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Biochemistry

Monensin prevents terminal glycosylation of the N- and O-linked oligosaccharides of the HLA-DR-associated invariant chain and inhibits its dissociation from the α–β chain complex

(carboxylic ionophores/class II antigens/post-translational processing)

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ABSTRACT **In B-lymphoblastoid cells, the HLA-DR-associated invariant chain is processed to a form containing O-linked as well as N-linked oligosaccharides. After neuraminidase treatment, the O-linked carbohydrate is susceptible to digestion with an endoglycosidase (endo-β-*N*-acetylgalactosaminidase) that cleaves glycans with the structure Gal(β1→3)-GalNAc-Ser/Thr, and sialic acid can be added back to this core oligosaccharide by specific sialyltransferases. Treatment of cells with the sodium ionophore monensin markedly affects the post-translational processing of the invariant chain, although that of associated α and β chains is minimally affected. Only a small portion of the N-linked carbohydrate on the invariant chain is processed to an endoglycosidase-H-resistant form. The sialic acid residues normally found on the O-linked glycans are not added, but at least the first residue, GalNAc, is added. In addition to the changes in glycosylation, an intracellular accumulation of HLA-DR antigens also occurs in monensin-treated cells. The accumulation of HLA-DR antigens and the overall slower turnover rates of the α, β, and invariant polypeptides observed after monensin treatment probably reflects the build-up of newly synthesized proteins in Golgi apparatus-derived vacuoles coupled with a decrease in normal degradation in lysosomes.**

At the cell surface, class II histocompatibility antigens consist of two noncovalently associated transmembrane glycoproteins, α and β, encoded within the major histocompatibility complex. They are expressed predominantly on B cells and monocytes (reviewed in ref. 1). The HLA-DR antigens are the best-characterized human class II antigens.

The HLA-DR antigens associate intracellularly with a third polypeptide called the invariant (I) chain (2–5). A similar polypeptide has been observed to associate with murine Ia antigens (6, 7). The I chain is glycosylated and methionine-rich, has a basic isoelectric point (3, 5, 8), and is not encoded in the major histocompatibility complex (9, 10). Its apparent intracellular and transient association with α and β chains suggests it may function in facilitating α–β association, intracellular transport of the complex, or both (4, 8).

The carboxylic ionophore monensin catalyzes the exchange of Na^+ and K^+ across biological membranes (11). It inhibits intracellular transport of newly synthesized polypeptides to the cell surface and endocytosis of ligands and receptors entering the cell (reviewed in ref. 12). In monensin-treated cells newly synthesized proteins accumulate in intracellular vacuoles that appear to be derived from the Golgi apparatus, and terminal glycosylation is incomplete in many cases. Monensin may define a subcompartment between the functionally defined *cis* and *trans* Golgi apparatus, recently termed *medial* (13, 14). The inhibitory effect of monensin on endocytosis is most likely a result of the increased pH inside prelysosomal endosomes observed in monensin-treated cells (15).

We previously described post-translational modifications of the N-linked glycan units of the invariant chain and suggested that O-linked carbohydrate was added during its transient association with HLA-DR antigens (5). Here we prove the addition of O-linked glycans and report the effects of monensin on the post-translational processing of the invariant chain and on its interaction with HLA-DR antigens in B-lymphoblastoid cells.

MATERIALS AND METHODS

Cells, Radiolabeling, Immunoprecipitation, and Electrophoresis. Growth and radiolabeling of the Swei cell line with [^{35}S]methionine, immunoprecipitation of HLA-DR antigens with serum 247/HSB, and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) utilizing nonequilibrium pH gradient separation in the first dimension were all performed as described (5). For experiments in which cells were cultured in the presence of monensin (Calbiochem), a 10 mM stock solution in ethanol was diluted into culture medium immediately before use.

Glycoprotein Preparation. *Lens culinaris* hemagglutinin-binding glycoproteins were prepared as described (5). Glycoproteins binding *Bandeireae simplicifolia* isolectin I (Pierce) were isolated in a similar fashion on Bio-Gel A-15m beads conjugated with the lectin at 1 mg/ml. Glycoproteins were eluted in 0.5 M galactose, dialyzed, and recovered by acetone precipitation.

