Impaired Processing and Presentation by MHC Class II Proteins in Human Diabetic Cells

Gang Yan, Lijia Shi, Alfred Penfornis and Denise L. Faustman

*J Immunol* 2003; 170:620-627; doi: 10.4049/jimmunol.170.1.620
http://www.jimmunol.org/content/170/1/620

**References**  This article cites 30 articles, 17 of which you can access for free at:
http://www.jimmunol.org/content/170/1/620.full#ref-list-1

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Impaired Processing and Presentation by MHC Class II Proteins in Human Diabetic Cells

Gang Yan, Lijia Shi, Alfred Penfornis, and Denise L. Faustman

The biochemical processing of and Ag presentation by MHC class II molecules were examined in B cell lines derived from pairs of identical twins discordant for type 1 diabetes. MHC class II defects detected exclusively in cells derived from the twins with autoimmunity included increased rates of transport to and subsequent turnover at the cell surface, inadequate glycosylation, and a reduced display at the cell surface of antigenic peptides. These defects appeared to be secondary to a decreased abundance of the p35 isoform of the invariant chain (II), a human-specific chaperone protein for MHC class II normally generated by use of an alternative translation start site. Stable transfection of diabetic B cell lines with an II p35 expression vector corrected the defects in MHC class II processing and peptide presentation. A defect in the expression of II p35 may thus result in impairment of Ag presentation by MHC class II molecules and thereby contribute to the development of type 1 diabetes in at-risk genotypes.

The Journal of Immunology, 2003, 170: 620–627.

Type I (insulin-dependent) diabetes mellitus is an autoimmune disease that culminates in destruction of the β cells of islets of Langerhans and consequently results in insulin deficiency. The elimination of β cells is thought to be mediated by autoreactive T cells, a process that probably results from improper education of CD4+ and CD8+ T cells.

The invariant chain (II) functions as a chaperone in the intracellular transport of MHC class II proteins as well as in the loading of these proteins with peptides for presentation to CD4+ T cells. The II chain forms a core trimer that associates with three MHC class II αβ subunit dimers in the endoplasmic reticulum (ER), resulting in generation of the nonameric complex (αβII), (2, 3). This complex is incapable of binding antigenic peptides, which is important because most endogenous peptide fragments present in the ER are destined for association with MHC class I molecules (4, 5). The MHC class II-II complex undergoes extensive glycosylation and is transported from the ER to the endosomal-lysosomal Ag-processing compartments, a process determined by the targeting motifs of the II cytoplasmic domain (6–8). Within these compartments, II is removed from MHC class II molecules by a series of highly regulated proteolytic cleavages, with the accessory protein HLA-DM catalyzing the final release of a remaining II fragment known as class II-associated invariant peptides (CLIP) from the MHC class II groove, thereby allowing the binding of antigenic peptides.

The rate of removal of CLIP from MHC class II proteins may be influenced by MHC class II allelic variation (9, 10). The high affinity interaction of MHC class II molecules with antigenic peptides results in the formation of a stable complex, as evidenced by its resistance to dissociation in SDS sample buffer. The cell surface expression of these MHC class II-peptide complexes then contributes to T cell education, selection, and stimulation.

In fresh human lymphocytes and EBV-immortalized B cells, two major alternative II isoforms function in the intracellular routing of MHC class II molecules to the endosomal-lysosomal compartments. The predominant II chain is the 33-kDa isoform (II p33), but an additional human-specific 35-kDa isoform (II p35) is generated as a result of the use of an alternative upstream translation initiation site in the shared mRNA (11). These two II isoforms are thus identical, except that II p35 contains an additional 16 aa at the cytoplasmically located NH2-terminus. The ratio of these II isoforms in cells of fixed lineage appears to be highly regulated, with the p35 isoform representing ~20% of the total II pool of human B cells. Most MHC class II-II complexes contain at least one II p35 polypeptide (2, 3, 12). Other minor isoforms of II (p41, p43) are generated as a result of alternative RNA splicing; II p43 with the expression of the additional exon, similar to II p35, also contains the upstream translation initiation site.

The chaperone functions of II p35 and p33 in MHC class II assembly and transport are distinct, with differences apparent in the route taken by MHC class II molecules to the cell surface and in peptide acquisition for Ag presentation. The ratio of the two II isoforms thus determines for MHC class II molecules the choice and timing of compartment localization, the rate of exit from the ER, the extent of glycosylation, the rate of intracellular peptide loading, and the degree of endosomal degradation. Indeed, MHC class II molecules complexed exclusively with II p33 are generally transported indirectly to the endosomal-lysosomal compartments via the plasma membrane, whereas MHC class II proteins complexed with II p35 move directly to endosomes-lysosomes without first appearing at the cell surface (13). The transport route of MHC class II complexes containing II trimers with at least one II p35 molecule, the predominant form of the (αβII)3 complex, is exclusively intracellular (13–16). The association of II with MHC class II proteins results in masking of the ER retention motif in II p35.

