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Phil. Trans. R. Soc. Lond. B 1993 **342**, 43-50
doi: 10.1098/rstb.1993.0133

Glycosylation of CD4 and Thy-1

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SUMMARY

The site-specific glycosylation of soluble recombinant variants of human and rat CD4 (sCD4) expressed in Chinese hamster ovary (CHO) cells has been characterized. The presence of identical oligosaccharides at the conserved glycosylation site in domain 3 of rat and human sCD4 and the greater abundance of oligomannose and hybrid type glycans at the non-conserved glycosylation site of rat sCD4 clearly indicate that the protein structure influences oligosaccharide processing. Comparisons of rat sCD4 glycopeptides with mutant molecules with only single glycosylation sites and with a truncated form containing only the two NH_2-terminal domains, indicate that independent processing occurs at each glycosylation site and that domain interactions can also affect oligosaccharide processing. These and other analyses of sCD2 expressed in CHO cells and Thy-1 purified from various tissues suggest that the diversity of oligosaccharide structures on a protein is regulated by the location of the glycosylation sites and the nature of the target protein, cell and tissue. The functional significance of this control remains to be determined.

1. INTRODUCTION

A fundamental characteristic of most leucocyte surface antigens is that they are glycoproteins. Few of the antigens are defined by monoclonal antibodies that recognize carbohydrate epitopes although it is known that different leucocyte populations differ in antigen glycosylation (Carlsson *et al.* 1986; Fukuda *et al.* 1986). These differences may be of functional importance since the surface glycoproteins are often well-suited for the display of potential epitopes to natural lectins. The selectins, for example, are now established as important receptors in determining the first interactions between endothelial cells and leucocytes (Lawrence & Springer 1991). It is clear that cell type specificity of glycosylation is crucial in these functions. A further important step is the determination of which surface glycoproteins express which carbohydrate structures followed by a knowledge of which molecules present carbohydrate accessible for cellular interactions.

There are therefore three levels at which understanding of protein glycosylation has assumed significance. Firstly, the available repertoire of glycosylation processing enzymes for the particular tissue or cell-type, secondly the physical characteristics of the glycoprotein being processed and thirdly the local conformation at the individual glycosylation sites. In this context we review our recent results obtained with two T cell markers, namely CD4 and CD2 (Ashford *et al.* 1993; Davis *et al.* 1993), together with another member of the immunoglobulin superfamily, Thy-1 (Parekh *et al.* 1987; Williams *et al.* 1993). The last named antigen is a major cell surface glycoprotein of rodent thymocytes but is also expressed abundantly in brain where it has been studied in detail.

2. CD4 AND CD2

CD4 is expressed on most thymocytes and on approximately two thirds of peripheral T lymphocytes (Parnes 1989). The extra-cellular portion of CD4 contains four immunoglobulin superfamily (IgSF) domains alternating between the V- (first and third) and C2-type (second and fourth) domains (figure 1). Rat and human CD4 share a conserved N-glycosylation site in the third domain at Asn 270/271. Each also has a second non-conserved glycosylation site at Asn 159 of the second domain in rat CD4 and at Asn 300, in the fourth domain in human CD4 (Maddon *et al.* 1985; Clark *et al.* 1987). A third glycosylation site in rat CD4 (Asn 365) is believed not to be glycosylated in view of its close proximity to the transmembrane region of the molecule, and is not included in the soluble rat CD4 variant.

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Phil. Trans. R. Soc. Lond. B (1993) **342**, 43–50
Printed in Great Britain

