Direct Transport of Newly Synthesized HLA-DR from the trans-Golgi Network to Major Histocompatibility Complex Class II Containing Compartments (MIICS) Demonstrated Using a Novel Tyrosine-sulfated Chimera*

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Binding of antigenic peptides to major histocompatibility complex (MHC) class II glycoproteins occurs in specialized endocytic compartments of antigen-presenting cells, which in man are termed MIICs. Newly synthesized MHC class II molecules are transported from the trans-Golgi network to MIICs, but previous studies of this important step in antigen processing have failed to conclusively determine whether most immature MHC class II complexes are transported directly to the processing compartments or are first transiently exposed at the cell surface. To attempt to resolve this question, I constructed a chimeric HLA-DRα chain containing two optimal tyrosine sulfation motifs. When expressed in a human B lymphoblastoid cell line lacking functional DRα chains, the chimera was correctly incorporated into complexes containing endogenous β and invariant chains, transported to the trans-Golgi network, and efficiently sulfated. Pulse-chase experiments showed that the sulfated complexes were rapidly transported to processing compartments with kinetics consistent with direct transport from the trans-Golgi network. The rate of maturation was not significantly altered in cells expressing a temperature-sensitive mutant of dynamin under conditions where the endocytosis of transferrin was inhibited by 95%, confirming that endocytosis was not required for delivery to MIICs. Maturation of MHC class II-containing complexes was inhibited by aluminum fluoride and brefeldin A, indicating the involvement of heterotrimeric G-proteins and ADP-ribosylation factor in the transport event(s). The procedure described provides a unique mechanism to study critical events in antigen processing and presentation.

Major histocompatibility complex (MHC) class II glycoproteins are heterodimers that bind antigenic peptides and present them to CD4+ “helper” T cells (reviewed in Ref. 1). In man, expression of MHC class II molecules is generally restricted to “professional” antigen-presenting cells such as dendritic cells, B lymphocytes, activated macrophages, granulocytes, and T cells, although expression by other cell types can be induced by γ-interferon. In each case endosomal compartments enriched in MHC class II and referred to as MIICs (MHC II compartments) can be detected, which are believed to be the sites of peptide loading (2–6). B lymphoblastoid and B lymphoma cell lines constitutively express high levels of intracellular and cell surface MHC class II. Therefore, the majority of studies of human MHC class II-related antigen processing has been conducted using such cells. Although peptide exchange can occur, it is generally accepted that in B cells most antigenic peptides are loaded onto newly synthesized class II molecules (7) and that correctly formed αβ-peptide complexes are subsequently long lived (8). Thus to understand fully the biochemistry of antigen processing and presentation, it is necessary to determine the nature and number of compartments through which newly synthesized MHC class II molecules pass.

In most instances the intracellular trafficking of newly synthesized proteins can be defined by pulse-chase analysis of [35S]methionine-labeled molecules. However, investigation of MHC class II trafficking by this technique is severely complicated by the relatively asynchronous movement of MHC class II chains within the secretory pathway. In the endoplasmic reticulum (ER) newly synthesized class II α and β chains associate with the invariant chain (Ii) (9), a chaperone that acts both to mask the peptide binding groove (10, 11), and direct complexes to endosomal compartments (12, 13). Export from the ER requires the formation of a nonameric complex comprising an Ii trimer and three associated α and β chains. Assembly occurs via trimeric, pentameric, and heptameric intermediates and has an overall half-time of approximately 60 min (14). Thus export of radiolabeled molecules is asynchronous, since some labeled α or β chains associate with heptameric complexes and are exported within 10 min of the chase period, whereas others associate with Ii trimers and might still be retained in the ER 2 h later.

Nonameric complexes rapidly traverse the Golgi stack but cannot readily be detected at the cell surface until 1–2 h after they have acquired terminal oligosaccharide modifications indicative of delivery to the trans-Golgi network (TGN) (15). It is accepted that the complexes are retained in MIICs until peptide loading has occurred; however, there is still dispute as to whether the majority of αβIi complexes are transported directly from the TGN to MIICs (16) or transiently expressed at, and rapidly endocytosed from, the cell surface (17).

