Structure of the *N*-Linked Oligosaccharides of MHC Class I Molecules from Cells Deficient in the Antigenic Peptide Transporter

Implications for the Site of Peptide Association[1]

Bradley K. Hayes,[2]* Fernando Esquivel,[3†] Jack R. Bennink,[†] Jonathan W. Yewdell,[†] and Ajit Varki[4]*

Class I molecules are *N*-linked glycoproteins encoded by the MHC. They carry cytosolic protein-derived peptides to the cell surface, displaying them to enable immune surveillance of cellular processes. Peptides are delivered to class I molecules by the transporter associated with Ag processing (TAP). Peptide association is known to occur before exposure of class I molecules to the medial Golgi-processing enzyme α-mannosidase II, but there is limited information regarding the location or timing of peptide binding within the earlier regions of the endoplasmic reticulum (ER)-Golgi pathway. A reported association of newly synthesized class I molecules with the ER chaperonin calnexin raises the possibility of persistence of the monoglycosylated *N*-linked oligosaccharide (NLO) $Glc_1Man_8GlcNAc_2$, known to be recognized by this lectin. To explore these matters, we determined the structure of the NLOs on the subset of newly synthesized class I molecules awaiting the loading of peptide. We pulse-labeled murine MHC H-2D^b class I molecules in RMA/S cells, which lack one of the TAP subunits, causing the great majority of the molecules to be retained for prolonged periods in an early secretory compartment, awaiting peptide binding. MHC molecules pulse-labeled with [^{3}H]glucosamine were isolated, the NLOs specifically released and structurally analyzed by a variety of techniques. Within the chosen window of biosynthetic time, most D^b molecules from parental RMA cells carried mature NLOs of the biantennary complex-type, with one to two sialic acid residues. In RMA/S cells, such chains were in the minority, the majority consisting of the precursor forms $Man_8GlcNAc_2$ and $Man_9GlcNAc_2$. No glucosylated forms were detected, nor were the later processing intermediates $Man_{5-7}GlcNAc_2$ or $GlcNAc_1Man_{4-5}GlcNAc_2$. Thus, most D^b molecules in TAP-deficient cells are retained in an early compartment of the secretory pathway, before the point of first access to the Golgi α-mannosidase I, which trims α1–2 linked mannose residues, but beyond the point where the α1–3-linked glucose residue is finally removed by the ER glucosidase II. Thus, structural analysis of NLOs on class I molecules within a defined biosynthetic window has established a biochemical measure of the timing of peptide association. ***The Journal of Immunology,* 1995, 155: 3780–3787.**

T cells expressing CD8 play an important role in eradicating viruses and other intracellular parasites, recognizing antigenic peptides in a complex with class I molecules of the MHC. Class I molecules are heterodimeric glycoproteins consisting of a small invariant nonglycosylated soluble subunit (β2 microglobulin) complexed to a larger polymorphic glycoprotein (α-chain), which is anchored to the membrane by a hydrophobic carboxy-terminal sequence (1, 2). Both subunits are co-translationally translocated into the endoplasmic reticulum (ER),[5] where α-chains rapidly acquire one to three *N*-linked oligosaccharides (NLOs). The antigenic peptides most abundantly presented by class I molecules are derived from cytosolic proteins (reviewed in Refs. 3 and 4). The evolutionary wisdom of this arrangement is obvious because viral replication has an absolute requirement for protein synthesis, which occurs primarily in the cytosol. However, this imposes a topologic hurdle, with a lipid bilayer separating the Ags from class I molecules. Most Ags clear this hurdle via the action of TAP (transporter associated with Ag presentation), a member of the ABC superfamily of transporters that functions to transport antigenic peptides from the cytosol into the secretory compartment (5–14). TAP consists of two MHC-encoded subunits termed TAP1 and TAP2, both of which are required to create a functional transporter. In TAP-deficient cells, most class I molecules are loosely assembled, and incubation of cells or detergent extracts at 37°C results in the dissociation of α-chains from β2 microglobulin, with loss of reactivity to most class I-specific mAbs (15–21).

Due to their central role in the immune system, it is important to understand the biogenesis of class I molecules. Additionally, their

*Glycobiology Program, University of California-San Diego Cancer Center, and the Department of Medicine, University of California, San Diego, La Jolla, CA 92093; and †Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

Received for publication May 31, 1995. Accepted for publication August 3, 1995.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by National Institutes of Health Grants CA38701 (A.V.) and PO1CA58689 (M.F.).

[2] Current address: Department of Biochemistry and Molecular Genetics, University of Alabama, Birmingham, AL 35294.

[3] Current address: Instituto de Biotecnologia, Cuernavaca, Morelos, Mexico.

[4] Address correspondence and reprint requests to Ajit Varki, Cancer Center, 0687, UCSD School of Medicine, La Jolla, CA 92093-0687.

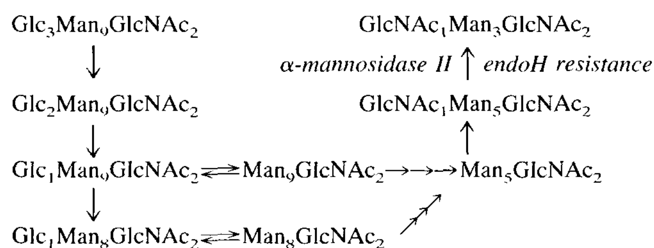
[5] Abbreviations used in this paper: ER, endoplasmic reticulum; TAP, transporter associated with Ag processing; NLO, *N*-linked oligosaccharide; endo-H, endo-β-*N*-acetylglucosaminidase H; PNGaseF, Peptide:*N*-glycosidase F.

Copyright © 1995 by The American Association of Immunologists

0022-1767/95/$02.00

biosynthesis and assembly serves as a model for the general problem of assembling multimeric membrane glycoproteins and regulating their export from the ER. Most peptides appear to bind to pre-assembled α-β_2 microglobulin complexes (originally referred to as "empty" class I molecules; a more precise term is "peptide-receptive") (16, 22). In detergent extracts or on the cell surface, peptide-receptive class I molecules are unstable at temperatures of >30°C and rapidly dissociate, causing conformational alterations in α-chains reflected in the loss of mAb reactivity. Earlier reports indicate that at least a portion of these molecules are retained in the ER (23) associated with the chaperonin calnexin (21, 24–28), which is now known to be a lectin (29–31) recognizing a monoglycosylated intermediate in the NLO processing pathway (32, 33). More recent findings indicate that most of these newly synthesized peptide-receptive complexes are bound to TAP (13, 14), rather than to calnexin. Regardless, in both models the MHC complexes are released upon peptide binding, resulting in rapid export to the cell surface via the Golgi complex.

The sequential modification of NLOs by enzymes concentrated in subcompartments of the secretory pathway provides the most precise means available for defining the location of events that occur in the assembly and maturation of glycoproteins (34). After the initial en bloc transfer of $Glc_3Man_9GlcNAc_2$ units to a suitable Asn residue, the sugar chains are processed by several different enzymes before being modified into an endo-β-*N*-acetylglucosaminidase H (endo-H)-resistant form ($GlcNAc_1Man_3GlcNAc_2$) by the Golgi-processing enzyme α-mannosidase II, which is first encountered in the medial Golgi.



