Eur. J. Biochem. *251*, 288–294 (1998)

Structural study of the O-linked sugar chains of human leukocyte tyrosine phosphatase CD45

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(Received 18 July/4 November 1997) – EJB 97 1026/5

The O-linked sugar chains of the human leukocyte cell surface glycoprotein CD45 were released as tritium-labeled oligosaccharides by *β*-elimination in the presence of NaB^3H_4. Mono Q column chromatography revealed that they comprise neutral (64%) and acidic (36%) oligosaccharides, the latter of which were converted to neutral ones by *Arthrobacter ureafaciens* sialidase treatment. Structural studies of each oligosaccharide fractionated on a Bio-Gel P-4 column by sequential exoglycosidase digestion and by methylation analysis revealed that human leukocyte CD45 contains mainly core 1 and core 2 oligosaccharides, 15% of which are modified with poly (*N*-acetyllactosamine) chains in different extensions. CD45 consists of several isoforms which were isolated after cell surface sialic acid residues were labeled by periodate/NaB^3H_4 treatment. Bio-Gel P-6 column chromatography of a mixture of the tritium-labeled glycopeptide/oligosaccharides obtained by pronase-digestion followed by mild alkaline borohydride treatment showed that distribution of the sialylated core 2 oligosaccharides is different among CD45 isoforms.

Keywords: human leukocyte; CD45; O-linked sugar chain.

CD45 is a receptor-like leukocyte cell surface glycoprotein with protein tyrosine phosphatase activity in its cytoplasmic domain [1–6]. Analysis of mature T-cell and B-cell lines deficient in the expression of CD45 revealed that the activation signals mediated by T-cell antigen receptor and B-cell antigen receptor are impaired [7–11]. Similarly, in CD45-gene disrupted mutant mice, the maturation of T cells was severely disturbed and the peripheral T cells failed to respond to T-cell-antigen-receptor–mediated signaling [12]. These results indicate that CD45 is indispensable for exerting antigen-specific activation of T and B cells. CD45 exists as multiple isoforms generated from the single gene by alternate splicing of exons which encode a part of the extracellular domain [13–16]. The expression of distinct CD45 isoforms varies among T-cell subgroups and through T-cell ontogeny, but all the isoforms contain the identical transmembrane and cytoplasmic domains [17–19]. The individual isoforms showed differences in the interaction with CD4-T-cell antigen receptor-complex [20] and in the activation of intracellular signals [21–24].

Recently, the carbohydrate moiety of CD45 isoforms has attracted considerable interest since it has been shown to be involved in specific cellular interactions. Terminal galactose residues of T-cell surface CD45 are recognized by galectin-1 expressed on the stromal cells of human thymus and lymph nodes, and may be involved in apoptosis of T-lineage cells [25]. Similarly, mannose residues of CD45 expressed on mouse immature thymocytes are recognized by the serum mannan-binding protein, and may be important for the development and maturation of thymocytes [26], presumably by interaction with DEC-205, a membrane-bound protein which is homologous to the macrophage mannose receptor, expressed on thymic dendritic cells [27]. The sialylated N-linked sugar chains of CD45 contain only *α*-2,6-linked sialic acid residues [28] and may be involved in cell adhesion mediated by the Ig superfamily member CD22 [29], a sialic-acid-binding lectin [30]. Binding studies of oligosaccharides with different sialylation levels to a column immobilized with CD22-IgG chimeric protein revealed that CD22 has higher affinity toward highly sialylated complex-type oligosaccharides [31]. Structural studies of the N-linked sugar chains of CD45 from human peripheral blood leukocytes revealed that they are mostly of complex-type with highly branched tri- and tetra-antennary structures and poly(*N*-acetyllactosamine) units [28].

CD45 also contains O-linked sugar chains, most of which are distributed in the domains encoded by exons 4–6, the alternative splicing of which generates eight different isoforms [13–16]. Because the expression pattern of CD45 isoforms is different among T-cell subgroups or functions and because CD45-associated carbohydrates seem to be important for cellular functions [25, 26, 29], we have determined the structures of O-linked sugar chains attached to CD45 isolated from human peripheral blood leukocytes.

EXPERIMENTAL PROCEDURES

Chemicals. Diplococcal *β*-galactosidase, diplococcal and jack bean *β*-*N*-acetylhexosaminidase were obtained from Seika-

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Note. The subscript OT is used to indicate an NaB^3H_4-reduced oligosaccharide. All sugars mentioned in this paper have the D-configuration except for fucose which has the L-configuration.

gaku Kogyo Co. NaB^{3}H$_4$ (490 mCi/mmol) was purchased from Du Pont-New England Nuclear. *Arthrobacter ureafaciens* sialidase was obtained from Nacalai Tesque Co. *Psathyrella velutina* lectin-Affi-Gel 10 was kindly donated by Dr N. Kochibe, University of Gunma, Maebashi, Japan.