In order to address the precise post-Golgi trafficking of MHC class II molecules, an alternative strategy is required that restricts labeling to those molecules that have already been...
exported from the ER. Protein tyrosine sulfation is a ubiquitous late Golgi modification in mammalian cells that satisfies this requirement (reviewed in Ref. 18). In most cells it is confined to the trans-Golgi/TGN (19), and sulfate labeling has been used to study the biogenesis and trafficking of other TGN-derived vesicles (20, 21). A consensus sulfation motif has been defined (22), and Spiess and colleagues (23) showed that fusion of a non-antipeptide derived from procholecystokinin to the carboxyl terminus of either a soluble protein or a type II membrane protein allowed efficient sulfation of the resulting chimera. None of the MHC class II chains contain any recognizable sulfation motif, so in order to obtain labeling I have created a novel chimeric HLA-DRα chain containing two optimal tyrosine sulfation motifs. I have expressed this chimera in a B lymphoblastoid cell line lacking functional DR molecules, and I show that it correctly associates with β2 and β1 chains, is efficiently sulfated, and rapidly exported from the TGN, To inhibit clathrin-mediated endocytosis, without perturbing export from the TGN, I have utilized a temperature-sensitive mutant of dynamin 1 (24–26). I show that under conditions where clathrin-dependent endocytosis of transferrin is inhibited by at least 95%, the rate at which sulfated MHC class II is delivered to protease-containing compartments is unchanged. This indicates that most newly synthesized MHC class II molecules transit directly from the TGN to MHCs in this B cell line.

MATERIALS AND METHODS

Cells and Reagents—Tissue culture media and supplements were obtained from Sigma. HM2y2.DRN cells (27) were provided by Dr. P. Travers (University of London, UK) and maintained in RPMI 1640 containing 10% fetal calf serum, 1% minimum Eagle’s medium non-essential amino acids (Life Technologies, Inc.), 2 mM Glu, 1 mM sodium pyruvate, 50 IU/ml penicillin, and 50 μg/ml streptomycin in a humidified 95% air, 5% CO2 atmosphere at 37 °C. Hybridomas L243 (28), DA6.147, and DA6.231 (29) were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK) and propagated as described above. Supernatant from cultures of TALI.4.1 (30) was generously provided by Dr. J. Bodmer (Oxford, UK). Monoclonal antibody Bu45 (31) was obtained from the Binding Site (Birmingham, UK), LN2 (32) from Sigma, and 12CA5 (33) from Roche Molecular Biochemicals. Oligonucleotides were supplied by Genosys (Cambridge, UK) and molecular biology enzymes by New England Biolabs (Hitchin, UK). Other reagents were obtained from Sigma except where indicated.

Construction of pYY-DR—Plasmid pCD1 contains cDNA encoding human preprocathepsin D (34) between the HindIII and XbaI sites of pBluescript (Stratagene, Cambridge, UK). Oligonucleotides encoding the secretogranin I tyrosine sulfation site (sense 5′ TCAGAAGGATCTCCCTTTCAGGAACTGTCCAGGAAAGGAATCGAGAGTAGTGTTGAGTTTCTGTG 3′; antisense 5′ CTGAGACATTGGGCGGCTATGCAGTCTCTTCCTGCAAAGGGATCTCCG 3′) were annealed, digested with BamHI, and ligated into pCD1 that had been digested with BamHI and XbaI. The product (pCS1) contained the tyrosine sulfation site in frame with the cathepsin D signal peptide. A second aliquot of the annealed oligonucleotides was then digested with NcoI and ligated into pSCU with NcoI and XbaI to produce pCS2.

The mature DRα chain was amplified by polymerase chain reaction from plasmid pE.DRα (gift of Dr. N. Holmes, University of Cambridge) using Puu DNA polymerase (Stratagene) and primers designed to introduce unique ClaI and XbaI sites (sense 5′ CCATCGATGCACCCGGGACAGAAGGATCTCCCTTTCAGGAAAGGAATCGAGAGTAGTGTTGAGTTTCTGTG 3′; antisense 5′ GGAATTCTACCCCGGCGGCTATGCAGATCCACGTCCGTCTG 3′). The product was gel-purified, ligated into the EcoRV site of pBluescript, excised with ClaI and XbaI, and ligated into pCS2 cut with NcoI and XbaI to produce pCS3. The entire coding sequence of pCS3 was excised using XhoI and NdeI and ligated into the mammalian expression vector pB3 (gift of Dr. M. Jackson, Scripps Research Institute) that contains the SRα promoter (35), bovine growth hormone polyadenylation signal, and aminoglycoside phosphotransferase gene (36), to form pYY-DR. Plasmid for transfection was purified using Qiagen columns (Hibyand, Crawley, UK) according to the manufacturer’s instructions.

Transfection of HMy2.DRN—HMy2.DRN cells (2 × 106) were collected from culture, washed once with serum-free Iscove’s modified Dulbecco’s medium, and resuspended in 0.5 ml of the same medium. After transfer to a 0.4-cm electroporation cuvette (Bio-Rad) and incubation on ice for 10 min, 10 μg of pYY-DRα was added, and the incubation was continued for an additional 2 min. The cells were then electroporated at 230 V and 960 microfarads using a Bio-Rad Gene pulser, allowed to recover on ice for 10 min, then returned to culture. After 24 h the media were supplemented with G418 (Calbiochem), initially at 500 μg/ml, and after 48 h at 1,000 μg/ml. After incubation of 1.8 mg/ml. Resistant colonies were isolated by limiting dilution and, after 2 months at 1.8 mg/ml G418, maintained in media containing 0.9 mg/ml. A single clone expressing the modified HLA-DRα chain at high levels (YY1) was obtained and used for all subsequent experiments.