Previous work using endo-H (15, 16) indicated that the site of peptide loading is proximal to the point where the MHC class I molecule encounters α-mannosidase II (see scheme above). On the other hand, Williams and colleagues (24, 26, 35, 36) reported that before peptide loading, class I molecules are associated predominantly with the chaperone calnexin. The oligosaccharides recognized by the lectin function of calnexin ($Glc_1Man_9GlcNAc_2$ and $Glc_1Man_8GlcNAc_2$) are known (37–42) to undergo a deglucosylation-reglucosylation cycle (shown in the scheme above) that may prolong their association with calnexin in the ER (29, 30). Thus, structural characterization of the NLOs on newly synthesized class I molecules associated with calnexin and/or TAP is of interest and may help to define further the intracellular location of class I assembly.

Structural characterization of the NLOs of mature MHC class I molecules has been performed previously (43, 44), but these studies did not address the nature of the early biosynthetic intermediates. Radioactive monosaccharides can be used to pulse-label a cohort of class I molecules in normal cells, but the speed at which they pass though the secretory pathway makes it difficult to isolate the form awaiting peptide association (45). Furthermore, it is technically difficult to obtain and study the detailed structure of the oligosaccharides from a single protein during a narrow window of biosynthesis. To obtain sufficient quantities of TAP-associated radiolabeled class I molecules for analysis, we used RMA/S cells. These cells were selected from chemically mutagenized mouse RBL-5 lymphoma cells on the basis of low steady-state levels of cell surface class I expression (46). RMA/S cells synthesize normal H-2 K^b and D^b α-chains and β_2-microglobulin, but fail to assemble them properly because of a point mutation resulting in premature termination in the single copy of *TAP2* they express (47). Although the cells maintain a limited ability to present cytosolic Ags to T_{CD8+} (48–51), >90% of new synthesized class I molecules are shed or degraded without ever having bound to a peptide with sufficient affinity to stabilize their structure (17, 22). Characterization of [^{35}S]methionine-radiolabeled class I molecules showed that they are retained by TAP1 in an endo-H-sensitive form for 5 to 10 times as long as class I molecules synthesized by RMA cells (the mutagenized parent cells used for selection of RMA/S cells) (24). Consistent with this, NLOs on class I molecules in RMA cells reach the *trans*-Golgi network much more rapidly than those synthesized by RMA/S cells, as measured by their acquisition of sialic acids (16). These findings indicate that most class I molecules in RMA/S cells are retained in the more proximal regions of the secretory pathway, i.e., the ER, the intermediate compartment, or the most proximal regions of the Golgi complex. By inference, peptide association normally occurs in these compartment(s). Also consistent with this, chimeric class I molecules retained in an endo-H-sensitive form by virtue of cytosolic sequences obtained from adenovirus E3/19K glycoprotein can be assembled with peptide with similar efficiency as unmodified class I molecules (52). Here we have determined the structure of newly synthesized NLOs associated with murine MHC H-2D^b class I molecules derived from RMA/S cells.

Materials and Methods

Materials

Peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase (PNGase F) was purified by gel filtration (53) and characterized as described previously (54). Jack Bean β-hexosaminidase and α-mannosidase were from V-Labs (Covington, LA) and had activities of 0.5 and 0.23 U/μl respectively. *Arthrobacter ureafaciens* sialidase from Calbiochem (La Jolla, CA) was dissolved at 4 U/ml in 100 mM sodium citrate, pH 4.6, and stored at −20°C. The MicroPak AX-5 HPLC column was from Varian (San Fernando, CA). The TSK-GEL DEAE-2SW HPLC column was from TosoHaas (Montgomeryville, PA). Sephacryl S-200 HR, Biobeads SM-2, Sephadex G-15, and concanavalin A (Con A) Sepharose were from Pharmacia (Piscataway, NJ). Oligosaccharide standards were obtained from Dionex (Sunnyvale, CA) and labeled at the reducing terminus with Na[^{3}H]BH_4. All other reagents obtained from commercial sources were of the highest quality available.

Radiolabeling and isolation of D^b class I MHC

RMA and RMA/S cells (2×10^7) were pulse-radiolabeled with 1 mCi of [^{3}H]$GlcNH_2$ for 20 min in 0.5 ml of glucose-free media, washed, and incubated in complete media for an additional 3 h at 37°C. Detergent lysates were precleared with protein A-Sepharose preloaded with an irrelevant Ab. Supernatants were then incubated with protein A-Sepharose preloaded with the mAb 28-14-8S (ATCC# HB27, directed against D^b) and washed extensively. Beads were boiled in SDS-PAGE sample buffer, and the supernatant was loaded onto a 10% polyacrylamide gel. After electrophoresis, the region of the gel containing H-2 D^b was excised and mascerated and proteins were recovered by electroelution into 25 mM Tris, 192 mM Glycine, 0.1% (w/v) SDS, pH 8.6. Protein was precipitated by adding 50 μg of BSA (essentially fatty acid-free) as a carrier and 10 volumes of cold acetone. After overnight incubation at 4°C, the precipitated protein and salts were recovered by centrifugation at 12,000 × *g* for 5 min. Ten milliliters of 70% (v/v) ethanol was added to the pellets, and the samples were placed at −20°C for up to 2 wk. Under these conditions, the salts were solubilized but the protein remained insoluble. After centrifugation in an IEC centrifuge at 2000 rpm for 30 min, ~70% of the radioactivity was recovered in the pellet.

Release and purification of N-linked oligosaccharides

The ethanol pellets were resuspended in 200 μl of 20 mM HEPES, 1% (w/v) SDS, 20 mM 2-ME, pH 8.5, by heating to 95°C for 15 min with

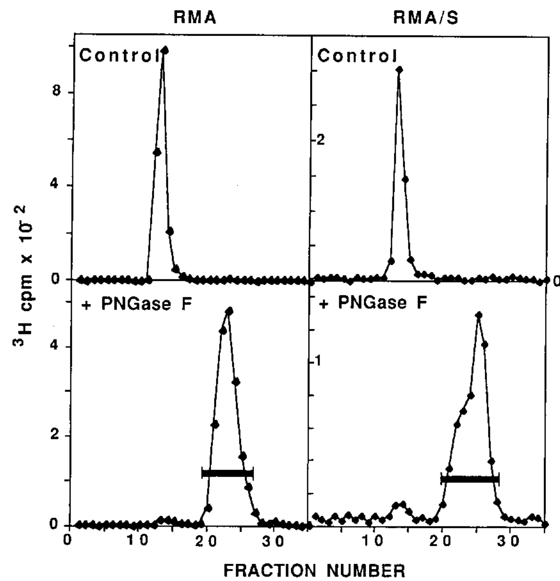


FIGURE 1. Gel filtration chromatography for the isolation of PNGaseF-released *N*-linked oligosaccharides. Gel-purified H-2 D^b molecules were incubated with or without PNGaseF and analyzed by gel permeation chromatography with Sephacryl S-200HR as described in *Materials and Methods*. The released oligosaccharides separated from the polypeptide backbone (shown by the bar) were pooled and analyzed further.

occasional venting to allow residual ethanol to evaporate and subsequently using a sonicating water bath at room temperature for 20 min. After adding 800 μl of 20 mM HEPES, 1.25% (w/v) Nonidet P-40, 20 mM 2-ME, pH 8.5, followed by 10 μl of 1 M 1,10-phenanthroline in methanol, a 50-μl aliquot of the 1-ml sample was saved as a minus PNGaseF control. NLOs were released by adding 10 μl of PNGaseF (2.5 mU) to the remaining 950 μl and incubating at 37°C. After 3 h, another 10 μl of PNGaseF was added, and the release was allowed to proceed for an additional 2 h, at which time the sample was heated to 95°C for 5 min. The released NLOs were separated from the polypeptide backbone by gel permeation chromatography (54) on a Sephacryl S-200 HR column (1.5 × 18 cm^2) run in 20 mM HEPES, 0.2% (w/v) SDS, pH 8.0. Fractions (0.93 ml) were collected, and radioactivity in 5% of the volume was monitored. Fractions with released oligosaccharides were identified by comparison with the profile of the control incubation sample, pooled, saturated KCl added to 2% (v/v), and the potassium dodecyl sulfate was allowed to precipitate overnight at 4°C. After centrifugation at 2600 × *g* for 15 min, the supernatant liquid was removed and any remaining Nonidet P-40 eliminated using a 5-ml Biobead SM-2 resin column as described previously (54). The column flow through and 5 column volumes of water wash were collected, lyophilized, redissolved in 1 ml of water, and desalted on a 1.5 × 27 cm Sephadex G-15 column run in water.

