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Modification of CD43 and other lymphocyte O-glycoproteins by core 2 N-acetylglucosaminyltransferase

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CD43, the major leukocyte sialoglycoprotein, is expressed on T lymphocytes in two predominant glycoforms. CD43 115 kDa is a pan T cell marker and is specifically recognized by the monoclonal antibody S7. CD43 130 kDa is associated with T cell activation and is specifically recognized by the monoclonal antibody 1B11. The thymoma EL-4 has been identified to express mainly CD43 115 kDa and little or no CD43 130 kDa. Transfection of EL-4 cells with core 2 β1→6N-acetylglucosaminyltransferase (C2GnT), an enzyme in the O-glycan biosynthesis pathway, resulted in an enhanced expression of the 1B11 epitope, CD43 130 kDa, and a loss of expression of the S7 epitope, CD43 115 kDa. Analysis of CD43 by SDS–PAGE revealed that CD43 in C2GnT transfected EL-4 cells has a molecular weight of 125 kDa compared to 115 kDa in nontransfected or control transfected EL-4 cells. SDS–PAGE analysis of three other lymphocyte O-glycoproteins, CD44, CD45, and RPTPα, revealed that C2GnT expression resulted in a molecular weight increase of approximately 3–5 kDa for each of these three cell surface glycoproteins. Our data indicate that, while CD43 may be a predominant substrate for C2GnT, other lymphocyte O-glycoproteins are also modified by this glycosyltransferase. Increased reactivity of cells with the monoclonal antibody 1B11, which specifically detects the expression of murine CD43 130 kDa, may thus be a marker of increases in branching of O-linked glycans generally.

Key words: CD43/core 2 N-acetylglucosaminyltransferase/ lymphocyte glycoproteins

Introduction

CD43 (leukosialin or in mouse also Ly-48) carries extensive O-linked glycosylation and is heavily sialylated. It is expressed on the surface of many hemopoietic cells including T lymphocytes, macrophages, and neutrophils (Fukuda, 1991). Although in human and mouse the CD43 polypeptide is encoded by a single gene that contains no introns (Baecher *et al.*, 1990; Dorfman *et al.*, 1990), there is considerable molecular weight heterogeneity due to differential glycosylation of the CD43 polypeptide. CD43 has only one site of N-linked glycosylation but has approximately 75 O-linked glycans attached (Fukuda, 1991). On human cells, two major CD43 glycoforms have been described, which differ in their O-glycan structure. The predominant CD43 glycoform associated with resting T cells (Carlsson and Fukuda, 1986; Baecher *et al.*, 1988) carries mainly O-linked tetrasaccharide side chains (NeuNAcα2→3 Galβ1→3 (NeuNAcα2→6) GalNAcα1→Ser/Thr) and migrates on SDS–PAGE with a MW of 115 kDa (referred to as CD43 115 kDa). A second major glycoform of CD43 has a MW of 130kDa (referred to as CD43 130kDa), carries mainly hexasaccharide side chains (NeuNAcα2→3 Galβ1→3 (NeuNAcα2→3 Galβ1→4 GlcNAcβ1→6) GalNAcα1→Ser/Thr), and is expressed on neutrophils, platelets and activated T cells (Fukuda, 1991). Increased expression of CD43 130 kDa has also been associated with immune disorders such as graft-versus-host disease, rheumatoid arthritis, AIDS and the Wiscott-Aldrich syndrome (Fox *et al.*,1983).

Modulation of CD43 glycoforms in T cell activation is thought to result from the induction of UDP-GlcNAc: Galβ1→3GalNAc (GlcNAc to GalNAc) β1→6N-acetylglucosaminyltransferase (core 2 N-acetylglucosaminyltransferase, C2GnT) (Piller *et al.*, 1988; Higgins *et al.*, 1991). Expression of C2GnT activity causes a shift from CD43 115 kDa to the synthesis of CD43 130 kDa (Bierhuizen *et al.*, 1994).

C2GnT is a member of the O-glycan β1,6 N-acetylglucosaminyltransferase family along with the I-branching enzyme (IGnT). Both enzymes are thought to modulate cell–cell interaction, IGnT has been associated with embryogenesis (Kapadia *et al.*, 1981) and C2GnT expression is associated with T cell activation (Piller *et al.*, 1988, Higgins *et al.*, 1991, Ellies *et al.*, 1994). The C2GnT promoter sequence contains potential binding sites for transcription factors (NF-IL-6, GATA-3, and TCF-1) which are specifically active in T-lymphocytes (Bierhuizen *et al.*, 1995). Studies using *in situ* hybridization in mouse embryos showed that C2GnT is widely expressed in day 7 embryo but then becomes restricted to mucin and cartilage producing tissue (Granovsky *et al.*, 1995). C2GnT transcript could not be detected in murine embryonic thymus but in a different study was found in human postnatal thymus (Baum *et al.*, 1995).