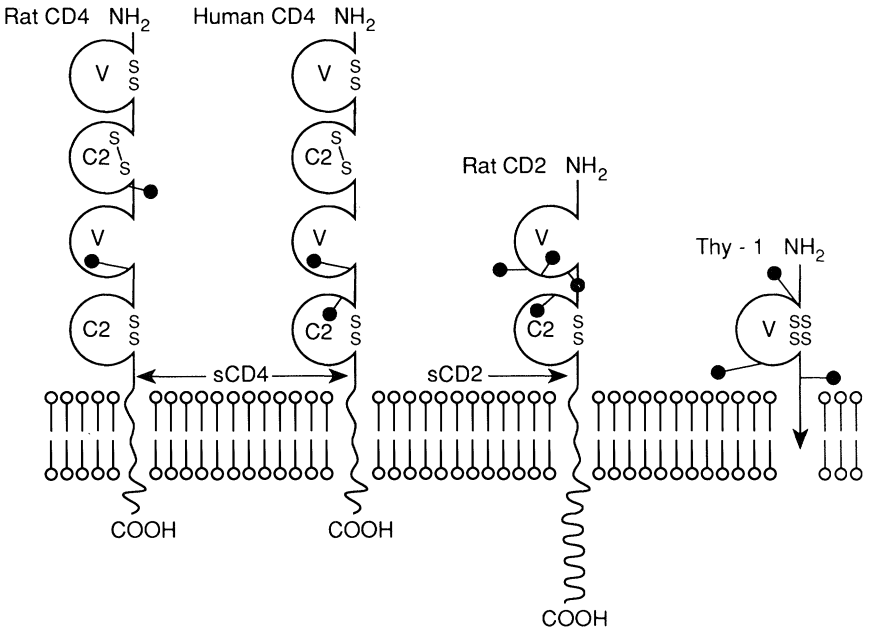

Figure 1. Schematic drawings of rat and human CD4, rat CD2 and Thy-1. The molecules are drawn with the circles representing immunoglobulin superfamily (IgSF) domains and the 'lollipops' N-linked oligosaccharides. The glycosylphosphatidylinositol membrane anchor of Thy-1 is depicted as a vertical arrow. The IgSF domains are designated as V set (V) or C2 set (C2) on the basis of sequence analysis (Williams *et al.* 1989). The positions of the mutations introduced in the CD4 and CD2 molecules to produce the recombinant soluble forms are indicated by horizontal arrows.

CD2, which is expressed on virtually all T lymphocytes, NK cells and thymocytes (Williams *et al.* 1987; Beyers *et al.* 1989; Bierer & Burakoff 1989), possesses two extra-cellular IgSF domains equivalent to the first and second domains of CD4. The NH_2-terminal domain shows a V-like fold while the second domain is C2-like (Lang *et al.* 1988). There are four potential N-glycosylation sites in rat CD2, three in domain 1 and one in domain 2 (figure 1) (Williams *et al.* 1987).

Soluble recombinant forms of human and rat CD4 and rat CD2 were expressed in Chinese hamster ovary (CHO) cells (Davis *et al.* 1990, 1993). The glycosylation potential in CHO cells has been well characterized (Sasaki *et al.* 1987; Kagawa *et al.* 1988; Takeuchi *et al.* 1988, 1989; Parekh *et al.* 1989; Spellman *et al.* 1989; Smith *et al.* 1990; Davidson & Castellino 1991) and will allow processing to multi-antennary and poly-N-acetyllactosamine oligosaccharides. However, despite the available repertoire of processing enzymes in this cell line the N-linked glycans of rat and human soluble CD4 (sCD4) expressed therein showed restrictions in size and type. Most of the oligosaccharides were of the biantennary complex, hybrid or oligomannose type (Ashford *et al.* 1993). The chromatographic profiles are shown in figure 2 and typical oligosaccharide structures in figure 3. These results were supported by similar findings from other studies of the glycosylation of human sCD4 expressed in CHO cells (Carr *et al.* 1989; Harris *et al.* 1990; Yuen *et al.* 1990; Spellman *et al.* 1991) and indicate the importance of the protein contribution in determining glycosylation.

In contrast, rat soluble CD2 (sCD2) expressed in CHO cells shows typical glycosylation of this cell line, with mainly biantennary complex structures and also tri- and tetra-antennary and poly-N-acetyllactosamine species (figures 2 and 3) (Davis *et al.* 1993). Thus the overall degree of processing is greater than in sCD4. As CD2 is structurally very similar to the first two domains of CD4 (Jones *et al.* 1992) the general three-dimensional conformation of these members of the immunoglobulin superfamily cannot be the only factor influencing their glycosylation. Clearly the local amino acid sequence and micro-environment of the glycosylation site must also be an important determinant.

The site specificity of glycosylation in CD4 was probed in detail through the preparation of a series of glycosylation variants of the rat soluble form (Davis *et al.* 1990). A comparison was made of intact mutant rat sCD4 molecules containing either the conserved (Asn 270) or the non-conserved (Asn 159) glycosylation sites with the corresponding rat sCD4-derived glycopeptides (Ashford *et al.* 1993). The results indicated that specific and independent processing occurred at each glycosylation site. Thus the glycosylation patterns for a particular site showed a strong similarity between the glycopeptide of the wild-type molecule and its corresponding glycosylation variant (figure 4). In particular, oligomannose and hybrid structures were restricted to the non-conserved site in rat sCD4. Glycosylation at the conserved site was characterized by exclusively biantennary complex oligosaccharides. This was identical to that reported for the equivalent site in human sCD4 (Spellman *et al.* 1991). Therefore overall differences in glycosylation between the rat and human glycoproteins were