Enzyme Treatments. Neuraminidase digestions were performed on immunoprecipitates with 0.02 international units of *Vibrio cholerae* enzyme (Calbiochem) (5). For digestion with endoglycosidase H, immunoprecipitates were first eluted in 50 μl of 1% $NaDodSO_4$ at 100°C for 3 min, diluted to 0.5 ml with citrate/phosphate-buffered saline, pH 6.5, and incubated with 15 milliunits of endo-β-*N*-acetylglucosaminidase H from *Streptococcus plicatus* (endo-H, Miles) for 16 hr at 37°C. Samples were recovered by acetone precipitation.

Endo-β-*N*-acetylgalactosaminidase (endo-GalNAcase) purified from *Streptococcus pneumoniae* (16) was the generous gift of T. Beyer. Immunoprecipitates were treated with neuraminidase, washed, and incubated in citrate/phosphate-buffered saline, pH 6.5, with 30 milliunits of endo-GalNAcase for 16 hr at 37°C. A control precipitate was incubated without enzyme. In some experiments, the immunoprecipitates were denatured with $NaDodSO_4$ before digestion with endo-GalNAcase.

For treatment with sialyltransferases, 247/HSB precipi-

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Abbreviations: endo-GalNAcase, endo-β-*N*-acetylgalactosaminidase; endo-H, endo-β-*N*-acetylglucosaminidase H; I chain, invariant chain; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis.

tates were first digested with neuraminidase. After several washes, pellets were resuspended in 75 μl of 0.1 M sodium cacodylate buffer, pH 6.5, containing bovine serum albumin at 1 mg/ml, Triton X-100 at 10 mg/ml, and 12 mM CMP-*N*-acetylneuraminic acid (substrate). Approximately 10–15 milliunits of both β-galactoside (α2→3)sialyltransferase and α-*N*-acetylgalactosaminide (α2→6)sialyltransferase were added. These enzymes were purified from porcine submaxillary gland (17, 18) and were given by A. Eckhardt and R. Mullin. A control precipitate was incubated in buffer containing substrate only. After 8 hr at 37°C, another aliquot of each enzyme and 5 nmol more substrate were added, and incubation was continued for a further 12 hr.

Binding Assay and Solid-Phase Radioimmunoassay. HLA-DR antigens on the cell surface or in total cell extracts were quantitated with the monoclonal antibody XD5.A11, which recognizes a monomorphic determinant on class II β chains (unpublished data). Surface antigen expression was determined with a cellular binding assay using Swei cells performed as described (19).

Total cellular HLA-DR antigens were quantitated with a solid-phase competitive radioimmunoassay. Cells were extracted in 1% Triton X-100 at 5×10^6 cells per ml as described (5). Dilutions of the extracts in 10 mM Tris·HCl, pH 8.0/0.15 M NaCl/0.5% deoxycholate/1% bovine serum albumin were prepared and a standardized amount of XD5.A11 antibody was added to each tube. While this incubation continued for 60 min, 50 μl of *Lens culinaris* hemagglutinin-binding B-cell line membrane glycoproteins (50 μg/ml in 5.5 M urea) were adsorbed to the wells of flexible microtiter plates (Dynatech, Alexandria, VA). The glycoprotein solution was removed, and the plate was incubated in 10 mM Tris·HCl, pH 8.0/0.15 M NaCl/1% bovine serum albumin for 10 min, followed by three washes with deionized water. Fifty microliters of each cell extract/XD5.A11 mixture was added to the glycoprotein-coated wells (in duplicate). After 60 min at 22°C and three water washes, the uninhibited XD5.A11 antibody bound to the glycoprotein-coated wells was detected by incubation with affinity-purified ^{125}I-labeled rabbit anti-mouse immunoglobulin F(ab′)$_2$ (2×10^5 cpm per well in 50 μl). After 45 min, the plate was washed with water three times and dried, and the wells were sliced and their bound radioactivities were measured. The relative inhibition titer of the extract is the reciprocal of the minimal dilution at which XD5.A11 binding is inhibited by 50%.

