O-Glycan Sialylation and the Structure of the Stalk-like Region of the T Cell Co-receptor CD8*

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Studies of mucins suggest that the structural effects of O-glycans are restricted to steric interactions between peptide-linked GalNAc residues and adjacent polypeptide residues. It has been proposed, however, that differential O-glycan sialylation alters the structure of the stalk-like region of the T cell co-receptor, CD8, and that this, in turn, modulates ligand binding (Daniels, M. A., Devine, L., Miller, J. D., Moser, J. M., Lukacher, A. E., Altman, J. D., Kavathas, P., Hogquist, K. A., and Jameson, S. C. (2001) *Immunity* 15, 1051–1061; Moody, A. M., Chui, D., Reche, P. A., Priatel, J. J., Marth, J. D., and Reinherz, E. L. (2001) *Cell* 107, 501–512). We characterize the glycosylation of soluble, chimeric forms of the αα- and ββ-isofoms of murine CD8 containing the O-glycosylated stalk of rat CD8αα, and we show that the stalk O-glycans are differentially sialylated in CHO K1 versus Lec3.2.8.1 cells (82 versus 6%, respectively). Sedimentation analysis indicates that the Perrin functions, Pexp, which reflect overall molecular shape, are very similar (1.61 versus 1.54), whereas the sedimentation coefficients (s) of the CHO K1- and Lec3.2.8.1-derived proteins differ considerably (3.73 versus 3.13 S). The hydrodynamic properties of molecular models also strongly imply that the sialylated and non-sialylated forms of the chimera have parallel, equally highly extended stalks (∼2.6 Å/residue). Our results indicate that, as in the case of mucins, the overall structure of O-glycosylated stalk-like peptides is sialylation-independent and that the functional effects of differential CD8 O-glycan sialylation need careful interpretation.

The early responses of T lymphocytes are determined by interactions of both the T cell receptor (TCR)b and CD8 or CD4 molecules with major histocompatibility complex (MHC) molecules on antigen-presenting cells. CD8 is required by mature T cells restricted to recognizing foreign antigenic determinants (peptides) complexed with class I MHC antigens (MHCp), whereas CD4 is involved in MHC class II-restricted T cell responses (1). Because CD8 and CD4 are believed to interact with the same MHCp complex as the TCR (2), these proteins are commonly referred to as T cell co-receptors.

CD8 is a glycoprotein consisting of disulfide-linked subunits, α and β, which are encoded by two closely linked genes near the immunoglobulin κ locus (3). Despite sharing relatively low sequence similarity (∼20%), the CD8 subunits are structurally related and predicted to have identical topologies (4). Each chain consists of an extracellular immunoglobulin superfamily (IgSF), V-set domain attached to hydrophobic transmembrane sequences and short cytoplasmic tails via extended, disulfide-linked stalk-like peptides of 48–51 (α-chain) or 37–42 (β-chain) residues (reviewed by Gao et al. (5)). The IgSF domains in CD8 exhibit highly variable N-linked glycosylation; in humans, only a single site on the β-chain is glycosylated, whereas in mice the α- and β-chains have three and one sites, respectively. The ratio seen in the mouse is reversed for rat CD8. In all species, the stalk-like region of each chain is rich in proline, serine, and threonine residues and is O-glycosylated. Amino acid sequencing of rat CD8α contains the four threonine residues 122, 126, 132, and 134 clustered at the membrane-distal end of the stalk are occupied with O-glycans (6). The cytoplasmic domain of CD8α is attached to the tyrosine kinase p56lck (reviewed in Ref. 7). Most T cells express CD8 as an αβ heterodimer, although a homodimeric (CD8αα) is found on subsets of intraepithelial T lymphocytes of the gut, γδ T cells, NK cells, and lymphoid-related dendritic cells (reviewed by Zamoyska (8)). CD8αα and CD8ββ T cells undergo different pathways of selection, perhaps reflecting an underlying functional specialization within the CD8 T cell population (9).

The interaction between CD8αα and class I MHCp ligands was initially investigated using cell adhesion assays (10). More

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recently, the affinities and kinetics of the interaction of soluble forms of human and mouse CD8α with human and mouse class I MHC, respectively (11, 12), and of human CD4 and murine MHC class II (13), have been determined using surface plasmon resonance-based methods. In both cases, the interactions were shown to have extraordinarily low affinities and extremely rapid dissociation kinetics. Crystallographic analyses of the complexes of CD8αα with MHC class I from humans and mice (14, 15) indicate that CD8 binds the membrane-proximal α2 and α3 domains of MHCp and makes additional contacts with β2-microglobulin. An analogous interaction was revealed by the crystal structure of the complex of human CD4 domains 1 and 2, and murine MHC class II (16), insofar as CD4 binds a membrane-proximal cavity formed by residues from both the α2 and β2 domains of class II. It has been proposed that the weak interactions between the TCR and MHCp may be enhanced by simultaneous interactions involving CD8 (17). However, CD8 interactions are generally at least 10-fold weaker than those involving the TCR, and CD4 interactions are possibly even weaker. Therefore, although they will contribute somewhat to the overall interaction, it seems more likely that the key function of the co-receptors is to recruit sufficient p56Lck to pre-formed TCR-MHCp complexes to consolidate the early signaling response (11).

