Post-translational Processing of an O-Glycosylated Protein, the Human CD8 Glycoprotein, during the Intracellular Transport to the Plasma Membrane*

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The biosynthesis of human CD8 glycoprotein in transfected rat epithelial cells produces an unglycosylated precursor, an intermediate species only initially O-glycosylated, and a doublet mature form carrying neutral and sialylated O-linked oligosaccharides with the core-2 structure (Pascale, M. C., Malagolini, N., Serafini-Cessi, F., Migliaccio, G., Leone, A., and Bonatti, S. (1992) J. Biol. Chem. 267, 9940–9947). In this study, the most relevant post-translational event analyzed is dimerization, addition of the first O-linked GalNAc, fulfillment of O-linked chains, as well as expression of involved glycosyltransferases, have been examined and correlated with the localization and transit rate of CD8 through the exocytic pathway. The glycosyltransferase activities measured in rat epithelial cells transfected with human CD8 DNA are entirely consistent with the primary structure assigned to CD8 oligosaccharides. The half-time of appearance of the initially O-glycosylated precursor and mature form was estimated to be 4 and 14 min, respectively, and the half-time for delivery of mature CD8 to the cell surface was found to be about 30 min, indicating a very fast routing. Pulse experiments with [35S]cysteine at 37 °C followed by chase-periods at low temperatures showed that folding/dimerization occurs before routing to the Golgi apparatus, whereas the addition of O-linked GalNAc appears to take place later, very likely in cis-Golgi cisternae. Treatment of cells with monensin accumulated the intermediate CD8 form carrying non-elongated O-linked GalNAc, whereas brefeldin A treatment produced a sialylated glycoprotein species with a mobility faster than the mature CD8. These results indicate that the two drugs affect assembly of O-linked chains at different time of their processing.

Much of the current knowledge on organization and functioning of the secretory pathway in animal cells has been obtained indirectly from study of the post-translational modifications of glycoproteins traveling from the endoplasmic reticulum (ER) to the plasma membrane (1–4). Perhaps the best example is given by the Golgi complex; the morphological localization of several maturing enzymes, and the temporal sequence of the modification steps, largely contribute to definition of multiple functional compartments in this organelle (4–6). At present, most post-translational modifications have been ordered with respect to their sequence in the intracellular pathway; thus, it is possible to infer the position in the pathway of a transiting protein either by the extent of N-linked oligosaccharide chain remodeling (1) or by the occurrence of palmitylation (7), sulfation (8), etc. On the other hand, the timing and location of O-glycosylation are still a controversial matter (for references see Ref. 9). This multi-step process, which is usually initiated by the transfer of GalNAc to serine/threonine residues, comprises the addition of Gal, GlcNAc, fucose, and sialic acid in a linear or branched sequence (10). Most of the studies reported thus far have focused on the complex biochemical aspects of the process (11–14); in an attempt to better insert O-glycosylation in the frame of current knowledge of the intracellular transport pathway and other modification processes, we chose to study the maturation of human CD8 glycoprotein. CD8 is expressed on the surface of T-lymphocyte subclasses and participates to recognition processes and signal transduction events (15); it occurs mainly in the form of homodimer held together by disulfide bonds and bears only O-linked oligosaccharides (16, 17). We recently isolated clones of rat epithelial cells after co-transfection experiments (18); these clones express high levels of human CD8 protein and thus allowed study of the biosynthesis of the protein and determination of the structure of its oligosaccharide chains (17). In the present work we address the question of the timing and topology of CD8 oligosaccharide initiation and maturation; these results are discussed in the light of current debate on the functional organization of the ER-plasma membrane transport pathway (4–6).