Analysis of N*-linked oligosaccharides*

Purified, desalted oligosaccharides were fractionated into neutral and anionic species by anion exchange chromatography, using 1.8 ml of QAE Sephadex prewashed with at least 10 column volumes of 2 mM Tris base. The desalted oligosaccharides in water were adjusted to 2 mM Tris base from a stock solution, applied to the column, and the flow through and an 11-ml wash of 2 mM Tris base was collected into a 50-ml tube. Anionic oligosaccharides were eluted with 5 × 1 ml aliquots of 1 M NaCl in 2 mM Tris base and collected into a single tube. The fractionated oligosaccharides were lyophilized, desalted on Sephadex G-15 as described above, lyophilized, resuspended in water, and stored at −20°C. The neutral oligosaccharides were analyzed using an AX-5 amine-bonded HPLC column (55). The column was equilibrated in 65% acetonitrile:35% water (flow rate 1 ml/min), the sample injected, and a gradient of 65% acetonitrile to 30% acetonitrile developed over 70 min. Fractions (0.4 min) were monitored for radioactivity after adding 5 ml of Liquiscint liquid scintillation mixture. Anionic oligosaccharides were analyzed by ion exchange chromatography using a DEAE HPLC column. The sample was injected onto the column equilibrated in water (flow rate 0.6 ml/min), eluted with water for 5 min, and then developed with a gradient of 0 to 100 mM NaCl over 70 min. Fractions (1 min) were monitored for radioactivity. The percent of radiolabel in [^{3}H]NeuAc was determined by treating the anionic oligosaccharides with 20 mU of *A. ureafaciens* sialidase for 5 h at 37°C in 40 mM sodium citrate, pH 4.6, in a final volume of 25 μl. The samples were placed in a boiling water bath for 5 min and applied to a Biogel P-4 gel permeation chromatography column (0.7 × 40 cm^2) equilibrated in 100 mM ammonium formate, pH 6.5. The column was eluted with the same buffer at a flow rate of 0.53 ml/min, and fractions (0.75 min) were monitored for radioactivity. The anionic oligosaccharides were also analyzed by lectin affinity chromatography (56) on a 1.8-ml column of Con A Sepharose 4B washed with 20 ml of Con A column buffer (10 mM HEPES, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.02% (w/v) NaN_3, pH 8.0). The oligosaccharide sample in 7.5 μl of water was diluted with 0.5 ml of Con A column buffer, applied to the column, and washed in 1.5 ml of column buffer followed by 4 × 2 ml aliquots of column buffer. The column was eluted first with 5 × 2 ml aliquots of Con A column buffer containing 10 mM α-methyl-glucopyranoside and then with 5 × 2 ml aliquots of Con A column buffer containing 100 mM α-methyl-mannopyranoside prewarmed to 60°C. The 2-ml fractions were monitored for radioactivity.

Table I. *PNGaseF release and charge distribution of [^{3}H]GlcNH$_2$-labeled oligosaccharides from H-2 D^b molecules*[a]

Cell Line	Radioactivity Release by PNGaseF	Anionic Oligosaccharides
RMA	98%	83%
RMA/S	95%	32%

[a] *N*-linked oligosaccharides of [^{3}H]GlcNH$_2$-labeled H-2 D^b were released by PNGaseF, isolated, and fractionated into neutral and anionic species as described in *Materials and Methods*.

Liquid scintillation counting

Appropriate background subtraction was determined with blank vials. In all cases, the samples were counted at a constant quench level in aqueous-compatible scintillation fluid for a period long enough to give a 95% confidence level. Thus, even with the low levels of radioactivity available for some analyses, signal-to-noise ratios were acceptable.

Results

Release, purification, and fractionation of N*-linked oligosaccharides*

Newly synthesized D^b molecules were pulse-labeled with [6-^{3}H]GlcNH$_2$ for 20 min, followed by a 3-h chase period. Under these conditions, most molecules in RMA cells are expected to have passed through the Golgi apparatus, whereas a substantial portion of those in RMA/S cells are retained in an early secretory compartment, still awaiting peptide loading. The radiolabeled molecules were immunoaffinity-purified and either analyzed directly or after SDS-PAGE separation and electroelution of D^b α-chains from excised gel slices. Both approaches gave generally similar results, but the data obtained with electroeluted molecules are presented here, because it cannot be affected by minor contaminants (of different M_r than α-chains) seen on the fluorographs of gels. Oligosaccharides were released with PNGaseF, which cleaves most known NLOs from glycoproteins (57), and separated from the polypeptide backbone by gel permeation chromatography (Fig. 1, Table I). Of the [^{3}H]GlcNH$_2$ incorporated into D^b molecules in both cells, >95% was released by PNGaseF. This was anticipated because D^b is not known to be *O*-glycosylated and [^{3}H]GlcNH$_2$ is incorporated into NLOs almost exclusively as [^{3}H]GlcNAc or [^{3}H]sialic acids (45, 58). The slightly altered profile of elution of the RMA/S oligosaccharides is likely due to the increased proportion of high mannose-type oligosaccharides (see below). The radiolabeled, purified NLOs were fractionated into neutral and anionic species by ion exchange chromatography on QAE Sephadex (Table I). The percent associated with the latter in RMA and

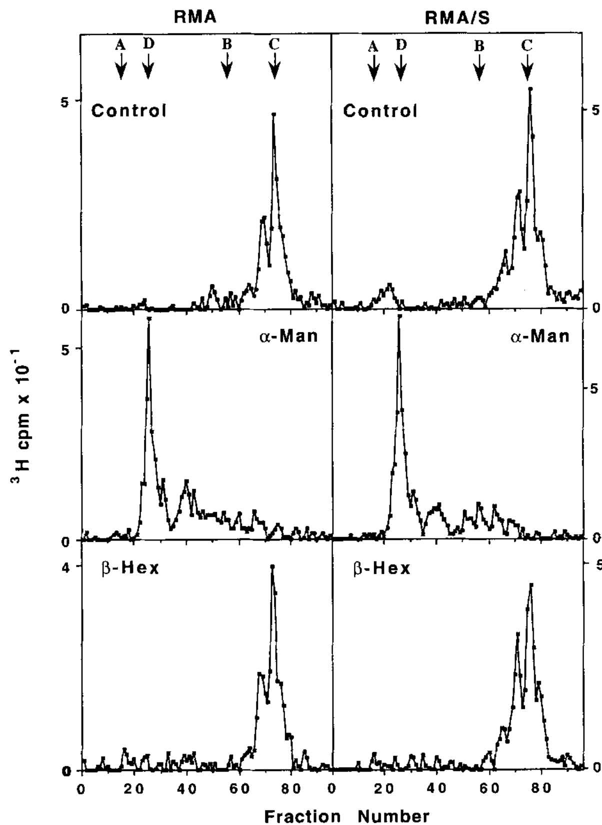
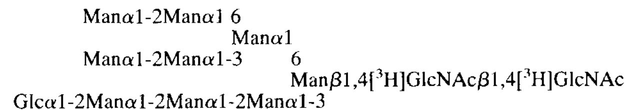


FIGURE 2. HPLC size analysis of neutral oligosaccharides on an amine-bonded AX-5 HPLC chromatography column. The neutral NLOs released from H-2 D^b molecules were analyzed with or without digestion by α-mannosidase or β-hexosaminidase as described previously (54). The position of elution of authentic oligosaccharide standards are indicated: *A*, GlcNAc; *B*, $Man_5GlcNAc_2$; *C*, $Man_9GlcNAc_2$; and *D*, Manβ1-4$GlcNAc_2$ (α-mannosidase-digested $Man_9GlcNAc_2$).