FACS Analysis—Cells were collected from culture, washed twice with ice-cold buffer containing 5 mg/ml BSA (PBS/BSA), and resuspended in PBS/BSA containing 5% (v/v) normal rabbit serum (1 × 106 cells/ml). After incubation at room temperature for 30 min to block nonspecific binding sites, the cells were collected by centrifugation, and resuspended in ice-cold PBS/BSA. Fluorescein isothiocyanate-conjugated mouse anti-human HLA-DR (clone G46-6; Becton Dickinson, Cowley, UK), or an appropriate isotype control (Sigma), was added, and the cells were incubated on ice with occasional mixing for 90 min. They were then washed three times with ice-cold PBS/BSA, once with PBS, and fixed in PBS containing 2% paraformaldehyde. Fixed cells were analyzed using a Becton Dickinson FACSort.

Radiolabeling and Immunoprecipitation—Clone YY1 cells (1.5 × 107) were collected from culture, washed twice with PBS/BSA, resuspended in medium Eagle’s containing 2% (v/v) heat-denatured newborn calf serum at 1.5 × 106 cells/ml, and incubated in a gassed incubator at 37 °C for 2 h. The cells were then collected by centrifugation at room temperature (4 min at 250 × g), washed once with sulfate-free medium lacking bicarbonate, and supplemented with 20 mM NaHepes, pH 7.4 (Life Technologies, Inc.), and resuspended in 200 μl of the same medium. After preincubation at 37 °C for 5 min, 50 μl of medium containing 0.25 mCi Na35SO4 (lyophilized stock; Amersham Pharmacy Biotech) was added, and the incubation was continued for an additional 5–10 min with occasional mixing. The cells were then transferred to ice, diluted to 10 ml with ice-cold PBS/BSA containing 10 mM sodium sulfate, and collected by centrifugation at 4 °C. A further wash in PBS/BSA-sulfate the cells were resuspended at 2.5 × 106 cells/ml in RPMI 1640 containing 10% newborn calf serum, 20 mM Hepes, pH 7.4, adjusted to 1 mM sulfated, and chased at 37 °C for times as indicated. Finally the cells were collected by centrifugation at 4 °C, the medium discarded, and the cells lysed as described previously (37). Radiolabeled class II was recovered with L243 + protein A (“mature”), or DA6.231 + protein G (total), and li with a combination of Bu45 and LN2 + protein G. Bound proteins were separated by SDS-PAGE using 12.5% gels and analyzed by autoradiography using a Fuji BAS2000 system.

N-Glycanase Digestion—In some experiments eluted proteins were precipitated with 10 volumes of acetone previously chilled to −80 °C and collected by centrifugation. The precipitates were resuspended in 25 μl of 0.1 M sodium phosphate, pH 6.5, containing 10 mM EDTA and 0.1% SDS, and heated to 100 °C for 10 min. The solutions were then cooled to room temperature, CHAPS added to a final concentration of 0.5%, and the mixture was centrifuged at 37 °C in the presence of 1 unit of N-glycanase (Roche Molecular Biochemicals). An equal volume of twice concentrated SDS-PAGE sample buffer was then added, and the samples were analyzed as described above.

Expression of Dynamin—Plasmid pTM1-BA-Hα-Dyn (kindly provided by Dr. S. Schmid, La Jolla, CA) contains human dynamin 1 having an amino-terminal hemagglutinin epitope tag and the mutation G273D (25). Plasmid pMEP4 (Invitrogen) contains the human metallocathepsin Iα promoter allowing inducible expression of heterologous proteins in mammalian cells after treatment with heavy metal ions (38). It also contains elements conferring resistance to hygromycin B treatment and the ability to replicate episomally in some cells. The entire coding sequence was excised with SpeI and SaFI and ligated into the Nhel and Xhol sites of pMEP4 to form pMEY. Clone YY1 cells were transfected with pMEY as described above and selected in medium supplemented with G418 (0.9 mg/ml) and 0.6 mg/ml hygromycin B (Roche Molecular Biochemicals) at 37 °C. A representative clone (YY1:DY8) was used for subsequent experiments. Mutant dynamin was induced by culturing clone YY1:DY8 in medium additionally supplemented with 100 μM ZnCl2 for 18–24 h at 31 °C. Following induction, high level expression was observed throughout the cell cycle. Reduced dynamin was not detectable in YY1 cells.