An additional level of complexity regarding these interactions arises from the recent suggestion that ligand binding by CD8 is regulated in vivo by changes in its O-glycosylation. Specifically, Moody et al. (18) and Daniels et al. (19) each report that the binding of tetrameric forms of MHCp in a CD8-dependent, non-cognate manner to double-positive thymocytes diminishes as O-glycan sialylation increases and the thymocytes progress through positive selection. To account for this effect, sialylation-induced changes in the structural properties of the stalk, either involving electrostatic repulsion between the two chains (18) or chain extension effects (19), were proposed to affect the ligand-binding site even though this is located ~30 Å from the stalk. As has already been noted (20), thermodynamic considerations make an electrostatic repulsion-based mechanism for these effects extremely unlikely. In the present study, we test the second proposed mechanism, i.e. that O-glycan sialylation modulates the extension of the stalk-like region of CD8. Our findings suggest that sialylation has little, if any, effect on the overall structure of CD8.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents—**Anhydrous hydrazine was prepared by distillation from reagent-grade hydrazine (Fierce, catalog number 21515-1) mixed with calcium oxide and toluene. Reagents for automated hydrazinolysis were from Oxford GlycoSciences (Abingdon, UK). Chromatography (grade 3MM) paper was from Whatman. Acetonitrile and methanol were from Riedel-de-Haën, Germany. Sequencing-grade exoglycosidase and peptide glycan sialylase modulates the extension of the stalk. As has already been noted (20), thermodynamic considerations make an electrostatic repulsion-based mechanism for these effects extremely unlikely. In the present study, we test the second proposed mechanism, i.e. that O-glycan sialylation modulates the extension of the stalk-like region of CD8. Our findings suggest that sialylation has little, if any, effect on the overall structure of CD8.

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distilled water/acetonitrile containing 0.1% formic acid at about 20 nL/min from borosilicate capillary needles. For MSMS, the collision energy was 35–40 V for the doubly charged [M + 2H]^2+ ions from the C-terminal glycopeptide ions and 100–110 V for the singly charged ions. The mass window for parent ion selection was about 4 Da. Argon at 20 pounds/square inch was used as the collision gas. Sample acquisition and processing were performed with the Micromass MassLynx data system.

Analytical Ultracentrifugation—Analytical ultracentrifugation experiments were performed using a Beckman Optima XL/I analytical ultracentrifuge, which is equipped with absorbance and interference optics. Samples of sCD8αx, sCD8αxK1, and sCD8αxLec were spun at 40,000 rpm at 20 °C. Sample distributions were recorded, using the interference or absorbance systems, at 2-min intervals. The data were analyzed using the Sedfit software (34, 35). The partial specific volumes and masses of sCD8αxK1, sCD8αxLec, and sCD8αxE were calculated using the known amino acid composition of CD8 and the glycosylation analysis of sCD8αxK1 and sCD8αxLec reported herein. The calculation was accomplished using a derivative program of AtoB (36). The sedimentation coefficients of sCD8 calculated over a concentration range were extrapolated to infinite dilution and corrected for buffer viscosity and density compared with water as a standard solvent. The program Sedfit was used to calculate the frictional ratio of each sample, which being the ratio between the experimental frictional coefficient and the frictional coefficient of a spherical species of the same mass and partial specific volume. The frictional and sedimentation coefficients obtained were corrected for hydration effects for a series of different levels of hydration as previously described (37), yielding “dry” Perrin functions P_exp and sedimentation coefficients S_20,w.5(0) versus S_20,w.5(0) (Eq. 1): (38)

\[ P_{exp} = \frac{f}{f_0} \left[ 1 + \frac{\delta_{exp}}{f_0 P_{T_0}} \right]^{1/3} \]  

(Eq. 1)

and

\[ S_{20,w.5}(0) = S_{20,w.5}(0) \left[ 1 + \frac{\delta_{exp}}{f_0 P_{T_0}} \right]^{1/3} \]  

(Eq. 2)

where \( \nu \) is the partial specific volume of the protein (ml/g), \( P_{T_0} \) the buffer density (g/ml), and \( \delta_{exp} \) is the hydration of the protein (g/g). The bead molecules used to predict hydrodynamic properties were calculated using the program AtoB (36). The solution properties of the bead models were calculated using SOLTRO (39).

