Sialic Acid Capping of CD8β Core 1-O-Glycans Controls Thymocyte-Major Histocompatibility Complex I Interaction*

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Bidentate interaction of a T-cell receptor and CD8αβ heterodimer with a peptide-MHCI complex is required for the generation of cytotoxic T-lymphocytes. During thymic development, the modification of CD8β glycans influences major histocompatibility complex class I binding to T-cell precursors called thymocytes. ES mass spectrometry (MS) and tandem MS/MS analysis were used to identify the changes occurring in the CD8β-glycopeptides during T-cell development. Several threonine residues proximal to the CD8β Ig headpiece are glycosylated with core-type 1 O-glycans. Non-sialylated glycoforms are present in immature thymocytes but are virtually absent in mature thymocytes. These results suggest how sialylation in a discrete segment of the CD8β stalk by ST3Gal-1 sialyltransferase creates a molecular developmental switch that affects ligand binding.

Within complex biological systems, glycans serve as key structural and functional elements (1). Cell surface glycans are altered during cell differentiation and activation in conjunction with changing glycoprotein expression pattern (2). The precise chemistry of glycan modification requires that the vertebrate genome encodes a variety of enzymes that modify various structural and functional elements (1). Cell surface glycans are virtually absent in mature thymocytes. These results suggest how sialylation in a discrete segment of the CD8β stalk by ST3Gal-1 sialyltransferase creates a molecular developmental switch that affects ligand binding.

The CD8 cell surface molecule is critical for the development and activation of T-cells whose T-cell receptors (TCR) recognize peptides bound to major histocompatibility complex class I (MHC) molecules (6, 7). This co-receptor is encoded by two distinct genes, α and β, whose polypeptide products are expressed in one of two forms, CD8αa homodimers or CD8αβ heterodimers (8, 9). Most T-cells mature within the thymus and express cell surface CD8αβ receptors. Previously, we and others (10, 24) have shown that immature thymocytes bind peptide-MHCI (pMHC) tetramers more avidly than mature thymocytes. The binding difference is the result of a developmentally regulated glycosylation modification involving sialic acid residues. Evidence of this is the increased CD8β-MHCI avidity of mature thymocytes following treatment with neuraminidase, an enzyme that removes sialic acid residues from cell surface glycans. Moreover, the sialyltransferase ST3Gal-1, which specifically sialylates core 1-O-glycans, is involved in controlling the differential binding as evidenced by decreased CD8αβ-MHCI avidity after induction of ST3Gal-1 (10). Given that CD8β glycans change during thymic development (10, 11), we examined the physical nature of CD8β O-glycosylation.

Through the application of recent advances in mass spectrometry (12), we have been able to identify a developmental change in CD8β stalk glycosylation, which functions as a molecular switch to critically affect ligand binding.

**EXPERIMENTAL PROCEDURES**

CD8β Sample Preparation—Unfractionated thymocytes from C57BL/6 mice (~2–4 × 10^7/experiment) were lysed by buffer containing 1% Triton X-100. Resulting lysates were processed as described previously (10) using anti-CD8β mAb YTS 156.77 for immunoprecipitation. After separation on two-dimensional non-reducing/reducing SDS-PAGE, the proteins were stained with Gel-Code Blue reagent and the double positive (DP) and single positive (SP) CD8β bands were excised. The gel slices were digested with either trypsin or N-glycanase (PNGase) followed by trypsin using conditions described previously (13).

Mass Spectrometry Analysis—The tryptic peptide extracts were cleaned by reverse-phase (C18) trapping and eluted by 1:1 MeOH:HOAc with 1% trifluoroacetic acid or acetonitrile:0.01% trifluoroacetic acid or acetonitrile:0.01% formic acid in various experiments. The eluted volume (2–3 μl) was loaded in a nanopray tip and analyzed by ES mass spectrometry (MS) and tandem MS/MS analysis on either ABI QStar or ABI QSTARXL ion trap mass spectrometers. The peptide mass fingerprints for the tryptic fragments were searched using the Mascot search engine (matrix science). The following parameters were used: Cysteine was set as fixed modification, peptide mass tolerance was ± 0.2 Da, and one missed cleavage was allowed.