Purification of CD45. Packed human buffy coat cells, which are enriched in T cells, were prepared by Ficoll-Isopaque gradient centrifugation from pooled human blood supplied by the Finnish Red Cross Blood Transfusion Service, Helsinki. The cells were homogenized using a Potter-Elvehjem homogenizer in 10 mM sodium phosphate, pH 7.4 containing 0.15 M NaCl, 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride. The cell homogenates were centrifuged at 20 000×*g* for 15 min, and the resulting supernatants were further centrifuged at 100 000×*g* for 45 min. CD45 was purified from the final supernatants by affinity chromatography using a column containing the anti-CD45 monoclonal antibody MEM-28 immobilized to Sepharose 4B. The MEM-28 antibody has previously been characterized [32]. The column was washed with 20 mM glycine/NaOH, pH 9.0, containing 0.1% sodium deoxycholate to remove nonspecifically absorbed proteins. The bound material was eluted with 50 mM diethylamine, pH 11.5. The eluates were neutralized and salts were removed by ultrafiltration. CD45 thus prepared contained four bands migrating with apparent molecular masses of 180 kDa, 190 kDa, 205 kDa and 220 kDa as determined by SDS/PAGE [33] followed by autoradiography (Fig. 5).

Liberation of O-linked sugar chains from CD45. The CD45 preparation (0.8 mg) was dissolved in 0.5 ml of 0.05 M NaOH containing 0.5 M NaBH$_4$ and 12.5 mCi NaB^{3}H$_4$, and incubated at 45 °C for 20 h according to the previously published method [34] with modification. After acidification with acetic acid, the reaction mixture was passed through an AG50 W X 12 (H$^+$) column and the effluent was evaporated. Boric acid in the residue was removed by repeated evaporation with methanol. The residue was subjected to paper chromatography using a solvent of 1-butanol/ethanol/water (4:1:1, by vol.). The area of the paper from the origin to the position at which authentic *N*-acetylgalactosaminitol migrated was extracted with water.

Surface-labeling of sialic acid residues on human peripheral blood leukocytes. Human peripheral blood leukocytes were surface-labeled by the NaIO$_4$/NaB^{3}H$_4$ method [35], which specifically labels sialic acid residues. In brief, the cells (0.5−1×10^8) were suspended in 0.5 ml 10 mM sodium phosphate, pH 7.4, containing 0.15 M NaCl (NaCl/P$_i$) and treated with 10 ml of 0.1 M NaIO$_4$ solution at 0 °C for 10 min. The cells were then washed several times with cold buffer, and the pellet was incubated with 10 ml of 0.01 M NaOH containing 500 µCi of NaB^{3}H$_4$ at room temperature for 5 min. After extensive washing, the cells were lysed with NaCl/P$_i$ containing 1% Triton X-100 and the cell lysates were immunoprecipitated with MEM-28 monoclonal antibody as described previously [36]. The CD45 isoforms were purified by preparative SDS/PAGE. A mixture of the tritium-labeled CD45 isoforms and each isoform were subjected to pronase digestion and then mild alkaline borohydride treatment [34]. The resulting tritium-labeled glycopeptide/oligosaccharide mixtures were analyzed on a Bio-Gel P-6 column equilibrated with 0.1 M ammonium carbonate solution containing 0.1% SDS.

Analytical methods. The radioactive oligosaccharides were applied to a Mono Q column equilibrated with 5 mM acetate, pH 4.0, and eluted with a 5 mM−300 mM acetate, pH 4.0, gradient. Fractionation of the radioactive oligosaccharides by Bio-Gel P-4 column chromatography was performed as described previously [37]. Sugar composition analysis was performed according to the method described previously [38]. Lectin column chromatography using immobilized *P. velutina* lectin-Affi-Gel 10 was performed as described previously [39]. In brief, samples on the column were eluted at room temperature with 10 mM Tris/HCl, pH 7.4, containing 0.1 M NaCl and 1 mM each of CaCl$_2$, MgCl$_2$ and MnCl$_2$, and allowed to incubate at room temperature for 30 min. Oligosaccharides bound to the column were eluted with the same buffer containing 1 mM *N*-acetylglucosamine. Methylation analysis of oligosaccharides, which were released from the protein by treatment with 0.05 M NaOH/1 M NaBH$_4$, was conducted as described previously [40].