RMA/S cells (80 and 32%, respectively) may not be a completely accurate measure of actual amounts, because neutral and anionic chains may have different specific activities. After uptake, [^{3}H]$GlcNH_2$ is first converted to UDP-[^{3}H]GlcNAc, which can be used either directly or indirectly (see below) as a GlcNAc donor in oligosaccharide biosynthesis (45, 58). Alternatively, it can be epimerized to UDP-[^{3}H]GalNAc (incorporated primarily into *O*-linked oligosaccharides) or converted to [^{3}H]ManNAc, the precursor to CMP-[^{3}H]sialic acids. Anionic oligosaccharides should have higher specific activities than neutral chains for three reasons. First, they simply have more monosaccharides that can be radiolabeled. Second, the [^{3}H]GlcNAc on the external antennae comes directly from UDP-[^{3}H]GlcNAc, whereas that in the chitobiose core comes from the lipid-linked oligosaccharide Glc_3Man_9[^{3}H]$GlcNAc_2$-pyrophosphate-dolichol. The [^{3}H]GlcNAc in the latter may be of lower specific activity because it must pass through additional biosynthetic intermediates. Finally, Endo-H-sensitive oligosaccharides have only one or no *N*-acetyl-lactosamine antennae, whereas sialylated complex type oligosaccharides have two or more such antennae (with higher specific activity [^{3}H]GlcNAc residues). Thus, the percent of radioactivity associated with anionic chains represents an upper limit of the percent of total oligosaccharides that are anionic. Regardless of the precise ratios, it is clear that class I molecules from RMA cells have a much higher proportion of anionic chains than those from RMA/S cells. This fits previous knowledge that a larger proportion of the latter are retained in an early compartment of the secretory pathway.

Analysis of neutral oligosaccharides

The neutral oligosaccharides were analyzed by amine-adsorption HPLC, wherein separation occurs on the basis of size, with smaller molecules eluting first. Neutral oligosaccharides from both RMA and RMA/S were resolved into two major species, eluting in positions expected for $Man_9GlcNAc_2$ and $Man_8GlcNAc_2$ (Fig. 2). However, early intermediates such as $Glc_1Man_8GlcNAc_2$ could comigrate with the $Man_9GlcNAc_2$ standard, and the minor peaks eluting ahead could represent later processing intermediates such as $GlcNAc_1Man_5GlcNAc_2$. The possibility of terminal GlcNAc residues was ruled out easily by treatment with β-hexosaminidase, which gave no release of [^{3}H]GlcNAc, that would elute very early (Fig. 2). Thus, all of the [^{3}H]GlcNAc residues are in the chitobiose core of these oligosaccharides, inaccessible to the enzyme. Particularly because of the recent interest in the role of glucose units in calnexin binding (29–32), it was important to know whether such residues were present in a terminal position. If unprocessed glucose residues were present in the form of $Glc_1Man_8GlcNAc_2$ (see scheme below) or $Glc_1Man_7GlcNAc_2$, such oligosaccharides should be partially resistant to digestion with the exoglycosidase Jack Bean α-mannosidase (a well characterized enzyme that is free of any α-glucosidase activity).



In fact, α-mannosidase treatment caused most of the radioactivity to collapse to a species that co-migrated with the $Man_1GlcNAc_2$ (the last mannose residue is ß-linked and resistant to this enzyme). A small amount of radioactivity eluting immediately after the major peak is likely due to incomplete digestion, because this was also seen after treatment of a $Man_9GlcNAc_2$ standard (data not shown). The smallest possible size of a glucose-containing α-mannosidase resistant species is expected to be $Glc_1Man_4GlcNAc_2$, which should elute very close to the standard $Man_5GlcNAc_2$ (*B* in Fig. 2). However, only a very small fraction of the total radioactivity migrated in this area after α-mannosidase treatment (~3% with the RMA sample, and ~6% with the RMA/S sample). Thus, if any glucose residues are remaining, they are present only on a very small fraction of the total oligosaccharides. The structure of the major neutral oligosaccharides ($Man_9GlcNAc_2$ and $Man_8GlcNAc_2$) indicates that the class I molecules are retained in compartments lacking Golgi α-mannosidase I, which normally removes further mannose residues (the ER, the intermediate compartment, or possibly the most proximal elements of the Golgi complex). The presence of $Man_8GlcNAc_2$ is consistent with the removal of a single mannose residue from $Man_9GlcNAc_2$ by the α-mannosidase(s) located in the ER (59).

Analysis of the anionic oligosaccharides

The most common anionic substituent on the oligosaccharides of class I molecules is expected to be sialic acid. The anionic oligosaccharides from the D^b molecules of RMA and RMA/S cells were compared to determine whether the degree of sialylation was different. Analysis by DEAE HPLC chromatography (Fig. 3) showed that the majority of oligosaccharides had one or two negative charges, with a small fraction having three negative charges. Treatment with *A. ureafaciens* sialidase converted most of the oligosaccharides into neutral species, although 13% of the radiolabel retained a single negative charge. The distribution of radiolabel in the different anionic species of oligosaccharide from D^b from RMA and RMA/S cells are virtually identical (Table II). The 13% of radiolabel that remained anionic after sialidase digestion could

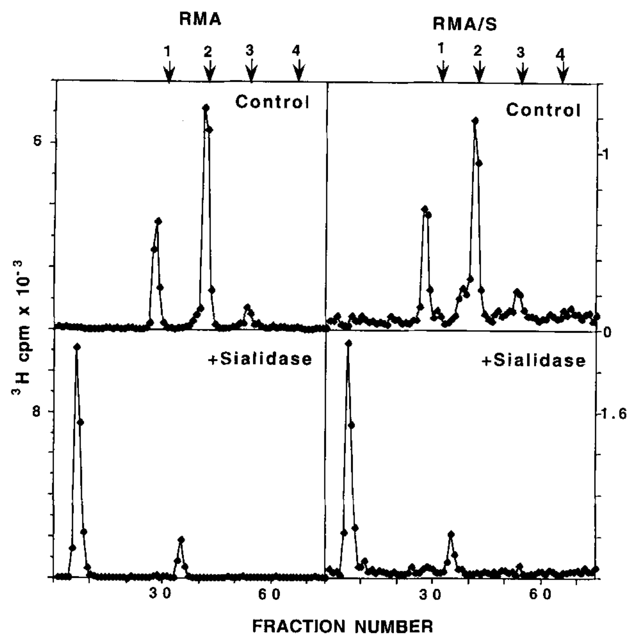


FIGURE 3. DEAE-HPLC analysis of anionic oligosaccharides. Anionic oligosaccharides released and purified from H-2 D^b molecules were analyzed by anion exchange chromatography with and without sialidase treatment as described previously (54). The elution position of oligosaccharide standards is indicated: 1) monosialylated *N*-acetyllactosamine; 2) disialylated biantennary NLO; 3) trisialylated triantennary NLO; and 4) tetrasialylated tetra-antennary NLO.