RESULTS

The structure/function analysis of protein glycosylation can be problematic because preventing glycosylation, either genetically or with inhibitors, can lead to protein misfolding and/or non-secretion (for example see Ref. 40). In the case of O-glycans, post-expression enzymatic deglycosylation is difficult because commercially available endo-β-N-acetylgalactosaminidases do not cleave all types of O-glycan chains (41). The O-glycosylation of proteins secreted by CHO cell-derived Lec3.2.8.1 cells has not been characterized previously in detail, but these cells are known to be deficient in enzymes required for sialylation (Lec2 mutations; Refs. 42 and 43) and galactosylation (Lec8 mutations; Ref. 44). It was therefore anticipated that these cells would produce soluble forms of sCD8 αa and ββ with substantially modified O-glycans. We confirm this proposal here and examine its consequences for the structure of sCD8 expressed in Lec3.2.8.1 versus non-mutant CHO K1 cells using velocity sedimentation analysis, which is one of the few techniques that presently yield structural data for native glycoproteins with the properties of CD8. To allow reliable hydrodynamic modeling we also characterize the N-glycosylation of the soluble forms of CD8.

CD8 Constructs—For the present study, four recombinant soluble forms of glycosylated CD8 were prepared as described previously (22). Briefly, sCD8αx consists of the mouse CD8α chain (residues 1–130 of the mature polypeptide) expressed as a soluble, homodimeric fusion protein with 17 residues of the rat CD8α stalk-like peptide (residues 122–138) containing the O-linked sugars and the OX-8 anti-rat CD8 monoclonal anti-body epitope (Fig. 1a). Overall, the stalk-like region of this protein, measured from the first residue beyond β-strand G of the mouse α-chain V-set IgSF domain (i.e. Val-121), consists of 26 residues. b, the mouse CD8ββ chain (residues 1–116, boxed) was fused to the 23 residues forming the C terminus of the sCD8α construct (residues 125–147 of construct a).

Glycosylation Analysis, N-Linked Glycans—The N-glycans were released by hydrazinolysis from purified sCD8αxaK1, sCD8αβK1, and sCD8ββK1 expressed in CHO K1 cells, and sCD8αxLec and sCD8αβLec expressed in Lec3.2.8.1 cells. The glycan pools were analyzed directly by MALDI-TOF mass spectrometry to obtain the composition of the constituent isobaric monosaccharides (Tables I–III) or labeled with 2-AB for analysis by NP-HPLC (Fig. 2 and Tables I–III). The HPLC procedure separates glycans on the basis of their hydrophilicity which, in practice, corresponds very closely to molecular weight (29). HPLC chromatograms were calibrated against a standard dextran hydrolysatse, enabling sample retention times to be expressed as glucose units. Preliminary structural assignments were made by comparison with glucose unit values in a data base of standard N-glycans (21). The glycosylation profiles of the αα and ββ constructs, and of a non-physiological form of CD8 consisting of the ββ homodimer, were all very similar (Fig. 2a, top 3 panels), indicating that there is little difference in the glycan processing of the α- and β-chains and that the N-glycosylation sites are equally accessible to the processing enzymes.

The identity of the N-glycans was confirmed by exoglycosidase digestion of the pool of labeled glycans and re-analysis by NP-HPLC (representative data for sCD8αa is shown in Fig. 2b). The relative proportions of each glycan, derived from integration of the fluorescence intensities of the peaks (Fig. 2a), are
The N-glycan pool from sCD8αK1 was subjected to exoglycosidase sequencing and MALDI-TOF mass spectrometry, as described under "Experimental Procedures," to derive the structures shown. The relative abundance was calculated from the fluorescence intensities of the peaks from the NP-HPLC analysis (Fig. 2a, top panel).

### Table I

**Analysis of N-glycans of sCD8α expressed in CHO K1 cells**

The N-glycan pool from sCD8αK1 was subjected to exoglycosidase sequencing and MALDI-TOF mass spectrometry, as described under "Experimental Procedures," to derive the structures shown. The relative abundance was calculated from the fluorescence intensities of the peaks from the NP-HPLC analysis (Fig. 2a, top panel).

| Peak no. | Structure | GU [M+Na]+ | Composition | Trivial name | Relative amount (%) |
|----------|-----------|------------|-------------|--------------|---------------------|
| N.D.     | -         | -          | -           | -            | -                   |
| N1       | 6.21      | 1257.4     | H$_5$N$_4$  | Man$_3$      | 18                  |
| N2       | 7.07      | 1419.5     | H$_5$N$_4$  | Man$_3$      | 15                  |
| N3       | 7.60      | 1809.6     | H$_5$N$_4$F$_1$ | A$_5$G$_5$F$_1$ | 8                   |
| N4b      | 7.94      | 1606.3     | H$_5$N$_4$  | A$_5$O$_2$   | 19                  |
| N4c      | 7.96      | 1581.5     | H$_5$N$_4$S$_1$ | A$_5$G$_5$F$_1$ | 8                   |
| N5       | 8.31      | 2100.7     | H$_5$N$_4$F$_1$ | S$_1$A$_5$G$_5$F$_1$ | 27                  |
| N6       | 8.86      | 2391.8     | H$_5$N$_4$F$_1$ | A$_5$G$_5$F$_1$ | 4                   |
| N7       | 8.90      | 1743.6     | H$_5$N$_4$  | Man$_3$      | 1                   |

* N.D., not detected by NP-HPLC.
* N.D., H$_5$N$_4$ was not detected by NP-HPLC but was produced by desialylation of H$_5$N$_4$S$_1$ during the MALDI ionization process.
† Compositional abbreviations are: H, hexose; N, N-acetylaminohexose; F, fucose; and S, sialic acid.
‡ G$_n$ refers to antennae number (n); G$_n$ is the number of galactose residues; F$_n$ is the number of fucoses; and S$_n$ is the number of sialic acids; Man$_n$ refers to the number of mannose residues. The scheme for glycan representation is as follows: •, N-acetylgalactosamine; ○, galactose; diamond with dot = fucose; □ = mannose; *, N-acetylatedneuraminic acid; dashed line, $\alpha$-linkage; full line, $\beta$-linkage.