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1 The abbreviations used are: ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; BFA, brefeldin A; BSM, bovine submaxillary mucin; GalNAcβ1→3Gal-T, UDP-GalGalNAcβ1→3-galactosyltransferase; GalNAcβ1→6GlcNAc-N-T, UDP-GlcNAcGalNAcβ1→6-N-acetylglucosaminyltransferase; GlcNAcβ1→4Gal-T, UDP-GalGlcNAcβ1→4-galactosyltransferase; GalNAcα2→6NeuAcα2→3-sialyltransferase; CMP-NeuAc-GalNAcα2→6-sialyltransferase; Galβ1→4GlcNAcα2→3-sialyltransferase; Galβ1→4GlcNAcα2→6NeuAcα2→3-sialyltransferase; CMP-NeuAcGalβ1→4GlcNAcα2→6-sialyltransferase.
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**EXPERIMENTAL PROCEDURES**

All culture reagents were supplied by GIBCO. Solid chemicals and liquid reagents were obtained from E. Merck, Darmstadt, Federal Republic of Germany (FRG); Farmitalia Carlo Erba, Milan, Italy; Serva Feinbiochemica, Heidelberg, FRG. SDS was purchased from BDH, Poole, United Kingdom. \[^{[S]}\text{Cysteine (Cysteine)}(>1000\text{ mcCi/mmol})\] was obtained from Du Pont-New England Nuclear, UDP-\[^{[4]}\text{C}\text{GlcNAc (GlcNAc)}(304\text{ mCi/mmol})\], UDP-\[^{[4]}\text{C}\text{galactose (325 mCi/mmol)}\], and CMP-\[^{[3]}\text{C}\text{NeuAc (262 mCi/mmol)}\] were from Amersham, Buckinghamshire, United Kingdom. Mouse monoclonal OKT8 was supplied by Ortho, Raritan, NJ. Neuraminidase (Vibrio cholerae) was obtained from Boehringer, Mannheim, FRG. Protein A-Sepharose CL-4B was from Pharmacia, Uppsala, Sweden. Monensin and brefeldin A (BFA) were from Calbiochem, San Diego, CA.

**Cell Culture and Transfection**

Parental FRT cells (19) and the FRT-U10 clone isolated after transfection (18) were cultured in Coon’s modified Ham’s F-12 medium containing 10% fetal calf serum and maintained in a 95% air, 5% CO\(_2\) incubator.

**Glycosyltransferase Assays**

Assays from preconfluent FRT cells were prepared as detailed by Piller et al. (20). In all assays 50 \(\mu\)g of micromolar proteins were used as the enzyme source.

\text{UDP-Gal} Gal\text{GalNAc} \beta-1\-3\text{galactosyltransferase (GalNAc:Gal}\beta-1\-3Gal-T) — The reaction mixture with asialo-BSM as an acceptor was prepared as described by Piller et al. (20). Incubation was stopped by addition of 1 ml of 1% phosphotungstic acid in 0.5 M HCl and the precipitate collected and counted as previously described (21).

\text{UDP-GlcNAc GalNAc} \beta-1\-4\text{n-acetylglucosaminyltransferase (GalNAc:Gal}\beta-1\-4GlcNAc-T) — The incubation mixture and the isolation of \[^{[1]}\text{C}\text{GlcNAc (14C)}\] were described by Piller et al. (20). The radioactivity incorporated into asialo-BSM was quantitated as described for the GalNAc:Gal\beta-1\-4GlcNAc-T.

\text{CMP}-\text{NeuAcGalNAc} \alpha-2\-3\text{sialyltransferase (GalNAc:Gal}\alpha-2\-3NeuAc-T) — The assay mixture with asialo-BSM as an acceptor was as described (20). The radioactivity incorporated into asialo-BSM was quantitated as described for the GalNAc:Gal\beta-1\-4GlcNAc-T.

\text{CMP}-\text{NeuAcGalNAc} \alpha-2\-3\text{sialyltransferase (GalNAc:Gal}\alpha-2\-3NeuAc-T) — The assay mixture with 2 mM Gal\beta-1\-3GalNAc\alpha-O-benzyl as an acceptor was as described by Piller et al. (20). The radioactive incorporation into asialo-BSM was quantitated as described for the GalNAc:Gal\beta-1\-4GlcNAc-T.