Table II. *Distribution of negative charge on [^{3}H]GLcNH$_2$-labeled oligosaccharides from H-2 D^b molecules*[a]

Cell Line	Sialidase Treatment	Net Negative Charge			
		0	−1	−2	−3
RMA	−		30%	62%	8%
	+	87%	13%		
RMA/S	−		30%	59%	11%
	+	87%	13%		

[a] Anionic [^{3}H]GlcNH$_2$-labeled oligosaccharides from H-2 D^b were isolated and analyzed by DEAE-HPLC before and after sialidase digestion, as described in *Materials and Methods*.

be due to incomplete release of sialic acids by the sialidase, to the presence of anionic moieties other than sialic acid, or to the conversion of [^{3}H]GlcNH$_2$ to [^{3}H]Sia. To distinguish these possibilities, anionic oligosaccharides from RMA-derived D^b were treated with *A. ureafaciens* sialidase and the products were analyzed by gel permeation chromatography on Biogel P-4. The intact anionic oligosaccharide eluted quantitatively with the void volume of the column and, after sialidase treatment, 13% of the radiolabel eluted in the included volume where [^{3}H]Sia is expected to run (Fig. 4). Thus, the 13% of radiolabel that retains a single negative charge after sialidase digestion can be accounted for completely by the conversion of [^{3}H]GlcNH$_2$ to [^{3}H]Sia. These results also confirm that the sole anionic constituent on the oligosaccharides from Class I MHC is sialic acid.

Affinity chromatography with Con A Sepharose was used to analyze further the structure of the anionic oligosaccharides. Tri- and tetra-antennary oligosaccharides are unretained by this column, and digalactosylated biantennary molecules and some monogalactosylated biantennary chains are eluted with 10 mM

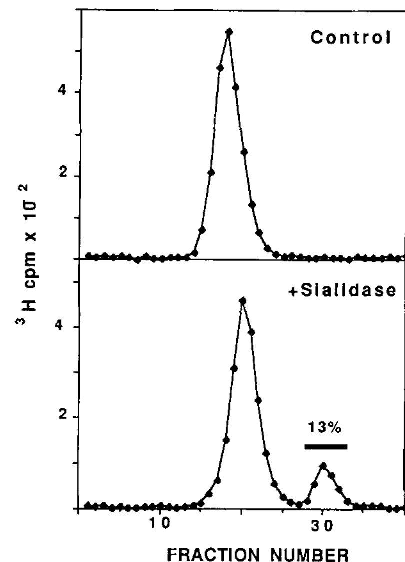


FIGURE 4. Gel permeation chromatography of sialidase-digested anionic oligosaccharides. The anionic oligosaccharides released and purified from H-2 D^b molecules of RMA cells was digested with sialidase and analyzed by gel permeation chromatography on BioGel P-4 as described in *Materials and Methods*. The label co-migrating with authentic NeuAc after sialidase digestion represented 13% of the applied radioactivity.

Table III. *Con A Sepharose analysis of anionic [^{3}H]GlcNH$_2$-labeled oligosaccharides from H-2 D^b molecules*[a]

Cell Line	Unretained	10 mM αMe-Glc-eluted	100 mM αMe-Man-eluted
RMA	17%	80%	3%
RMA/S	35%	62%	3%

[a] Anionic [^{3}H]GlcNH$_2$-labeled oligosaccharides from H-2 D^b were isolated and analyzed, as described in *Materials and Methods*.

α-methyl glucopyranoside, whereas hybrid chains and the remaining monogalactosylated biantennary oligosaccharides are eluted with 100 mM α-methyl mannopyranoside (56). As shown in Table III, very little radioactivity eluted with 100 mM α-methyl mannopyranoside; this is consistent with previous steady-state analyses of the related class I molecules K^k and D^k derived from the mouse B cell lymphoma cell line AKTB-1b (43). Comparison of Tables II and III reveals that the percentage of tri- and tetra-antennary anionic oligosaccharides (unretained by Con A) from D^b derived from either RMA or RMA/S cells is greater than the percentage of anionic oligosaccharides with three sialic acids. Thus, these oligosaccharides must be sialylated only partially. Likewise, a lower percentage of radiolabel than was associated with monosialylated oligosaccharides eluted from Con A Sepharose with 10 mM α-methyl mannopyranoside, indicating that some of the biantennary chains from either cell line are also partially sialylated. Interestingly, a greater percentage of RMA/S- than RMA-derived anionic oligosaccharides are unretained by Con A, indicating that a greater percentage of D^b molecules that do manage to pass through the Golgi complex in RMA/S cells acquire tri- or tetra-antennary oligosaccharides.

Discussion

In most previous studies of MHC class I biosynthesis, the structures of the NLOs were inferred indirectly on the basis of endo- or

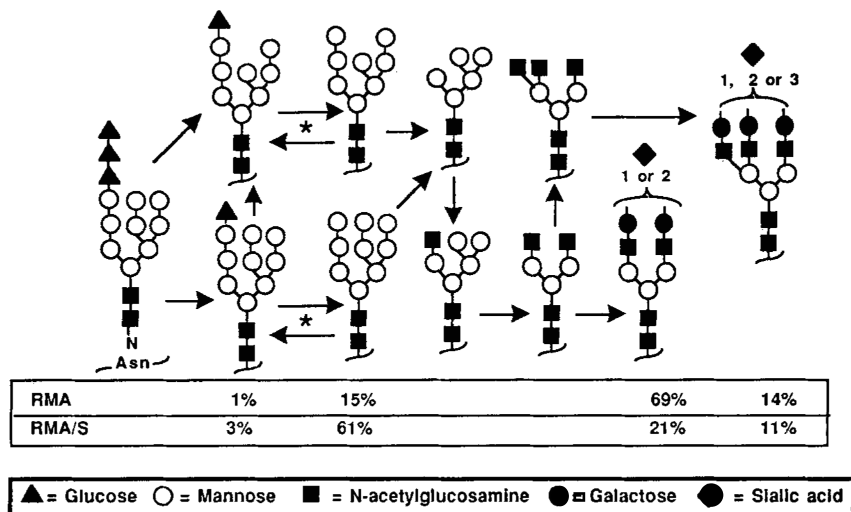


FIGURE 5. Distribution of radioactivity in *N*-linked oligosaccharide structures from H-2 D^b molecules of RMA and RMA/S cells after a 3-h chase. Key steps in the well established pathway for processing for NLOs (34) are shown, without specification of linkages. The asterisks indicate the steps of deglucosylation and reglucosylation (39–42) that are suggested by others to determine calnexin binding (29, 30, 32). The identification of the monoglucosylated molecules is indirectly based on α-mannosidase resistance and is a maximum estimate. The percentages shown are the results from the representative experiments presented in this paper. The totals add up to slightly less than 100% because of the small amount of unidentified neutral oligosaccharides (see Fig. 2).

exoglycosidase digestions, monitoring shifts in mobility on SDS-PAGE, or isoelectric focusing (15, 16). Although such approaches are extremely valuable, they do not define precisely the structure of the oligosaccharides in question. On the other hand, fine structural analyses are performed rarely on immunoprecipitates of metabolically labeled glycoproteins (43, 44, 60), because of the small quantities of material available and the potential for contamination by other glycoproteins. Here we have optimized several technologies to determine directly the structure of *N*-linked chains of class I molecules radiolabeled in a relatively brief pulse-chase experiment, thus avoiding late changes in the oligosaccharide caused by terminal degradation.