Immunobiology Laboratory, Massachusetts General Hospital and Harvard Medical School, Charlestown, MA 02129

Received for publication June 7, 2002. Accepted for publication October 29, 2002.

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

1 This work was supported by The Iacocca Foundation.
2 G.Y. and L.S. contributed equally to this work.
3 Current address: Endocrinologie, Hopital Jean Minjoz, 25030 Besancon Cedex, France.
4 Address correspondence and reprint requests to Dr. Denise L. Faustman, Immunobiology Laboratory, Massachusetts General Hospital, East, Building 149, 13th Street, CNY-5, Charlestown, MA 02129. E-mail address: faustman@helix.mgh.harvard.edu
5 Abbreviations used in this paper: II, invariant chain; CLIP, class II-associated invariant peptide; EndoH, endoglycosidase H; ER, endoplasmic reticulum; HA, hemagglutinin; NSH-SS-biotin, N-hydroxy succinamide-S-S-biotin.

Copyright © 2003 by The American Association of Immunologists, Inc.

0022-1767/03/$02.00
thus allowing the transport of $\alpha \beta$ dimer containing this isoform of Ii out of the ER (17, 18). The transport of MHC class II-Ii nonamers containing exclusively Ii p33 is to the cell surface before peptide loading. This delays Ag presentation by these MHC class II molecules and results in a cell surface phenotype characterized by abundant class II proteins that are not loaded with antigenic peptides (13–16).

The patterns of protein glycosylation and phosphorylation for MHC class II-Ii complexes also differs between those containing only Ii p33 and those containing at least one molecule of Ii p35 (12, 17). The ER retention signal of Ii p35 delays the exit of MHC class II assemblies containing this isoform and thereby facilitates complex glycosylation (7). In contrast, association of MHC class II molecules exclusively with Ii p33 results in the rapid development of resistance to endoglycosidase H (EndoH) and simple patterns of N-glycosylation, signs of fast transport out of the ER. In addition, to the glycosylation pattern representing a marker of intracellular transport rate and route, it may affect the rate of degradation survival of some proteins in the endosomes-lysosomes (19).

Cells expressing recombinant MHC class II proteins in the absence of either Ii p33 or p35 exhibit rapid delivery of the class II proteins to the cell surface as well as enhanced loading with exogenous peptides as a result of the surface expression of these molecules with empty peptide-binding grooves (20). The rate of intracellular transport of MHC class II molecules is thus slowest in cells expressing exclusively the p35 isoform of Ii, is faster in cells expressing exclusively the p33 isoform of Ii, and is faster still in cells lacking both Ii isoforms (18).

The MHC class II region of the human genome, also more commonly known as the HLA region, contains up to 14 different class II loci that are clustered into three major subregions: HLA-DR,-DQ, and -DP. Each of these subregions contains at least one functional β locus and one α locus. Although the MHC class II region of the genome confers the greatest statistical risk for type 1 diabetes as well as for other autoimmune diseases, the complexity of this genetic association is revealed by the fact that identical twins exhibit <40% concordance for type 1 diabetes expression (21), and for other autoimmune disease the concordance rate is even less. Furthermore, diverse human and murine functional studies suggest that CD4+ T cells might contribute to the initiation of autoimmunity, including type I diabetes. Given that CD4+ T cell education is determined by Ag presentation by MHC class II molecules, we have now investigated biochemically the assembly of MHC class II molecules and their presentation of antigenic peptides in established EBV-immortalized B cell lines derived from human identical twins pairs discordant for autoimmune type 1 diabete. The study of cells from such twins controls for the possible confounding influence of the MHC class II haplotype on disease-specific defects in the intracellular transport or peptide loading of class II molecules.

We now show that B cells derived from diabetic twins exhibit rapid delivery of poorly glycosylated and peptide-empty MHC class II molecules to the cell surface as well as delayed peptide loading. These Ag processing defects were associated exclusively with disease penetrance and were secondary in part to a reduced abundance of the p35 isoform of Ii. Stable transfection of cells from diabetic twins with a vector encoding Ii p35 corrected these defects in the assembly of and peptide presentation by MHC class II molecules. The reduced production of a chaperone protein (Ii p35) that facilitates MHC class II assembly may thus be an important phenotype tracking with disease expression of type 1 diabetes in individuals with disease-conferring class II haplotypes.