RESULTS

HLA-DR-Associated I Chain Contains Both N- and O-Linked Carbohydrate. The presence of two complex N-linked oligosaccharides on the I chain has been well documented (3–5). Tunicamycin-resistant glycosylation of the invariant chain suggested the presence of O-linked carbohydrate as well (5). When HLA-DR antigens are precipitated with a rabbit anti-DR serum (247/HSB) from Swei cells labeled with [^{35}S]-methionine and analyzed by 2D-PAGE, the characteristic α–β–I chain pattern is observed (Fig. 1*A*). The charge heterogeneity of the I chain is due to sialylation (5). When the immunoprecipitate is treated with neuraminidase before 2D-PAGE, a doublet is resolved at the position of the I chain (Fig. 1*B*), the lower spot (I) representing invariant chain with only N-linked oligosaccharides, and the upper spot (Ip) thought to represent the I chain with both N- and O-linked carbohydrate (5).

The upper spot of the I doublet was found to be sensitive to endo-GalNAcase, a glycosidase that cleaves the structure Gal(β1→3)GalNAc-Ser/Thr between the serine or threonine residue and GalNAc (16). Digestion of a neuraminidase-treated immunopecipitate with endo-GalNAcase reduced the molecular weight of the upper spot of the doublet, although the doublet was still resolved (Fig. 1*C*). When the precipitate

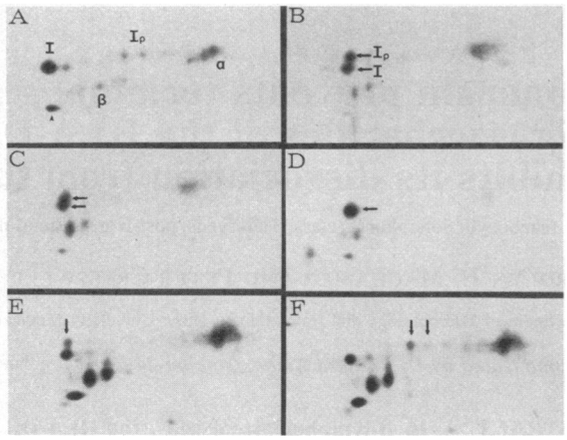

FIG. 1. The I chain possesses O-linked carbohydrate. Swei cells were labeled with [^{35}S]methionine for 4 hr, and HLA-DR antigens were immunoprecipitated from detergent extracts with serum 247/HSB and analyzed by 2D-PAGE. Electrophoresis was from right to left (acidic to basic) in the first dimension and from top to bottom in 10.5% polyacrylamide/NaDodSO$_4$ gels for the second dimension. α, β, and I chains are indicated. Ip is the I chain processed to a form containing sialylated N- and O-linked oligosaccharides. The arrowhead marks an immunoglobulin light chain present in the immunoprecipitates, which serves as a convenient marker on the gels. The precipitates were treated in the following manner: (*A*) untreated control; (*B*) neuraminidase-treated; (*C*) neuraminidase- and endo-GalNAcase-treated; (*D*) neuraminidase-treated and denatured in NaDodSO$_4$ followed by endo-GalNAcase digestion; (*E*) neuraminidase-treated and incubated with substrate for sialyltransferases only; (*F*) neuraminidase-treated and incubated with substrate plus α-*N*-acetylgalactosaminidase (α2→6)sialyltransferase and β-galactoside (α2→3)sialyltransferase.

was denatured in NaDodSO$_4$ before treatment with endo-GalNAcase, the doublet was no longer resolved (Fig. 1*D*). Therefore, at least two O-linked oligosaccharide cores are present on the processed I chain, one readily susceptible to endo-GalNAcase and one that is not cleaved unless the polypeptide is first denatured. Since the carbohydrate added to the I chain in the presence of tunicamycin normally contains four sialic acid residues (5), they are probably linked α2→6 to the GalNAcase and α2→3 to the Gal residues in the O-linked oligosaccharide cores (20).

Further evidence for the presence of O-linked carbohydrate was obtained with the use of two highly purified sialyltransferases that are specific for glycans with the structure Gal(β1→3)GalNAc-Ser/Thr. One, α-*N*-acetylgalactosaminide (α2→6)sialyltransferase, adds a sialic acid residue α2→6 to GalNAc, and the other, β-galactoside (α2→3)sialyltransferase, adds a sialic acid residue α2→3 to Gal (17, 18). When a mixture of these two sialyltransferases was incubated with substrate and a neuraminidase-treated 247/HSB precipitate, the upper spot of the I doublet was shifted to a more acidic form (arrows, Fig. 1*F*), indicating addition of sialic acid residues.