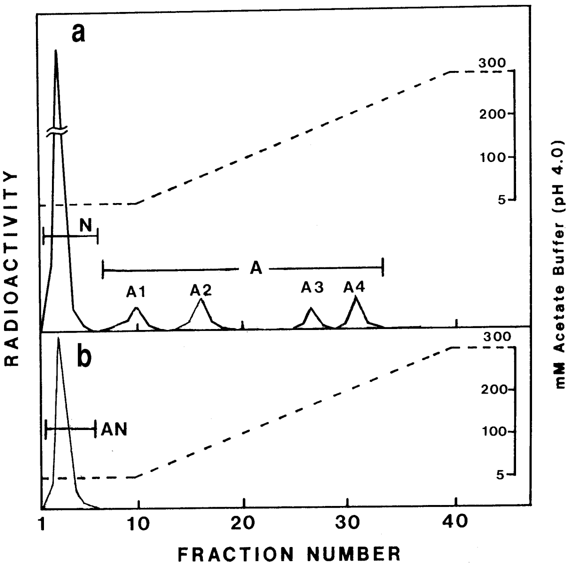

Fig. 1. Mono Q column chromatography of oligosaccharides released from CD45 by alkaline borotritide/borohydride treatment (a), and of sialidase digests of the acidic oligosaccharides as indicated by bar A in (a) (b).

Oligosaccharides. Tritium-labelled *N*-acetylgalactosaminitol, lactitol and sialyl lactitol were prepared from *N*-acetylgalactosamine, lactose and sialyl lactose by reduction with NaB^{3}H$_4$. The origins of the following oligosaccharides were as follows: Neu5Acα2→3Galβ1→3(Neu5Acα2→6)GalNAc$_{\mathrm{OT}}$ (Neu5Ac · Gal · Neu5Ac · GalNAc$_{\mathrm{OT}}$) and Neu5Acα2→3Galβ1→3GalNAc$_{\mathrm{OT}}$ (Neu5Ac · Gal · GalNAc$_{\mathrm{OT}}$) were prepared from glycophorin and fetuin according to the method described previously [41], and Galβ1→3GalNAc$_{\mathrm{OT}}$ (Gal · GalNAc$_{\mathrm{OT}}$) was from those digested with *A. ureafaciens* sialidase. Galβ1→4GlcNAcβ1→3-Galβ1→3GalNAc$_{\mathrm{OT}}$ (Gal · GlcNAc · Gal · GalNAc$_{\mathrm{OT}}$) was obtained from porcine *zona pellucida* glycoproteins [42].

RESULTS

Fractionation of oligosaccharides by Mono Q column chromatography. The radioactive oligosaccharides obtained from CD45 by alkaline borohydride/borotritide treatment were separated into a neutral (N) and an acidic (A) fraction (Fig. 1a). The percentage molar ratios of fractions N and A calculated from their radioactivities were 64% and 36%, respectively. The acidic fraction contained four main peaks A1, A2, A3 and A4 (Fig. 1a). Under the identical conditions, standard oligosaccharides, Neu5Acα2→3[Galβ1→4GlcNAcβ1→6(Galβ1→3)]GalNAc$_{\mathrm{OT}}$, Neu5Acα2→6(Galβ1→3)GalNAc$_{\mathrm{OT}}$, Neu5Acα2→3Galβ1→4-GlcNAcβ1→6(Neu5Acα2→3Galβ1→3)GalNAc$_{\mathrm{OT}}$, and Neu5Acα2→6(Neu5Acα2→3Galβ1→3)GalNAc$_{\mathrm{OT}}$, were reported to be eluted at the same positions as peaks A1, A2, A3 and A4, respectively [43]. When fraction A was digested with *A. ureafaciens* sialidase, all of the acidic oligosaccharides were converted