Western Blotting—YY1 cells (2 × 106) were collected from culture, washed once with PBS, and lysed in PBS containing 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride (10 mM/ml) at 0 °C. Protein in the clarified lysates was precipitated by the addition of 6 volumes of acetone previously cooled to −80 °C and incubated on ice for 10 min. After centrifugation at 17,000 × g for 15 min, precipitates were solubi-
RESULTS

Expression of a Tyrosine-sulfatable HLA-DRα Chain—In order to focus upon movement of newly synthesized MHC class II between the late Golgi and compartments involved in antigen processing and peptide loading, I constructed a chimeric DRα chain that could be efficiently labeled with sulfate. Consideration of the crystal structure of HLA-DR1 (41) suggested that a hydrophilic peptide fused to the amino terminus of the non-polymorphic α chain would be accessible to post-translational modification but unlikely to interfere with binding of Ii.

Analysis of the kinetic properties of protein tyrosine sulfotransferases has shown that the apparent K_m of peptide substrates decreases with increasing number of sulfation sites (42). Accordingly, I constructed a chimera comprising the human cathepsin D signal peptide and two repeats of a sequence related to the sulfation site of bovine secretogranin 1, fused to Glu^α of HLA-DRα. The predicted structure of this chimera, including the expected amino terminus following signal peptide cleavage and sites of protein tyrosine sulfation, is indicated in Fig. 1A.

LICR-LON-HMy2 is a variant of the plasma cell leukemia-derived B lymphoblastoid cell line ARH-77 (43). HMy2.DRN was generated by two rounds of γ-irradiation each followed by antibody and complement-mediated selection. The initial round was directed toward the HLA-A3 locus and isolated a line (HMy2.A3M) lacking an entire MHC haplotype. The second round was directed toward HLA-DR and identified a line (HMy2.DRN) in which the remaining DRα gene was mutated to encode a truncated protein that is rapidly degraded prior to export of αβIi complexes from the ER (27). The cell expresses cell surface immunoglobulin G (44), HLA-DP, and HLA-DQ but no HLA-DR.

Western blot analysis of the HMy2.DRN-derived clone YY1 generated by transfection with cDNA encoding the chimeric α chain, using the DRα-specific antibody DA6.147 (29), demonstrated the stable expression of the chimeric chain (Fig. 1B, lane 2). Similarly, FACS analysis confirmed that the chimeric chain restored surface expression of mature HLA-DR molecules (Fig. 1C).

As shown in Fig. 2, [35S]sulfate was rapidly incorporated into complexes containing the chimeric α chain. Without a subsequent chase incubation, radiolabeled proteins were efficiently precipitated with antibodies toward the luminal domain of Ii (Fig. 2, lane 1) but could not be recovered using L243 (lane 2), an antibody whose epitope is masked in HLA-DR molecules which are associated with intact Ii (45). Most of the radiolabel recovered using anti-Ii antibodies was present as a smear of apparent molecular mass of 45–80 kDa. This material was sensitive to digestion with chondroitinase A, B, C, consistent with previous observations that Ii is the protein core for B cell chondroitin sulfate proteoglycan (46). After a 2-h chase incubation less than 5% of the radiolabel was associated with intact Ii (lane 3) but was efficiently recovered with L243 (lane 4). Radiolabel precipitated with L243 was entirely resistant to chondroitinase digestion (data not shown). This suggests that the chondroitin sulfate present in the un-chased precipitates is exclusively associated with intact Ii chains, consistent with the conclusion that the Ii chain must be cleaved at a site membrane proximal to the site of proteoglycan addition in order to reveal the L243 epitope.

Characterization of Sulfated HLA-DR—At early time points the chimeric α chain migrated with an apparent molecular mass of 42 kDa (Fig. 2). Subsequently it was converted in a time-dependent fashion to a 40-kDa form (Fig. 2, lane 4, and Fig. 5A). This chain could be precipitated with monoclonal DA6.147 (which recognizes both β chain-associated, and free, α
characterized under “Materials and Methods” either directly (lanes 1–4) or after re-precipitation with DA6.147 (lane 2) or TAL14.1 (lane 3). B, clone YY1 (lane 1) or HMy2.DRN stably expressing wild-type DRa (lane 2) were labeled with [35S]sulfate for 10 min. MHC class II molecules were recovered using anti-class II antibody DA6.231. C, clone YY1 cells were radiolabeled with [35S]methionine as described previously (37) (lanes 1–4) or [35S]sulfate as described under “Materials and Methods” (lanes 5 and 6). Cells were chased for 4 h (lanes 1–4) or 1 h (lanes 5 and 6) in an excess of “cold” label, and MHC class II molecules were collected with L243. Samples were treated with N-glycanase as described under “Materials and Methods” either directly (lanes 5 and 6) or after re-precipitation of the α chains (lanes 1 and 2) or β chains (lanes 3 and 4) chains, respectively. Lanes 1, 3, and 5 show the results of mock incubations, and lanes 2, 4, and 6 show the effects of N-glycanase digestion. The arrow shows the position of deglycosylated β chains determined in lane 4.