In mouse, monoclonal antibodies S7 (Gulley *et al.*, 1988, Baecher-Allan *et al.*, 1993) and 1B11 (Tomlinson Jones *et al.*, 1994) specifically recognize the 115 kDa and 130 kDa forms of CD43, respectively. Analysis of expression of these CD43 glycoforms suggests that similar regulation of CD43 glycosylation occurs in human and murine systems (Tomlinson Jones *et al.*, 1994). Activation of CD4 and CD8 single positive T cells in the periphery correlates with upregulated expression of CD43 130 kDa, recognized by mAb 1B11. In graft versus host dis-

ease, donor CD4 and CD8 single positive T cells show increased expression of CD43 130 kDa and in parallel, a higher C2GnT activity is observed in the spleen (Ellies *et al.*, 1994). In the thymus, double negative, double positive, and CD8 single positive thymocytes express both major glycoforms of CD43. However, CD4 single positive thymocytes are negative for the 1B11 reactive form, indicating a selective downregulation of CD43 130 on CD4 single positive cells during thymic maturation (Tomlinson Jones *et al.*, 1994). This parallels data from human thymus, where it was shown, by *in situ* hybridization, that C2GnT mRNA is found in the cortex (immature thymocytes) but not in the medulla (mature thymocytes) (Baum *et al.*, 1995). Recently, we have shown that positive selection in the thymus correlates with a downregulated expression of the 1B11 form of CD43 in double positive thymocytes (Ellies *et al.*, 1996).

Deletion of the CD43 gene leads to altered T cell properties including hyperadhesion, hyperproliferation and impaired viral clearance, but it does not affect the numbers or the ratio of CD4 and CD8 single positive cells (Manjunath *et al.*, 1995). Modulation of CD43 glycosylation by C2GnT might thus be important for the maturation of functionally competent cells.

While CD43 is considered to be a major target for C2GnT, PSGL-1 (P-selectin glycoprotein ligand-1) is another mucin type cell surface protein which has been reported to be a substrate of C2GnT (Li *et al.*, 1996). Expression of core 2 structures together with α1–3 fucosyltransferase activity was found to be required for PSGL-1 to act as ligand for P- and E-selectins. In addition to these examples, other glycoproteins such as CD45, RPTPα and CD44 may potentially also be affected by C2GnT. The leukocyte common antigen CD45, a transmembrane protein tyrosine phosphatase, is heavily N- and O-glycosylated and involved in T cell activation. RPTPα is an ubiquitous protein tyrosine phosphatase of unknown function which has sites for extensive O-linked glycosylation in its extracellular domain (Daum *et al.*, 1994). CD44 represents a heterogeneous group of cell surface and secreted proteins generated by alternate splicing of a single gene (Screaton *et al.*, 1992). The O-glycosylation of the protein depends on the splicing of the extracellular domain and is suggested to regulate lectin activity (Bennett *et al.*, 1995).

We report here the analysis of C2GnT overexpression in the EL-4 T lymphoma cell line and its effects on CD43 glycoform expression. The effects of C2GnT overexpression on the glycoproteins CD44, CD45, and RPTPα are also examined.

Results

MSCV/C2GnT retrovirus production

Murine C2GnT cDNA was cloned into the murine stem cell virus (pMSCV*neo*EB) retroviral vector in both sense and antisense orientations. Infectious virus was produced by introducing the sense construct into the PA317 helper-free packaging cell line followed by G418 selection. Virus producing cells were assayed for C2GnT activity to verify viral expression of the C2GnT gene. Vector alone infected cells are essentially negative for C2GnT activity (<0.02 pmol/mg/h) whereas cells infected with C2GnT show considerable activity (0.234 pmol/mg/h, Table I). Viral titers were amplified by transferring virus producing PA317 culture supernatants onto the ecotropic packaging cell line ψ2. Following G418 selection, resistant populations were cloned by limiting dilution. C2GnT activity measured in the transfected ψ2 cells (1.653 pmol/mg/h) was almost 8-fold increased over C2GnT activity measured in transfected PA317 cells and even higher than the C2GnT levels found in the cytotoxic cell line CTL-2c (1.534 pmol/mg/h) which consistently expresses high levels of C2GnT activity (Table I). Interestingly, cultures of the two C2GnT transfected fibroblast cell lines PA317 and ψ2 were less securely attached to the culture dish in monolayer culture, suggesting that C2GnT expression may significantly alter their adhesive properties.

Table I. C2GnT activity in transfected retroviral packaging cell lines

	C2GnT activity (pmol/mg/h)	
	PA317	ψ2
Uninfected	ND	ND
pMSCV*neo*	<0.02	0.065
pMSCV*neo*:C2GnT	0.234	1.653

C2GnT overexpression in EL-4 cells

In a previous study, we have shown that the lymphoma EL-4 expresses CD43 115 kDa, recognized by mAb S7, and only very low levels of CD43 130 kDa, recognized by mAb 1B11 (Tomlinson Jones *et al.*, 1994). We transfected these cells with C2GnT using the ecotropic C2GnT retrovirus, and G418 resistant cells were cloned by limiting dilution. Transfected EL-4 cells were analyzed for their C2GnT activity and for the expression of T cell activation and/or differentiation associated O-glycoproteins CD43, CD44, CD45, and RPTPα.

C2GnT transfected EL-4 cells showed identical doubling time and morphology as untransfected cells or cells transfected with MSCV vector alone. The transfected cells continued to grow in a nonadherent fashion and form larger cell aggregates.

Flow cytometric analysis revealed that MSCV control transfected EL-4 cells express medium levels of the S7 reactive CD43 115 kDa glycoform and have low levels of the 1B11 reactive CD43 130 kDa glycoform, whereas C2GnT transfected EL-4 cells showed a marked increase in the expression of the 1B11 reactivity and lost reactivity with mAb S7 (Figure 1). Nontransfected EL-4 cells have a similar profile of anti-CD43 antibody reactivity as MSCV transfected EL-4 cells (data not shown).