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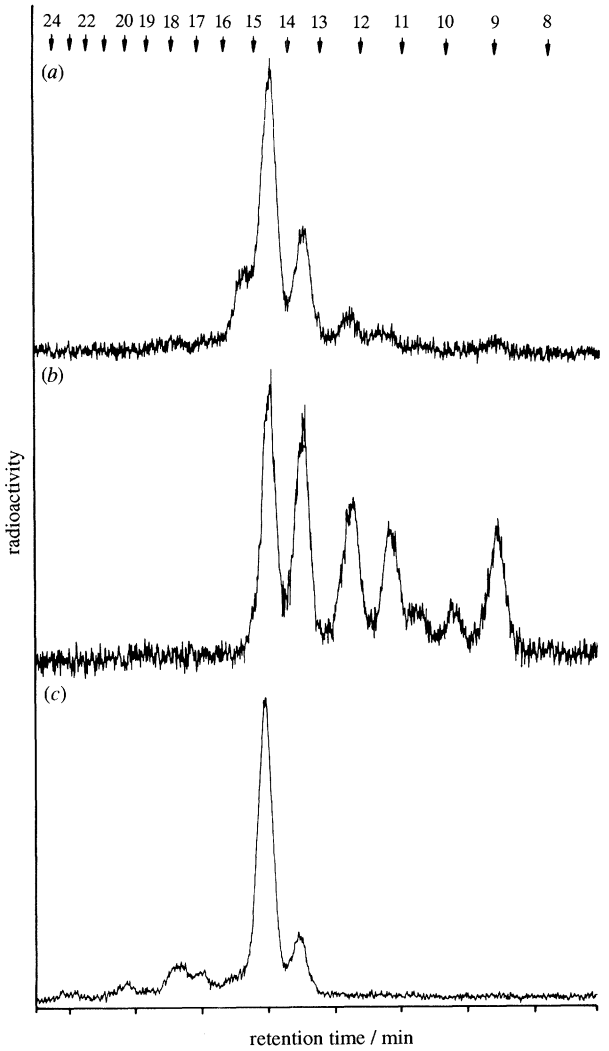

Figure 2. Bio-Gel P-4 gel filtration profiles of the desialylated, tritium-labelled oligosaccharides of recombinant soluble CD4 and CD2 expressed in CHO cells. (*a*) Total oligosaccharides of human sCD4. (*b*) Total oligosaccharides of rat sCD4. (*c*) Total oligosaccharides of rat sCD2. The vertical arrows indicate the elution positions of isomaltooligosaccharides containing the corresponding number of glucose units. The time axis is marked at 100 min intervals. (Data from Ashford *et al.* 1993 and Davis *et al.* 1993)

accounted for by site-specific glycosylation at the non-conserved sites. These results again implicate local protein structure as a major influence on oligosaccharide processing and indicate that no site to site interactions were occurring in rat sCD4.

In an extension of this study a truncated form of rat sCD4 was prepared consisting of domains 1 and 2, which contained the non-conserved glycosylation site at Asn 159 (Davis *et al.* 1990). In the absence of the third and fourth domains there was now more processing at this glycosylation site than was observed with the glycopeptide and the full length glycosylation variant containing this site (figure 4) (Ashford *et al.* 1993). It seems therefore that the presence of domains 3 and 4 affect processing at this site in the intact molecule. The precise packing of the two COOH-terminal domains and their potential influence on the glycosylation site at Asn 159 in rat sCD4 will not be known until the structure of the whole molecule is solved.

An unexpected finding was the presence of terminal α-galactose residues on approximately 20% of the oligosaccharides from human sCD4 (Ashford *et al.* 1993). Although oligosaccharides terminating in α-galactose residues are known to occur normally in glycoproteins from non-primate mammals (Spiro & Bhoyroo 1984; Galili *et al.* 1987), examination of the glycosylation of other recombinant glycoproteins expressed in CHO cells has not revealed the presence of these moieties (Sasaki *et al.* 1987; Kagawa *et al.* 1988; Takeuchi *et al.* 1988, 1989; Carr *et al.* 1989; Parekh *et al.* 1989; Spellman *et al.* 1989, 1991; Smith *et al.* 1990; Yuen *et al.* 1990; Davidson & Castellino 1991).