To confirm that the 30-kDa sulfated species was indeed the DRβ chain, I re-precipitated denatured eluates previously recovered with L243 (Fig. 3A, lane 2), confirming that it was indeed derived from the chimera. Conversion to the faster migrating form could be prevented by treatment with the membrane-permeant thiol proteinase inhibitor E64d, indicating that it was the result of proteolytic activity. Surprisingly, in addition to the 40–42-kDa band corresponding to the chimeric α chain, efficient labeling of the DRβ chain was also observed in immunoprecipitates of both immature and mature molecules (Fig. 2, lanes 1 and 4). This was unexpected since none of the tyrosine residues in this chain are located within the context of an optimal motif, and only minor labeling was observed in sulfate-labeled human tonsil cells (47). I decided to investigate this further.

To confirm that the 30-kDa sulfated species was indeed the DRβ chain, I re-precipitated denatured eluates previously recovered with L243 (Fig. 3A, lane 2) with either DA6.147 or the DRβ chain-specific antibody TAL14.1 (30). As predicted, DA6.147 recognized the 40-kDa band and TAL14.1 the 30-kDa band in the denatured eluates (Fig. 3A, lanes 2 and 3), confirming that both HLA-DR subunits were efficiently sulfated in clone YY1 cells. In contrast, neither the α nor the β chains were labeled with sulfate when HMY2.DRN cells stably expressing a DRα chain lacking the sulfation motifs were examined under identical conditions to those used for clone YY1 cells (Fig. 3B). This suggests that efficient incorporation of sulfate into the β chain requires the optimal motifs contained in the chimeric α chain.

In addition to tyrosine residues, sulfate can also be incorporated into N- and O-linked oligosaccharide chains (reviewed in, 37). To confirm that the 30-kDa sulfated species was indeed the DRβ chain, I re-precipitated denatured eluates previously recovered with L243 (Fig. 3A, lane 2) with either DA6.147 or the DRβ chain-specific antibody TAL14.1 (30). As predicted, DA6.147 recognized the 40-kDa band and TAL14.1 the 30-kDa band in the denatured eluates (Fig. 3A, lanes 2 and 3), confirming that both HLA-DR subunits were efficiently sulfated in clone YY1 cells. In contrast, neither the α nor the β chains were labeled with sulfate when HMY2.DRN cells stably expressing a DRα chain lacking the sulfation motifs were examined under identical conditions to those used for clone YY1 cells (Fig. 3B). This suggests that efficient incorporation of sulfate into the β chain requires the optimal motifs contained in the chimeric α chain.

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molecules that had already been exported from the TGN during the labeling period, since a similar degree of processing was observed after prolonged incubation at 20 °C (Fig. 4, 1st column), a temperature at which further export is blocked. Consistent with this hypothesis, nearly 50% maximal L243 binding of sulfated complexes was obtained when brefeldin A addition was delayed until 15 min of the chase at 37 °C had been completed (Fig. 4, 5th column). Together these data strongly suggest that proteolysis of Ii requires prior export of αβIi complexes from the TGN and that similar mechanisms govern the formation of vesicles destined for MIICs as those destined for constitutive or regulated secretion.

Rapid Movement of Newly Sulfated HLA-DR Molecules to MIICs—I next examined the time course of the delivery of sulfated αβIi complexes to protease-containing compartments. After an initial lag of 5–10 min, incubation at 37 °C led to a rapid time-dependent increase in the proportion of sulfated α and β chains that could be recovered with L243 (Fig. 5A, odd lanes). Initially the 42-kDa form of the α chain was the sole form detected (lanes 1–4). After a delay of approximately 20 min, label was also present in the 40-kDa species, which was the major form detected at all times after the first 40 min (lanes 5–12). At present the precise amino terminus of the 40-kDa form has not been determined. However, the decrease in apparent molecular weight implies removal of at least 13 amino acids, which would include the first sulfated tyrosine residue. The most likely scenario therefore is that both of the potential tyrosine residues in the amino-terminal extension of the 42-kDa chimera are sulfated but that only one tyrosine sulfate residue is present in the 40-kDa form (summarized in Fig. 8). Consistent with this conclusion, appropriate correction of the quantitated amount of L243 precipitable radiolabel associated with both α chain species produced a result that closely followed that obtained by direct quantitation of the β chain (compare solid squares in Fig. 5, B and C).

A recent study using MoJuSo cells concluded that Ii degradation and peptide loading occurs in distinct intracellular compartments (53). Thus the appearance of L243-precipitable species of 42 and 40 kDa, respectively, may reflect delivery to sequential processing compartments (Fig. 8). Consistent with this hypothesis, virtually all of the BFA-resistant signal observed in the experiments shown in Fig. 4 (5th column) was present as the 42-kDa form (data not shown).