The abbreviations used are: TCR, T-cell receptor; MHC, major histocompatibility complex class I; pMHC, peptide-MHCI; mAb, monoclonal antibody; DP, double positive; SP, single positive; PNGase, N-glycanase; ES, electrospray; MS, mass spectrometry; LC, liquid chromatography; DN, double negative; PNA, peanut agglutinin; Sia, sialic acid; NeuAc, N-glycolyneuraminic acid; NeuAs, N-acetylenuraminic acid; HexNAc, N-acetylhexosamine; GalNAc, N-acetylgalactosamine.
CD8β Core 1-O-Glycan Sialylation Linked to T-cell Development

RESULTS

The Peanut Agglutinin (PNA) Lectin Detects Glycosylation Differences on Developing Thymocytes and Binds CD8β—Within the thymus, T-cell precursors move through a series of developmental stages distinguishable by different CD4 and CD8 cell surface expression patterns (14). CD4+CD8+ double negative (DN) cells progress to the CD4+CD8− DP stage in the thymic cortex, and upon successful selection, mature into either CD4+ or CD8+ SP T-cells in the thymic medulla (Fig. 1a). The DP to SP transition is dependent on TCR ligation by pMHC molecules containing self-peptides (15). Varied T-cell surface glycosylation patterns detected by the plant lectin, PNA, also mark thymic developmental progression. PNA binds to core 1-O-glycans bearing terminal galactose residues (Galβ1→3GalNAcSer-Thr), staining immature cortical thymocytes strongly (PNAhigh) and mature medullary thymocytes weakly (PNAlow) (Fig. 1b) (16, 17). The change in PNA reactivity is attributable to the induction of the ST3Gal-1 sialyltransferase within the hematopoietic compartment that catalyzes the addition of sialic acid (Sia) residues in a α2→3 linkage to terminal galactose (Siaα1→3Galβ1→3GalNAcSer-Thr), capping the PNA binding site in medullary thymocytes (Fig. 1, b and c) (18). Genetic disruption of ST3Gal-1 causes PNAhigh reactivity to persist into the medullary thymocyte compartment (Fig. 1b) (2). Five likely possibilities for O-glycan structures that could bind PNA in the cortex are presented in Fig. 1c. Among the small group of thymocyte cell surface molecules identified as being PNA-reactive are CD45, CD43, and CD8(19). In particular, the CD8 chain is a major component of the differential PNA binding observed on immature thymocytes (Fig. 1d).

Mass Spectrometry Analysis of CD8β Glycans—To identify N- and O-linked glycan sites on CD8β and define glycosylation changes associated with the DP to CD8 SP thymocyte transition, mass spectrometry was used to analyze tryptic peptides of CD8β prepared from immunoprecipitates. CD8β proteins were immunoprecipitated from lysates of cell surface-labeled DP and CD8 SP thymocytes sorted by MoFlo, using Sepharose-
coupled anti-CD8β mAb and separated on two-dimensional non-reducing/reducing SDS-PAGE gels as described previously (10). Whereas three distinct pairs of CD8αβ heterodimers (α38Kd β30Kd, α38Kd β29Kd, and α33Kd β29Kd) are evident on DP thymocytes, CD8β heterogeneity is reduced upon DP to SP maturation (Fig. 2a). By the CD8 SP stage, a single 38-kDa CD8α subunit is paired with a major 30-kDa CD8β glycoform. Note that aside from the 33-kDa CD8α cytoplasmic RNA splice variant found in DP thymocytes, CD8α is not detectably altered during thymic maturation as assessed by the two-dimensional gel analysis. A composite pattern is obtained from silver staining of proteins immunoprecipitated from unfractonated thymocytes and run in the two-dimensional gel system (see “Experimental Procedures”). Since DP thymocytes comprise 80% of thymocytes while the CD8 SP fraction accounts for merely 3–5%, the total thymocyte CD8β immunoprecipitation pattern is most similar to that of the isolated DP thymocytes. The two-dimensional gel pattern of CD8β proteins precipitated from sorted DP and CD8 SP thymocytes provided a ready basis to obtain “DP” and “CD8 SP” thymocyte-derived gel slices as indicated in the Fig. 2b inset. The excised gel slices were then digested with either trypsin or PNGase followed by trypsin using the conditions described previously (13). Following the in-gel digestion and extraction, the purified peptide mixture was analyzed using ES on Q-TOF geometry tandem hybrid instrumentation (20) in both MS and MS/MS modes (see “Experimental Procedures”).