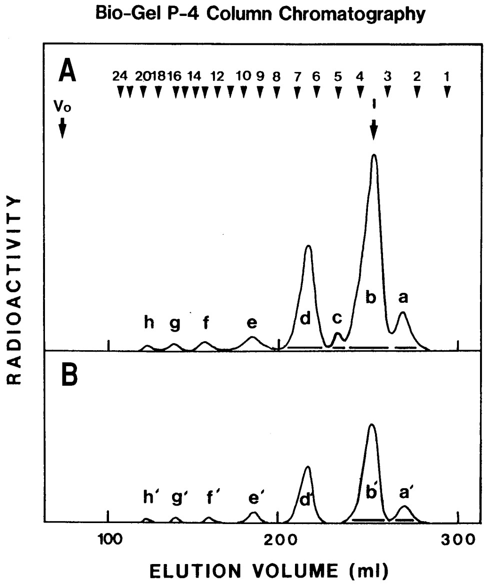

Fig. 2. Bio-Gel P-4 column chromatography of neutral (A) and sialidase-treated acidic (B) oligosaccharides. Arrow-heads at the top of the figure indicate elution positions of glucose oligomers used as internal standards, and the numbers indicate glucose units. A bold arrow indicates the elution position of an authentic oligosaccharide, Galβ1→3GalNAc$_{\mathrm{OT}}$.

Table 1. Methylation analysis of O-linked sugar chains from CD45. Molar ratios were calculated by taking the value of the total 2-*N*-methylacetamido-2-deoxygalactitol as 1.0.

Methylated sugars	Molar ratio of	
	peaks b + b′	peaks d + d′
Galactitol		
2,3,4,6-Tetra-O-methyl-1,5-di-O-acetyl	1.2	1.9
2,3,4,6-Tri-O-methyl-1,3,5-tri-O-acetyl	–	0.3
2-N-Methylacetamido-2-deoxyglucitol		
3,6-Di-O-methyl-1,4,5-tri-O-acetyl	–	1.2
2-N-Methylacetamido-2-deoxygalactitol		
1,4,5,6-Tetra-O-methyl-3-monoO-acetyl	1.0	0.2
1,4,5-Tri-O-methyl-3,6-di-O-acetyl	–	0.8

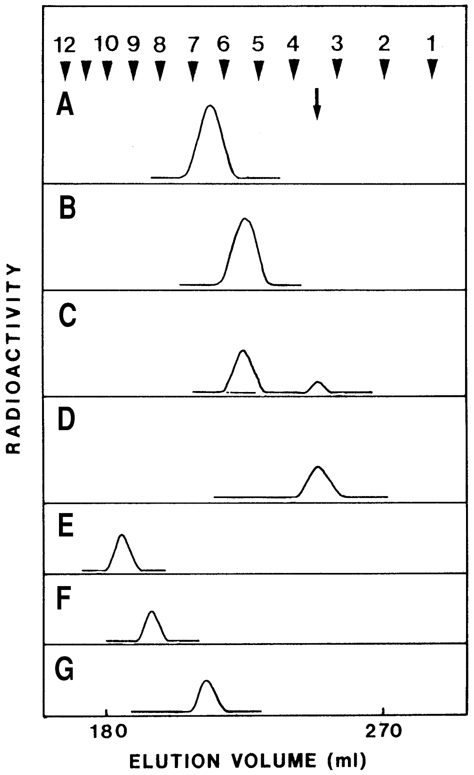

Fig. 3. Sequential exoglycosidase digestion of peaks d and e. The oligosaccharides and their digestion products were analyzed by Bio-Gel P-4 column chromatography: (A) peak d in Fig. 2A; (B) peak d in (A) digested with diplococcal β-galactosidase; (C) the peak in (B) digested with diplococcal β-*N*-acetylhexosaminidase; (D) the peak indicated by a bar in (C) digested with jack bean β-*N*-acetylhexosaminidase; (E) peak e in Fig. 2A; (F) peak e in (E) digested with diplococcal β-galactosidase; (G) the peak in (F) digested with diplococcal β-*N*-acetylhexosaminidase. The arrowheads and bold arrow at the top of the figure are the same as in Fig. 2.

to neutral ones (AN) (Fig. 1b). Therefore, the acidic nature of the oligosaccharides could be ascribed to their sialic acid residues.

Fractionation of oligosaccharides by Bio-Gel P-4 column chromatography. Oligosaccharides in fractions N and AN were subjected to Bio-Gel P-4 column chromatography. Oligosaccharides in fraction N were separated into eight peaks (named a–h, respectively) with elution positions of 2.5, 3.5, 5.0, 6.5, 9.5, 12.5, 15.7 and 18.7 glucose units (Fig. 2A). Oligosaccharides with the same elution positions as peaks a, b, d, e, f, g and h were also obtained from fraction AN (named a′, b′, d′, e′, f′, g′ and h′, respectively, Fig. 2B), suggesting that they are desialylated forms of the peaks in Fig. 2A.