Measurement of C2GnT activity in transfected EL-4 cells revealed that the change in CD43 glycoform expression correlates with altered C2GnT activity (Figure 2). C2GnT transfected EL-4 cells showed elevated levels of C2GnT activity (0.366 ± 0.004 pmol/mg/h), whereas MSCV transfected EL-4 cells had similar low levels of C2GnT activity as nontransfected EL-4 cells (0.066 ± 0.043 pmol/mg/h and 0.030 ± 0.009 pmol/mg/h, respectively). Although C2GnT levels in C2GnT transfected EL-4 cells were considerably increased over the activity levels measured in EL-4 controls, they were still approximately 4-fold lower than the C2GnT activity measured in CTL-2c cells (1.534 ± 0.058 pmol/mg/h). In control experiments, we used GlcNAcβ1→3Gal-NAcαpNp instead of Galβ1→3GalNAcαpNp as acceptor for ^{3}H-GlcNAc. No transfer of radioactivity was detected, indicating that core 4 N-acetylglucosaminyltransferase (C4GnT, GlcNAcβ1→3GalNAc β1→6N-acetylglucosaminyltransferase) activity was absent in the cell extracts (data not shown). We can therefore

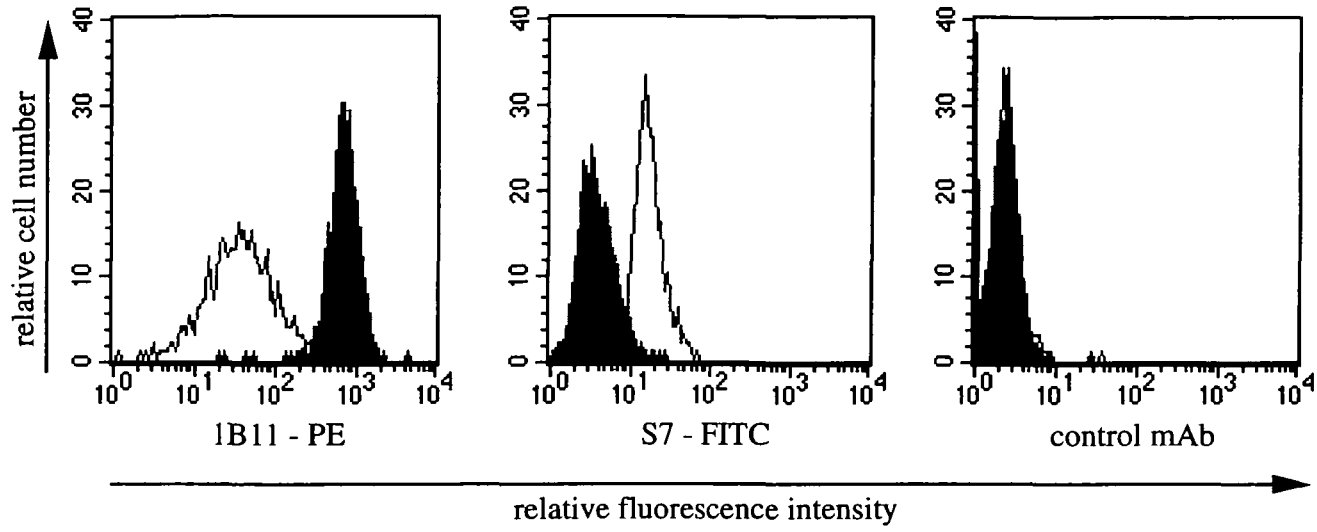

Fig. 1. CD43 glycoform expression is altered in C2GnT transfected EL-4 cells. C2GnT transfected (filled area) and control MSCV transfected (solid line) EL-4 cells are analyzed using mAb 1B11, which recognizes CD43 130 kDa, and mAb S7, which recognizes CD43 115 kDa. MAb 1F10 is an isotype matched control to mAbs S7 and 1B11.

conclude that the transfer of radiolabeled GlcNAc was due to C2GnT activity.

FACS analysis of other cell surface glycoproteins which potentially may also be modified by C2GnT revealed no change in levels of cell surface expression of CD44 between C2GnT transfected, MSCV control transfected EL-4 cells (Figure 3A) or untransfected cells (data not shown). To examine possible changes in CD45 isoform expression, we used RB and RC isoform specific mAbs 23G2 and DNL1.9, respectively, as well as the isoform-independent mAb 30F11.1. Again, no significant differences in CD45 isoform expression between C2GnT transfected and MSCV transfected EL-4 cells (Figure 3B) were found. However, untransfected EL-4 cells gave a slightly increased CD45 signal with each of the three CD45 specific mAbs tested.