The occurrence of terminal α-galactose residues in the oligosaccharides from human sCD4 is probably not associated specifically with this molecule but is more likely to be caused by the activation of a latent, endogenous α-galactosyltransferase by the transfection process. This phenomenon was also observed recently when the two COOH-terminal domains of rat sCD4 were expressed in CHO cells (D. A. Ashford & A. N. Barclay, unpublished observation). A similar finding

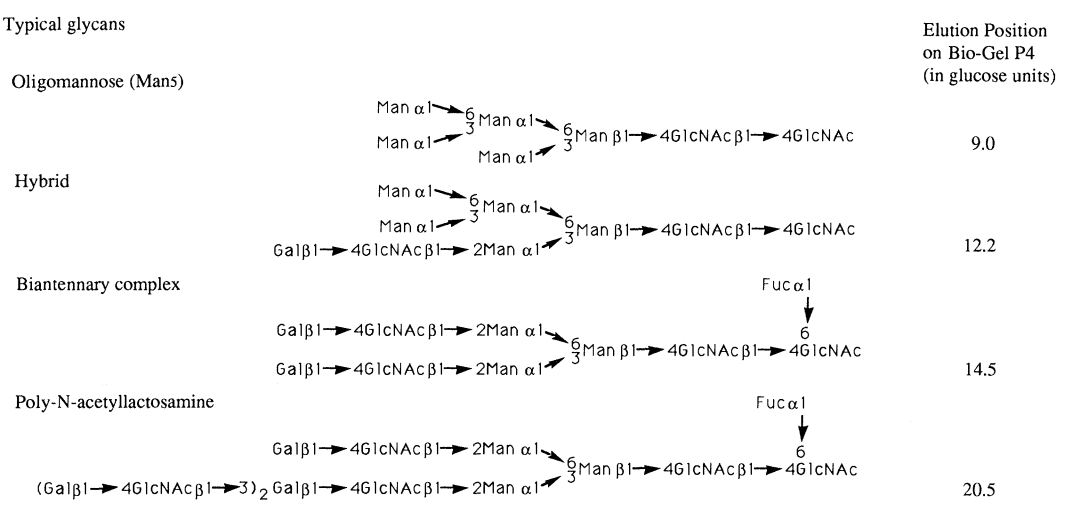

Figure 3. Examples of the structure of the different types of neutral desialylated N-linked oligosaccharides. Fuc, L-fucose; Gal, D-galactose; GlcNAc, D-N-acetylglucosamine; Man, D-mannose.

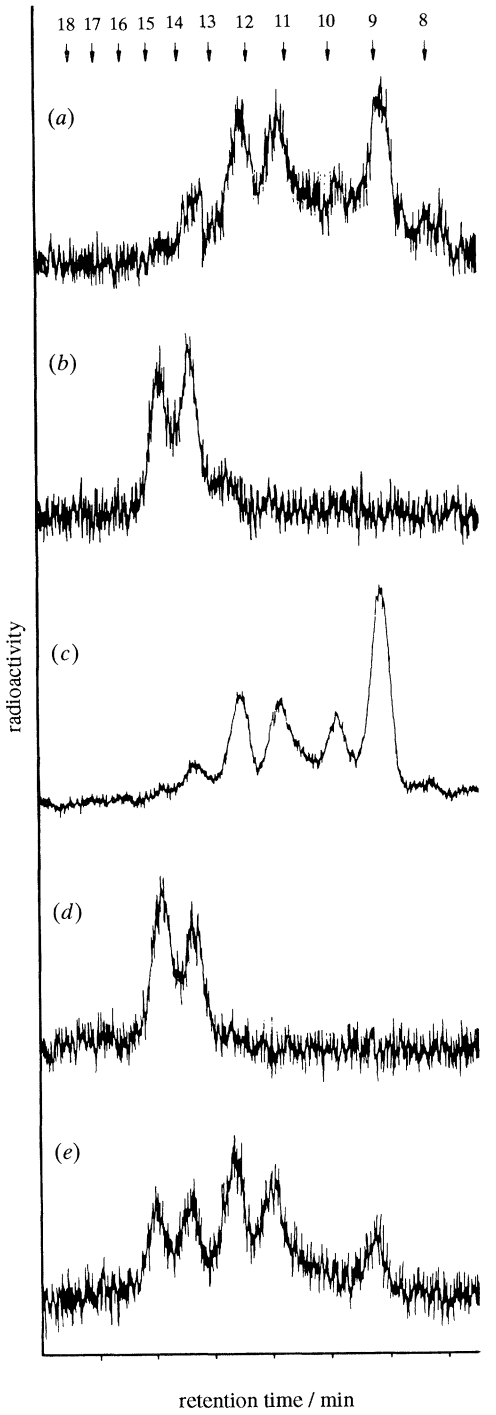

Figure 4. Bio-Gel P-4 gel filtration profiles of the desialylated, tritium-labelled oligosaccharides of the glycopeptides and glycosylation mutants of rat soluble CD4. (*a*) Total oligosaccharides of rat sCD4-derived glycopeptide from the region of the first glycosylation site. (*b*) Total oligosaccharides of rat sCD4-derived glycopeptide from the region of the second glycosylation site. (*c*) Total oligosaccharides of rat sCD4 with the second glycosylation site removed. (*d*) Total oligosaccharides of rat sCD4 with the first glycosylation site removed. (*e*) Total oligosaccharides of rat sCD4, NH_2-terminal-two IgSF domain form. For details of the annotation see legend to figure 2. (Reproduced from figure 8 of Ashford *et al.* (1993).)