Effects of Monensin on Glycosylation of HLA-DR Antigens. Treatment of B lymphoblastoid cells with 1 μM monensin markedly affected the post-translational processing of the I chain (Fig. 2). Viability and overall protein synthesis in these cells were not affected. Far fewer sialic acid residues are added to the invariant chain in monensin-treated cells (Fig. 2*D*). The charge heterogeneity observed for the I chain from monensin-treated cells could not be reduced with neuraminidase (Fig. 2*E*), even after denaturation of the precipitate with NaDodSO$_4$ (not shown). Similar effects were observed in a number of other B-lymphoblastoid cell lines (unpublished data).

Both N-linked oligosaccharides are added to the invariant

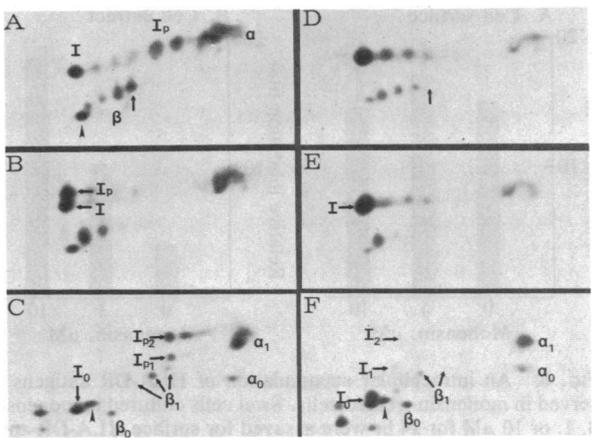

FIG. 2. Effects of monensin on the glycosylation of HLA-DR antigens. Swei cells were labeled for 4 hr with [^{35}S]methionine in the absence (*A–C*) or presence (*D–F*) of 1 μM monensin, and HLA-DR antigens were precipitated with 247/HSB. (*A* and *D*) Untreated precipitates; (*B* and *E*) neuraminidase-treated precipitates; and (*C* and *F*) endo-H-treated precipitates. In *C* and *F*, the subscripts refer to the number of N-linked oligosaccharides that have been processed to an endo-H resistant form. The arrowhead notes the immunoglobulin light chain marker. The first-dimension tube gels from both samples *C* and *F* were run side by side with control (untreated) precipitates on the same second-dimension slab gel, and background spots were used to line up the fluorograms and identify the spots in the endo-H-treated sample.

chain in monensin-treated cells, but only a very small proportion acquire resistance to digestion with endo-H (I_1 and I_2 in Fig. 2*F*). The α and β chains are less obviously affected than the I chain. One less sialic acid residue is added to β chains in monensin-treated cells (Fig. 2*D*), but endo-H-resistant oligosaccharides are present (β_1 in Fig. 2*F*). The use of nonequilibrium pH gel electrophoresis in the first dimension did not allow clear resolution of the acidic α chain spots. Nevertheless, one of the two N-linked oligosaccharides is processed to an endo-H-resistant form in monensin-treated cells (α_1 in Fig. 2*F*), as is observed in untreated cells (Fig. 2*C*). The rate of acquisition of resistance to endo-H (as determined in pulse–chase experiments) was somewhat slower in monensin-treated cells compared to untreated cells, however (unpublished data).

Although no I doublet is resolved in Fig. 2*E*, some O-linked carbohydrate appears to be added to the invariant chain in monensin-treated cells. The invariant chain binds *Bandeireae simplicifolia* lectin (21), which is specific for α-D-Gal and α-D-GalNAc residues (Fig. 3*B*). This binding is due to O-linked carbohydrate, as it occurs when cells were labeled in the presence of both tunicamycin and monensin (Fig. 3*C*). A narrowly spaced doublet can be resolved at the I spot in monensin-treated cells when the N-linked oligosaccharides are not present [in tunicamycin-treated cells or endo-H-treated precipitates (Fig. 2*F*)]; this doublet is not susceptible to digestion with endo-GalNAc (not shown). This is probably because only the first residue of the O-linked oligosaccharide cores, GalNAc, is added to the I chain in monensin-treated cells and would not be susceptible to cleavage with the enzyme.