In contrast, sCD8αLec and sCD8αβLec contained only oligomannose glycans (Fig. 2a, two lower profiles), which digested with jack bean $\alpha$-mannosidase to Man$_5$GlcNAc$_2$. The remaining glycans were all bi-antennary complex-type glycans. Of these, 57% were sialylated, mostly as the mono-sialylated form. Thirty nine percent of the bi-antennary glycans were core-fucosylated (Table I).

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### Table II

**Analysis of N-glycans of sCD8α expressed in Lec3.2.8.1 cells**

The N-glycan pool from sCD8αLec was subjected to exoglycosidase sequencing and MALDI-TOF mass spectrometry as described under "Experimental Procedures" to derive the structures shown. The relative abundance was calculated from the fluorescence intensities of the peaks from the NP-HPLC analysis (Fig. 2a, 4th panel). The scheme for glycan representation is as follows: •, N-acetylgalactosamine; ○, galactose; diamond with dot = fucose; □ = mannose; *, N-acetylatedneuraminic acid; dashed line, $\alpha$-linkage; full line, $\beta$-linkage.

| Peak no. | Structure | GU [M+Na]+ | Composition | Trivial name | Relative amount (%) |
|----------|-----------|------------|-------------|--------------|---------------------|
| N1       | 5.21      | 933.3      | H$_5$N$_4$  | Man$_3$      | 72                  |
| N2       | 6.24      | 1257.4     | H$_5$N$_4$  | Man$_3$      | 16                  |
| N3       | 7.12      | 1419.5     | H$_5$N$_4$  | Man$_3$      | 8                   |
| N4       | 7.90      | 1581.5     | H$_5$N$_4$S$_1$ | A$_5$G$_5$F$_1$ | 4                   |

### Table III

**Relative proportions of N-glycans of sCD8 α and β chains expressed in CHO K1 cells**

The N-glycan pools from sCD8 α and β chains, separated on SDS-PAGE (Fig. 3a), were subjected to exoglycosidase sequencing as described under "Experimental Procedures" to derive the structures shown. The relative abundance was calculated from the fluorescence intensities of the peaks from the NP-HPLC analysis (Fig. 3b). The scheme for glycan representation is as follows: •, N-acetylgalactosamine; ○, galactose; diamond with dot = fucose; □ = mannose; *, N-acetylatedneuraminic acid; dashed line, $\alpha$-linkage; full line, $\beta$-linkage.

| Peak no. | Relative amount α (%) | Relative amount α2 (%) | Relative amount β (%) |
|----------|-----------------------|------------------------|-----------------------|
| N1       | 6.21                  | 18                     | 28                    | 20                    |
| N2       | 7.60                  | 9                      | 11                    | 48                    |
| N3       | 7.94                  | 32                     | 26                    | 12                    |
| N4       | 8.31                  | 22                     | 15                    | -                     |
| N5       | 8.80                  | 4                      | 3                     | 5                     |

The relative proportions of N-glycans of sCD8 α and β chains expressed in CHO K1 cells were determined by mass spectrometry and compared with the results from SDS-PAGE. The bands corresponding to the α-chains (bands α1 and α2) and β-chain (band β) were excised, sCD8α chains from both the αα and αβ constructs ran as doublets on SDS-PAGE (Fig. 3a). Protein sequencing analysis of α1 and α2 by mass spectrometry confirmed that both bands were derived from the α-chain (data not shown). The doublet was not due to the existence of distinct sCD8α glycoforms because it remained after digestion with PNGaseF (data not shown). Importantly, identical heterogeneity was also apparent in Lec3.2.8.1-derived protein (data not shown), but its...
The structures identified are given in Tables I and II. Glycans present in the sCD8 glycan pool from sCD8ase; bovine kidney hydrazinolysis. Absence of the core 2-branching N-acetylgalactosaminyltransferase in CHO K1 cells (48), which are predicted (Fig. 4b, Table IV) indicated that these glycans consisted of the type 1 core Gal-β1,3GalNAc disaccharide (O1) and its sialylated tri- (O2) and tetrasaccharide derivatives (O3). This pattern is consistent with the known absence of the core 2-branching N-acetylgalactosaminyltransferase in CHO K1 cells (48), which is required for the synthesis of larger O-glycan structures branching at C6 of the core GalNAc.