Final studies were performed on D^b molecules isolated after a 20-min pulse label and a 3-h chase period. This time period was chosen because previous work (28) indicated that most molecules in RMA cells should have been delivered to the cell surface, whereas most from the RMA/S cell line should be awaiting peptide loading, tethered by TAP1 (and/or calnexin) in an early compartment. We found that a majority of oligosaccharides from RMA/S cells were of the neutral high mannose type, whereas most of those from RMA cells were of the sialylated complex type. Structural analysis of these oligosaccharides indicates that neutral and charged species represent, respectively, the endo-H-sensitive and -resistant species described in previous reports (16, 17). The small amount of precursor forms persisting even in RMA cells after 3 h is not surprising and fits previous descriptions that it is difficult to completely "chase" these molecules out of the early compartment (24). Likewise, the small fraction of sialylated oligosaccharides found in the mutant cells is expected, because a substantial minority of the class I molecules in these cells do pass through the Golgi apparatus over the 3-h chase period, although they rapidly denature upon reaching the plasma membrane (16, 22, 24).

Williams and colleagues reported that in RMA/S cells, D^b molecules with endo-H-sensitive oligosaccharides remained associated with calnexin for at least 4 h after synthesis (24, 36). Although only a fraction of D^b was detected in chemically cross-linked complexes with calnexin, the efficiency of cross-linking was thought to be low, and it was concluded that a high percentage of retained D^b is complexed to calnexin. This conclusion conflicts with findings that human class I heavy chains associate with calnexin only for a very short period before being transferred to TAP (13, 14, 32). Moreover, the conclusion that a majority of class I molecules in RMA/S cells remains associated with calnexin in anticipation of peptide binding can be reconciled with more recent findings of TAP1 association only if it is assumed that TAP, calnexin, and class I molecules comprise a supercomplex. To our knowledge, there is no direct evidence for such a complex.

In any event, given the possibility that class I molecules are bound by both TAP and calnexin in RMA/S cells, it is important to relate our present findings to the emerging information about the lectin-like specificity of calnexin. Ou et al. first showed that the association of newly synthesized glycoproteins with calnexin was dependent upon *N*-linked glycosylation (29). Hammond et al. (30) then reported that treatment of cells with inhibitors of ER-glucosidases blocked the association of vesicular stomatitis virus G protein and influenza virus hemagglutinin (HA) with calnexin. Based on partial mobility shifts of [^{35}S]methionine pulse-labeled HA observed in SDS-PAGE after Jack Bean α-mannosidase digestion of calnexin-associated HA, they concluded that many of the oligosaccharides were monoglucosylated. This would be consistent with a previous report that oligosaccharides associated with VSV G protein retained in the ER (and presumably bound to calnexin) were predominantly monoglucosylated (61). Based on the more rapid turnover of radiolabeled glucose than glucosylated oligosaccharides, it was proposed that glucose residues were regenerated continuously through the opposing actions of ER glucosidase II and UDP-glucose:glycoprotein transferase. The latter enzyme was discovered by Parodi and colleagues, who demonstrated its presence in the ER and, appropriately, that it demonstrates a marked preference for oligosaccharides present on denatured glycoproteins (37–42). More recently, further evidence for this hypothesis has appeared (33), including the direct demonstration of a specific lectin-like recognition of $Glc_1Man_{8-9}GlcNAc_2$ by calnexin (32). Our own preliminary studies suggest that at least some class I-associated oligosaccharides in RMA/S cells remain glucosylated for a period of time, because after pulse radiolabeling with

[1-^{3}H]galactose (which can be converted to [1-^{3}H]glucose), a portion of the D^b-associated label is sensitive to endo-H digestion (J. Yewdell, unpublished observations). However, the data in Figure 2 indicate that such glucosylated oligosaccharides must represent a very small fraction of the neutral chains associated with D^b molecules, even in the RMA/S cells. Assuming that each molecule has two glycosylation sites, 12% or less of the D^b molecules from RMA/S cells carry even one glucosylated oligosaccharide at a time point when most of the molecules should be associated with calnexin (24). This indicates that either possession of monoglucosylated oligosaccharides is not a requirement for continued D^b binding to calnexin or that only the 12% or less of glucosylated D^b molecules are bound to calnexin. The former possibility is consistent with the recent demonstration that the lectin-like activity of calnexin is needed to initiate, but not to perpetuate, its interactions with glycoproteins (32).

Whatever the precise mechanism that accounts for retention of D^b, our findings directly demonstrate that H-2 D^b molecules are retained in a compartment of the secretory pathway that lacks the Golgi-processing enzyme α-mannosidase I (See Fig. 5). A simplified model of the Golgi apparatus has been proposed (62) in which it is divided into three general compartments; the *cis*-Golgi network (CGN), the medial Golgi, and the *trans*-Golgi network (TGN). In this model, the CGN is comprised of the *cis*-most compartment of the Golgi that is selectively stained with osmium tetraoxide and includes the ER/Golgi transitional elements referred to as the salvage compartment or the intermediate compartment (63); the medial Golgi is the site where most of glycosylation reactions occur and may be comprised of several distinct cisternae; and the TGN is the site of protein sorting and may also be involved in terminal glycosylation. Although Golgi mannosidase I is present in this medial Golgi complex, it is not completely absent from the CGN. This suggests that D^b molecules await peptide loading in the ER, the early part of the CGN, or in both of these compartments. TAP has been localized to both of these compartments by immunoelectron microscopy, although the specificity of staining of the *cis*-Golgi complex was not as clear as the specificity of ER staining (10). More recently, we found that TAP expressed via recombinant vaccinia viruses extensively co-localizes with BiP, a molecular chaperone often used as a marker of the ER (Russ et al., unpublished data). Moreover, we have shown that a fluorescein-tagged class I-binding peptide specifically binds class I molecules in the ER of fixed and permeabilized cells (22). Taken together, the preponderance of the evidence suggests that much, if not most, of peptide binding occurs in the ER.

Our results also indicate that after the final removal of glucose residues from *N*-linked glycoproteins, they are not transported immediately to the location of Golgi α-mannosidase I action, but can be retained in an earlier compartment. This is particularly relevant to the recent demonstration by Arar et al. that ERGIC-53, a membrane protein of the ER-Golgi intermediate compartment, is identical to a previously recognized intracellular mannose-specific lectin (64). This type I integral membrane protein is known to recycle between the Golgi apparatus and ER (64). Thus, this mannose-binding protein could be interacting with the $Man_8GlcNAc_2$ and $Man_9GlcNAc_2$ intermediates found on early class I molecules, aiding in their retention and/or recycling between the ER and the early part of Golgi apparatus. Another fact that must be reconciled eventually with these models is the finding that complete inhibition of glucose trimming by castanospermine results in rapid degradation of unassembled MHC class I molecules (65), as well as other newly synthesized glycoproteins (66). In this situation, one can hypothesize that prevention of exposure of the inner glucose residue abrogates the initial recognition by calnexin, marking the protein for destruction.

Acknowledgments

The authors acknowledge Carolyn Machamer for the original suggestion that prompted this work and Leland Powell for helpful suggestions during the course of this work and for his careful review of the manuscript.