Structures of oligosaccharides. When peaks a and a′, which contained 6.9% and 2.5% of the total oligosaccharides, respectively, were applied to a Shodex SP-1010 column for the analysis of sugar compositions, the radioactivity was eluted with *N*-acetylgalactosaminitol (data not shown), indicating that peaks a and a′ are *N*-acetylgalactosaminitol.

Peaks b and b′ contained 36.9% and 13.2% of the total oligosaccharides, respectively, and were eluted at the same position as Galβ1→3GalNAc$_{\mathrm{OT}}$ (Fig. 2A and B, respectively). Methylation analysis of a mixture of peaks b and b′ showed the presence of 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetyl galactitol and 1,4,5,6-tetra-*O*-methyl-3-mono-*O*-acetyl 2-*N*-methylacetamido-2-deoxygalactitol with almost the same molar ratio (Table 1). These results indicated that oligosaccharides b and b′ are Gal1→3GalNAc$_{\mathrm{OT}}$.

Methylation analysis of a mixture of peaks d and d′, which contained 20.1% and 6.7% of the total oligosaccharides, respectively, showed the presence of 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetyl galactitol and 2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetyl galactitol, 3,6-di-*O*-methyl-1,4,5-tri-*O*-acetyl-2-*N*-methylacetamido-2-deoxyglucitol, and 1,4,5,6-tetra-*O*-methyl-3-mono-*O*-acetyl 2-*N*-methylacetamido-2-deoxygalactitol and 1,4,5-tetra-*O*-methyl-3,6-di-*O*-acetyl 2-*N*-methylacetamido-2-deoxygalactitol in the ratio of 9.5:1.5:6:1:4 (Table 1). When peak d in Fig. 2A was digested with diplococcal β-galactosidase, one galactose residue was released and the product was eluted at 5.5 glucose units (Fig. 3B). The peak in Fig. 3B was then digested with diplococcal β-*N*-acetylhexosaminidase, and 20% of it was eluted at 3.5 glucose units releasing one *N*-acetylglucosamine residue, while the remaining 80% was resistant to the enzyme digestion (Fig. 3C). The peak indicated by a bar in Fig. 3C was digested

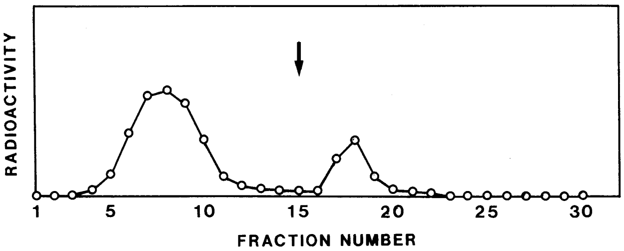

Fig. 4. *P. velutina* lectin-Affi-Gel 10 column chromatography of β-galactosidase–treated oligosaccharide d. The products shown in Fig. 3B were applied to a *P. velutina* lectin-Affi-Gel 10 column. An arrow indicates the position where the elution buffer was changed to buffer containing the haptenic sugar.

with jack bean β-*N*-acetylhexosaminidase, and the product was eluted at 3.5 glucose units (Fig. 3D). The peaks eluted at 3.5 glucose units in Fig. 3C and D were shown to be Gal1→3GalNAc$_{\mathrm{OT}}$ by methylation analysis (data not shown). When the peak in Fig. 3B was subjected to *P. velutina* lectin-Affi-Gel 10 column chromatography, 80% of the oligosaccharide was eluted in the retarded fraction and the remaining 20% was bound and eluted with 1 mM *N*-acetylglucosamine (Fig. 4). Since GlcNAcβ1→6(Galβ1→3)Galβ1→4Glc$_{\mathrm{OT}}$ is eluted in the retarded fraction and GlcNAcβ1→3Galβ1→4Glc$_{\mathrm{OT}}$ is bound and eluted with 1 mM *N*-acetylglucosamine from a *P. velutina* lectin-Affi-Gel 10 column [39], peak d should contain the branched and unbranched oligosaccharides. Similar results were obtained from peak d′ in Fig. 2B (data not shown).

These results indicated that peaks d and d′ contain two oligosaccharides: Galβ1→4GlcNAcβ1→6(Galβ1→3)GalNAc$_{\mathrm{OT}}$ and Galβ1→4GlcNAcβ1→3Galβ1→3GalNAc$_{\mathrm{OT}}$ with the molar ratio of 4:1.

When peak e (Fig. 3E), which contained 3.4% of the total oligosaccharides, was digested with diplococcal β-galactosidase followed by diplococcal β-*N*-acetylhexosaminidase, the product was eluted at 6.5 glucose units releasing one galactose residue (Fig. 3F) and one *N*-acetylglucosamine residue (Fig. 3G). Sequential exoglycosidase digestion revealed that the peak in Fig. 3G is the same as that in Fig. 3A. Similar results were also obtained from peak e′ in Fig. 2B, which contained 1.3% of the total oligosaccharides.