The NP-HPLC profile of sCD8aseLec O-glycans (Fig. 4a) was characterized by the complete absence of the di-sialylated O3-glycan structure and a substantial reduction in the monosialylated structure (Fig. 4c and Table IV). Therefore, the key difference between the two forms of sCD8α is that although 86% of the core 1 O-glycans are mono- and di-sialylated in CHO K1 cells, 82% of the Lec3.2.8.1-derived core 1 O-glycans are non-sialylated. This glycan composition is consistent with the known defects in Lec3.2.8.1 cells (42–44), which are predicted to result in the production of single GalNAc residues. However, although hydrazinolysis cleaves single GalNAc residues from proteins, some monosaccharides are removed along with peptides at a subsequent paper chromatography step. Therefore, in principle, the NP-HPLC analysis could overestimate the degree of sialylation of sCD8aseLec. In order to determine whether or not single GalNAc residues occupy any of the sites in sCD8aseLec, mass spectrometric analysis of the tryptic fragments of sCD8α was undertaken.

Electrospray mass spectrometry and subsequent MS/MS
fragmentation of the \([M + H]^+\) ions of tryptic peptides derived from sCD8αLec and sCD8αK1 produced a cleavage profile consistent with that of a 15-amino acid C-terminal α-chain glycopeptide with the sequence APTPVPPPTGTPRPL (examples are given in Fig. 5). This peptide contains three of the four threonine residues in the sCD8 stalk likely to be sites of glycosylation (6). The fragmentation pattern indicated that, when present, the hexoses are attached to the HexNAc residues.

MALDI mass spectrometry (Fig. 6a) showed that in excess of 92% of the tryptic glycopeptides of sCD8αLec had all three sites occupied (m/z = 2107.1, 2269.1, 2431.3, and 2593.3); 7% had two sites occupied (m/z 1903.2, 2066.1, and 2227.2), and 1% had one site occupied (m/z 1701.6 and 1863.6). The relative amounts of the fully occupied, sCD8αK1 (Fig. 4a, top panel), and sCD8αLec glycopeptide glycoforms are also apparent in the MALDI-MS profile of the whole tryptic digest. Relative to the amount of the glycoform consisting of single GalNAc residues at each of the three glycosylation sites (m/z = 2107.1), glycopeptides with one (m/z = 2269.1), two (m/z = 2431.3), and three (m/z = 2593.3) additional hexose sugars were present at 107, 62, and 13%, respectively. By taking these ratios into account, and the results of the NP-HPLC analysis (Fig. 4, a and c; Table IV), ~70% of the O-glycans present on Lec3.2.8.1-derived CD8α were monosialylated and 30% were disialylated. Thus, at least 82% of all the O-glycans present in the CHO K1-derived CD8αa were sialylated (i.e. 85% of the 96% core 1 O-glycans detected by MALDI-MS).

Analytical Ultracentrifugation—sCD8αK1, sCD8αLec, and sCD8aaE have apparent sedimentation coefficients at 293 K (s₂₀,₅₀) that vary inversely with concentration, presumably due to the limiting effects of macromolecular crowding on diffusion (Fig. 7) (49). Extrapolation to zero protein concentration yields values for s₀,₅₀ unaffected by crowding effects, s₀,₀(0).

### Table IV

| Peak no. | Structure | GU     | Relative amount (%) |
|----------|-----------|--------|---------------------|
| sCD8αK1  | O         | 1.79   | 15                  |
|          | O         | 2.97   | 55                  |
|          | O         | 4.4    | 30                  |
| sCD8αLec | O         | 1.79   | 82                  |
|          | O         | 2.97   | 18                  |

**Experimental Procedures**

The O-glycan pools from sCD8αK1 (Fig. 4a, top panel) and sCD8αLec (Fig. 4c, top panel) were subjected to exoglycosidase sequencing as described under "Experimental Procedures" to derive the structures shown. The relative abundance was calculated from the fluorescence intensities of the peaks from the NP-HPLC analyses. The scheme for glycan representation is as follows: ●, N-acetylglactosamine; ○, galactose; *, N-acetyleneuraminic acid; dashed line, α-linkage; full line, β-linkage.
hydrodynamic properties of low resolution molecular models of Ntially different sedimentation coefficients (3.73 $\pm$ 0.5) and the Perrin function ($P_{\text{exp}}$). Where necessary, the calculations are based on hydron weights of 0.1–0.5 g of $H_2O/g$ protein. The calculated mass values are sufficiently close to the mass of sCD8K1 obtained by MALDI-TOF (56,871 Da, data not shown) to be used in the calculations. As expected, sCD8K1 and sCD8Lec have much larger s and P values than sCD8E, reflecting the presence of the stalk-like region and N- and O-glycosylation of the CHO K1- and Lec2.8.1-derived proteins. Crucially, the P values, which provide shape information, are very similar for sCD8K1 and sCD8Lec (1.61 versus 1.54) even though the two proteins have substantially different sedimentation coefficients (3.73 versus 3.13), due to the different sizes of the N- and O-glycans attached to each protein.