\text{CMP}-\text{NeuAcGalNAc} \alpha-2\-3\text{sialyltransferase (GalNAc:Gal}\alpha-2\-3NeuAc-T) — The assay mixture was as previously described (24) using 0.65 \(\mu\)mol of Gal\beta-1\-4GlcNAc as an acceptor. The two sialylated isomers were separated from the reaction mixture by high performance liquid chromatography as described (24). Radioactive Labeling and CD8 Immunoprecipitation

In all experiments the cells were allowed to grow at sub-confluence and then manipulated as follows. One hour before labeling, normal medium was replaced with Dulbecco’s modified Eagle’s medium containing 1% fetal calf serum (labeling medium) but lacking cysteine, and cells were then labeled for 2.5–30 min with labeling medium containing 100 \(\mu\)Ci/ml \[^{[S]}\text{Cysteine (Cysteine)}\] (22). Cells were chased for variable times at different temperature with labeling medium containing a 10-fold excess of cold cysteine and 10 \(\mu\)g/ml cycloheximide. Preparation of cell extracts, immunoprecipitation, SDS-PAGE, and fluorography were performed as described previously (7, 18). Throughout this study, monoclonal OKT8 antibody was used. To immunoprecipitate CD8 present on the plasma membrane, the antibody was loaded on the FRT-U10 monolayer and left at 4°C for 45 min; the antibody was then removed, and the washed monolayer was lysed and concentrated to protein A-Sepharose as described (7). In the case of neuraminidase treatment, washed protein A-Sepharose beads were treated as described in Ref. 17 except that sodium acetate buffer (50 mM), pH 4.8, and neuraminidase (15 units/ml) were used. The experiments involving incubations at low temperature were performed in a water bath as detailed previously (7).

**RESULTS**

**Glycosyltransferase Activities of FRT Cells**—Different precursor and mature forms are produced during biosynthesis of CD8 in transfected rat epithelial cells (FRT-U10): an unglycosylated precursor (CD8u), a palmitylated and initially O-glycosylated intermediate (CD8i) and a doublet mature form with an apparent \(M_r\) of 32–34 kDa (CD8m) carrying neutral and monosialylated O-linked chains with the core-2 structure and few disialylated chains (17). The analysis of glycosyltransferase activities of FRT cells, involved in the O-glycosylation, is largely consistent with the structure we previously assigned to CD8 oligosaccharides (Table I). In particular, the high activities of the GalNAc:Gal\beta-1\-4GlcNAc-T (responsible for core-2 assembly) and GlcNAc:Gal\beta-1\-4Glc-T account for the assembly of the major O-linked chain of CD8 from FRT-U10 cells, which has the following structure: Gal\beta-1\-4GlcNAcGal\beta-1\-3GalNAc (17). Among the sialyltransferase activities tested, the highest one was found to be the \(\alpha-2\-6\) sialyltransferase toward N-acetyllactosamine (Table I).

**Timing of CD8 Processing**—Pulse-chase labeling experiments with \[^{[S]}\text{Cysteine (Cysteine)}\], followed by immunoprecipitation and SDS-PAGE analysis, were performed to ascertain the maturation rate from CD8u to CD8m. As shown in Fig. 1, the newly synthesized CD8u form, labeled with a 2.5-min pulse and chased for increasing periods, disappeared with a half-time of about 9 min, while the intermediate CD8i appeared quickly, with a half-time of about 4 min. The mature doublet of CD8 accumulated at later time points, with a half-time of about 14 min. No significant differences in the ratio between the 32- and the 34-kDa forms of CD8m were observed late in the chase; thus, both forms represent final products of CD8 biosynthesis. In all experiments we noticed that newly synthesized CD8 was labeled to a higher extent, with \[^{[S]}\text{Cysteine (Cysteine)}\].

**Table I**

| Enzyme | Acceptor | Transferase activity | nmol/mg protein/h |
|--------|----------|---------------------|------------------|
| GalNAc\beta-1\-3Gal-T | Asialo-BSM | 11.40 |
| GalNAc\beta-1\-4GlcNAc\alpha-O-benzyl | Gal\beta-1\-3GalNAc\alpha-O-benzyl | 26.30 |
| GlcNAc\beta-1\-4Gal-T | GlcNAc | 39.20 |
| Gal\beta-1\-3NeuAc-T | Gal\beta-1\-3NeuAc-T | 0.07 |
| Gal\beta-1\-3Gal\beta-1\-3NeuAc-T | Asialo-BSM | 1.00 |
| Gal\beta-1\-4GlcNAc | Gal\beta-1\-4GlcNAc | 3.55 |
| Gal\beta-1\-3NeuAc-T | Gal\beta-1\-3NeuAc-T | 0.16 |