Western blots were carried out to determine whether C2GnT overexpression alters the electrophoretic mobility of CD43 and

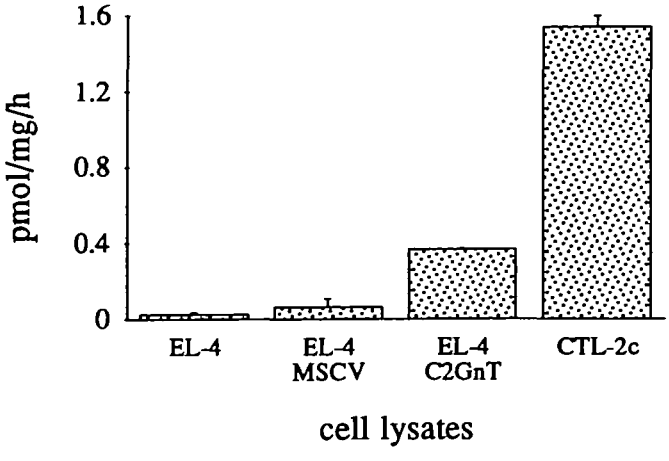

Fig. 2. C2GnT transfected EL-4 cells express increased C2GnT activity. Whole cell lysates from untransfeced, control MSCV or C2GnT transfected EL-4 cells and from untransfected CTL-2c cells were measured for C2GnT activity. Data are the mean ± SEM of duplicate samples and are representative for one of two independent assays.

other potential targets of C2GnT. For comparison of the glycoproteins from C2GnT and MSCV transfected El-4 cells, we used fractions purified by wheat germ agglutinin (WGA) affinity columns, because this step enabled enrichment of glycoproteins and resulted in cleaner blots. Western blots of whole cell lysates of untransfected EL-4 cells are shown to demonstrate that neither WGA concentration of glycoproteins nor transfection with MSCV control vector resulted in altered electrophoretic mobility of the glycoproteins examined.

An antipeptide antibody raised against the C-terminus of CD43, which recognizes CD43 independent of its glycosylation status, confirmed that a higher molecular weight form of CD43 (~125 kDa) is expressed in C2GnT transfected EL-4 cells when compared to untransfected or vector alone transfected EL-4 cells (Figure 4). Analysis of CD44 revealed that the electrophoretic mobility of this cell surface molecule is also affected by C2GnT. Western blotting using anti-CD44 mAb KM 201 identified CD44 as a 85 kDa protein in MSCV control transfected and in untransfected EL-4 cells, whereas in EL-4 cells overexpressing C2GnT, CD44 was seen as a 88 kDa band (Figure 5A). Western blots using antipeptide antibodies specific for CD45 revealed that the molecular weight of CD45 is similarly altered by C2GnT overexpression. In untransfected EL-4 cells, two isoforms of CD45 are detected as 190 kDa and 200 kDa isoforms, whereas in C2GnT transfected cells the two bands have a 5 kDa higher molecular weight with 195 kDa and 205 kDa, indicating that C2GnT affects similarly both CD45 isoforms expressed on EL-4 cells (Figure 5B). RPTPα, the fourth O-glycoprotein examined in Western blots, showed also a 5 kDa molecular weight increase in C2GnT transfected EL-4 cells. RPTPα, detected as a 130 kDa band in untransfected cells, was seen as a 135 kDa band in C2GnT transfected EL-4 cells (Figure 5C).

Discussion

The present study investigates the overexpression of C2GnT in the murine T cell lymphoma EL-4 and its effect on CD43 glycoform expression. EL-4 cells express very low levels of C2GnT and transfection of EL-4 cells with C2GnT re-