An early comparative study of CD8β SP and DP preparations run by nanospray ES-MS from a formic acid/acetonitrile solution showed a clear quadruply charged signal at m/z 823.88 (corresponding to M – 3,291.49) in the SP sample, which was virtually absent in the corresponding DP analysis. Fig. 3 shows the MS/MS spectrum of this ion at moderate collision energies of 30–50 eV. The spectrum is the sum of data obtained at 30, 40, and 50 eV. The spectrum shows definitive evidence of glycosylation via major signals at m/z 204 (HexNAc), 366 (HexHexNAc), 290 (NeuGc minus H2O), 308 (NeuGc), and 673 (NeuGcHexHexNAc). Hex denotes any six-carbon neutral sugar, including glucose, galactose, and mannose, whereas HexNAc is a six-carbon sugar with an N-acetylated amino group at position 2. NeuGc is formed by an enzyme that catalyzes the hydroxylation of the N-acetyl group attached to C5 of the nine-carbon sialic acid backbone. The mouse CD8β sialic acids identified were of the glycolyl variety as expected rather than the N-acetyl (NeuAc)-type that predominates in mammalian brain tissue (1). The collision energies were chosen to provide both carbohydrate and peptide backbone fragmentation (21) in an effort to identify the CD8 peptide sequence carrying the glycosylation. Signals observed at m/z 215, 314, 413, 528, 627, and 740 were interpreted as N-terminal peptide fragments, b ions (20), assignable to a sequence . . . VVDV(L/I) . . . , which is present in CD8β in tryptic peptide-(112–125), LTVVDVLPTTAPTKK (Figs. 3 and 4). The threonines at positions 113, 120, 121, and 124 represented possible sugar attachment sites via O-glycosylation. A (non-glycosylated) peptide of this sequence would be expected to show intense C-terminal ammonium ion (y′) fragmentation (20, 21) corresponding to fragmentation at the labile L-P and A-P bonds, giving calculated nominal masses of 715 and 345, respectively. Neither of these signals is present in Fig. 3. However, with increasing collision energy, which causes preferential cleavage of sugar residues, prominent signals at m/z 843 and 473 begin to appear, which are 128 Da higher in mass. These signals were assigned to C-terminal proline cleavage fragments PTTPKK and PTKK, respectively, thus proving that the peptide backbone for the 823.88+ peptide is in fact the CD8β-(112–126) sequence LTVVDVLPTTAPKK. The fact that the 473 ion was not only created at higher collision energies showed that at least threonine 124 is glycosylated.

Subtracting this peptide mass (1581.93 Da) from the experimentally determined mass of the glycopeptide (3291.49 Da) then allowed the total carbohydrate mass to be calculated as 1709.56 corresponding to NeuGc3HexHexNAc3 in agreement with the sugar fragment ions described earlier. The b ion peptide fragment series (m/z 215, 314, 413, 528, 627, and 740) is visible at relatively low collision energies, which in our experience would not cause total carbohydrate elimination from the fragments. This strongly suggests that threonine 113 is not glycosylated (Fig. 4). In additional variable collision energy experiments, the proline y′ ion fragments at m/z 843 and 473 were seen to carry glycolyl substituents via signals at m/z 1046 (843 + HexNAc), 1208 (843 + HexHexNAc), 1249 (843 + HexHexNAc), 676 (473 + HexNAc), and 838 (473 + HexHexNAc). A consideration of these data together with the sugar fragment ions observed (both at low mass and as neutral losses from the quasimolecular ion) suggested that threonines 120, 121, and 124 are each O-linked to core 1 (HexHexNAc) structures, two of which are capped with N-glycolylneuraminic acid. The virtual absence of the m/z 823.88 signal from the corresponding DP preparation provided the first molecular evidence of differential sialylation of the CD8β SP/DP glycoproteins.