Effects of Monensin on the Dissociation of the I Chain from the α–β Complex. The effect of monensin treatment on the transient association of I chain with α–β complexes (3–5) was investigated with pulse–chase labeling techniques. After 10-min labeling with [^{35}S]methionine, Swei cells were chased with unlabeled methionine for various periods of time. As seen in Fig. 4 *A–D*, only a very small level of I chain is detected in association with pulse-labeled HLA-DR α and

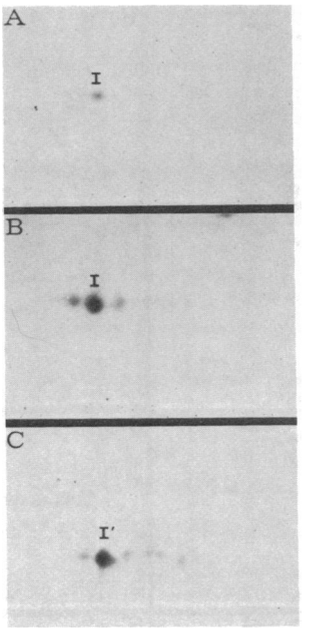

FIG. 3. The I chain from monensin-treated cells binds *Bandeiraea simplicifolia* isolectin I. Extracts from Swei cells labeled with [^{35}S]methionine for 4 hr were incubated with Bio-Gel A-15m beads conjugated with the lectin and washed, and glycoproteins were eluted in 0.5 M α-D-galactose. (*A*) Untreated cells; (*B*) monensin-treated cells; (*C*) tunicamycin- and monensin-treated cells. I′ is the invariant chain lacking its two N-linked oligosaccharides.

β chains in untreated cells after 4 hr of chase. In cells treated with monensin, however, I chain is still present in HLA-DR immunoprecipitates from cells chased up to 8 hr (Fig. 4 *E–H*).

The turnover times of α, β, and I polypeptides were also compared by analyzing total *Lens culinaris* hemagglutinin-binding glycoproteins from the pulse–chase-labeled cell ex-

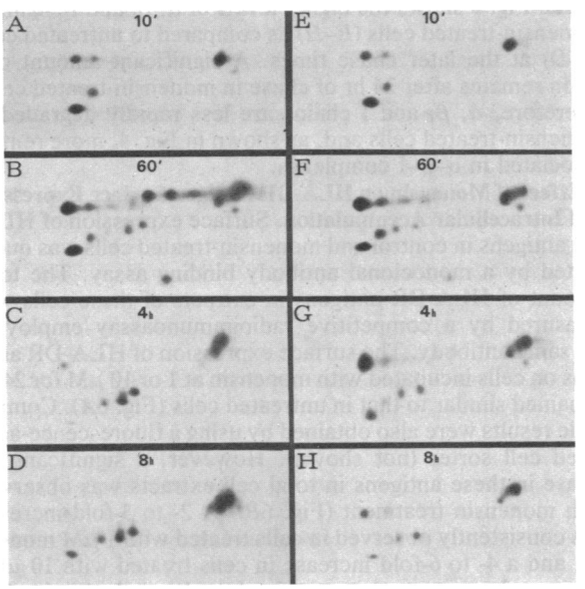

FIG. 4. Dissociation of the I chain from α–β complexes is delayed in monensin-treated cells. Swei cells were pulse-labeled for 10 min with [^{35}S]methionine and chased in the presence of excess unlabeled methionine for various periods of time. HLA-DR antigens were precipitated with 247/HSB and analyzed by 2D-PAGE. Samples in *E–H* were labeled and chased in the presence of 1 μM monensin. Chase periods were 10 min (*A* and *E*); 60 min (*B* and *F*); 4 hr (*C* and *G*); and 8 hr (*D* and *H*).