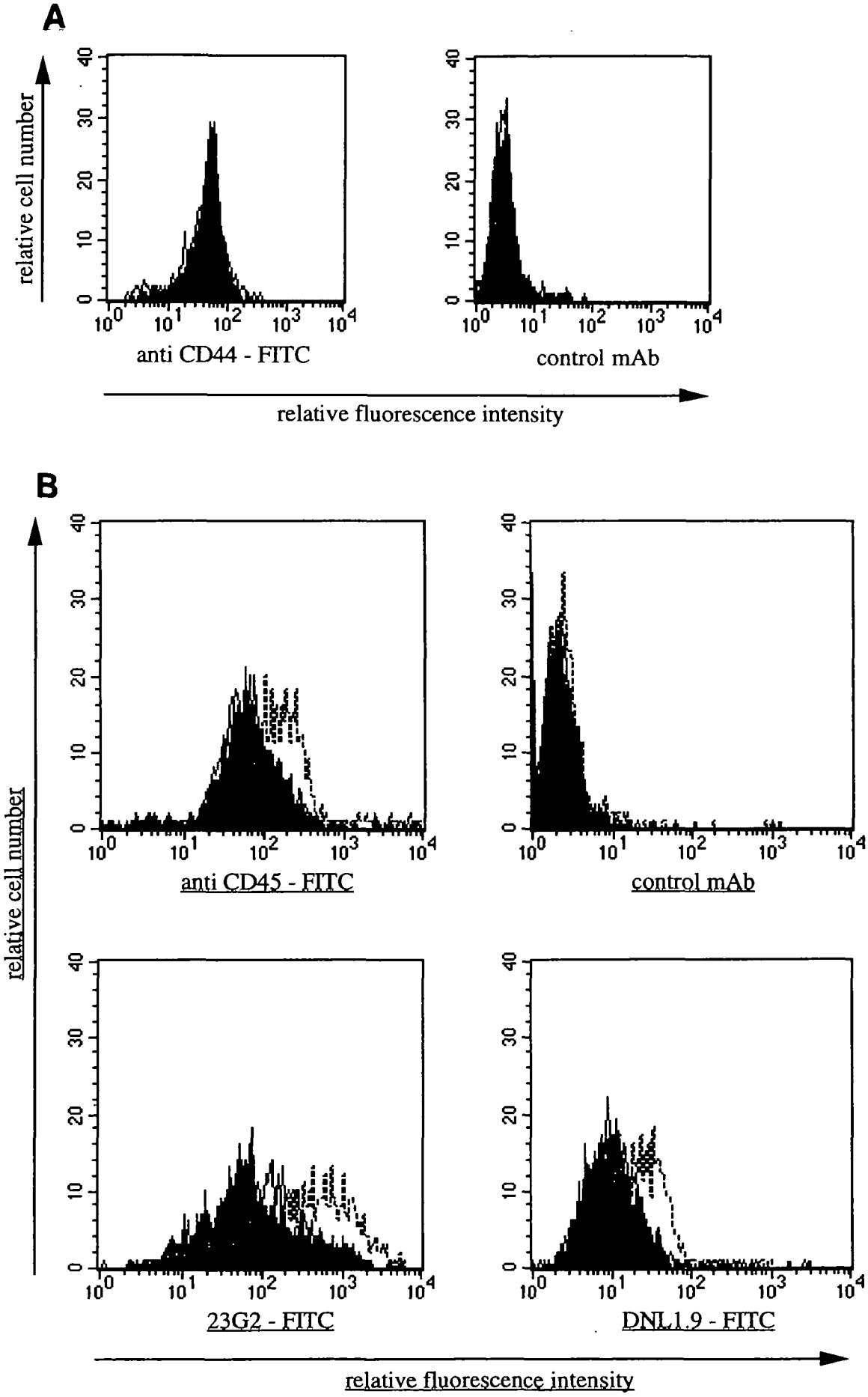

Fig. 3. Expression of CD44 and CD45 is not affected by C2GnT. (A) Flow cytometric analysis of CD44 expression in EL-4 cells transfected with C2GnT (filled area) or MSCV control vector (solid line). Control mAb 1F10 is isotype matched. (B) Flow cytometric analysis of CD45 expression in untransfected EL-4 cells (broken line) and cells transfected with C2GnT (filled area) or control MSCV (solid line). Anti CD45 is an isoform-independent mAb, mAb 23G2 recognizes CD45 isoform RB, mAb DNL1.9 recognizes CD45 isoform RC. The control mAb 1F10 is isotype matched.

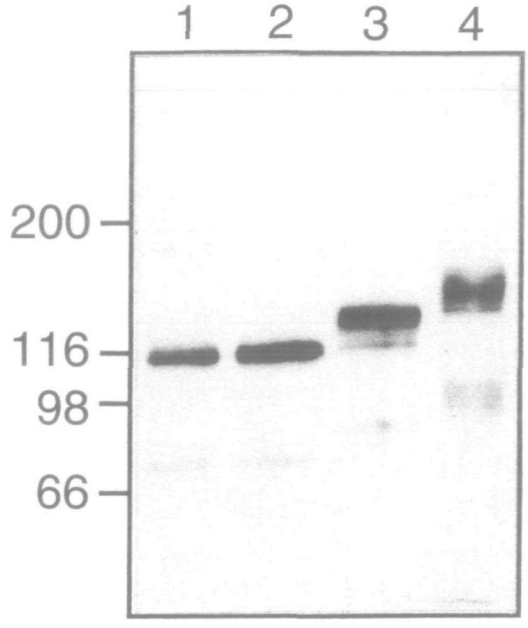

Fig. 4. C2GnT transfected EL-4 cells express CD43 with an increased molecular weight. Whole cell lysates of untransfected EL-4 cells (lane 1) and CTL-2c cells (lane 4) and wheat germ agglutinin purified cell lysates of MSCV transfected (lane 2) and C2GnT transfected (lane 3) EL-4 cells were electrophoresed on a reducing 7.5% SDS–polyacrylamide gel. Expression of CD43 glycoforms was detected by immunoblotting with a polyclonal rabbit anti-peptide Ab against CD43.

sulted in a significant increase in C2GnT activity. Our data demonstrate that expression of C2GnT in EL-4 cells causes a shift in CD43 glycoform expression, as indicated by the altered reactivity of C2GnT transfected cells with the two anti-CD43 mAbs S7 and 1B11, which are specific for CD43 115 kDa and CD43 130 kDa, respectively. The levels of C2GnT activity in transfected EL-4 cells were still only about 25% of the levels of activity found in CTL-2c, a cytotoxic T cell line that consistently expresses high level of C2GnT activity and carries exclusively CD43 130 kDa. This lower C2GnT activity could be due to a number of host cell factors including transcription rate, transcript stability, or an unknown posttranslational control of C2GnT in transfected EL-4 cells.

SDS–PAGE revealed that CD43 from C2GnT transfected EL-4 cells has a MW of 125 kDa which is approximately 5 kDa less than the MW of CD43 naturally expressed on activated thymocytes (Tomlinson Jones *et al.*, 1994). This apparent lower molecular weight of CD43 in C2GnT transfected EL-4 cells when compared to CD43 in CTL-2c could be due to the lower levels of C2GnT activity seen in C2GnT transfected EL-4 cells relative to the C2GnT activity expressed in CTL-2c cells, reflecting that not all of the approximately 75 O-linked side chains are in fact modified by C2GnT. Alternatively, induction of other not yet determined glycosyltransferases may be required in addition to C2GnT for expression of the high molecular weight glycoform of CD43 expressed on CTL-2c cells.