Materials and Methods

Cells

The EBV-transformed B cell lines used in the present study were prepared from four sets of identical twins discordant for type 1 diabetes (for >15 years) and from random control individuals. All paired twin sets were derived on the same day, although different twin sets were derived at totally different times. At the time the cell lines were made for the twin sets presented in this paper, the diabetic twins no longer had autoantibody expression indicative of active autoimmune disease. The nondiabetic twins and controls were autoantibody negative and exhibited both normal insulin secretion and fasting blood glucose concentrations at yearly examinations over a period of >10 years. One of the diabetic twins of a single pair of twins developed autoimmune hypothyroidism 17 years after the diagnosis of type 1 diabetes. Permission for blood drawing was obtained after full institutional review of the protocol. In the case of experiments for which data obtained from only one twin set are presented, similar results were obtained for all four twin pairs unless indicated otherwise.

Additional derivative B cell lines in this study included T1 and T2 lymphoblastoid cells purchased through American Type Culture Collection (Manassas, VA) and were donated by Dr. P. Cresswell (New Haven, CT). T2 cells are a mutant cell line derived from T1 cells; they lack a large segment of chromosome 6 that encodes MHC class II genes, the Lmp2 and Lmp7 proteins, and the ATPase peptide transporters Tap1 and Tap2. T2 cells do not express surface MHC class II due to direct gene deletion or MHC class I due to deletion of the obligatory transporter and proteasome assembly genes.

Peptide synthesis

The peptide hemagglutinin (HA)907–919 (PKYVKQNTLKLAT) was derived from influenza A/Texas/77/77 virus H3 HA and is HLA-DR restricted. The peptide IgC-H2-D1 (KVKTVKVDALGQNS) is derived from the Igk chain and is also HLA-DR restricted. The peptide SGPLKAEIQRLY was eluted from HLA-DQ, matches the sequence of an uncharacterized human protein, and binds the products of all HLA-DQ alleles with varying affinity. All peptides were biotinylated at the NH2 terminus (Quality Control Biochemicals, Hopkinton, MA). They were purified by HPLC, lyophilized, and reconstituted in water.

Assay of peptide binding to the cell surface

The binding of exogenous peptide to MHC class II molecules on the surface of B cells was performed basically as previously described (22). EBV-transformed cells (3 × 106 cells/ml) in 50 μl of complete culture medium were incubated for 4 h at 4°C with 50 μM biotinylated peptide, washed, and then incubated for 30 min at 4°C with FITC-conjugated streptavidin (4.22 μg/ml; Life Technologies, Gaithersburg, MD). As an alternative to incubation at 4°C, in some experiments cells were treated with 12 mM sodium azide to prevent endocytosis and were incubated with peptide for 2 h at 37°C. Cell fluorescence was analyzed (2000 cells/sample) with an EPICS Elite flow cytometer (Coulter, Hialeah, FL); background fluorescence was simultaneously quantified and subtracted. For greater sensitivity of detection of peptide binding, after incubation with biotinylated peptide the cells were sequentially exposed at 4°C to FITC-conjugated avidin D, to biotinylated Abs to avidin D, and again to FITC-avidin D (Vector, Burlington, CA).

For evaluation of the stability of MHC class II-peptide complexes at the cell surface, EBV-immortalized B cells were incubated with 50 μM biotinylated peptide for 4 h at 37°C, washed, and then incubated for various times at 37°C in the presence of 500 μM unlabelled peptide. Fluorescence was determined by flow cytometry.

Antibodies

Among the various mAbs to human MHC class II used in the present study, L243 recognizes predominantly αβ dimers of HLA-DR devoid of intact Ii (American Type Culture Collection), 9.3F10 (Coulter) recognizes all HLA class II proteins (DR, DP, and DQ), 1-2 (Coulter) recognizes HLA-DR, 16.23 (provided by R. Wank) recognizes the HLA-DR3 dimer, 1-3 (Coulter) recognizes a nonpolymorphic region and therefore binds to all class II proteins (DR, DP, and DQ), and DA6.147 (provided by K. Guy) recognizes DRα and DR dimers. mAb PIN.1 was generated in response to a peptide corresponding to aa 12–28 of the p33 isoform of human Ii, but detects the cytoplasmic NH2-terminal regions of both Ii p33 and Ii p35. The mAb CerCLIP.1 recognizes human CLIP in association with MHC class II. Rabbit polyclonal Abs to Ii p35 (R.Ip35N) and Lp41 (R.Ip41) and control rabbit polyclonal Abs to HC3 were obtained from Affiniti Research (Mamhead, U.K.). mAb W6/32 recognizes all human MHC class I molecules and was purchased through ATCC.
Analysis of surface expression of MHC class II and CLIP

EBV-transformed B cells (1 × 10⁷) in the log phase of growth (>95% viability) were incubated for 30 min at room temperature in a final volume of 500 µl with various mAbs to MHC class II or CLIP (1.5–2.0 µg/ml). For nonfluorescent primary Abs, immune complexes were detected with FITC-conjugated goat Abs to mouse IgG (Coulter).

