and analysed by reverse-phase h.p.l.c. Amino acid sequencing of P2B was carried out on an Applied Biosystems 477A protein Sequencer by Dr. M. West at Allelex Inc., Toronto, Canada. Three independent runs were performed and the repetitive yield based on Thr residues was approx. 87% for P2B.

Monosaccharide composition

In parallel with amino acid analysis, aliquots of the glycoproteins were precipitated with ethanol and hydrolysed with 2 M-trifluoroacetic acid for 4 h at 80 °C. The amino sugars and hexoses and fucose were separated on a carboxypac PA1 h.p.l.c. column with a mobile phase of 15 mM-NaOH or 100 mM-NaOH for NeuNAc at a flow rate of 1 ml/min. The monosaccharides were detected with a Dionex pulse amperometric detector II, and amounts were calculated based on the separation of standard monosaccharide mixtures.

RESULTS

Purification of the two major L-PHA binding glycoproteins from MDAY-D2

The major L-PHA-binding glycoproteins in plasma membrane preparations isolated from MDAY-D2 cells also bound ¹²⁵I-WGA and were sensitive to digestion

Fig. 1. Affinity chromatography of MDAY-D2 cell lysate on WGA-Sepharose

A detergent lysate prepared from 50 ml of packed MDAY-D2 cells pre-mixed with [³H]mannose- and [¹⁴C]galactose-labelled MDAY-D2 cells was applied to a 25 ml column of WGA-Sepharose, washed extensively and eluted with 5% *N*-acetylglucosamine. Sample aliquots (25 μ l) were precipitated in cold acetone, spotted on to nitrocellulose and incubated with ¹²⁵I-L-PHA (10⁶ c.p.m./ml). After extensive washing, blots were exposed to X-ray film (upper right). Run-through and eluted fractions were monitored at 280 nm.

Fig. 2. Chromatography of L-PHA-binding glycoproteins on Biogel A5m

L-PHA-binding glycoproteins eluted from WGA-Sepharose were pooled, concentrated by ultrafiltration and applied to a 2.5 cm $\times$ 100 cm column of Biogel A5m. Aliquots were also assayed for L-PHA-binding material, and fractions were pooled as indicated by the bars.

Fig. 3. ^{125}I -labelled P2A and P2B separated by SDS/PAGE

Undigested P2A and P2B are shown in lanes 1 and 2 respectively. Aliquots of P2A were predigested with: nil, lanes 3 and 6; neuraminidase, lane 4; glycopeptidase F, lanes 5 and 7; glycopeptidase F plus neuraminidase, lane 8. 10% gels were used.

Fig. 4. L-PHA lectin blotting and Western blotting with rabbit anti-P2B antiserum

Purified P2A and P2B (1 μg of each) were digested with neuraminidase (N) or neuraminidase plus endo- β -galactosidase (N,E) prior to analysis by Western blotting with anti-P2B or L-PHA/anti-L-PHA, as indicated. C indicates the position of an immunoreactive contaminant observed in all Western blots using the anti-P2B antiserum. P is pyrocin dye to mark the lanes on the Western blot.

with endo- β -galactosidase [15]. Based on this information, a WGA-Sepharose affinity column was used as the initial purification step and material eluted from the column was monitored for L-PHA reactivity (Fig. 1). A detergent lysate of [^3H]mannose- and [^{14}C]galactose-

Fig. 5. PNA reactivity of purified P2A and P2B

Aliquots containing 5 μg of protein were separated by SDS/PAGE (12% gels) and electroblotted on to nitrocellulose. Duplicate blots were blocked with bovine serum albumin then incubated with either 50 mM-sodium acetate buffer alone or the same buffer containing neuraminidase (Nanase). The blots were then reacted with PNA followed by rabbit anti-PNA and finally with alkaline phosphatase-coupled goat anti-(rabbit IgG) for detection of PNA-reactive material.

labelled MDAY-D2 cells containing 5 g of protein was applied to a WGA-Sepharose column. More than 95% of the L-PHA-reactive glycoproteins bound to the column and were recovered in the eluted fraction (i.e. 120 mg). Glycoproteins eluted from the WGA-Sepharose affinity column were then separated on a Biogel A5m column into two major peaks, an early-eluting fraction referred to as P2A (3.4 mg), and a late-eluting fraction, referred to as P2B (4.3 mg) (Fig. 2). The ratio of [^3H]mannose to [^{14}C]galactose was different in the two fractions, suggesting that P2B had a higher proportion of N-linked than O-linked oligosaccharide compared to P2A. Both P2A and P2B separated by SDS/PAGE stained poorly with Coomassie Blue and Silver Stain, consistent with a high degree of glycosylation (results not shown). Radioiodinated P2A and P2B fractions from the Biogel column each migrated on SDS/PAGE as a single broad band with molecular masses of approx. 110 kDa and 130 kDa, respectively. As a final step in purification, P2A and P2B were applied to preparative SDS/PAGE and electroeluted producing yields of 1.3 mg and 2.3 mg respectively.