These results indicated that oligosaccharides included in peaks e and e′ in Fig. 2 contain the following structures:

Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1↘
6
GalNAc$_{\mathrm{OT}}$
3
Galβ1↗

and

Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→3GalNAc$_{\mathrm{OT}}$

Peaks f, g and h in Fig. 2A, which contained 2.5, 2.0 and 1.2% of the total oligosaccharides, respectively, were combined and digested repeatedly with diplococcal β-galactosidase and then with diplococcal β-*N*-acetylhexosaminidase. About 80% of them were eluted at the positions of 5.5 and 3.5 glucose units, which corresponded to GlcNAcβ1→6(Galβ1→3)GalNAc$_{\mathrm{OT}}$ and Galβ1→3GalNAc$_{\mathrm{OT}}$, with the molar ratio of 4:1 (data not shown), indicating that the oligosaccharides in peaks f, g and h have the same core structures as those of peak d and contain two, three and four repeats of poly(*N*-acetyllactosamine) unit. The remaining 20% of the oligosaccharides in peaks f, g and h were not converted to the core structures by digestion with these enzymes. Similar results were obtained from the combined oligosaccharides in peaks f′, g′ and h′ in Fig. 2B, which contained 0.6, 0.5 and 0.3% of the total oligosaccharides, respectively.

Table 2. Proposed structures of neutral (N) and desialylated-neutral (AN) O-linked sugar chains of CD45. Peaks f, f′, g, g′, h and h′ show their major oligosaccharides.

Fractions	Structures	Molar ratio of	
		N	AN
		%	
a and a′	GalNAc$_{\mathrm{OT}}$	6.9	2.5
b and b′	Galβ1→3GalNAc$_{\mathrm{OT}}$	36.9	13.2
c	n.d.	1.9	[a]
d and d′	Galβ1→4GlcNAcβ1↘ 6 GalNAc$_{\mathrm{OT}}$ 3 Galβ1↗	16.1	5.3
	Galβ1→4GlcNAcβ→3Galβ1→3GalNAc$_{\mathrm{OT}}$	4.1	1.3
e and e′	Galβ1→4GlcNAcβ1→3 (d and d′)	3.4	1.3
f and f′	(Galβ1→4GlcNAcβ1→3)$_2$ (d and d′)	2.5	0.6
g and g′	(Galβ1→4GlcNAcβ1→3)$_3$(d and d′)	2.0	0.5
h and h′	(Galβ1→4GlcNAcβ1→3)$_4$(d and d′)	1.2	0.3

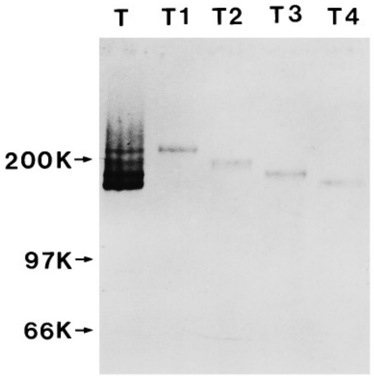

Fig. 5. Autoradiogram of CD45 isoforms isolated by preparative SDS/PAGE. Lane T contained a mixture of CD45 isoforms (8000 cpm) purified from peripheral blood leukocytes, and lanes T1−T4 indicate purified individual CD45 isoforms (1000−1400 cpm). The positions of molecular-mass markers are shown.

Small radioactive peaks were also eluted from a Bio-Gel P-4 column periodically by 3 glucose units at the positions larger than 20 glucose units (Fig. 2). Although the exact structures of the contents of these peaks were not determined due to the limited amounts of the samples available for the analysis, the periodic elution patterns of the oligosaccharides started from peaks d and d′ in Fig. 2, indicated that they contain different numbers of poly(*N*-acetyllactosamine) units.

The oligosaccharide structure of peak c was not determined due to the lack of sufficient material.

The proposed oligosaccharide structures of human leukocyte CD45, as determined by sequential exoglycosidase digestion and methylation analysis, are summarized in Table 2.