References

1. Bjorkman, P. J., and P. Parham. 1990. Structure, function, and diversity of class I major histocompatibility complex molecules. *Annu. Rev. Biochem. 59:253.*
2. Bijlmakers, M. J., and H. L. Ploegh. 1993. Putting together an MHC class I molecule. *Curr. Opin. Immunol. 5:21.*
3. Townsend, A., and H. Bodmer. 1989. Antigen recognition by class I-restricted T lymphocytes. *Annu. Rev. Immunol. 7:601.*
4. Yewdell, J. W., and J. R. Bennink. 1992. Cell biology of antigen processing and presentation to major histocompatibility complex class I molecule-restricted T lymphocytes. *Adv. Immunol. 52:1.*
5. Deverson, E. V., I. R. Gow, W. J. Coadwell, J. J. Monaco, G. W. Butcher, and J. C. Howard. 1990. MHC class II region encoding proteins related to the multidrug resistance family of transmembrane transporters. *Nature 348:738.*
6. Trowsdale, J., I. Hanson, I. Mockridge, S. Beck, A. Townsend, and A. Kelly. 1990. Sequences encoded in the class II region of the MHC related to the "ABC" superfamily of transporters. *Nature 348:741.*
7. Spies, T., M. Bresnahan, S. Bahram, D. Arnold, G. Blanck, E. Mellins, D. Pious, and R. DeMars. 1990. A gene in the human major histocompatibility complex class II region controlling the class I antigen presentation pathway. *Nature 348: 744.*
8. Monaco, J. J., S. Cho, and M. Attaya. 1990. Transport protein genes in the murine MHC: possible implications for antigen processing. *Science 250:1723.*
9. Spies, T., V. Cerundolo, M. Colonna, P. Cresswell, A. Townsend, and R. DeMars. 1992. Presentation of viral antigen by MHC class I molecules is dependent on a putative peptide transporter heterodimer. *Nature 355:644.*
10. Kleijmeer, M. J., A. Kelly, H. J. Geuze, J. W. Slot, A. Townsend, and J. Trowsdale. 1992. Location of MHC-encoded transporters in the endoplasmic reticulum and *cis*-Golgi. *Nature 357:342.*
11. Androlewicz, M. J., K. S. Anderson, and P. Cresswell. 1993. Evidence that transporters associated with antigen processing translocate a major histocompatibility complex class I-binding peptide into the endoplasmic reticulum in an ATP-dependent manner. *Proc. Natl. Acad. Sci. USA 90:9130.*
12. Androlewicz, M. J., B. Ortmann, P. M. van Endert, T. Spies, and P. Cresswell. 1994. Characteristics of peptide and major histocompatibility complex class I/beta 2-microglobulin binding to the transporters associated with antigen processing (TAP1 and TAP2). *Proc. Natl. Acad. Sci. USA 91:12716.*
13. Ortmann, B., M. J. Androlewicz, and P. Cresswell. 1994. MHC class I/beta 2-microglobulin complexes associate with TAP transporters before peptide binding. *Nature 368:864.*
14. Suh, W. K., M. F. Cohen-Doyle, K. Fruh, K. Wang, P. A. Peterson, and D. B. Williams. 1994. Interaction of MHC class I molecules with the transporter associated with antigen processing. *Science 264:1322.*
15. Ljunggren, H. G., S. Paabo, M. Cochet, G. Kling, P. Kourilsky, and K. Karre. 1989. Molecular analysis of H-2-deficient lymphoma lines: distinct defects in biosynthesis and association of MHC class I heavy chains and beta 2-microglobulin observed in cells with increased sensitivity to NK cell lysis. *J. Immunol. 142:2911.*
16. Ljunggren, H. G., N. J. Stam, C. Ohlen, J. J. Neefjes, P. Hoglund, M. T. Heemels, J. Bastin, T. N. Schumacher, A. Townsend, K. Karre, and H. L. Ploegh. 1990. Empty MHC class I molecules come out in the cold. *Nature 346:476.*
17. Townsend, A., C. Ohlen, J. Bastin, H. G. Ljunggren, L. Foster, and K. Karre. 1989. Association of class I major histocompatibility heavy and light chains induced by viral peptides. *Nature 340:443.*
18. Townsend, A., T. Elliott, V. Cerundolo, L. Foster, B. Barber, and A. Tse. 1990. Assembly of MHC class I molecules analyzed in vitro. *Cell 62:285.*
19. Baas, E. J., H. M. van Santen, M. J. Kleijmeer, H. J. Geuze, P. J. Peters, and H. L. Ploegh. 1992. Peptide-induced stabilization and intracellular localization of empty HLA class I complexes. *J. Exp. Med. 176:147.*
20. Anderson, K. S., J. Alexander, M. Wei, and P. Cresswell. 1993. Intracellular transport of class I MHC molecules in antigen processing mutant cell lines. *J. Immunol. 151:3407.*
21. Song, E. S., Y. Yang, M. R. Jackson, and P. A. Peterson. 1994. In vivo regulation of the assembly and intracellular transport of class I major histocompatibility complex molecules. *J. Biol. Chem. 269:7024.*
22. Day, P. M., F. Esquivel, J. Lucsko, J. R. Bennink, and J. W. Yewdell. 1995. Effect of TAP on the generation and intracellular trafficking of peptide-receptive major histocompatibility complex class I molecules. *Immunity 2:137.*
23. Williams, D. B., S. J. Swiedler, and G. W. Hart. 1985. Intracellular transport of membrane glycoproteins: two closely related histocompatibility antigens differ in their rates of transit to the cell surface. *J. Cell Biol. 101:725.*
24. Degen, E., M. F. Cohen-Doyle, and D. B. Williams. 1992. Efficient dissociation of the p88 chaperone from major histocompatibility complex class I molecules requires both beta 2-microglobulin and peptide. *J. Exp. Med. 175:1653.*
25. Capps, G. G., and M. C. Zuniga. 1994. Class I histocompatibility molecule association with phosphorylated calnexin: implications for rates of intracellular transport. *J. Biol. Chem. 269:11634.*