Purified P2A and P2B each migrated on SDS/PAGE as a single broad band centering at 110 kDa and 130 kDa respectively (Fig. 3). Both glycoproteins contained L-PHA-reactive oligosaccharides and endo- β -galactosidase-sensitive polylactosamine sequences (Fig. 4). A rabbit polyclonal antiserum raised against SDS/PAGE-purified P2B showed negligible reactivity for

Table 1. Monosaccharide composition of P2A and P2B

The GlcNAc and GalNAc content was obtained by mild acid hydrolysis: 4 M-HCl at 80 °C for 4 h, and amino acid analysis. Amino sugar and hexosamine content was determined by separation of hydrolysates on a Dionex carbopac PA1 column and detection with a pulsed amperometric detector II. The two methods differed by less than 10 % for amino sugar content. n.d., not done.

Sugars	Monosaccharide composition (% of total weight)	
	P2A	P2B
Glc	8.0	17.0
GalNAc	2.0	0.9
Gal	6.9	9.9
Man	3.2	7.2
Fuc	0.2	0.3
NeuNAc	n.d.	18.9

Table 2. Amino acid composition of P2A and P2B

Results are shown for a single determination. The mean composition of 207 proteins is from ref. [42], gpL115 data is from ref. [19] and leukosialin data is from ref. [17]. n.d., not done.

Amino acid	Residues/100 amino acids				
	Average of 207 proteins	P2A	P2B	gpL115	Leuko-sialin
Asx	10.7	9.2	10.0	6.5	7.0
Glx	10.6	10.8	11.0	9.1	10.7
Ser	6.3	12.6	14.0	12.5	16.9
Gly	8.1	9.3	11.0	9.3	13.7
His	2.2	2.0	1.6	1.9	2.0
Arg	4.4	3.9	4.6	3.9	3.5
Thr	5.7	9.5	6.6	12.5	9.7
Ala	8.5	7.2	7.0	7.1	7.1
Pro	4.8	6.9	5.3	9.9	7.4
Tyr	3.3	2.2	3.3	1.4	1.2
Val	6.8	5.8	5.1	7.0	5.7
Met	1.9	1.6	1.5	1.8	1.3
Ile	5.0	3.5	3.1	3.5	3.1
Leu	8.1	7.7	7.9	7.8	6.2
Phe	3.7	3.0	3.1	1.8	1.9
Lys	6.5	4.3	3.9	3.3	2.8
Cys	2.3	n.d.	n.d.	n.d.	n.d.
Trp	1.3	n.d.	n.d.	n.d.	n.d.

SDS/PAGE-purified P2A in a Western blots, suggesting that the glycoproteins are structurally different (Fig. 4).

Structural characterization of P2A

Digestion of P2A with glycopeptidase F reduced the apparent molecular mass to 70 kDa, and further digestion with neuraminidase increased the molecular mass to 100–110 kDa (Fig. 3). The 20–30 kDa increase in apparent molecular mass of P2A following neuraminidase digestion is characteristic of glycoproteins bearing a high level of sialylated *O*-linked oligosaccharides such as

Fig. 6. Glycosidase digestions of ¹²⁵I-labelled P2B

Aliquots were incubated with: nil, (lane 1); endo- β -galactosidase (lane 2), glycopeptidase F (lanes 3 and 5) or glycopeptidase F plus neuraminidase (lane 4).

leukosialin [17]. Even after glycopeptidase F and neuraminidase digestion, P2A migrated on SDS/PAGE as a broad band suggesting that considerable amounts of large *O*-linked oligosaccharides may be present. This interpretation was supported by the high degree of PNA reactivity of P2A following removal of sialic acid with neuraminidase (Fig. 5). PNA binds to unsubstituted Gal β 1-3GalNAc disaccharides commonly found in *O*-linked oligosaccharides [29]. The monosaccharide composition of P2A showed more GalNAc and less mannose than in P2B (Table 1). Similarly, L-PHA reactivity was at least 2-fold greater for P2B than for P2A, also indicating that the two glycoproteins differ in their proportions of *O*- and *N*-linked oligosaccharides (Fig. 4).