Development of EndoH resistance and stable complex formation by MHC class II

Cells (8 × 10⁷) were labeled with 2 mCi of [³⁵S]methionine (DuPont Biotechnology Systems, Boston, MA) in 15 ml of methionine-free RPMI 1640 for 30 and 90 min at 37°C and then were incubated for various times in complete culture medium containing a 100-fold excess of unlabeled methionine. Cells were lysed at 4°C in a solution containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.5 mM PMSF, and 0.1 mM TLCK (Nα-tosyl-L-lysine chloromethyl ketone), and lysates were subjected to immunoprecipitation with mAbs (L243) to MHC class II and protein A-Sepharose beads (Pharmacia, Piscataway, NJ). For determination of EndoH resistance, the immunoprecipitates were eluted from the beads in 15 µl of EndoH digestion buffer (0.1 M sodium phosphate (pH 6.8), 2% SDS, and 2% glycerol) and divided into two portions, one of which was heated at 100°C in a solution containing 10 mM Tris-HCl (pH 6.8), 2% SDS, and 0.1% NaN₃, and the other was maintained at room temperature before analysis by SDS-PAGE and autoradiography.

Determination of the rates of synthesis and surface appearance of MHC class II

Cells (−3 × 10⁷) were labeled with 1 mCi of [³⁵S]methionine in 5 ml of methionine-free RPMI 1640 for 30 min at 37°C and then incubated for various times at 27°C in complete culture medium containing a 100-fold excess of unlabeled methionine. The cells were suspended in 1 ml of Hank’s solution containing N-hydroxy succinimide-S-S-biotin (NHS-S-biotin; Pierce, Rockford, IL) at a concentration of 3 mg/ml, incubated for 3 min on ice, and then lysed for 30 min on ice in the lysis solution described above. Lysates were subjected to immunoprecipitation with mAb to HLA-DR and protein A-Sepharose beads. Proteins were eluted from the beads in 100 µl of PBS containing 2% SDS. A portion (20 µl) of the eluted proteins was used for total MHC class II synthesis (results multiplied by 5). A second portion (80 µl) of the eluted proteins was diluted into 1 ml of PBS containing 1% Triton X-100 and subjected to precipitation with streptavidin-Sepharose beads (Pierce) for 2 h at room temperature. The resulting precipitates were washed three times with precipitation buffer and suspended in SDS sample buffer for cell surface MHC class II (results multiplied by 0.8). Cells (−3 × 10⁷) were labeled with 10 µCi/ml of [³⁵S]methionine in 5 ml of methionine-free RPMI 1640 for 15–20 min at 37°C and then incubated for 15, 30, 45, 60, 90, 120, 180, or 240 min at 27°C in complete culture medium with a 100-fold excess of unlabeled methionine. After labeling, using buffers similar to those used for MHC class II cells, cells were subjected to immunoprecipitation with mAb to MHC class I and protein A-Sepharose beads.

Immunoblot analysis

Cells (2 × 10⁷) were lysed in 500 µl of a solution containing 10 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.15 M NaCl, 0.2 mM EDTA, 0.02% NaN₃, PMSF (100 µg/ml), aprotenin (1 µg/ml), and leupeptin (1 µg/ml), and equal amounts of lysate protein were subjected to SDS-PAGE on a 12.5% gel. The separated proteins were transferred to a polyvinylidene difluoride membrane, which was then subjected to immunoblot analysis with polyclonal Abs to li p35, li p41, or mAb HC3. Immune complexes were detected with appropriate secondary Abs labeled with HRP and an ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ).

Stable transfection of B cell lines with an li p35 vector

The human li p35 cDNA (in which the second translation start site was mutated) was provided by E. Long (11) and was cut from the expression vector Sp64 to construct the expression vector pBabe li p35 out of BamHI sites. EBV-transformed B cells (1 × 10⁷) were incubated for 5 min on ice in a volume of 100 µl containing 30 µg of pBluescript li p35 plasmid DNA and were then subjected to electroporation at 220 V and 960 Ω in a 0.4-cm cuvette. Transfected cells were cultured for 2 days without selection and then were maintained in the presence of puromycin (0.25 µg/ml) for >2 mo. At least three stable and independently derived transfected cell lines were established from each EBV cell line.