**FIG. 1.** Pulse-chase analysis of \[^{[S]}\text{Cysteine (Cysteine)}\]-labeled CD8 immunoprecipitated from FRT-U10 cells. Cells were pulse-labeled at 37°C for 2.5 min and chased at 37°C for increasing times (in min) as indicated on the top of each lane. Immunoprecipitation, SDS-PAGE, and fluorography were performed as described by Bonatti et al. (7).
thesized CD8u was poorly immunoprecipitated by the antibody, while a larger immunoprecipitation was observed very quickly during the chase (Fig. 1, compare lane 1 with the others). This phenomenon has been observed previously with influenza virus hemagglutinin (25), and it probably reflects a poor capability of the monoclonal antibody to recognize newly made CD8 chains not yet completely folded. Alternatively, a large intracellular pool of [35S]cysteine and some delay prior of the action of cycloheximide could explain this result.

A faster kinetic of CD8 processing was observed in cells pulse-labeled at 37 °C then incubated for 60 min at 15 °C, and eventually shifted for different times to 37 °C (Fig. 2, compare A with B). Under these conditions, CD8i and CD8m were detectable at the first time-point of chase (2.5 min), and the half-time of CD8u disappearance and of CD8m formation were shortened (6 and 12 min versus 9 and 14 min). These results very likely depend on the fact that during the stay at 15 °C some events, e.g. the protein folding, required for the routing to the Golgi compartment had already occurred (see below).

CD8m was the only form expressed on plasma membrane, as shown by surface immunoprecipitation of labeled cells (data not shown). Surface immunoprecipitation of pulse-chase labeled cells followed by SDS-PAGE analysis revealed that CD8m is transported to the surface with a linear kinetic, reaching the plasma membrane with a half-time of about 30 min (Fig. 3). All together, these observations suggest that CD8 is processed and transported to the cell surface quickly, with no apparent rate-limiting steps apart from the formation of the CD8i form.

**FIG. 2.** Effect of lowering cell temperature on the kinetics of disappearance of CD8u, CD8i, and appearance of CD8m. Panel A, FRT-U10 cells were pulse-labeled with [35S]cysteine at 37 °C for 2.5 min and chased at 37 °C for increasing time (in min) as indicated on the abscissa axis. The amount of CD8u (■), CD8i (▲), and CD8m (●) isolated by SDS-PAGE was determined by liquid scintillation counting of the gel portions containing the relative bands. Panel B, cells were pulse-labeled with [35S]cysteine at 37 °C for 2.5 min, chased for 60 min at 15 °C, and finally chased at 37 °C for increasing time (in min) as indicated on the abscissa axis. Quantitation of CD8u (■), CD8i (▲), and CD8m (●) was as in A.

**FIG. 3.** Kinetics of appearance of mature CD8 at the cell surface. FRT-U10 cells were pulse-labeled with [35S]cysteine at 37 °C and chased at 37 °C for increasing times (in min) indicated in the abscissa axis. The immunoprecipitation of CD8 present at the cell surface was performed as described under "Experimental Procedures." The immunoprecipitates were subjected to SDS-PAGE and fluorography, and the radioactivity found in CD8m band was determined as described in the legend of Fig. 2.

**FIG. 4.** Effect of lowering the cell temperature to 20 °C on the CD8 maturation. SDS-PAGE analysis of the immunoprecipitated products from FRT-U10 cells labeled with [35S]cysteine with a 2.5-min pulse (lane 1) followed by a 15-min chase at 37 °C (lane 2) or 60-min chase at 20 °C (lane 3).

**FIG. 5.** Effect of lowering cell temperature to 15 °C on the CD8 maturation. SDS-PAGE analysis of the immunoprecipitated products from FRT-U10 cells labeled with [35S]cysteine with a 2.5-min pulse (lane 1) followed by a 15-min chase at 15 °C (lane 2) or 60 min at 37 °C (lane 3).