The amino acid composition of P2A was very similar to that reported for human gpL115 [19] or leukosialin [18], with characteristically high Ser, Thr and Pro contents, and low Ile and Lys compared to an average of 207 proteins (Table 2). In addition, an *N*-terminal sequence for P2A yielded DVEQNMKMLGE which showed some similarity with the recently sequenced rat cDNA for leukosialin (i.e. ENLPNTMTMLPE [30]).

Structural characterization of P2B

Glycopeptidase F digestion yielded a single tight band of 40 kDa (Fig. 6) for P2B. The large shift to an apparent molecular mass of 40 kDa observed following glyco-

peptidase F digestion of P2B suggested that the glycoprotein consists of > 50% Asn-linked carbohydrate. Monosaccharide analysis showed that P2B was 54.2% carbohydrate with a composition characteristic of Asn-linked structures containing polylactosamine sequences. The small *N*-acetylgalactosamine content suggested that P2B may also have *O*-linked carbohydrate chains. Neuraminidase digestion of endoglycosidase F-treated P2B reduced the apparent molecular mass on SDS/PAGE by an additional 2 kDa, suggesting that a small amount of sialylated *O*-linked chains may also be present on P2B (Fig. 6). The desialylated P2B fraction showed weak reactivity with PNA; however, this may also be due to small amounts of contaminating P2A in the P2B fraction (Fig. 5).

Although P2B contained Arg and Lys, the purified glycoprotein was resistant to trypsin (results not shown), which may be due to the high carbohydrate content. Following reductive alkylation with iodoacetamide, P2B was cleaved by trypsin, Protease V8 and chymotrypsin into three major peptides, each heavily glycosylated based on their diffuse nature on SDS/PAGE. This suggests that intrachain disulphide bonds contribute in part to the stability of P2B against proteolytic degradation.

The anti-P2B antiserum appeared to recognize conformation-dependent determinants in P2B which could only be destroyed with a combination of SDS and reduction/alkylation of the glycoprotein. Boiling purified ¹²⁵I-P2B in 1% SDS or reduction and alkylation alone did not reduce the efficiency of immunoprecipitation. However, reduced, alkylated and SDS-denatured P2B was not immunoreactive (results not shown). This also suggested that the tertiary structure of purified P2B was stabilized in the presence of SDS by disulphide bonds.

N-terminal sequence analysis for P2B produced the sequence DFEVKNDGTTTIMASFCAMF and was confirmed by independent sequencing runs. A comparison of this sequence with that deduced from a cDNA of murine lysosomal associated membrane glycoproteins (LAMP-1) showed five residue differences in the first 20 amino acids (i.e. residues 1,7,11,17 and 19 in LAMP-1 are L,N,C,S and S respectively) [31]. In addition, we have recently confirmed the exact murine LAMP-1 *N*-terminal sequence in four cDNAs selected with the rabbit anti-P2B antiserum from a λ gt11 pre B cell cDNA library (M. Heffernan & J. Dennis, unpublished work). These results suggest that the P2B glycoprotein is the murine LAMP-1 glycoprotein.

DISCUSSION

Increased β 1-6 branching of complex-type oligosaccharides has been directly linked to increased metastatic potential. The major L-PHA-staining glycoproteins in membrane preparations of MDAY-D2 cells were 80–160 kDa in size, very similar to that observed in oncogene-transfected clones of the SP1 carcinoma and rat 1 fibroblast cells [12]. Interestingly, the membrane glycoproteins most readily detected by lectin binding (i.e. WGA, L-PHA, concanavalin A, PNA), and shown to be aberrantly glycosylated in malignant cells, are diffuse species in the 80–160 kDa size range [12,32,33]. We have isolated and characterized the major L-PHA-binding glycoproteins found in the metastatic lymphoreticular

tumour cell line called MDAY-D2. The glycoproteins, designated P2A and P2B, migrated at approx. 110 kDa and 130 kDa respectively on SDS/PAGE, were immunologically distinct, had different proportions of *O*- and *N*-linked oligosaccharides and produced different sized peptides following glycopeptidase F digestion.

P2A had both L-PHA-reactive Asn-linked oligosaccharides and a high proportion of sialylated *O*-linked structures. The amino acid composition of P2A showed atypical features that were very similar to those of gpL115 [19] or leukosialin from human leucocytes [17] and the W3/13 antigen or rat leukosialin [18] also known as CD43 [34]. In addition, there was some similarity between the *N*-terminal sequence of P2A and the *N*-terminal sequence of rat leukosialin deduced from the recently cloned cDNA [30]. Another characteristic feature of these sialoglycoproteins is the apparent increase in their molecular mass (i.e. 30–40 kDa) following neuraminidase digestion [17,35]. Leukosialin appears to be altered in size in the lymphocytes from individuals with the inherited immunodeficiency called Wiskott–Aldrich syndrome. G1b, a heavily *O*-linked glycosylated glycoprotein found in platelets, is also abnormal in Wiskott–Aldrich patients, suggesting that the biochemical defect may be related to altered *O*-linked glycosylation [35,36]. This is particularly intriguing as P2A has also been shown to bind specifically to collagen type I, and this activity was dependent on sialylation of the *O*-linked oligosaccharides [21]. Increased *O*-linked glycosylation of several unidentified glycoproteins in the B16 melanoma has recently been correlated with enhanced cell adhesion *in vitro* and increased lung colonization potential *in vivo* [37].