To aid in the interpretation of these effects, we calculated the hydrodynamic properties of low resolution molecular models of each protein. The calculated s and P values for sCD8E, i.e. 2.61 and 1.14, respectively, modeled explicitly on the crystal structure of this protein (14) for $\delta = 0.3 g/g$, are very similar to the experimental values (i.e. 2.65 and 1.12) and indicate that in solution, as in crystals, this form of sCD8 lacking the stalk-like region is very compact (Table V; Fig. 8a). The sCD8K1 and sCD8K2 models were based on the crystal structure of the ligand-binding domain of murine CD8 (15), the sequence of the stalk-like regions, and the foregoing glycosylation analysis. The hydrodynamic properties of a model of sCD8K1 with a highly extended parallel stalk (i.e. $\sim 2.6A/\text{per residue}$, the likely upper limit for mucin-like polypeptides) are also in good agreement with the experimental values (i.e. s and P values of 4.05 and 1.53 at $\delta = 0.3 g/g$, versus 3.73 and 1.61, respectively; Fig. 8c). Crucially, a model of sCD8K2 with the same highly extended, parallel stalk also gives calculated s and P values that closely match the experimental values (3.23 and 1.53 at $\delta = 0.3 g/g$, versus 3.13 and 1.54, respectively; Fig. 8b).

To confirm that hydrodynamic modeling is sensitive to the overall structure of CD8, the predicted hydrodynamic properties of models of sCD8K2, in which the stalk is absent to the shortest possible, were determined (Fig. 8d). In combination, models 4–6 in Fig. 8d approximate the hydrodynamic properties of an essentially unconstrained, flexible stalk. Along with the comparisons between the models of the unglycosylated and glycosylated forms of sCD8K1 (Fig. 8, a–c) this shows that, in combination, s and P are capable of discriminating between models of CD8 whose stalk-like regions have distinct conformational properties.

**DISCUSSION**

We have expressed proteins in glycosylation mutant and wild-type Chinese hamster ovary cells in order to circumvent the pitfalls inherent in analyzing the biological effects of glycosylation indirectly with inhibitors or by mutagenesis, or after incomplete enzymatic deglycosylation. We characterize the glycosylation of the two isoforms of recombinant sCD8 and the O-glycosylating capacity of the Lec3.2.8.1 cell line. We show that Lec3.2.8.1 cells are considerably restricted in forming the type 1 core disaccharide and profoundly deficient in O-glycan sialylation. We use these observations to characterize the effects of O-glycan sialylation on the structure of the stalk-like region of CD8. Our results suggest that relatively few of the shortest possible O-glycans profoundly affect the extension of the stalk-like region of this cell surface molecule and that sialylation has little or no additional effect.

The presence of the $\beta$-chain in sCD8 $\alpha$,$\beta$, or its absence in $\alpha$, might have been expected to give rise to different N-glycosylation patterns, particularly given the very low level of protein sequence conservation. Although differing slightly in proportion, the N-glycans of CHO K1 cell-derived sCD8 $\alpha$ and $\beta$ were essentially of the same type, suggesting that the glycosylation sites are equally well exposed on both chains and accessible to the relevant processing enzymes. The N-glycan structures are restricted and consist of oligomannose and bi-antennary complex-type glycans. This glycan profile is presumably a consequence of the three-dimensional structure of the protein, as CHO K1 cells are known to be capable of processing tri- and tetra-antennary sialylated glycans on other glycoproteins, such as recombinant tissue plasminogen activator (50) and erythropoietin (51). Overall, however, our results argue strongly against the possibility that structure-based N-glycosylation differences are responsible for any functional differentiation of the $\alpha$- and $\beta$-isoforms of CD8.

The O-glycosylation in CHO cells is also very restricted and probably much simpler than for CD8 expressed on lymphocytes. In CHO K1 cells, the O-glycans consist of mono- and di-sialylated core 1 structures. Differences in O-glycosylation in the Lec3.2.8.1 cell line are likely due to the Lec2 and Lec8 mutations, which result in defective CMP-sialic acid and UDP-galactose translocation into the Golgi, respectively (43, 44). Lectin-binding studies of the O-glycosylation of recombinant glycophorin A expressed in Lec8 cells (52) indicated that the glycans are truncated and non-sialylated, and this is confirmed here for sCD8 expressed in Lec3.2.8.1 cells. Most of the O-linked glycans were predicted to be restricted to single GalNAc residues, although leakiness of the Lec2 and Lec8 phenotypes may generate O-linked structures with galactose and/or sialic acid residues added to the GalNAc. A 15-amino acid fragment, identified as the glycopeptide from the C terminus (position 133–147), was present as three distinct glycoforms, with the dominant species bearing a single GalNAc sugar at each of the three threonine residues. However, this glycopeptide is also present as glycoforms that contain an additional hexose residue at one or more of these sites, and mono-sialylated core 1 disaccharide was detected by sialidase digestion and HPLC analysis (Fig. 4c), confirming that the Lec3.2.8.1 mutant is indeed leaky.