Bio-Gel P-6 column chromatography of glycopeptides/oligosaccharides from CD45 isoforms. The CD45 molecules were immunoprecipitated from the surface-labeled leukocyte lysates and subjected to preparative SDS/PAGE. Our leukocyte preparation contained four CD45 isoforms with apparent molecular

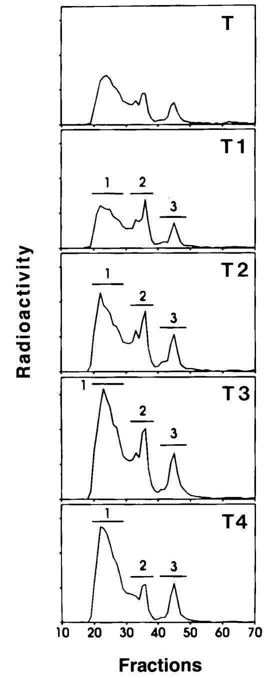

Fig. 6. Bio-Gel P-6 column chromatography of a mixture of tritium-labeled glycopeptides and oligosaccharides obtained from a mixture and individual isoforms of CD45. Samples containing 9000−16000 cpm were applied to the column. The bars 1, 2 and 3 indicate the elution positions of N-linked oligosaccharides, O-linked oligosaccharides with tetra-pentasaccharides and O-linked oligosaccharides with di-trisaccharides, respectively.

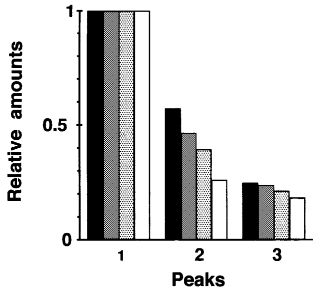

Fig. 7. Diagram of the relative amounts of tritium-labeled glycopeptides/oligosaccharides from each CD45 isoform. The relative amounts were calculated by taking the amount of peak 1 as 1.0 in each isoform. The solid, hatched, spotted and open bars indicate the oligosaccharides derived from T1, T2, T3 and T4, respectively.

masses of 180, 190, 205 and 220 kDa (Fig. 5 lane T). Each CD45 isoform was extracted from the gel and again subjected to analytical SDS/PAGE. Lanes T1−T4 in Fig. 5 show that each CD45 isoform was isolated in a radiochemically pure form.

The mixed and individual isoforms of CD45 were then digested with pronase followed by mild alkaline/borohydride treatment. To avoid generation of glycopeptides with a single amino acid residue which are resistant to alkaline treatment [44], samples were digested mildly with pronase. The resulting glycopeptide/O-linked sugar chain mixtures were analyzed by Bio-Gel P-6 column chromatography. In all samples, they were separated into three peaks 1, 2 and 3 as indicated in bars in Fig. 6. The elution positions of standard oligosaccharides indicated that peak 1 contains mainly N-linked oligosaccharides and O-linked oligosaccharides larger than hexasaccharide, the latter of which may correspond to sialylated forms of peaks e′ to h′ in Fig. 2B, and peaks 2 and 3 contain mainly O-linked oligosaccharides with tetra-pentasaccharides and di-trisaccharides, respectively.

Although exact N-glycosylation sites have not been elucidated in CD45, more potential N-glycosylation sites are found in the extracellular domains other than those encoded by exons 4−6 [4]. When taking the amount of peak 1 in each isoform as 1.0, the relative amounts of peaks 2 decreased as the molecular masses of the isoforms decreased, while those of peaks 3 were constant among the isoforms (Fig. 7). These results indicated that each CD45 isoform contains a different amount of sialylated core 2 oligosaccharides.

DISCUSSION

CD45 is a heavily glycosylated transmembrane protein with tyrosine phosphatase activity, and is involved in T-cell-receptor−mediated signaling [7−12]. CD45 is expressed as multiple isoforms (180−220 kDa) denoted as RA, RB, RC and RO [4, 5], which are generated by alternative splicing of exons 4−6, and their expression patterns are cell-type and differentiation-stage specific. The putative O-glycosylation sites are clustered in the domains encoded by exons 4−6, which are absent in the smallest form [4−6]. The present study indicates that these domains contain more sialylated O-linked sugar chains with core 2 structure, because twice as many O-linked sugar chains with core 2 structure were present on CD45T1 (220 kDa) which contains all these domains than CD45T4 (180 kDa) which does not have such domains. This differential distribution of the sialylated oligosaccharides could be important for CD45-specific or isoform-specific functions. The expression of sialylated O-linked sugar chains with core 2 structure has been reported to increase in CD43 of peripheral blood leukocytes from healthy individuals by activation with phytohaemagglutinin or anti-CD3 antibody and of peripheral blood leukocytes from patients with Wiskott-Aldrich syndrome [45]. Therefore, further work is necessary to elucidate how O-linked sugar chains with core 2 structure relate to the functions of these molecules.