was the activation of a normally silent fucosyltransferase when CHO cells were transfected with a human α-1,3-fucosyltransferase gene (Potvin *et al.* 1990).

3. THY-1

Additional studies have been carried out to probe the characteristics of the natural expression of N-glycosylation at individual glycosylation sites within the immunoglobulin-like domain but in the absence of inter-domain effects. Thy-1, a glycosylphosphatidylinositol membrane-anchored molecule, was used as the model since it possesses just one immunoglobulin-like domain (V-type) and three N-glycosylation sites (figure 1) (Williams & Gagnon 1982). As indicated above it is expressed on all mouse and rat thymocytes, on mouse but not rat T cells, and, in both rodents and human, the antigen is abundantly expressed in brain.

A comparative study of rat Thy-1 from the thymocyte with that from the brain showed that there was tissue specificity of N-glycosylation (Parekh *et al.* 1987). This occurred despite the amino acid sequences being identical for the molecule in the two tissues. Furthermore, the differential effects of tissue glycosylation were expressed at the level of individual glycosylation sites in the different tissue-derived Thy-1 molecules (Parekh *et al.* 1987). The site distribution of oligosaccharides was such that no Thy-1 molecules were found to be in common between the two tissues (figure 5). Thus each tissue created unique sets of glycoforms, (i.e. the same polypeptides but carrying oligosaccharides that differ either in structure or in site location or both).

In an extension of this work N-glycosylation in brain-derived Thy-1 has been analysed comparatively across the three species, rat, mouse and human. It was found that the tissue specificity of brain Thy-1 N-glycosylation outlined above was remarkably conserved across these species (figure 6*a*). Considering that the amino acid sequence homology compared with the rat is 82% for the mouse and only 66% for the human, this is a striking result.

Tryptic digests were made of the intact Thy-1 glycoprotein from each species so that N-glycosylation analysis could be made from the isolated glycopeptides that corresponded to the three glycosylation sites. It was found that the differences between the N-linked glycan pattern observed for the intact Thy-1 molecule in human brain and those for rat and mouse (figure 6*a*) could be explained by variation in glycosylation at site 1 (proximal to the NH_2-terminus at Asn 23 in all three species). Thus rat and mouse contained only oligomannose-type structures at this site, whereas essentially only complex- and hybrid-type glycans were present at this location in the human-derived molecule (Williams *et al.* 1993). However, at the second site (at Asn 74/75 in rat and mouse respectively, and at Asn 60 in human brain Thy-1) very similar glycan profiles, mainly of complex oligosaccharides, were found in all species (Williams *et al.* 1993). At the third site (at Asn 98/99/100 for rat, mouse and human respectively) almost identical patterns containing complex-, hybrid- and oligomannose-