Results

Surface expression of MHC class II on diabetic B cell lines

The abilities of APC to stimulate T cells as well as to induce positive or negative T cell selection are dependent on the total number of surface MHC class II peptide complexes. With the use of flow cytometry with four different mAbs to MHC class II molecules, we therefore initially examined the total surface density of these molecules on EBV-immortalized B cell lines from identical twin pairs discordant for type 1 diabetes as well as on control cell lines. The MHC class II surface density revealed by the three mAbs, L243, I-2, and I-3, did not differ significantly between diabetic twin cell lines and either matched nondiabetic twin cells or non-HLA-matched random control EBV cell lines (Table I). In contrast, the MHC class II surface density revealed by mAb 9.3F10, which recognizes HLA-DR, -DP, and -DQ, was significantly greater for the diabetic cell lines than for the matched twin cells or control cells. Although the exact conformation of MHC class II molecules recognized by mAb 9.3F10 is not known, the Ab detects a subset of APC with an enhanced ability to present exogenous Ag, an indirect indication of its preferential binding to peptide-empty forms of MHC class II (23). These results suggested that the overall surface density of MHC class II molecules did not differ between B cells from diabetic or nondiabetic twins or between twin cells and control cells, but that the expression of peptide-empty MHC class II molecules on the surface of the diabetic B cells might be increased.

Table I. Surface density (mean channel fluorescence) of MHC class II and of CLIP on diabetic twin, nondiabetic twin, and control B cell lines

| Ab          | Diabetic | Nondiabetic | p Value* | Control | p Value† |
|-------------|----------|-------------|----------|---------|----------|
| Anti-class II |          |             |          |         |          |
| L243 (n = 12) | 17.00 ± 0.32 | 17.02 ± 0.18 | 0.915    | 17.05 ± 0.05 | 1.000    |
| I-2 (n = 12)  | 15.71 ± 0.86 | 16.09 ± 1.09 | 0.079    | 16.25 ± 0.35 | 0.967    |
| 9.3F10 (n = 12) | 25.20 ± 0.68 | 20.5 ± 0.825 | 0.001    | 21.88 ± 0.18 | 0.009    |
| I-3 (n = 12)  | 12.30 ± 1.30 | 13.40 ± 0.66 | 0.470    | 12.50 ± 0.01 | 0.100    |
| Anti-CLIP     |          |             |          |         |          |
| CerCLIP.1 (n = 8) | 6.80 ± 2.20 | 8.80 ± 3.20 | 0.062    | 7.10 ± 1.5  | 0.060    |

* Data are the means ± SD for the indicated number (n) of cell lines examined.
† Comparison by Student’s t test between diabetic and nondiabetic twins.
‡ Comparison between diabetic twin and control cells. n represents the total number of diabetic, nondiabetic, and control cells examined.
Defects in MHC class II processing in cell lines expressing mutant chaperone proteins often result in the retention by MHC class II molecules on the cell surface of the CLIP fragment of Ii (24). Conversely, a low level of CLIP retention by surface MHC class II is a characteristic of human lymphoid cells that are developmentally immature or exhibit enhanced endosomal-lysosomal processing (25). We therefore examined surface occupancy of MHC class II by CLIP with the mAb CerCLIP. Although all diabetic cell lines examined showed a reduced level of CLIP retention by surface MHC class II compared with the matched nondiabetic or the control cell lines, this difference was not statistically significant with this sample size (Table I).

*Increased surface density of peptide-empty MHC class II on diabetic B cells*

The peptide occupancy of surface MHC class II molecules can be directly quantitated by measurement of the binding of exogenous labeled peptides (20, 26). With the use of flow cytometry, we therefore examined the binding of two biotinylated HLA-DR-specific antigenic peptides (HA307–319 and IgC37–51) at saturating concentrations to MHC class II molecules on the surface of diabetic and nondiabetic twin B cells and on mutant T2 B cells that lack surface MHC class II proteins. The binding of the HA307–319 peptide to B cells derived from diabetic twins was markedly increased compared with that to B cells from the matched nondiabetic twins (Fig. 1A). This difference was apparent both for cells incubated at 4°C for 4 h (Fig. 1A) and for cells incubated at room temperature in the presence of sodium azide for 2 h (data not shown), both of which procedures were chosen to prevent the turnover of surface MHC class II. Similar results were obtained with the peptide IgC37–51 (Fig. 1B), but only background peptide binding fluorescence was evident on T2 cells, confirming the specificity of peptide binding to MHC class II. These results were thus indicative of a defect in intracellular peptide loading of MHC class II molecules in the B cells derived from diabetic twins.