Cleavage of the carboxyl-terminal region of Ii was essentially complete after a 2-h chase (Fig. 5). At this time 20–30% of the radiolabel was present in complexes that were resistant to dissociation by incubation in SDS sample buffer at room temperature (data not shown). This is indicative of conformational changes that occur following antigenic peptide loading of MHC class II molecules (54). Sulfated molecules could also be detected by cell surface immunoprecipitation using L243 at this time. Together these data suggest that the sulfated amino-terminal extension does not significantly alter the normal trafficking or maturation of MHC class II molecules.

Sulfated αβIi Complexes Are Transported Directly to Proteolytic Compartments—At present there is still some debate as to the precise post-Golgi trafficking routes followed by newly synthesized MHC class II glycoproteins (for example see Refs. 16, 17, 24, and 55). The rapid delivery to protease-containing compartments observed in the present study suggested that in this case trafficking from the TGN to MIICs was largely independent of prior delivery to the cell surface. Consistent with this conclusion no significant population of immature molecules could be detected at the cell surface using antibodies directed to the luminal domain of Ii or following biotinylation with membrane-impermeant reagents (data not shown). However, these experiments could not eliminate the possibility that the half-time of immature complexes at the cell surface was too short to allow their detection. To address this point directly I examined the effect of expressing a temperature-sensitive mutant of dynamin 1.

Unlike other methods of blocking receptor-mediated endocytosis, the mutant dynamin does not alter export from the TGN (26). To minimize the time during which endocytosis was inhibited, I utilized a temperature-sensitive mutant that is functional at temperatures below 32 °C but acts as a dominant inhibitor at 38 °C (25). Although mutant protein synthesized at 37 °C does not fold properly and so is essentially inert (25), my attempts to isolate cells constitutively expressing high levels of the mutant were unsuccessful, either at 31 or 37 °C. To overcome this I utilized the pMEP4 vector that contains the human metallothionein IIA promoter and in Epstein-Barr virus transformed cells replicate episomally. As shown in Fig. 6A, a stable clone capable of showing a 10–20-fold induction of the mutant
The membrane transport pathways. The expression of the mutant protein at 37 °C was approximately twice that of the endogenous dynamin II (data not shown) and did not grossly interfere with cell growth. To confirm that the induced mutant protein was functional, I examined the endocytosis of transferrin. As previously shown for HeLa cells (25), endocytosis was identical in uninduced and induced cells assayed at 30 °C. In contrast, uptake in the induced cells was rapidly inhibited (\( t_{1/2} \)) upon shift to the restrictive temperature (data not shown). As shown in Fig. 6B, YY1:D8 cells induced at the permissive temperature, shifted to 38 °C for 10 min to achieve the mutant conformation, and then cooled to 0 °C prior to transferrin binding, showed a 95% reduction in the rate of endocytosis upon return to 38 °C (open squares) as compared with uninduced controls (closed squares).

In contrast, induction of the dynamin-dependent block in endocytosis in YY1:D8 cells did not cause cell surface accumulation of immature \( \alpha \beta \)I complexes (data not shown). Similarly, no effect on the rate of proteolytic maturation of sulfated MHC class II was observed when compared with mock-treated YY1 cells (Fig. 7). In both cases the rate and extent of processing was somewhat reduced as compared with untreated controls. However this appeared to reflect a direct effect of the zinc ion on the processing enzymes, rather than any inhibition of the membrane transport pathways.

FIG. 6. Inhibition by mutant dynamin of transferrin endocytosis. A, YY1 cells (lane 1), un-induced YY1:D8 cells (lane 2), or induced YY1:D8 cells (lane 3) were processed for immunoblotting using antibody 12CA5 as described under “Materials and Methods.” The arrow indicates the epitope-tagged mutant dynamin. B, un-induced (■) or induced (□) YY1:D8 cells were collected from culture at 31 °C and incubated for 10 min at 38 °C. The cells were precipitated and resuspended in buffer containing biotinylated transferrin (5 μg/ml). After binding and washing at 4 °C, the cells were resuspended in media and chased at 38 °C for times as indicated and then processed for enzyme-linked immunosorbent assay as described under “Materials and Methods.”

FIG. 7. Effect of mutant dynamin on sulfated MHC class II processing. Mock-induced YY1 cells (■) or induced YY1:D8 cells (□) were collected from culture at 31 °C and depleted of endogenous sulfate at the same temperature for 3 h. The cells were labeled at 38 °C for 10 min, chased at the same temperature for times as indicated, and then immunoprecipitated with L243 and DA6.231 as described under “Materials and Methods.” Results are expressed as the percentage of the total \( \beta \) chain-associated radiolabel recovered with L243.