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utation—Much evidence indicates that incubation at 15 °C arrests intracellular transport in an intermediate compartment between ER and Golgi complex, whereas incubation at 20 °C leads to accumulation of transiting proteins in the trans-Golgi network (27, 28). It is also well established that folding and disulfide bond formation of secretory and plasma-membrane proteins are early events occurring (or starting) in the lumen of ER (29, 30). When FRT-U10 cells were pulse-labeled with [35S]cysteine at 37 °C, and either chased at 37 °C for 15 min or at 20 °C for 60 min, a very similar SDS-PAGE profile was observed, indicating that the normal maturation pathway took place, although more slowly (Fig. 4). On the contrary, the chase at 15 °C did not result in the conversion of CD8u in CD8i (Fig. 5), supporting the notion that transport to the Golgi apparatus is required for the O-glycan initiation. Since a significant amount of radioactivity in CD8i and CD8m was detected when [3H]glucosamine was given to the cells directly at 15 °C, one can exclude that the glycosylation machinery
was impaired at this temperature.

CD8 is a dimer stabilized by disulfide bridges (16); thus, the immunoprecipitated products from pulse-chase experiments at 15 °C were analyzed by SDS-PAGE in the absence of reducing and alkylating agents. As shown in Fig. 6, the monomeric and dimeric form of CD8u were already present in the pulsed cells; after the chase at 15 °C, the amount of the dimeric form increased drastically, whereas the monomeric form almost disappeared, and much more immunoprecipitable CD8 was detected. A very similar pattern was found when the chase at 15 °C was followed by another 5-min chase period at 37 °C (Fig. 6, lanes 2 and 3). These results indicate that the arrest of CD8 in a pre-Golgi compartment(s) allows the folding required for the dimerization, and support the notion that the completion of folding results in a better recognition of CD8 by OKT8 monoclonal antibodies.

**Effect of Monensin and BFA on CD8 Maturation**—To get an insight into the O-glycosylation processing of CD8 from FRT-U10 cells, the effect of monensin and BFA on the conversion of CD8 from precursor to mature forms was studied. When cells were pulsed for 5 min with [35S]cysteine and then chased for 90 min in the presence of monensin, two bands were visualized, the major one migrating as CD8i (Fig. 7, lane 5) and the second, with a mobility faster than that of CD8m, accumulating in the corresponding control cells. Even a smear between the two bands was visualized. Neuraminidase digestion of the immunoprecipitate from monensin-treated cells did not result in any modification of the mobility of the band migrating as CD8i, indicating that this form is synthesized before whatever sialylation process occurs. Conversely, the band of higher M, appeared to be sensitive to neuraminidase (Fig. 8, lane 4). After neuraminidase digestion this band still migrated faster than neuraminidase-treated CD8m from control cells (Fig. 8, cf. lanes 2 and 4). All together these results strongly suggest that the effect of monensin results in an arrest of the majority of CD8 molecules at the stage of CD8i (non-elongated O-linked GalNAC), whereas molecules that escape from this arrest did not reach full maturation.

A very different pattern was found in BFA-treated cells. After a [35S]cysteine pulse-chase of 5–90 min, only a single broad band was formed in between the mobility of CD8i and CD8m (Fig. 7, lane 4), indicating that the interference of this drug in O-linked oligosaccharide elongation is later than that of monensin. The addition of Gal residues in CD8 from BFA-treated cells was demonstrated by labeling of the band with [3H]galactose (results not shown). Finally, neuraminidase treatment reduced significantly the apparent molecular weight of the band accumulating in BFA-treated cells (Fig. 8, cf. lanes 5 and 6); this indicates that the glycoprotein has been, at least partially, sialylated.

**DISCUSSION**

Plasma membrane glycoproteins, as well as secretory proteins, move from the ER to the plasma membrane and extracellular space with different rates (29–33). Current results indicate that CD8 manufactured by FRT-U10 cells belongs to the fast moving proteins; the newly made unglycosylated form (CD8u) disappears with a half-time of about 9 min, whereas mature CD8 is produced and delivered to the plasma membrane of the cell with a half-time of about 14 and 30 min, respectively. Interestingly, another intermediate form, named CD8i, is generated in between CD8u and CD8m. This form, which corresponds to palmitoylated and initially O-glycosylated chains (17), appears with a half-time of about 4 min and lasts an average time of 5 min.