The P2B glycoprotein contained more than 50% Asn-linked oligosaccharide structures with sialylated polylactosamine sequences. The size of glycosidase F-treated P2B and the carbohydrate content of the glycoprotein were very similar to that of LAMP-1 previously identified in murine fibroblasts and macrophage cell lines [20]. Immunoreactive LAMP-1 from 3T3 and P388 cells cultured in the presence of tunicamycin was found to have a molecular mass of 42 kDa. The molecular mass of endoglycosidase F-treated P2B was estimated to be 40 kDa, and 2–5 kDa smaller when neuraminidase-treated in addition. The *N*-terminal peptide sequence of P2B and that of mouse LAMP-1 showed a high degree of similarity and this has recently been confirmed by sequencing of a cDNA selected from a λ gt11 library using rabbit anti-P2B antiserum. Four lambda clones containing full length cDNA inserts coding for LAMP-1 were selected using the rabbit anti-LAMP-1 antiserum (M. Heffernan & J. Dennis, unpublished work).

LAMP-1 is concentrated in the lysosomal compartment of fibroblasts and it has also been detected on the cell surface of lymphoid cells [38]. The glycoprotein constitutes approx. 0.05–0.1% of cellular protein in MDAY-D2 cells and appears to be the major carrier of complex-type oligosaccharide chains in the cell. Using fluorescently labelled anti-(P2B/LAMP-1) polyclonal sera, permeabilized rat2 fibroblast cells displayed a perinuclear ring characteristic of lysosomal localization (J. W. Dennis, unpublished results). To a lesser degree, fluorescent staining of the plasma membrane was also detected in rat2 cells, particularly subconfluent cultures. Lippincott-Schwartz & Fambrough [39], have demonstrated that chicken LAMP-1 (LEP-100) shuttles between the plasma membrane, endosomes and lysosomes

in chick-embryo fibroblast cells. LAMP-1 has also been detected on the surface of the murine macrophage-like cell line P388 [38], and the human U937 and HL60 myelomonocytic leukaemia cell lines, but was absent on a variety of other tissue culture cells or normal peripheral blood monocytes [40]. LAMP-1 was no longer detectable at the cell surface in U937 cells induced to differentiate with phorbol ester *in vitro*. It is possible that malignant transformation may alter not only Asn-linked oligosaccharide processing on P2B/LAMP-1 but also its subcellular distribution. In this regard, tumour cells are known to secrete increased levels of lysosomal hydrolases [41] which may be linked to increased cycling and expression of P2B/LAMP-1 glycoproteins at the cell surface.

Western blotting analysis suggested that P2B was present in all normal mouse tissues but ranged in size from approx. 90 to 150 kDa (results not shown). Since the carbohydrate constitutes a large proportion of the mass of P2B, the size heterogeneity between tissues may be due to tissue-specific differences in Asn-linked oligosaccharide processing.

P2B purified from MDAY-D2 cells has been shown to bind weakly to immobilized collagen, fibronectin and laminin *in vitro*. Enzymic removal of sialic acid, polylactosamine or all Asn-linked chains in P2B-enhanced binding to these ECM proteins [21]. These results suggest that transformation-related changes in oligosaccharide processing on P2B/LAMP-1 expressed at the cell surface may alter cellular contacts with ECM components and contribute to the malignant phenotype of the cells. In particular, increased β 1-6 branching at the trimannosyl core of Asn-linked oligosaccharides and associated polylactosamine sequences on P2B/LAMP-1 may reduce tumour-cell adhesion to ECM proteins. In support of this hypothesis, inhibition of Asn-linked oligosaccharide processing by swainsonine has been shown to block experimental metastasis [13,14], increase tumour cell adhesion to ECM proteins, and decrease invasion of human amnion basement membrane *in vitro* (S. Yagel & R. Feinmesser, C. Waghorne, P. Lala, M. Breitman & J. Dennis, unpublished work). Further studies on purified glycoproteins which are subject to oncodevelopmental changes in oligosaccharide processing may help clarify the role of these carbohydrate structures in cell-cell interactions.

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