Some monoclonal antibodies raised against CD45 recognize carbohydrate epitopes. The UCHL-1 antibody binds to sialylated O-linked sugar chains enriched in CD45RO [23]. Possibly, antibodies raised against CD45RA may recognize the carbohydrates because they reacted differently with CD45 molecules expressed on T and B cells [46]. Differences in glycosylation of CD45 between T and B cells have been described, where the reactivities with lectins [47, 48], anti-carbohydrate antibodies [49] and glycosidases [50, 51] were quite different. The O-linked sugar chains of B cell CD45 reacted more strongly with anti-I and anti-i antibodies and were more susceptible to digestion with endo-*β*-galactosidase than those of T-cell CD45 [50], indicating that B-cell CD45 contains more O-linked sugar chains with poly(*N*-acetyllactosamine). The present study showed that the O-linked sugar chains of CD45 isolated from human buffy coat cells, which are enriched in T cells, also contain poly(*N*-acetyllactosamine) mainly present on the core 2 structure, the amount of which is about 15% of the total oligosaccharides. Quite interestingly, the amounts of the oligosaccharides with poly(*N*-acetyllactosamine) repeats decreased with an increase in the molecular size of the O-linked sugar chains. Since not all of the poly(*N*-acetyllactosamine) were susceptible to digestion with a mixture of diplococcal *β*-galactosidase and diplococcal *β*-*N*-acetylhexosaminidase, and since a small amount of 2,3,4-tri-*O*-methy-1,5-di-*O*-acetyl fucitol was detected by methylation analysis of the whole CD45 O-linked sugar chains (data not shown), some of the poly(*N*-acetyllactosamine) chains could be fucosylated and, therefore, resistant to the digestion with the enzymes. Furthermore, poly-*N*-acetyllactosamine was also found to be associated with the core 1 structure present in oligosaccharides d, d′, e and e′, and most possibly in oligosaccharides f, f′, g, g′, h and h′. These results clearly demonstrated the presence of i-antigenic structures in CD45 O-linked sugar chains. In addition, when a mixture of oligosaccharides f, f′, g, g′, h and h′ were subjected to *P. velutina* lectin-Affi-Gel 10 column chromatography, about 10% of them was retarded in the column after washing with 1 mM *N*-acetylglucosamine only when they were initially treated with diplococcal β-galactosidase. Since GlcNAcβ1→6(GlcNAcβ1→3)Galβ1→4Glc$_{OT}$ binds to a *P. velutina* lectin-immobilized column and is retarded in the column after washing with 1 mM *N*-acetylglucosamine [37], the high-molecular-mass O-linked sugar chains may also contain I-antigenic structures.

Sialic acid residues of CD45 have been shown to interact with CD22, a sialic-acid-binding lectin [29, 30], expressed on mature B cells, thus promoting T-B cell interaction [52]. CD22 recognizes an α-2,6-linked sialic acid and binds more strongly to the multiply sialylated complex-type than the mono-sialylated oligosaccharides [31]. Structural analysis of the N-linked sugar chains of CD45 from human peripheral blood revealed that the major oligosaccharides are of tri-antennary and tetra-antennary complex-type with and without poly(*N*-acetyllactosamine) [28]. However, most of them were at most disialylated as determined by the electrophoretic mobility of them, and may not interact well with CD22. None of these N-linked oligosaccharides interacted with CD22 (unpublished results). CD22 also binds clustered O-linked sugar chains with Neu5Acα2→6GalNAc structure. The estimated amount of Neu5Acα2→6($\pm$Galβ1→3)-GalNAc in the O-linked sugar chains of CD45 from human peripheral blood leukocytes is less than 10% of them. Although these O-linked sugar chains constitute a relatively small proportion of the total sugar chains, they may appreciably contribute to the binding to CD22. Previous studies have shown that the O-glycosylation will extend the structure of CD45 molecule [53, 54] and the clustering of the O-linked sugar chains will increase an affinity toward their receptors [55]. Therefore, the localization and clustering of the O-linked sugar chains at the defined N-terminal part of the CD45 external domain may increase and enhance the interaction between the carbohydrate ligands on CD45 and their receptors.

The present work was supported by Grants-in-Aid for Scientific Research (06680578) and that for Priority Areas (09240104) from the Ministry of Education, Science, Sports and Culture of Japan, and by research grants from the Academy of Finland, the Finnish Cancer Society, the Sigrid Juselius Foundation and the Emil Aaltonen Foundation.

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