To overcome this problem I designed a strategy based upon protein tyrosine sulfation. This modification occurs in late Golgi compartments, allows labeling to high specific activity, and has been used by others (20, 21) to study post-Golgi trafficking events. A previous study demonstrated that human MHC class II \( \alpha \) and \( \beta \) chains could be sulfated (47); however, labeling periods of 5–7 h were required suggesting that the modification was considerably sub-stoichiometric. Consistent with this hypothesis, neither chain contains a recognizable tyrosine sulfation motif (22). Other studies (23) have shown that a nonapeptide derived from procholecystokinin can be efficiently sulfated when fused to both soluble and membrane proteins and that a synthetic protein containing 12 repeats of a heptapeptide from bovine secretogranin I can be sulfated stoichiometrically (57). Accordingly I elected to create an MHC class II chimera containing optimal sulfation sites.

A key requirement was that the sulfation motif should be accessible to modification in the TGN but not interfere with the correct assembly of (\( \alpha \)II)\( _{3} \) complexes in the ER. Examination of space-filling models generated from the crystal structures of HLA-DR molecules associated with either an antigenic peptide or CLIP (the fragment of Ii that occupies the peptide-binding groove) (41, 58) revealed that the amino terminus of the \( \alpha \) chain was exposed at the surface of the molecule and projected from the face mainly comprised of the \( \beta \) chain below, and apparently perpendicular to, the peptide-binding groove. As sulfation motifs are hydrophilic I concluded that, if fused to the amino terminus of the \( \alpha \) chain, such motifs would probably be accessible to tyrosine sulfotransferases. Similarly as only 25 amino acid residues in Ii are located between the transmembrane domain and CLIP, and they apparently lack any ordered structure (59), I considered it unlikely that a short hydrophilic peptide fused to the \( \alpha \) chain amino terminus would interfere

**DISCUSSION**

A central event in the generation of an adaptive immune response is the binding of peptide fragments of antigen to MHC class II glycoproteins and their subsequent presentation to CD4+ T lymphocytes (reviewed in Ref. 1). The critical reactions occur in multiple endocytic compartments variously termed MIICs and CIVs (reviewed in Refs. 3 and 56) and predominantly involve newly synthesized MHC class II molecules (7). However, the precise trafficking routes followed by newly synthesized \( \alpha \beta \)I complexes and the biochemical machinery governing their movement between the TGN and MIICs remain unclear. Export of newly synthesized MHC class II from the ER requires the formation of a nonameric complex comprising three \( \alpha \beta \) dimers associated with an Ii trimer (14). This is not complete for 1–2 h after a 10-min pulse, although labeled \( \alpha \) and \( \beta \) chains which have reached or passed beyond the trans-Golgi can readily be detected within 30 min (15). This means that reagents that might potentially be used to examine export of MHC class II from the TGN cannot easily be tested if they also affect export from the ER.

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with the correct assembly of \((αβIi)_2\) complexes. To increase the specific activity of the chimera I decided to include multiple copies of the sulfation motif. However, as a large amino-terminal extension might adversely affect \((αβIi)_2\) complex assembly, I focused upon a chimera containing two potential sites, and I utilized a heterologous signal peptide to facilitate the molecular cloning strategy. The data presented in this study clearly demonstrate that fusion of a 24-residue peptide to the amino terminus of the HLA-DR \(α\) chain does not interfere with the correct trafficking or maturation of MHC class II molecules. In addition to studies of HLA-DR maturation, this observation may also be applicable to other studies of antigen presentation. Efficient presentation can be achieved if the CLIP sequence in \(Ii\) is replaced with an antigenic peptide (60). My data suggest that an alternative strategy in which the antigenic peptide is fused to the \(α\) chain might also be successful.

After a brief labeling period sulfated MHC class II molecules could all be recovered using antibodies directed toward the luminal domain of \(Ii\). This is consistent with the hypothesis that proteolysis (and consequent loss of this epitope) does not occur until the newly synthesized complexes have reached MIICs. At early times the major radiolabeled species recovered was the chondroitin sulfate proteoglycan form of \(Ii\). Overall this is a minor \(Ii\) species, but it appears to have an important role since inhibition of its synthesis depresses antigen presentation (61). The proteoglycan is mainly located at the cell surface (46) but is rapidly degraded. This suggests that a population of \(Ii\) may be preferentially directed to the plasma membrane, although this may be independent of association with MHC class II (62). No evidence for such a route was provided by the present study, although the possibility that the sulfated glycosaminoglycan chains were rapidly released at the cell surface, and therefore did not accumulate, was not explored. Interestingly, a significant fraction of the sulfated \(Ii\) could not be recovered using antibodies to MHC class II despite repeated precipitations with reagents showing a variety of specificities. This suggests that multiple populations of chondroitin sulfate-modified \(Ii\) complexes may exist. This possibility has not been fully explored, but the YY1 cell line could facilitate such studies.