Because of the complexity of the nanospray MS spectra of the total CD8β digests, a further preparation of SP and DP CD8β was then examined by nanoLC-MS and nanoLC -MS/MS (see “Experimental Procedures”). These data were important in allowing the unambiguous confirmation of the glycosylation state of the remainder of the CD8β stalk region. This was achieved by locating N-glycolylneuraminic acid-containing signals at m/z 1040 + , 1117 + , and 1194 + . These glycopeptides were found eluting at 24.8 min, 26.7/27.4 min (doublet), and 28.1 min respectively, compared with the CD8β-(112–126) diasialyl glycopeptide described earlier in the nanospray experiment, which eluted at 30.2/31.1 min as evidenced by signals at 8234 + and 10985 + .

A consideration of the mass differences among the 1040, 1117, and 1194 signals together with the MS/MS data, which showed that the peptide portion of the differing molecules began with the same LTVDV . . . sequence, allowed the as-
A comparison of CD8 SP and DP glycopeptides revealed a
key difference in the O- but not the N-linked glycopeptides. This difference is the almost complete absence of non-sialylated Hex,HexNAc, Hex3HexNAc structures in CD8 SPs (Figs. 5 and 6). Conversely, double and triple Sia-capped Hex,HexNAc, Hex3HexNAc-(112–126)-peptides occur in greater abundance in SP rather than DP, although these differences are smaller by comparison. Unexpectedly, our structural studies have revealed that both SP and DP are mainly mono-sialylated in the stalk region despite the presence of five core-type 1 O-glycan substitutions (Thr-120, Thr-121, Thr-124, Thr-127, and Thr-128) (Fig. 4). Sialylation occurs principally within the 120–124 sequence, and there appears to be little or no additional sialylation of residues 127 and 128. Since SP thymocytes are PNA\textsuperscript{low}, this finding suggests that the majority of CD8\textbeta stalk O-glycans are inaccessible to this lectin. Thus, the key change between DP and SP is core-type 1 sialylation at a single site in the 121–124 stalk segment. This site is presumably that recognized by the peanut lectin.

**DISCUSSION**

CD8\textbeta rather than CD8\textalpha was previously shown to be the critical co-receptor on thymocytes for MHCI binding (10, 22). In CD8\textbeta knock-out mice, for example, CD8\textalpha homodimers are expressed on the surface of thymocytes but fail to support significant MHCI binding activity as assessed by pMHCI tetramers using flow cytometry. Moreover, the varied glycosylation pattern of CD8\textbeta at different thymic developmental stages correlates with the noted change in the CD8\textbeta ligand binding activity whereby DP thymocytes interact with MHCI more avidly than CD8 SP thymocytes. Several hypotheses have been offered to account for this developmentally programmed alteration in CD8\textalpha co-receptor MHCI ligand-binding function (10,
The structure of O-glycan adducts revealed herein also limits the likelihood that galectins are operating to cross-link galactose residues on neighboring CD8β stalk adducts. Galectin-1 has been implicated in thymocyte apoptosis through the recognition of core 2 O-glycans on CD43 and CD45 (27, 29). Galectin-3 binding to β1-6-branched lactosamine chains produced by the Mgaat1 gene on TCR N-glycans is reported to inhibit T-cell activation, perhaps by altering TCR clustering (30). Most mammalian galectins bind preferentially to galactose on polylactosamine, although some may bind to other galactose linkages (31, 32). Detailed site-specific assignment of Sia adducts on the CD8β stalk confirms that the addition of sialic acid per se modulates CD8β co-receptor function.