Within the life-span of CD8u two main events occur; these are, in order, folding/dimerization and export to the cis-Golgi region. Our results strongly suggest that folding and dimerization of CD8u are strictly coupled events, taking place very quickly in the lumen of ER; the dimer is held by disulfide bonds, and protein disulfide isomerase of ER facilitates formation of the correct set of disulfide bonds (34). We cannot exclude that this step is influenced by overexpression of CD8; indeed, a high concentration of CD8 in the lumen of the ER
might either favor inappropriate interchain interactions or facilitate the overall process simply increasing the interactions between the folding chains. Several lines of evidence suggest that the folding/oligomerization step is the crucial one in the "quality control" mechanism and only the proteins that have successfully passed this step may be exported from the ER (34, 35).

Two different steps are responsible for assembly of CD8i: the palmitylation and the addition of 7–8 residues of O-linked GalNAc (17). According to a previous study (7), palmitylation appears to be an early Golgi event, but different locations have also been suggested (see Ref. 7).

More conflicting results have been amassed on temporal aspects of initial O-glycosylation; addition of GalNAc to viral glycoproteins has been located by some workers before glycoproteins reach the Golgi apparatus (36, 37), but in non-infected cells a large body of evidence indicates that the O-glycosylation starts in the first Golgi cisernae (29, 33, 38–41). Current results are consistent with the latter interpretation; indeed, CD8u/CD8i conversion is blocked in a chase performed at 15 °C, a condition that prevents entry into the Golgi stack (26). We suggest that CD8u/CD8i conversion takes place in a post-ER location, which may coincide more likely with the cis-Golgi than with an intermediate compartment between the ER and the Golgi apparatus (26, 27). On morphological basis, the intermediate compartment is defined as an array of tubulo-vesicular structures spread in the cytoplasm (42). Recently, these structures have been considered as part of a cis-Golgi network compartment, which would include the cis-region of the Golgi complex (5). Our finding that CD8u and CD8i are two clearly distinct forms is more easily explained if the intermediate compartment and the cis-Golgi region were functionally different entities.

An important point to address is the accumulation of CD8i as a discrete precursor in the CD8 processing. It has to be considered that in the absence of a consensus sequence for attachment of GalNAc to Ser/Thr residues, a defined conformation of the peptide, particularly in the vicinity of hydroxyaminic acids, must occur in all molecules to obtain a constant extent of O-glycosylation (43). In fact, the analysis of O-glycosylated glycoproteins indicates that each glycoprotein has a defined number of O-linked chains. Therefore it is possible that glycoproteins, highly different in the primary sequence and extent of O-glycosylation, require different times to receive the full complement of GalNAc residues. In the case of leukosialin, the well known T-cell glycoprotein, an intermediate carrying only the innermost O-linked GalNAc has not been evidenced, except in Jurkat cells which are defective in the glycosyltransferase responsible for the second step of O-glycosylation (20). Conversely, for LDL receptors and glycophorin A a transient precursor initially O-glycosylated has been identified (29, 44). A reasonable hypothesis to explain this discrepancy is that the precursor initially O-glycosylated accumulates transiently only when the time required for addition of all GalNAc residues is significantly shorter than the average time required for the passage from the cis-Golgi to the next compartment. In this context, the relative low extent of CD8 O-glycosylation (7–8 oligosaccharides/molecule) should facilitate the accumulation of the initially O-glycosylated intermediate form. It is not yet known if the addition of GalNAc residues is a prerequisite to proceed further in the transport of the glycoprotein. Additional work should address these problematic, because it is possible that, by analogy with the events taking place in the lumen of the ER, multiple "quality control" steps may exist in the intracellular transport pathway. Indeed, major histocompatibility complex class I molecules have been found retained in mutant cells, but recycling between the cis-Golgi and the ER (45).