*Rapid exit of MHC class II from the ER of diabetic B cells*

We next examined the transport of newly synthesized MHC class II-Ii protein complexes in diabetic and nondiabetic B cell lines. Cells were pulse-labeled with [35S]methionine and subjected to immunoprecipitation with Abs to MHC class II at various times thereafter. The rate of exit of MHC class II molecules from the ER was assessed by subjecting portions of the immunoprecipitates to treatment with EndoH, which cleaves carbohydrate chains from proteins that have not yet been processed further in the Golgi apparatus (27). Cleavage of N-linked carbohydrates from a protein by EndoH was revealed by an increase in electrophoretic mobility and rate of sialylation of MHC class I lysates from two sets of discordant twins. Exit of MHC class II exit to the Golgi results

*FIGURE 1.* Binding of biotinylated MHC class II-specific peptides to the surface of B cells derived from matched diabetic and nondiabetic identical twins. A, Flow cytometric analysis of binding of the biotinylated HLA-DR-specific peptide HA307–319 to EBV-transformed B cells of three diabetic (solid traces) and nondiabetic (dashed traces) twin pairs (right column). Background fluorescence in the absence of biotinylated peptide was determined by incubation with FITC-conjugated streptavidin alone (left column). Fluorescein in the presence of biotinylated peptide was determined by incubation with both peptide and FITC-conjugated streptavidin (right column). B, Percent change in fluorescence intensity induced by the binding of the HLA-DR-specific peptides HA307–319 (left panel) and IgC37–51 (right panel) to B cell lines derived from three matched sets of diabetic (■) and nondiabetic (□) twins. Background fluorescence was subtracted from the data, which are presented as the mean ± SD of duplicate samples analyzed on the same day. The same experiment was performed on three separate occasions with similar results.

These data at this early time point of pulse-chase thus suggested that the MHC class II-Ii complexes of the diabetic twin had altered EndoH sensitivity and perhaps a reduced association with Iip35.

The MHC class II-Ii complexes of the diabetic cell line first exhibited substantial resistance to EndoH after 120 min of the chase incubation, with almost complete resistance apparent by 240 min (Fig. 2A). In contrast, such complexes of the nondiabetic cell line exhibited only partial resistance to EndoH and continued to show band heterogeneity at 240 min. The complexes from nondiabetic twin and control cells only exhibited total EndoH resistance at 360–480 and 600 min, respectively. All diabetic twin sets compared with their MHC nondisease cohorts (n = 3) exhibited similar MHC class II assembly defects in pulse-chase experiments (data not shown).

These results indicate the MHC class II-Ii complexes of diabetic B cells exit the ER more rapidly than do complexes from nondiabetic cells or control cells. This pulse-chase pattern of the diabetic cells was reminiscent of data previously obtained with cells expressing exclusively the p33 isoform of Ii and lacking Iip35 (7, 18).
in an increase in molecular mass when analyzed by SDS-PAGE. Published data show a reduced rate of MHC class I exit from the splenocytes isolated from spontaneously diabetic nonobese diabetic mice, but not unaffected murine cohorts (28). Diabetic B cells from two diabetic twins showed a 15- to 30-min slowing in the delivery of class I molecules into the trans-Golgi, as evidenced by a delay in achieving a higher molecular mass (data not shown). Nondiabetic twin B cell lines exhibited normal MHC class I exit times comparable to those of control cell lines. Opposing assembly rates of slowed MHC class I compared with accelerated MHC class II in only diabetic B cell lines confirmed that marked rapid MHC class II exit is a specific immune transport dysfunction.

Rapid transport of MHC class II in diabetic B cells

The stability of MHC class II-peptide complexes on the cell surface is an important determinant of the efficacy of Ag presentation to CD4+ T cells. We next examined the appearance and turnover of MHC class II at the surface of diabetic and nondiabetic twin B cell lines. Cells were pulse-labeled with [35S]methionine, incubated in the absence of [35S]methionine for various times, and, immediately before lysis, biotinylated with a membrane-impermeable reagent (NHS-SS-biotin) to allow detection of only those MHC class II molecules expressed on the cell surface. Cell lysates were then subjected to immunoprecipitation with Abs to MHC class II to reveal total pulse-labeled MHC class II molecules, and a portion of the resulting immunoprecipitates was subjected to further precipitation with streptavidin-agarose to reveal surface MHC class II. For the twin pair analyzed in Fig. 2B, MHC class II molecules began to appear on the surface of the diabetic B cells by 1 h into the chase incubation, with maximal surface expression apparent at 2 h. In contrast, the B cells from the paired nondiabetic twin and control B cells exhibited a minimal number of 35S-labeled MHC class II molecules on the surface at 2 h. The overall rate of MHC class II synthesis in the diabetic cell line appeared similar to that in the matched nondiabetic cell line.

The B cells from the diabetic twin also exhibited rapid turnover of surface MHC class II. At 3 h into the chase incubation, a time at which the surface expression of 35S-labeled MHC class II had not yet peaked in nondiabetic twin or control cells, the amount of newly synthesized MHC class II at the surface of the diabetic cells was already decreasing (Fig. 2B). This pattern of rapid delivery and turnover of MHC class II molecules at the cell surface was consistently apparent in all diabetic cells (compared with matched twin cells and random control cells) examined.