Our findings show that the genetically programmed alteration of CD8β glycosylation during thymocyte differentiation from immature DP to mature SP stages is restricted to the O-glycans without concurrent changes in the N-linked structures. These O-linked sites (Thr-120, Thr-121, Thr-124, Thr-127, and Thr-128) localize to a segment of the CD8 stalk immediately abutting the CD8β Ig-like domain. O-Linked glycans are attached to none of the 14 other serine or threonine residues in the examined tryptic fragments. Three of the five threonines (Thr-120, Thr-124, and Thr-128) are conserved in all of the CD8β homologues sequenced to date, residing within or adjacent to the lysine-rich segment that is unique to the CD8β stalk (10). In view of both the weak association between CD8α and CD8β head regions (10), an uncharacteristic feature for Ig-like domain heterodimers and the almost certain requirement for participation of CD8α and CD8β CDR-like loops in the binding to the MHC class I domain (by extension from crystallographic analysis of two CD8αp-MHCI complexes) (33, 34), sialylation in this specific region may impact significantly on CD8αβ binding to MHC class I. The addition of sialic acid to the CD8β stalk could facilitate neutralization of positive charges on the adjacent stalk lysine residues (Lys-125, Lys-126, Lys-130, and Lys-132 to Lys-135), probably permitting the stalk to assume a retracted rather than fully extended configuration or resulting in other conformational changes. By altering CD8αβ domain-domain association and/or disposition of the CD8 globular headpiece relative to the cell surface, CD8β stalk O-glycans create a molecular switch regulating MHC binding. That sialic acid addition to core 1 O-glycans during thymic ontogeny is a conserved feature of vertebrate development (22) with CD8β representing a major thymic PNA-binding protein (Fig. 1d) underscores the essential nature of this molecular switch. Additional chemical details regarding the dynamic glycobiology of CD8 will be important, not only for understanding the co-receptor function of thymocytes but that of naive, memory and effector CD8 peripheral T-cells.

Fig. 6. Expansion of the m/z 893 region of Fig. 5 showing the abundance between SP and DP and the relative absence of the key signal relating to non-sialylated HexHexNAc structures in SP compared with its presence in DP thymocytes.

23–25). First, the sialylation of the CD8β stalk may affect the orientation of the co-receptor globular head domains relative to the T-cell membrane and/or CD8αβ domain-domain association strength, modulating the ability of the stalk binding surface of the CD8αβ Ig-like domain to clamp MHC class I. Second, sialic acid residues might reduce the clustering of CD8αβ molecules on thymocyte surfaces because of repulsion of the negatively charged sugars, preventing MHC binding as detected by pMHC tetramers. Third, multivalent mammalian lectins such as galectins (26), known to control clustering of certain T-cell surface glycoproteins (27), might "pre-cluster" non-sialylated CD8 co-receptors in DP thymocytes but not sialylated glycan-bearing counterparts on CD8 SP thymocytes. Fourth, the effects might be attributed to sialylation of molecules other than CD8αβ given the previous lack of direct evidence for developmentally controlled sialic acid addition to the CD8 co-receptor itself.

The current biochemical analysis favors reorientation of the globular head domains of CD8. We find that on the noted stalk threonines, CD8β harbors a sialic acid linked to a core 1 disaccharide that lacks an N-acetyllactosamine, indicating an absence of both core 2 O-glycans and elongated core 1 glycans, which might alter lateral mobility of CD8αβ in the plasma membrane by virtue of larger hydrodynamic radii. Consistent with this view, we observed no differences in the distribution of CD8αβ co-receptors on the surface of DP and CD8 SP thymocytes (10). Furthermore, CD8β remains constitutively concentrated in cholesterol-sphingolipid-rich plasma membrane microdomains due, at least in part, to CD8β cytoplasmic tail palmitoylation (28).

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