A similar effect of C2GnT on human CD43 was observed in earlier experiments where Chinese hamster ovary cells, stably expressing human CD43, were transfected with human C2GnT. In these experiments C2GnT expression resulted also in a molecular weight increase of CD43 and a selective recognition of CD43 from C2GnT transfected cells by T305, a monoclonal antibody specific for the hexasaccharide form of human CD43 (Bierhuizen *et al.*, 1994).

In addition to CD43, the effects of C2GnT activity on other lymphocyte glycoproteins were tested by Western blot and flow cytometric analysis. Although CD43 is a major substrate of C2GnT, increases in molecular weight were consistently observed, in C2GnT transfected cells, for the other three glycoproteins examined, CD44, CD45, and RPTPα. The relatively smaller increases in molecular weight associated with these glycoproteins, compared to CD43, is most likely due to the lower amount of O-glycans present in these glycoproteins.

Our results suggest that C2GnT may alter O-glycan structures on many other glycoproteins including PSGL-1, which has been reported in the human system to be a substrate of C2GnT (Li *et al.*, 1996). We were not able to assess whether PSGL-1 is expressed on EL-4 cells and examine the effects of C2GnT overexpression in the murine system, because murine PSGL-1 was cloned only recently (Yang *et al.*, 1996) and no antibodies were available in our laboratory.

C2GnT overexpression did not affect overall levels of cell surface expression of any of the glycoproteins tested, indicating that altered O-glycan structures did not affect properties such as processing, turnover, or shedding. The somewhat lower level of expression of CD45 seen in C2GnT transfected EL-4 cells as well as in MSCV control transfected cells is likely to be an artifact caused by the retroviral vector itself.

The reactivity of the mAbs S7 and 1B11 with specific glycoforms of CD43 on T cells allows the assessment of relative levels of C2GnT activity in these cells. Activity of this enzyme is apparently a major control point in the biosynthesis of O-linked polylactosaminoglycan structures in murine (Yousefi *et al.*, 1991) and human cells (Maemura and Fukuda 1992). Since increases of 1B11 epitope expression correlate with increased C2GnT levels, this marker also indicates a raised potential of such cells to form these extended chains.

We have shown earlier, using mAb 1B11, that CD43 130 kDa is differentially regulated in T cell ontogeny (Tomlinson Jones *et al.*, 1994). Furthermore, our data from the murine system (Ellies *et al.*, 1994) as well as data from others examining the human system (Fox *et al.*, 1981) have identified CD43 130 kDa as an activation associated molecule. Based on our present data, these changes of CD43 130 kDa expression are likely to be paralleled by glycosylation changes of many other O-glycosylated proteins, indicating that glycoprotein ligand interactions may be altered on many cell surface molecules.

Addition of these core 2 O-glycan structures may modulate lymphocyte adhesion to epithelial cells of the thymic cortex during the double positive stage of T cell development (Baum *et al.*, 1995) and may play a role in the production of immune competent cells (Ellies *et al.*, 1996). Galectin-1, a member of the family of β-galactoside binding proteins, has been shown to bind to CD43 and CD45 (Baum *et al.*, 1995) and to induce apoptosis of activated T cells (Perillo *et al.*, 1995). Induction of apoptosis required expression of CD45 and, interestingly, was decreased when N-glycan elongation was blocked and increased when O-glycan elongation was blocked. Clearly, altered expression of glycoforms of CD43, CD45, and other glycoproteins may play a key role in the function of many of these molecules.

This study shows that the expression of C2GnT regulates O-glycan expression of multiple proteins and introduces a useful tool for future experiments to potentially regulate glyco-