Numerous studies have shown that, in leukocytes, O-glycosylation is very complex and varies in an activation-dependent and tissue-specific manner. For example, marked changes in core 2 branching and 6-GlcNAc transferase activities have been associated with T cell maturation, and a thymus-specific core 2–6GlcNAc-transferase has now been identified (53, 54). Of most relevance to the present study, the sialylation of core 1 O-linked glycans is known to be up-regulated during thymocyte maturation (55, 56). Changes in O-glycan processing of this

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**Fig. 7.** Variation in measured sedimentation coefficient ($s_{20,W}$) of sCD8K1, sCD8E, and sCD8K2 with concentration. The lines are first order polynomial fits to the data. The y intercept defines the sedimentation coefficient ($s_{20,W}(0)$) at infinite dilution in each case. Interference data are shown in b and c. Due to the relatively small amounts of sCD8K1 available, reliable interference data could not be collected, and absorbance data is shown in a instead.

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*P. Stanley, personal communication.*
TABLE V

| Sample        | $M_m$  | $\bar{v}$ | $s_{20,w}(0)$ | $s(c)$ | $b$   | $S_{20,w}(0)$ | $P_{exp}$ |
|---------------|--------|-----------|---------------|--------|-------|---------------|-----------|
| sCD8ααK1      | 54571  | 0.686     | 3.31 ± 0.17   | −0.3   | 1.82  | 0.1           | 3.46      |
|               |        |           |               |        |       |               | 1.74      |
|               |        |           |               |        |       | 0.3           | 3.73      |
|               |        |           |               |        |       | 0.5           | 3.97      |
|               |        |           |               |        |       | 0.5           | 3.32      |
| sCD8ααLec     | 42659  | 0.703     | 2.78 ± 0.06   | −0.2   | 1.73  | 0.1           | 2.91      |
|               |        |           |               |        |       | 0.3           | 3.13      |
|               |        |           |               |        |       | 0.5           | 3.45      |
|               |        |           |               |        |       | 0.5           | 3.24      |
| sCD8ααE       | 25500  | 0.737     | 2.37 ± 0.05   | −0.1   | 1.25  | 0.1           | 2.43      |
|               |        |           |               |        |       | 0.3           | 2.65      |
|               |        |           |               |        |       | 0.5           | 2.81      |

* $M_m$ is molar mass calculated from the known amino acid composition of sCD8αα dimer and the glycosylation analysis reported herein. $\bar{v}$ is the partial specific volume, and $\delta$ is the hydration in g of water/g of protein. These were calculated using the same data as for the $M_m$. The $s_{20,w}(0)$ is the sedimentation coefficient extrapolated to infinite dilution, and the $\delta$ in the second instance of this term refers to the correction to $s_{20,w}(0)$ for the calculated hydration level of the protein, $s(c)$ is the dependence of the sedimentation coefficient on concentration. $P_{exp}$ is the Perrin function, the frictional ratio corrected for hydration (in this case for the theoretical $\delta$ values listed here). The units of $s$ are Svedbergs (S).

Fig. 8. Molecular models of sCD8αα used to simulate hydrodynamic parameters, and the results of the simulations. a, the model of sCD8αα is based on the crystal structure of human sCD8αα from the sCD8αα−class I MHCp complex (14). The sCD8ααLec (b) and sCD8ααK1 (c) structures are based on the crystal structure of murine sCD8αα from the sCD8αα−class I MHCp complex (15), the amino acid sequence of the stalks, and the glycan analysis described herein. An extension of 2.6 Å per residue was used to model the stalk region. $s$ and $P$ are the calculated sedimentation coefficients and Perrin functions derived for the models (see text for details of the calculation), whereas $s_{exp}$ and $P_{exp}$ are the experimentally determined sedimentation coefficients and Perrin functions corrected for hydration using a value of $\delta = 0.3$ g/g (see Table V). In a, b, and c, the protein (purple) and N- and O-glycans (small red and dark blue spheres) are shown as “bead” models (assemblages of larger, transparent spheres). d, to demonstrate the extent to which the modeling is sensitive to the structure of CD8, the hydrodynamic properties are shown for models of sCD8ααLec (shown schematically) in which the stalk is fully extended as in b (model 1), absent to different degrees (i.e. by 50 and 80% in models 2 and 3) or not fully extended (models 4–6).

nature could have at least two functional outcomes. First, novel ligands for lectin-like receptors might be generated, such as the sialylated Leα determinant and the associated structures serving as ligands for selectin- and galectin-mediated cell-cell adhesion molecules (e.g. Ref. 57). A second possibility is that modifications of O-glycans alter the structure of O-glycosylated glycopeptides, indirectly modulating the presentation or conformation of the ligand-binding sites of cell surface molecules. Two groups have independently proposed that the binding function of CD8 is regulated in this way. Moody et al. (18) have suggested that the developmentally programmed sialylation of core 1 O-glycan structures by the galactose-sialyltransferase, ST3, alters the quaternary structure of the globular head domain of CD8, reducing its capacity to “clamp” MHC class I. Similarly, Daniels et al. (19) showed that CD8 binds MHC class I tetramers less avidly and that it becomes less effective as an adhesion molecule as sialylation increases during thymocyte maturation. They argue that changes in the flexibility or extension of the stalk-like region of CD8 may be critical for optimal ligand binding.