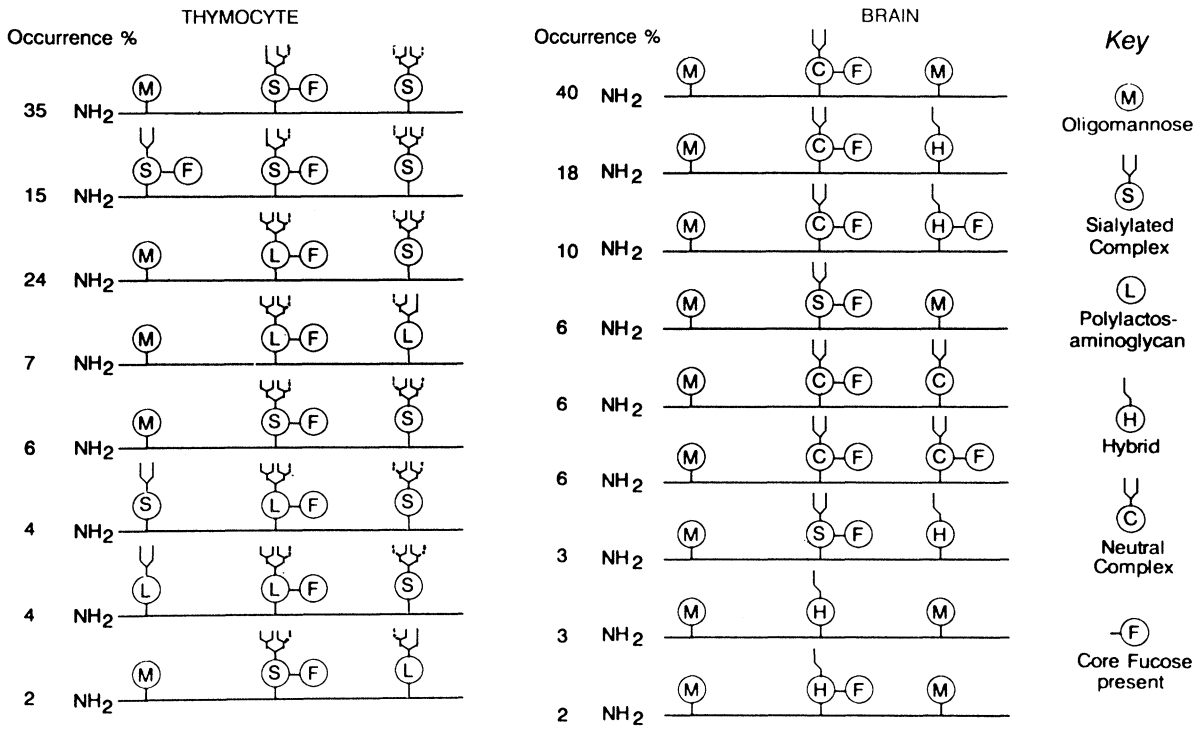

Figure 5. A comparison of the nature and percentage composition of the glycoforms of thymocyte- and brain-derived rat Thy-1 glycoprotein. Glycoforms representing less than 2% abundance are not illustrated.

type glycans were observed in each of the three species and these are illustrated in figure 6*b*.

A distinguishing feature of brain Thy-1 glycosylation in general is that, whereas the N-linked oligosaccharides of whole murine brain tissue are dominated by anionic glycans (Wing *et al.* 1992), the molecule itself expresses a majority of neutral oligosaccharides in all species studied. This may represent another aspect of protein-specific glycosylation but precisely how this particular aspect of conservation of glycosylation in brain Thy-1 is controlled across species is not known. It is, perhaps, noteworthy that timing of Thy-1 expression in brain can show locational specificity (Morris 1985) and this may be important for the available array of processing enzymes. It is of interest that Thy-1 expression on a neural cell line has been shown to inhibit selectively neurite outgrowth on mature astrocytes *in vitro* suggesting receptor-ligand interactions (Tiveron *et al.* 1992).

In general the remarkable degree of conservation of glycosylation in brain Thy-1 across the species suggests a common functional importance for the carbohydrate moieties in neural tissue and that the Thy-1 polypeptide serves as an appropriate carrier for these structures.

4. CONCLUSIONS

It is clear that a tissue or cell type shows specificity for N-glycosylation processes and that the protein undergoing glycosylation can itself make an important determining contribution. In particular the results described here support the importance of the local primary and secondary protein structures in influencing the differences in glycosylation between different sites on the same protein.

The truncated form of rat sCD4 showed more oligosaccharide processing at its glycosylation site than when the entire sCD4 variant was expressed, indicating that domain interections can affect glycosylation. Detailed analysis of the oligosaccharide patterns at the individual sites in brain Thy-1 and recombinant rat sCD4 support the hypothesis that the extent of processing may be determined primarily by the accessibility of the oligosaccharides (Hsieh *et al.* 1983; Hubbard 1988). Figure 7 shows the relationship between the glycosylation sites, N-linked oligosaccharides and peptide backbone of the first two domains of rat CD4 and of Thy-1.

The presence of only oligomannose structures at Asn 23 in rat and mouse brain Thy-1 is indicative of the inability of N-acetylglucosaminyltransferase I to act on $Man_5GlcNAc_2$. The additional presence of $Man_6GlcNAc_2$ and $Man_7GlcNAc_2$ structures is indicative of the α-mannosidases of the Golgi apparatus also encountering restricted access at this site. In contrast the virtual lack of oligomannose structures at the second site in Thy-1 from all species and at the conserved site in both rat and human sCD4 is complemented by a high percentage of complex glycans, suggesting little conformational restriction and that the action of N-acetylglucosaminyltransferase I was not rate limiting. The third glycosylation site of Thy-1 and the non-conserved site of rat sCD4 showed the presence of oligomannose structures, which were almost entirely of the $Man_5GlcNAc_2$ size.