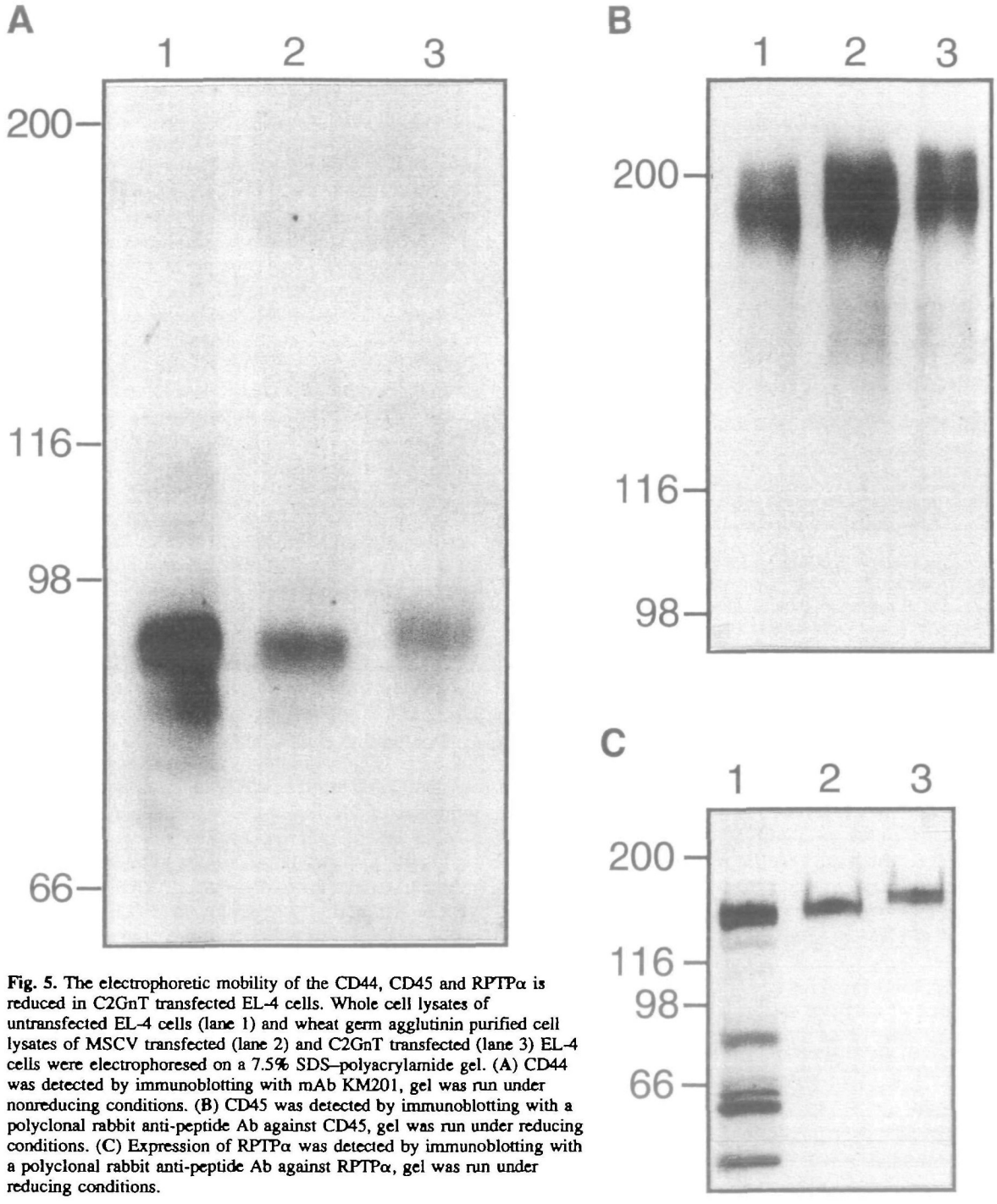

Fig. 5. The electrophoretic mobility of the CD44, CD45 and RPTPα is reduced in C2GnT transfected EL-4 cells. Whole cell lysates of untransfected EL-4 cells (lane 1) and wheat germ agglutinin purified cell lysates of MSCV transfected (lane 2) and C2GnT transfected (lane 3) EL-4 cells were electrophoresed on a 7.5% SDS–polyacrylamide gel. (**A**) CD44 was detected by immunoblotting with mAb KM201, gel was run under nonreducing conditions. (**B**) CD45 was detected by immunoblotting with a polyclonal rabbit anti-peptide Ab against CD45, gel was run under reducing conditions. (**C**) Expression of RPTPα was detected by immunoblotting with a polyclonal rabbit anti-peptide Ab against RPTPα, gel was run under reducing conditions.

protein–lectin interactions, cell signaling, and T lymphocyte development in the thymus.

Material and methods

Reagents

Phycoerythrin labeled mAb S7, 1B11 and fluorescein labeled mAb 30F11.1 (anti-CD45) were obtained from Pharmingen (Pharmingen, San Diego, CA). mAbs IM7.8.1 (anti-CD44) and 1F10 were produced as ascites in our laboratory. 23G2 (anti-CD45RB), DNL1.9 (anti-CD45RC), and anti-CD44 mAb (KM201) for Western blotting was kindly provided by P.Johnson (Department of Microbiology, UBC, Vancouver). 1F10 was used as an isotype control for mAbs S7 and 1B11; it is an IgG_{2a} monoclonal antibody reactive with a specific rabbit anti-peptide antibody idiotype. 1B11 and IF10 mAbs were FITC labeled in house according to the methods of Goding (Goding, 1986). Polyclonal rabbit anti-peptide antibodies recognizing cytoplasmic domains of CD43 and CD45 were produced as described for CD43 (Tomlinson Jones *et al.*, 1994) and CD45 (Chui *et al.*, 1994). Anti-RPTPα-1 was raised against a peptide corresponding to amino acid residues 512–558 and was the generous gift of F.Jirik (The Biomedical Research Centre, UBC, Vancouver). Galβ1–3GalNAcα-pNp and GlcNAcβ1–3Gal-NAcαpNp were obtained from Toronto Research Chemicals (Toronto, Ontario).

Murine stem cell virus C2GnT (MSCV/C2GnT) retrovirus construction

Murine C2GnT cDNA was obtained from the plasmid pmC2–251 (Warren *et al.*, unpublished observations; GenBank accession U19265). A 1715 bp *Xho*1 fragment encoding the C2GnT coding region was cloned into the polylinker site of the retroviral vector pMSCV*neo*EB (version 2.1, Hawley *et al.*, 1993).

Key features of the MSCV vector include sequences for efficient LTR-directed expression in primitive cells, the ψ+ packaging region for high viral titer of mutant viruses (which do not produce gag-related polypeptides) and the absence of all envelope sequences. The neomycin phosphotransferase (*neo*) gene in pMSCV*neo*EB contains a synthetic initiation sequence for translation in mammalian cells, and is under the control of an internal phosphoglycerate kinase (pgk) promoter for expression in a wide range of cells. Plasmids were constructed using standard methods (Sambrook *et al.*, 1989), and the orientation of the C2GnT cDNA was assessed by restriction analysis.