This proposal raises the question of whether or not the structural properties of CD8 can in fact be altered by sialylation. Previously, the structural effects of O-glycans had been thought to depend only on steric interactions between the peptide-linked GalNAc and the adjacent amino acids of the polypeptide (58–60). This conclusion was based on the analysis of mucins but was not confirmed for cell surface proteins with much shorter stalk-like polypeptides, such as CD8. Comparison of the hydrodynamic data for sCD8ααK1 and sCD8ααLec clearly indicates that O-glycans consisting of a single GalNAc are as effective as sialylated core 1 glycans in extending the stalk region of CD8. Therefore, our data generalize the concept that steric interactions between the peptide-linked GalNAc and the adjacent amino acids of the polypeptide account for the major structural effects of O-glycans, regardless of the length of the polypeptide.

The hydrodynamic modeling suggests that the degree of extension may be as great as 2.6 Å per residue. This is greater than for leukosialin (2 Å per residue (61)), comparable with that for bovine and porcine submaxillary gland mucins (2.5 Å per residue (59, 62)) but much less than the theoretical maximum (3.4 Å per residue). Remarkably, this apparent degree of extension of the CD8 stalk is achieved at half the density of leukosialin (63), i.e. approximately one glycan for every six amino acids. However, it is necessary to acknowledge the weaknesses of hydrodynamic modeling, wherein a static model is substituted for a dynamic molecule and the imperfectly understood hydration of proteins has to be arbitrarily fixed. Whereas our data indicate that the degree of extension is essentially indistinguishable for sCD8ααK1 and sCD8ααLec, systematic errors could confound a more quantitative analysis.

How can the results of Moody et al. (18) and Daniels et al. (19) be explained? The monomeric, non-cognate affinity of CD8...
for MHC class I molecules is extremely low (11, 15) and much weaker than for the TCR-MHCp interaction. Interactions that are this weak are very likely to be sensitive to avidity effects (i.e., density-dependent binding effects), which can occur in the absence of structural changes at the level of individual molecules. These effects are distinct from affinity changes, which are structure-dependent. Given that neither group directly tested the effect of CD8 sialylation on the monovalent binding affinity, in the light of our data, we suggest that the binding changes observed by Moody et al. (18) and Daniels et al. (19) are more likely the result of avidity changes. The simplest explanation is that de- or unsialylated CD8 tends to aggregate, increasing the likelihood of observable tetramer binding, for example. The differential sialylation of CD8 during thymopoiesis may simply occur coincidentally along with that of other glycoproteins for which sialylated O-glycans are of greater functional significance. The dominant thymocyte sialoglycoprotein is not CD8 but CD43 (64) after all. It could be argued that our results are not representative of the behavior of CD8 in vivo, where the αα-isoform predominates, given that we have characterized the solution properties of the αα-isoform. However, in all species, the analogous region of the β-chain of CD8 is also rich in proline and threonine residues and is likely to have a very similar structure to that of the α-chain. Therefore, although we cannot rule out the possibility that, in contrast to the α-chain, the structure of the β-chain is sensitive to sialylation, there is at present no obvious structural basis for suspecting that this is the case. More generally, as has been noted (65), because many proteins are affected by manipulations of the type employed by Moody et al. (18) and Daniels et al. (19) and glycosylation can affect proteins in several ways, it will often be difficult to establish that the glycosylation of a particular protein is important.

Finally, we note that the O-glycosylated region of the β-chain is significantly shorter than that of the α-chain (by nine residues in mouse CD8, measured to the inter-chain disulfide). Given the same degree of extension implied by the present hydrodynamic data, the β-chain is likely to cause the α-chain to arch, favoring a docking interaction with class I MHCp parallel with the cell surface, comparable with that seen in the crystal structures (14, 15). It is now accepted that the co-receptors and the TCR each have to bind the same MHCp molecule (2). If this is initiated by TCR binding to MHCp, as seems likely given the higher affinity of the TCR interaction (66), both co-receptors will be required to dock precisely to a binding site fixed —150 Å from the surface of the T cell. The question remains as to why CD4 and CD8 have evolved such different solutions to this docking problem. It seems relevant that cytototoxic CD8+ T cells are required to recognize their targets largely in the absence of interactions involving other similarly sized adhesive and co-stimulatory molecules, such as CD2 and CD28, that facilitate CD4+ T cell contacts with their targets. The orienting effects of the stalk, coupled with its inherent flexibility, may overcome this limitation and facilitate CD8-class I MHCp interactions during the very earliest stages of CD8+ T cell activation and immunological synapse formation. In this, O-linked sugars would appear to be key.

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O-Glycan Sialylation and the Structure of the Stalk-like Region of the T Cell Co-receptor CD8
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