The turnover of MHC class II molecules at the cell surface was further examined with a different approach (26). The half-life of surface MHC class II complexes loaded with exogenous peptides was assessed to determine whether peptide loading of the relatively large proportion of peptide-empty class II molecules on the surface of diabetic B cell lines would affect the kinetics of internalization or alter the durability of internalized MHC class II complexes perhaps deficient in select chaperone proteins. Previous studies have shown that at saturating peptide concentrations peptide dissociation is extremely slow and nonstoichiometric. Monitoring the disappearance of labeled peptide MHC class II provides a measure of surface internalization and internal stability of the MHC class II-peptide complexes, since fluorescence can persist until endosomal degradation. Cells were exposed for 4 h to 50 μM of a biotinylated HLA-DQ-specific peptide (SGPLKAEIAQRLEY), after which the cells were washed and then incubated for various times in the presence of 500 μM unlabeled peptide. At time zero of the chase incubation the fluorescence intensity of diabetic B cells was greater than that of the matched nondiabetic B cells or control cells, reflecting a larger number of MHC class II molecules newly filled with the exogenous peptide (Fig. 2C). The subsequent rate of disappearance of biotinylated peptide from the cell surface or internally was markedly greater for diabetic B cells than for nondiabetic or control B cell lines. Similar results were obtained with the HLA-DR-specific peptide IgCk(27-51), and B cell lines derived from four different identical twin pairs discordant for type I diabetes (data not shown). The loading of the empty peptide

![Image 61x457 to 285x742](http://www.jimmunol.org/)

**FIGURE 2.** Kinetics of MHC class II exit from the ER as well as its appearance and turnover at the cell surface in B cells lines derived from diabetic and nondiabetic twins. A, Pulse-chase analysis of the exit of MHC class II-Ii complexes from the ER. EBV-immortalized B cells derived from matched diabetic (DM) and nondiabetic (T) identical twins were labeled with [35S]methionine for 30 min and then incubated for the indicated times in the absence of radioactivity. The cells were lysed and subjected to immunoprecipitation with MHC class II Abs, and the resulting precipitates were incubated in the absence or the presence of EndoH, as indicated, before analysis by SDS-PAGE and autoradiography. The position of the p35 isoform of Ii is indicated by an arrow, and those of maturing MHC class II α- and β-chains are indicated with a vertical line. B, Rates of synthesis and appearance at the cell surface of MHC class II molecules. B cell lines from matched diabetic and nondiabetic identical twins as well as control cells (Con) were labeled with [35S]methionine for 30 min and incubated for the indicated times thereafter in the absence of radioactivity. The cells were then exposed to NHS-SS-biotin, lysed, and subjected to immunoprecipitation with Abs to MHC class II. Twenty microliters of the resulting immunoprecipitates were used for total MHC class II analysis. The rest of sample was subjected to further precipitation with streptavidin-Sepharose to recover surface class II; both samples were then analyzed by SDS-PAGE and autoradiography. C, Turnover of MHC class II-peptide complexes at the cell surface. B cell lines derived from a matched set of diabetic (●) and nondiabetic (□) twins as well as from an MHC class II-matched HLA-DR control subject (▲) were incubated for 4 h at 37°C with 50 μM of the biotinylated peptide SGPLKAEIAQRLEY, after which the cells were washed and incubated for the indicated times in the presence of 500 μM unlabeled peptide. The cells were then subjected to flow cytometric analysis for quantitation of surface biotinylation. Data are the mean ± SD of triplicate determinations and are expressed as a percentage of the fluorescence intensity for diabetic cells at time zero.

Downloaded from www.jimmunol.org by guest on August 14, 2017
binding grooves of MHC class II molecules on the surface of diabetic B cells thus did not normalize the increased rate of surface MHC class II internalization or the internal stability apparent in these cells.

Delayed formation of stable MHC class II complexes in some diabetic B cells

The binding of antigenic peptides by MHC class II molecules confers stability to the class II αβ dimer, as revealed by failure of SDS to induce its dissociation at room temperature (29). The formation of SDS-resistant MHC class II complexes, a phenomenon called dimer formation, in B cell lines from diabetic and unaffected twins was investigated by labeling the cells for 90 min with 0.5 mCi of [35S]methionine, chasing them in cold medium for 4 and 15 h, and then subjecting cell lysates to immunoprecipitation with Abs to MHC class II. In the twin sets the resulting precipitates were dissolved in SDS sample buffer and either boiled or not before analysis by electrophoresis and autoradiography. For one twin set the abundance of MHC class II complexes resistant to SDS at 4 h was markedly greater in the nondiabetic B cells than in diabetic B cells (Fig. 3A). Although virtually all MHC class II α- and β-chains in nondiabetic cells were present in stable complexes at 15 h, the diabetic cells at this time still contained substantial amounts of MHC class II not in the form of stable complexes. In this assay of MHC class II stability there was variability in the discordant diabetic twin sets. As presented, one twin set was markedly discordant in dimer formation, two other twin sets were mildly discordant, and one twin set showed no differences between the paired twin sample sets, thus suggesting that this assay less reliability tracks a phenotype of interrupted MHC class II assembly and disease expression.