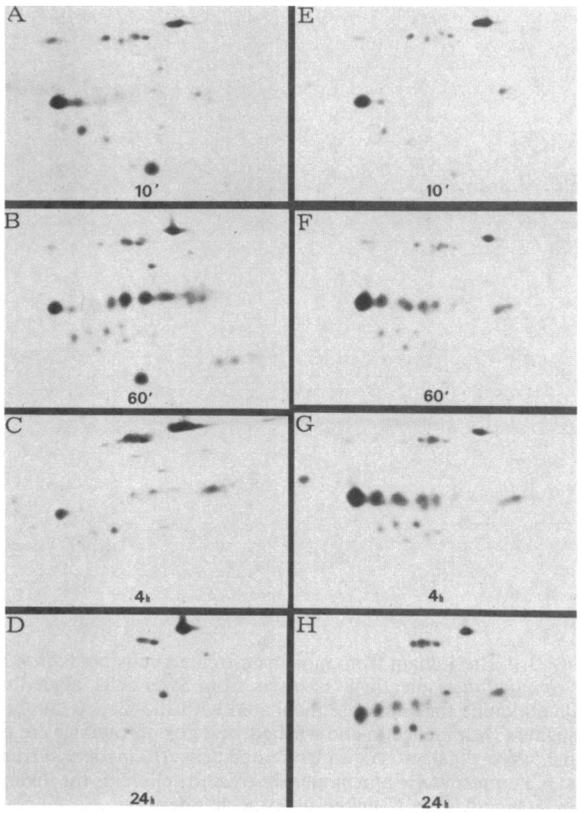

FIG. 5. Turnover of α, β, and I chains is slower in monensin-treated cells. Total *Lens culinaris* hemagglutinin-binding glycoproteins were prepared from the pulse-labeled extracts described in Fig. 4. Samples in *E–H* were from cells labeled and chased in the presence of 1 μM monensin. Chase periods were 10 min (*A* and *E*); 60 min (*B* and *F*); 4 hr (*C* and *G*); and 24 hr (*D* and *H*). The same number of cell equivalents per sample was used for preparation of glycoproteins from untreated and monensin-treated cell extracts.

tracts. Fig. 5 shows the higher levels of α, β, and I chains in monensin-treated cells (*E–H*) as compared to untreated cells (*A–D*) at the later chase times. A significant amount of I chain remains after 24 hr of chase in monensin-treated cells. Therefore, α, β, and I chains are less rapidly degraded in monensin-treated cells and, as shown in Fig. 4, more remain associated in α–β–I complexes.

Effect of Monensin on HLA-DR Antigen Surface Expression and Intracellular Accumulation. Surface expression of HLA-DR antigens in control and monensin-treated cells was quantitated by a monoclonal antibody binding assay. The total amount of HLA-DR antigens in extracts of these cells was measured by a competitive radioimmunoassay employing the same antibody. The surface expression of HLA-DR antigens on cells incubated with monensin at 1 or 10 μM for 24 hr remained similar to that in untreated cells (Fig. 6*A*). Comparable results were also obtained by using a fluorescence-activated cell sorter (not shown). However, a significant increase in these antigens in total cell extracts was observed with monensin treatment (Fig. 6*B*). A 2- to 3-fold increase was consistently observed in cells treated with 1 μM monensin, and a 4- to 6-fold increase in cells treated with 10 μM. Since surface expression remained unchanged, this implies an intracellular accumulation of HLA-DR antigens, as well as a lack of normal turnover of antigens already at the cell surface.

DISCUSSION

We previously showed that the invariant chain is transiently associated with HLA-DR antigens intracellularly and that it

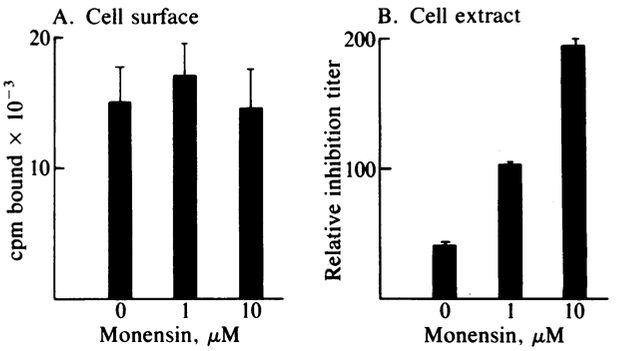

FIG. 6. An intracellular accumulation of HLA-DR antigens is observed in monensin-treated cells. Swei cells cultured in monensin at 0, 1, or 10 μM for 24 hr were assayed for surface HLA-DR antigens by a binding assay on intact cells (*A*). Detergent extracts from aliquots of these same cells were assayed for total cellular HLA-DR antigens by a solid-phase competitive radioimmunoassay (*B*); the average of two experiments is shown and error bars indicate the standard deviation.