26. Margolese, L., G. L. Waneck, C. K. Suzuki, E. Degen, R. A. Flavell, and D. B. Williams. 1993. Identification of the region on the class I histocompatibility molecule that interacts with the molecular chaperone, p88 (calnexin, IP90). *J. Biol. Chem. 268:17959.*
27. Zhang, Q., M. Tector, and R. D. Salter. 1995. Calnexin recognizes carbohydrate and protein determinants of class I major histocompatibility complex molecules. *J. Biol. Chem. 270:3944.*
28. Rajagopalan, S., and M. B. Brenner. 1994. Calnexin retains unassembled major histocompatibility complex class I free heavy chains in the endoplasmic reticulum. *J. Exp. Med. 180:407.*
29. Ou, W. J., P. H. Cameron, D. Y. Thomas, and J. J. Bergeron. 1993. Association of folding intermediates of glycoproteins with calnexin during protein maturation. *Nature 364:771.*
30. Hammond, C., I. Braakman, and A. Helenius. 1994. Role of *N*-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control. *Proc. Natl. Acad. Sci. USA 91:913.*
31. Helenius, A. 1994. How *N*-linked oligosaccharides affect glycoprotein folding in the endoplasmic reticulum. *Mol. Cell Biol. 5:253.*
32. Ware, F. E., A. Vassilakos, P. A. Peterson, M. R. Jackson, M. A. Lehrman, and D. B. Williams. 1995. The molecular chaperone calnexin binds $Glc_1Man_9GlcNAc_2$ oligosaccharide as an initial step in recognizing unfolded glycoproteins. *J. Biol. Chem. 270:4697.*
33. Hebert, D. N., B. Foellmer, and A. Helenius. 1995. Glucose trimming and reglucosylation determine glycoprotein association with calnexin in the endoplasmic reticulum. *Cell 81:425.*
34. Kornfeld, R., and S. Kornfeld. 1985. Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem. 54:631.*
35. Degen, E., and D. B. Williams. 1991. Participation of a novel 88-kD protein in the biogenesis of murine class I histocompatibility molecules. *J. Cell Biol. 112:1099.*
36. Jackson, M. R., M. F. Cohen-Doyle, P. A. Peterson, and D. B. Williams. 1994. Regulation of MHC class I transport by the molecular chaperone, calnexin (p88, IP90). *Science 263:384.*
37. Parodi, A. J., D. H. Mendelzon, G. Z. Lederkremer, and J. Martin Barrientos. 1984. Evidence that transient glucosylation of protein-linked $Man_9GlcNAc_2$, $Man_8GlcNAc_2$, and $Man_7GlcNAc_2$ occurs in rat liver and Phaseolus vulgaris cells. *J. Biol. Chem. 259:6351.*
38. Parodi, A. J., D. H. Mendelzon, and G. Z. Lederkremer. 1983. Transient glucosylation of protein-bound $Man_9GlcNAc_2$, $Man_8GlcNAc_2$, and $Man_7GlcNAc_2$ in calf thyroid cells. A possible recognition signal in the processing of glycoproteins. *J. Biol. Chem. 258:8260.*
39. Trombetta, S. E., M. Bosch, and A. J. Parodi. 1989. Glucosylation of glycoproteins by mammalian, plant, fungal, and trypanosomatid protozoa microsomal membranes. *Biochemistry 28:8108.*
40. Gañán, S., J. J. Cazzulo, and A. J. Parodi. 1991. A major proportion of N-glycoproteins are transiently glucosylated in the endoplasmic reticulum. *Biochemistry 30:3098.*
41. Sousa, M. C., M. A. Ferrero-Garcia, and A. J. Parodi. 1992. Recognition of the oligosaccharide and protein moieties of glycoproteins by the UDP-Glc:glycoprotein glucosyltransferase. *Biochemistry 31:97.*
42. Trombetta, S. E., and A. J. Parodi. 1992. Purification to apparent homogeneity and partial characterization of rat liver UDP-glucose:glycoprotein glucosyltransferase. *J. Biol. Chem. 267:9236.*
43. Swiedler, S. J., J. H. Freed, A. L. Tarentino, T. H. Plummer, Jr., and G. W. Hart. 1985. Oligosaccharide microheterogeneity of the murine major histocompatibility antigens. Reproducible site-specific patterns of sialylation and branching in asparagine-linked oligosaccharides. *J. Biol. Chem. 260:4046.*
44. Swiedler, S. J., G. W. Hart, A. L. Tarentino, T. H. Plummer, Jr., and J. H. Freed. 1983. Stable oligosaccharide microheterogeneity at individual glycosylation sites of a murine major histocompatibility antigen derived from a B cell lymphoma. *J. Biol. Chem. 258:11515.*
45. Varki, A. 1991. Radioactive tracer techniques in the sequencing of glycoprotein oligosaccharides. *FASEB J. 5:226.*
46. Ljunggren, H. G., and K. Karre. 1985. Host resistance directed selectively against H-2-deficient lymphoma variants: analysis of the mechanism. *J. Exp. Med. 162:1745.*
47. Yang, Y., K. Fruh, J. Chambers, J. B. Waters, L. Wu, T. Spies, and P. A. Peterson. 1992. Major histocompatibility complex (MHC)-encoded HAM2 is necessary for antigenic peptide loading onto class I MHC molecules. *J. Biol. Chem. 267:11669.*
48. Esquivel, F., J. Yewdell, and J. Bennink. 1992. RMA/S cells present endogenously synthesized cytosolic proteins to class I-restricted cytotoxic T lymphocytes. *J. Exp. Med. 175:163.*
49. Hosken, N. A., and M. J. Bevan. 1992. An endogenous antigenic peptide bypasses the class I antigen presentation defect in RMA-S. *J. Exp. Med. 175:719.*
50. Zhou, X., R. Glas, F. Momburg, G. J. Hammerling, M. Jondal, and H. G. Ljunggren. 1993. TAP2-defective RMA-S cells present Sendai virus antigen to cytotoxic T lymphocytes. *Eur. J. Immunol. 23:1796.*
51. Sijts, A. J., M. L. De Bruijn, J. D. Nieland, W. M. Kast, and C. J. Melief. 1992. Cytotoxic T lymphocytes against the antigen-processing-defective RMA-S tumor cell line. *Eur. J. Immunol. 22:1639.*
52. Lapham, C. K., I. Bacik, J. W. Yewdell, K. P. Kane, and J. R. Bennink. 1993. Class I molecules retained in the endoplasmic reticulum bind antigenic peptides. *J. Exp. Med. 177:1633.*
53. Plummer, T. H., Jr., and A. L. Tarentino. 1991. Purification of the oligosaccharide-cleaving enzymes of Flavobacterium meningosepticum. *Glycobiology 1:257.*
54. Hayes, B. K., H. H. Freeze, and A. Varki. 1993. Biosynthesis of oligosaccharides in intact Golgi preparations from rat liver. Analysis of *N*-linked glycans labeled by UDP-[6-^{3}H]*N*-acetylglucosamine. *J. Biol. Chem. 268:16139.*
55. Mellis, S. J., and J. U. Baenziger. 1981. Separation of neutral oligosaccharides by high-performance liquid chromatography. *Anal. Biochem. 114:276.*
56. Merkle, R. K., and R. D. Cummings. 1987. Lectin affinity chromatography of glycopeptides. *Methods Enzymol. 138:232.*
57. Tarentino, A. L., and T. H. Plummer, Jr. 1994. Enzymatic deglucosylation of asparagine-linked glycans: purification, properties, and specificity of oligosaccharide-cleaving enzymes from *Flavobacterium meningosepticum. Methods Enzymol. 230:44.*
58. Yurchenco, P. D., C. Ceccarini, and P. H. Atkinson. 1978. Labeling complex carbohydrates of animal cells with monosaccharides. *Methods Enzymol. 50:175.*
59. Roth, J., D. Brada, P. M. Lackie, J. Schweden, and E. Bause. 1990. Oligosaccharide trimming Man_9-mannosidase is a resident ER protein and exhibits a more restricted and local distribution than glucosidase II. *Eur. J. Cell Biol. 53:131.*
60. Tabas, I., and S. Kornfeld. 1980. Biosynthetic intermediates of beta-glucuronidase contain high mannose oligosaccharides with blocked phosphate residues. *J. Biol. Chem. 255:6633.*
61. Suh, K., J. E. Bergmann, and C. A. Gabel. 1989. Selective retention of monoglucosylated high mannose oligosaccharides by a class of mutant vesicular stomatitis virus G proteins. *J. Cell Biol. 108:811.*
62. Mellman, I., and K. Simons. 1992. The Golgi complex: in vitro veritas? *Cell 68:829.*
63. Hauri, H.-P., and A. Schweizer. 1992. The endoplasmic reticulum-Golgi intermediate compartment. *Curr. Opin. Cell Biol. 4:600.*
64. Arar, C., V. Carpentier, J.-P. Le Caer, M. Monsigny, A. Legrand, and A.-C. Roche. 1995. ERGIC-53, a membrane protein of the endoplasmic reticulum-Golgi intermediate compartment, is identical to MR60, an intracellular mannose-specific lectin of myelomonocytic cells. *J. Biol. Chem. 270:3551.*
65. Moore, S. E. H., and R. G. Spiro. 1993. Inhibition of glucose trimming by castanospermine results in rapid degradation of unassembled major histocompatibility complex class I molecules. *J. Biol. Chem. 268:3809.*
66. Kearse, K. P., D. B. Williams, and A. Singer. 1994. Persistence of glucose residues on core oligosaccharides prevents association of TCR alpha and TCR beta proteins with calnexin and results specifically in accelerated degradation of nascent TCR alpha proteins within the endoplasmic reticulum. *EMBO J. 13:3678.*