A surprising observation of this study was the efficient sulfation of the \(β\) chain, despite the absence of any recognizable motif. In contrast to the previous study of sulfation of endogenous molecules (47), which concluded that the peptide backbone of the \(β\) chain was modified, most if not all of the sulfate incorporated into the \(β\) chain in YY1 cells was added to the \(N\)-linked oligosaccharide chains. Sulfation of these residues was entirely dependent upon the presence of the sulfatable \(α\) chain, suggesting that the amino-terminal extension directly influenced the activity of the oligosaccharide sulfotransferase concerned. This appears possible since \(α\beta3\) (the site of my extension to this chain) is within 5–10 Å of \(β19\) (the single site of oligosaccharide addition) (41) and might therefore interact with elements of this chain. In contrast residues \(α78\) and \(α119\), the two sites of \(N\)-linked oligosaccharide addition to the \(α\) chain, are exposed at the opposite face to the amino-terminal extension and are therefore unlikely to be influenced by its presence. At present the substrate determinants that govern the activities of oligosaccharide-modifying enzymes are largely unknown. My results suggest that a cluster of negatively charged residues might enhance the activity of at least one such enzyme.

Incubation at 37°C led to the rapid degradation of \(Ii\) and consequent appearance of the L243 epitope. A brief lag was observed prior to the onset of proteolysis, which then occurred with a half-time of 20–30 min. Such kinetics are similar to those exhibited for movement between the ER and cis-Golgi (63). This suggests that delivery is directly to the site of proteolysis; the kinetics appear too rapid to involve prior exposure at the cell surface or movement through multiple endocytic compartments. To examine directly this conclusion, I investigated the effect of expressing a mutant form of dynamin 1 previously shown to inhibit receptor-mediated endocytosis in HeLa cells (25). It has previously been shown to be rapidly internalized in a clathrin-dependent manner (17, 64), and so I reasoned that a selective block in this process would resolve the question of post-Golgi targeting. Under conditions in which endocytosis of transferrin was almost completely abolished, no delay in processing was observed, demonstrating that the majority of MHC class II was directly targeted to MIICs in YY1 cells.

In contrast, a concurrent study conducted with transfected HeLa cells demonstrated that a significant proportion of immature class II was trapped at the plasma membrane by a mutant dynamin (24). However, unlike the present study, where the mutant phenotype was not induced until the commencement of the radiolabeling experiment, Wang and colleagues (24) induced their mutant dynamin 74 h prior to analysis. As the mutant phenotype is apparent within 48 h of tetracycline removal in these cells (26), the possibility exists that prolonged disruption of endocytosis may have indirectly affected multiple trafficking pathways in their study. This might also explain the discrepancy between their results and those of Lui and co-workers (55), who used an alternative method of disrupting clathrin-dependent events and concluded that most newly synthesized class II was directly targeted to the endocytic pathway. In addition, a major factor to be considered in the interpretation of these and similar studies is the level of expression of MHC class II in the various experimental systems used. Both tyrosine- and di-leucine-based TGN-sorting systems have finite capacities (65), and so it is likely that expression of high levels of immature \(αβIi\) complexes may saturate the sorting machinery. Typically the levels of expression of MHC class II molecules in B lymphoblastoid and B lymphoma cells are significantly greater than in resting B cells (66), although there is extensive variation between individual cell lines. Thus the apparent discrepancy between the results of previous studies (for example Refs 16 and 17) may simply reflect the relative capacities of the sorting machineries in the cell lines used. Although it is a B lymphoblastoid line, the YY1 cells developed in the present study have a lower level of HLA-DR expression relative to other Epstein-Barr virus-transformed cell lines (data not shown), presumably due to the absence of a second HLA-DR\(β\) gene. This, together with the
correspondingly reduced levels of HLA-DP and -DQ, might ensure that the relevant trafficking machinery in YV1 cells is not saturated and so explain the high efficiency of direct targeting observed. Under physiological conditions most, if not all, B cell antigens are delivered to MHC class II glycoproteins by membrane immunoglobulin. It appears biologically consistent that nascent MHC class II molecules should also be directly delivered to these compartments, rather than exposed at the cell surface where they might be aberrantly processed and hence acquire inappropriate peptides. This might not be the case for other antigen-presenting cells having different roles in the immune response.

In summary, the data obtained in the present study indicate that under conditions where the TGN-based sorting machinery is not saturated most newly synthesized MHC class II glycoproteins are directly transported to a processing compartment in which initial cleavage of I occurs (Fig. 8). Subsequently the partially processed complexes are either delivered to a more distal processing compartment or the MIIC in which they reside itself matures in a BFA-sensitive manner, allowing complete processing of the invariant chain and peptide loading to occur (53). Finally, the mature complexes are transported to the cell surface by an as yet poorly understood mechanism.

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