is post-translationally modified during its association by the addition of sialic acid residues (5). In this paper we have demonstrated that it is also modified by the addition of O-linked oligosaccharide prior to its dissociation from HLA-DR α–β complexes. With the use of a highly specific glycosidase and two sialyltransferases, we were able to demonstrate the presence of O-linked carbohydrate on the I chain and to predict its structure. The data presented in Fig. 1 suggest the presence of two O-linked oligosaccharide cores with the structure Gal(β1→3)GalNAc-Ser/Thr. Because a total of four sialic acid residues is normally found on the O-linked carbohydrate (5), it is likely that the I chain possesses two oligosaccharides with the structure

$$\begin{array}{l} \text{NeuNAc}(\alpha 2\rightarrow 3)\text{Gal}(\beta 1\rightarrow 3)\text{GalNAc-Ser/Thr.} \\ \qquad\qquad\qquad\qquad\qquad\qquad\quad 6 \\ \qquad\qquad\qquad\qquad\qquad\qquad\quad \uparrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\quad \alpha 2 \\ \qquad\qquad\qquad\qquad\qquad\qquad\quad \text{NeuNAc} \end{array}$$

The I chain also contains two N-linked oligosaccharides (3–5). As seen in Fig. 2*C*, both can be processed to the complex form, and variable sialylation of these glycans results in the marked charge heterogeneity of the I chain (Fig. 1*B* and ref. 5). In contrast, the two N-linked oligosaccharides found on the murine invariant chain appear to remain in the high-mannose state (22). Also, there is no evidence that O-linked carbohydrate is added to the murine invariant chain, although the murine and human polypeptides have been shown to be quite similar by peptide mapping (23).

The observed post-translational modifications are late or *trans* Golgi apparatus functions (24), and dissociation of the I chain from HLA-DR antigens does not occur until these modifications have taken place. This implies that the α–β complexes traverse the Golgi in association with I chain. Because of the known effects of monensin on Golgi function, we investigated the effects of the ionophore on the glycosylation and dissociation kinetics of HLA-DR antigens, hypothesizing that α–β complexes accumulating in the Golgi would remain associated with the I chain. We indeed were able to show a considerable prolongation of the association of the I chain with α–β complexes in monensin-treated cells (compare *C* and *G* in Fig. 4). Monensin also had striking effects on the glycosylation of the I chain. Cells treated with 1 μM monensin for 4 hr during the radiolabeling period contained I chains that demonstrated only a small amount of charge heterogeneity. These spots were not susceptible to

neuraminidase (Fig. 2 *B* and *D*), implying that sialic acid residues were not responsible for the charge heterogeneity. In a very small portion of the I chain molecules, one of the N-linked oligosaccharides was processed to an endo-H-resistant form, and in even less, both were endo-H resistant (Fig. 2*F*). The lectin-binding properties of the I chain from monensin-treated cells suggested that at least the first residue of the O-linked oligosaccharides was added (Fig. 3). In contrast to this result, it has been reported that the O-linked carbohydrate normally found on one of the herpes simplex virus glycoproteins was completely absent when that protein was purified from monensin-treated cells (25).

The absence of terminal glycosylation of polypeptides synthesized in monensin-treated cells is generally interpreted to mean that the glycoproteins do not reach the region of the cells where the appropriate glycosyltransferases reside, presumably the *trans* Golgi (12). The N-linked oligosaccharides of some polypeptides are processed to the complex form in monensin-treated cells, whereas those on other polypeptides remain endo-H sensitive (26–28). It has been suggested that this reflects differing rates of transport of glycoproteins in a given cell type (12). In the study reported here, the N-linked oligosaccharides on the α and β chains are processed from the high-mannose form to the complex form in a nearly normal manner, while those on the associated I chain remain predominately endo-H sensitive. Hence, other factors, such as the conformation of the polypeptide or of the complex, must contribute to the inhibition of processing of the I chain N-linked carbohydrate.

The I chain is not found associated with α and β chains in purified plasma membranes (3, 8), but whether it is transported to the cell surface and expressed separately is unresolved. Preliminary observations suggest that a small number of α and β chains still associated with I chain do reach the plasma membrane in monensin-treated cells (unpublished data). This suggests that the monensin-induced block in intracellular transport may not be absolute and also that some event other than arrival at the plasma membrane is responsible for the normal dissociation of the I chain from α–β complexes. An increase in pH in prelysosomal endosomes has been implicated in the effects of monensin on receptor-mediated endocytosis (12, 29). Increased lysosomal pH may account for the reduced degradation and consequent increased turnover time for α, β, and I chains as observed in Fig. 5. The inhibition of dissociation of the I chain from α–β complexes in monensin-treated cells could reflect a similar increase in pH inside the vesicles that transport the complex to the cell surface.

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