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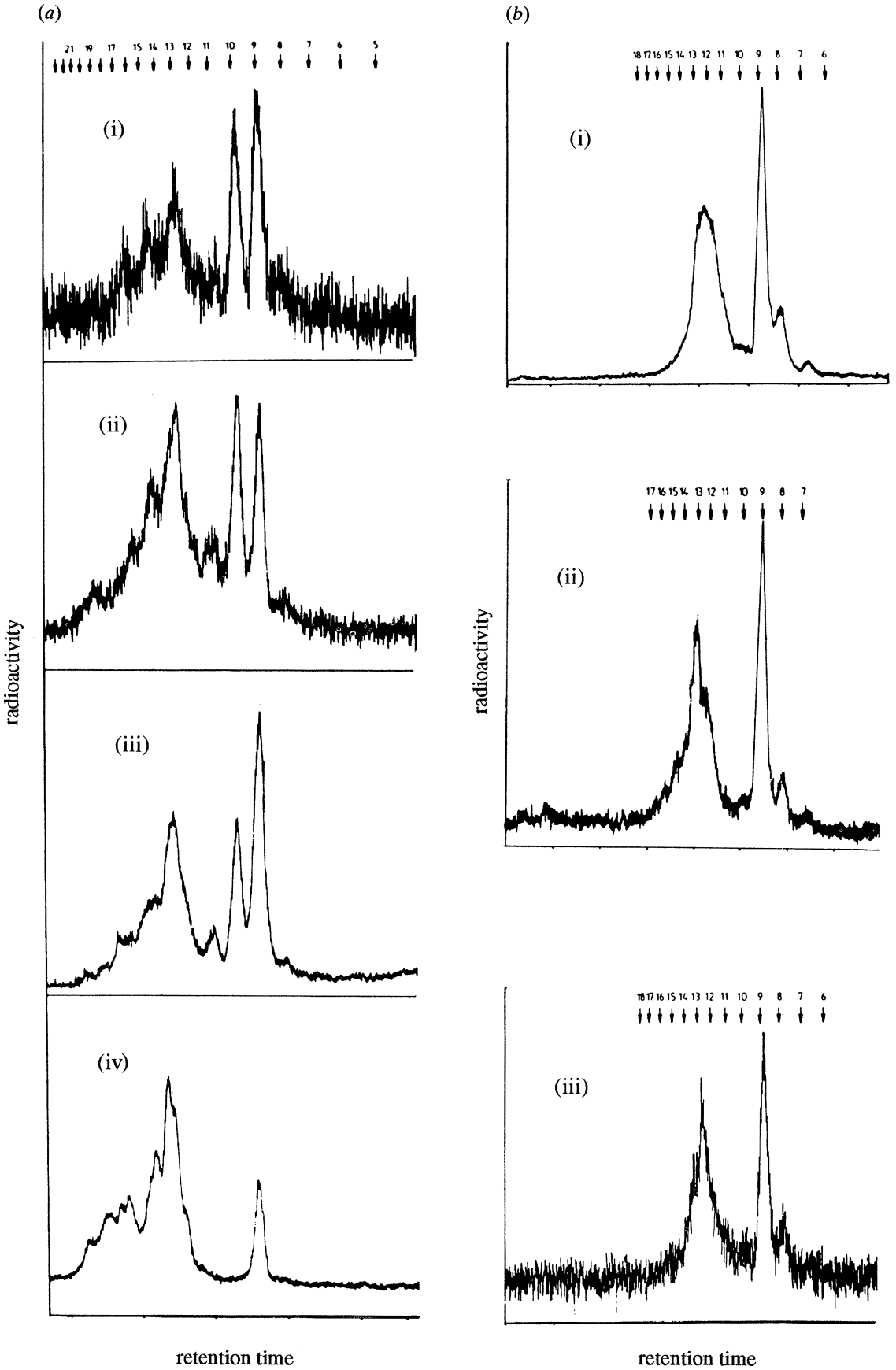

Figure 6. Bio-Gel P-4 gel filtration profiles of the desialylated, tritium-labelled oligosaccharides of brain Thy-1 preparations from rat, mouse and man. (*a*) Comparison of profiles of the intact Thy-1 molecules for: (i) rat; (ii) mouse 1.2; (iii) mouse 1.1; and (iv) human. For the mouse, Thy-1.1 and Thy-1.2 are two allelic forms of Thy-1 differing at a single amino acid residue (no 89) with an Arg–Gln interchange. (*b*) Comparison of profiles at the third N-glycosylation site in: (i) rat; (ii) mouse, from the major site 3 glycopeptide of Thy-1.1; and (iii) human. For details of the annotation see legend to figure 2. (Adapted from Williams *et al.* (1993).)