Recombinant virus was produced by introducing the sense pMSCV*neo*EB/C2GnT (referred to as C2GnT throughout this study) construct or control pMSCV*neo*EB (referred to as MSCV) into the amphotropic helper-free packaging cell line PA317 (Miller and Buttimore 1986) by standard $CaPO_4$ transfection. Cells were maintained in DMEM (Stem Cell Technologies, Vancouver, British Columbia) supplemented with 10% (v/v) FCS, 2 mM Glutamine, and 5 μM 2-mercaptoethanol in a humidified atmosphere containing 5% CO_2 at 37°C. After 24 h, culture media were supplemented with G418 (800 μg/ml) and incubation continued until a stable G418 resistant cell population resulted. Viral titers of 1×10^4 CFU/ml were obtained when assayed on NIH 3T3 fibroblasts.

Viral titers were amplified by transferring filtered PA317 culture supernatant containing virus onto subconfluent monolayers of the ecotropic helper-free cell line ψ2 (Mann *et al.*, 1983) in fresh media supplemented with 4 μg/ml polybrene. Twenty-four hours post-infection, G418 selection was performed as described above. G418 resistant populations were subcloned by limiting dilution. MSCV and C2GnT virus producing ψ2 clones produced virus titers of 1×10^6 G418 resistant CFU/ml when assayed on NIH 3T3 cells.

Retroviral infection of EL-4 cells

EL-4 cells were maintained in DMEM (Stem Cell Technologies, Vancouver, British Columbia) supplemented with 10% (v/v) FCS, 2 mM glutamine, and 5 μM 2-mercaptoethanol in a humidified atmosphere containing 5% CO_2 at 37°C.

Supernatants were collected from confluent dishes of MSCV and C2GnT virus producing ψ2 clones, filtered (0.45 μm membrane), and added to EL-4 cells growing exponentially in media supplemented with 4 μg/ml polybrene. After 48 h, the cells were subcultured into media supplemented with G418 at 500 μg/ml. Stable G418 resistant populations were cloned by limiting dilution and analyzed for C2GnT activity and expression of O-glycosylated proteins.

Immunofluorescence

Cells were suspended in fluorescence-activated cell sorting (FACS) buffer containing 2% (v/v) FCS in PBS and incubated for 40 min at 4°C with antibodies in 96-well round-bottom plates (Nunclon, InterMed, Denmark). Cells were washed twice and analyzed on a FACScan IV flow cytometer (Becton Dickinson, Mountain View, CA). Data on glycoprotein expression is presented relative to the binding of control mAb 1F10 within the same experiment.

C2GnT assay

Cells were washed in PBS and lysed in 150 mM NaCl and 0.25% Triton X-100 with protease inhibitors at 4°C. The lysates were adjusted to 10 mg protein/ml and transferase assays carried out according to established protocols (Saitoh *et al.*, 1991; Bierhuizen and Fukuda, 1992). The reaction mixtures for the C2GnT assay contained 50 mM MES (pH 7.0), 0.5 μCi of UDP-[^{3}H]GlcNAc, 1 mM UDP-GlcNAc, 0.1 M GlcNAc, 1 mM Galβ1–3GalNAcα-pNp, and 25 μl of cell lysate in a total volume of 50 μl. The reaction mixtures were incubated for 2 h at 37°C, diluted to 5 ml in H_2O, and processed by C18 Sep-Pak (Waters) column chromatography. After washing with water, product was eluted using methanol and the complete eluates counted in a scintillation counter.

Cell lysate preparation and Western blotting

Cells were lysed in 50 mM HEPES pH7.2, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100, 2 mg/ml BSA, and a standard cocktail of protease inhibitors. Lysates were separated from nuclei and debris by centrifugation. Membrane fractions were prepared by sonicating the cells in the above buffer without Triton X-100 and membrane fractions were isolated by differential centrifugation. Affinity chromatography was performed using wheat germ agglutinin and elution with N-acetylglucosamine (0.3 M).

SDS–PAGE in 7.5% gels was performed under reducing conditions for analysis of CD43, CD45 and RPTPα and nonreducing conditions for analysis of CD44. After electrophoresis, samples were transferred to nitrocellulose and stained with Ponceau S to verify protein transfer and position of M.W. standards (Bio-Rad, Richmond, CA). Blots were treated overnight in a blocking solution containing 5% BSA and 1% ovalbumin in Tris-buffered saline (TBS) and then incubated with 2–10 μg/ml of either anti-CD44 (mAb KM 201), anti-CD43, anti-CD45, or anti-RPTPα-1 antibodies in TBS for 1 h. The blots were washed in TBS containing 0.05% NP-40 (TBSN) and then incubated with a 1:20,000 peroxidase conjugated secondary Ab followed by washing in TBSN and TBS prior to adding ECL reagent (Amersham Canada Ltd., Oakville, Ontario) for 1 min. Autoradiography was performed using XAR-5 x-ray film (Eastman Kodak, Rochester, NY).

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Abbreviations

C2GnT, Core2 N-acetylglucosaminyltransferase; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; PE, phycoerythrin; RPTPα, receptor protein tyrosine phosphatase α.

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