Further information on intracellular glycoprotein traffic has been obtained by analyzing the effect of monensin and BFA treatment on CD8 maturation. Although the effect of monensin on O-glycosylation has been studied less than that on N-glycosylation, there is evidence that the effect of this drug results in a block of O-glycosylation at the first step, with a consequent accumulation of non elongated O-linked GalNAc (33, 46). Our results, showing that after a long pulse-chase period in the presence of monensin a band migrating as CD8i is accumulated, are consistent with these data. It is worth noting that, in our experimental conditions, arrest due to monensin was not complete and even partially elongated chains sensitive to the neuraminidase are synthesized. In K-562 cells the α2–6-sialyltransferase acting on O-linked GalNAc is not inhibited by monensin (33). Since even in FRT this sialyltransferase is well expressed (see Table I), it may be suggested that the CD8 molecules which escape from the block of monensin become substrate for this sialyltransferase. In the absence of monensin the majority of CD8 oligosaccharides synthesized by FRT-U10 cells appears to have the innermost GalNAc substituted in the O-6 position with GlcNAc, rather than with NeuAc (17). In agreement with a previous suggestion (14), we interpreted this event as dependent on a location in the Golgi apparatus of GalNAcα1→6GlcNAc-T earlier than GalNAcα2→6NeuAc-T, so that the latter cannot work, inasmuch as the O-6 position of GalNAc is already substituted (17). One can suggest that monensin, besides the block at the first step of O-glycosylation, interferes with the ordered localization and activity of Golgi glycosyltransferases, in particular GalNAcβ1→6GlcNAc-T and GalNAcα2→6NeuAc-T, so that altered carbohydrate sequences may be synthesized.

A similar but not identical impairment on the full maturation of CD8 has been observed after BFA treatment. In this case, the altered oligosaccharide elongation should depend on redistribution of the Golgi-specific enzymes into ER caused by the drug (47). In BFA-treated cells a band with an apparent molecular weight in between the initially O-glycosylated and the fully processed form and sensitive to neuraminidase digestion accumulates. This indicates that the arrest of O-linked chains due to BFA is later than that caused by monensin and that some addition of sialic acid takes place. Even the LDL receptor, which carries more O-linked than N-linked chains, has been demonstrated to undertake a partial sialylation in the presence of BFA (48). The exclusive presence of O-linked chains in CD8 indicates that the sialylation of O-glycosylated proteins is not abolished by BFA. Moreover, very recently it has been reported that even α2–3- and α2–6-sialyltransferases acting toward N-linked chains are redistributed from the Golgi to the ER in HepG2 cells exposed to BFA (49). Since we identified four different sialyltransferases that, at least in principle, may work in the biosynthesis of CD8 O-linked chains (see Table I), we are planning to use the model of FRT-U10 expressing CD8 to dissect the effect of BFA on the four different sialylation steps involved in the O-linked oligosaccharide biosynthesis. Recently, Leahy et al. (50) have established the crystal structure of a soluble form of CD8 expressed by CHO cells. According to this study the molecule is formed by a single immunoglobulin-like domain connected to a transmembrane segment by a "stalk" region in which O-linked chains are clustered. These authors assigned seven O-linked chains per molecule, a value quite consistent with our results (17). In contrast, the carbohydrate structure of CD8 from FRT cells was different from that synthesized by CHO
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...cells, in that the latter contain only trace amounts of GlcNAc (50). The discrepancy may be explained by the high expression of GalNAcβ1→6GlcNAc-T in FRT cells (see Table I), which shifts the elongation of CD8 chains to core-2 structure; this glycosylation step could not be active in CHO cells. It is worth noting that activation of T lymphocytes enhances the expression of core-2 GalNAcβ1→6GlcNAc-T (13); thereby, one can postulate that in T lymphocytes the oligosaccharides structure we identified is produced, at least in part, in activated cells. Since O-linked glycosylation appears to be crucial to keep the first ectodomain of CD8 in an extended form and to avoid proteolytic degradation of the glycoprotein (50), the recognition function of CD8 in resting and activated T cells might be modulated by the different processing of O-linked chains.

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