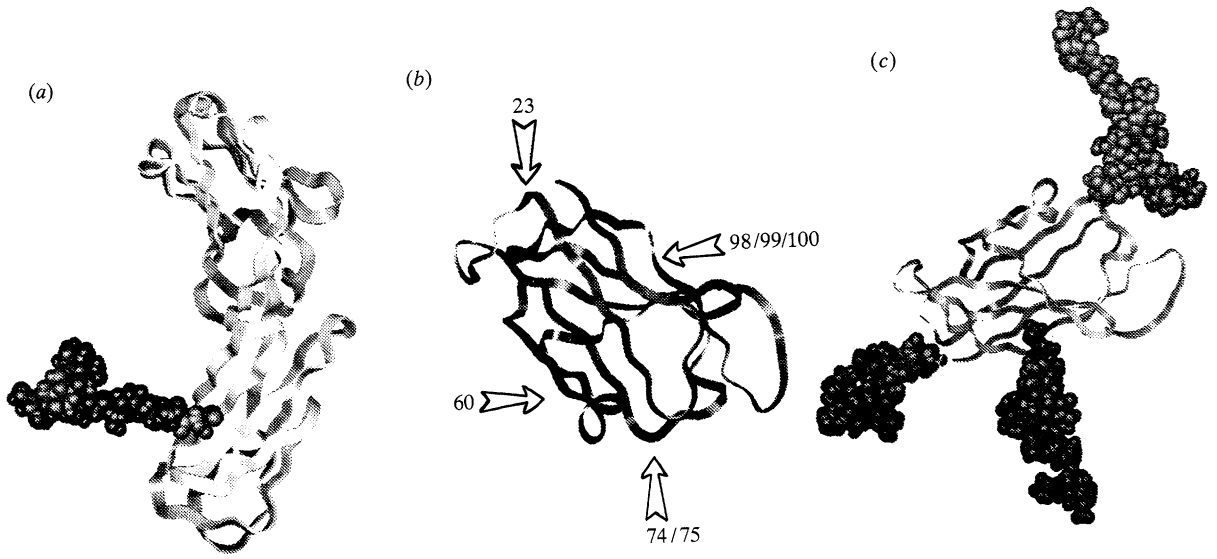

Figure 7. Models for the three-dimensional structure of rat CD4 domains 1 and 2 and Thy-1. (*a*) Rat CD4 domains 1 and 2 showing a $Man_5GlcNAc_2$ oligosaccharide at Asn-159. (*b*) Thy-1 polypeptide with arrows indicating the locations of the glycosylation sites of rat and mouse (23, 74, 75 and 98/99) and human (23, 60 and 100) Thy-1. (*c*) A view of Thy-1 showing an oligomannose oligosaccharide at Asn-23 (bottom left), a poly- N-acetyllactosamine oligosaccharide at Asn-74 (top) and a complex oligosaccharide at Asn-98 (bottom right). The polypeptide backbone is shown in the diagrams as a continuous ribbon and the N-linked oligosaccharides as space filled models.

The lack of larger oligomannose structures is suggestive of increased α-mannosidase accessibility at these sites. These findings are substantiated by the additional presence of complex and hybrid oligosaccharides, which reflect further processing.

The presence of complex- and hybrid-type, instead of oligomannose-type carbohydrate structures at site 1 in human brain Thy-1 reflects less restrictive oligosaccharide processing than occurred at this site in the rat and mouse glycoprotein. The reason for this is unknown but seems likely to relate to particular characteristics of the three-dimensional structure of the human glycoprotein. In this context it is of interest that the second glycosylation site in human brain Thy-1, located at Asn 60, is somewhat removed from the corresponding site in rat and mouse brain Thy-1 (at Asn 74/75 respectively) in terms of amino acid sequence but is located on the same surface in models of the three-dimensional structure (figure 7*b*).

The general conclusion therefore is that accessibility of the oligosaccharides to the processing enzymes is a principal factor determining site specificity of glycosylation. The prime determinant of this is the three-dimensional conformation of the protein as it relates to the environment of the glycosylation site and the attached oligosaccharide.

The authors acknowledge the special role of Alan Williams in this study of cell surface antigens. The Glycobiology Institute is supported by the Monsanto Co. A.N.B. is supported by the MRC and S.J.D. by the MRC AIDS Directed Programme. We are grateful to Susan Field and Ruth Preston for their preparation of the typescript. This paper is dedicated to the memory of Alan Williams.

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