Reduced abundance of Ii p35 in diabetic B cells

The altered transport kinetics and peptide loading of MHC class II molecules detected in diabetic B cell lines were suggestive of a defect in the expression or function of the p35 isoform of Ii (7, 13). We therefore subjected B cells derived from matched sets of diabetic and nondiabetic twins to immunoblot analysis with Abs to the p35 or p41 isoforms of Ii or to the HC3 proteasome subunit, as indicated.

Correction of MHC class II defects in diabetic B cells by restoration of Ii p35 expression

To define the possible role of the functionally reduced expression of Ii p35 in the MHC class II defects of diabetic B cells, we subjected B cells derived from matched diabetic and nondiabetic twins to stable transfection with an Ii p35 vector. Transfection restored the abundance of Ii p35 in diabetic B cells to that apparent in untransfected nondiabetic cells (Fig. 4A); transfection of the nondiabetic cells had little effect on the overall abundance of Ii p35.

We next examined the occupancy of surface MHC class II molecules with endogenous peptides in the transfected diabetic and nondiabetic twin sets. Cells were thus incubated for 4 h at 4°C with the biotinylated HA307–319 peptide and then analyzed by flow cytometry. Transfection of the diabetic cells with the Ii p35 vector resulted in a decrease in the extent of surface binding of the exogenous peptide to a level similar to those apparent with untransfected nondiabetic cells (Fig. 4B). These results indicated that restoration of Ii p35 expression in the diabetic cells corrected the defect in the loading of MHC class II molecules with endogenous peptides.

The effects of restoration of Ii p35 expression in diabetic cells on the increased rates of appearance and subsequent turnover of newly synthesized MHC class II at the cell surface were also evaluated. The time course of the appearance of MHC class II molecules at the cell surface and their subsequent internalization in stably transfected Ii p35 diabetic B cells did not differ substantially from that observed in the corresponding untransfected or transfected nondiabetic cells (Fig. 4C). These results support the critical functional role of deficient Ii p35 in diabetic cells in conferring altered MHC class II assembly and correction of these intracellular defects with restored protein expression.
cells is a direct cause of disease or whether it represents a phenotype potentially useful for tracking disease expression. Each paired twin set of affected and unaffected twin blood cells was transformed to EBV cell lines on the same day, and many sets of these paired samples were prepared multiple times over the past 10 years. Therefore, it is unlikely that a random increase in the number of activated B cells at the time of transformation could account for the consistent diabetic twin EBV cell line errors repeatedly observed.

Although not reported in this study, we have also examined EBV cell lines from other discordant twin pairs with differential autoimmune disease expression. Both diseased twin EBV cell lines from a rheumatoid arthritis and multiple sclerosis patient compared with their unaffected twin similarly display accelerated MHC class II transport to the cell surface and reduced peptide-loaded surface MHC class II structures. These data are consistent with possible insufficiencies in lip35 expression as a contributor to the development of diverse forms of autoimmunity without this gene translation defect determining target organ selection.

The avidity of T cells for MHC class II-peptide complexes is thought to be a determinant of T cell selection (30). MHC class II molecules are usually retained in the ER through the formation of mixed Ii trimers that contain at least one Ii subunit with an ER retention signal. One consequence of such multimerization is that Ii p35, which contains an NH2-terminal ER retention signal (7, 8), exerts an effect that is disproportionate to its relative abundance (18). Studies with transfected human B cells have shown that the ER residence time of Ii p33 is <3 h, whereas that of Ii p35 is 6–9 h (18). Cells expressing only the p33 isoform of Ii also do not exhibit complex patterns of protein glycosylation (7). Furthermore, expression of Ii p35 at normal or increased levels in transfected cells promotes Ag presentation by MHC class II molecules by preventing excessive degradation of these molecules in endosomes-lysosomes and thereby presumably increasing the opportunity for their interaction with antigenic peptides (18).

In summary, these findings describe a new epigenetic influence of altered abundance of an important human-specific chaperone protein, lip35, that possibly explains the selective influence of at-risk MHC class II genes to confer altered Ag presentation in only diabetic twins with disease.

Acknowledgments
We thank E. Long (National Institutes of Health, Bethesda, MD), P. Cresswell (Yale University, New Haven, CT), R. Wank (University of Munich, Munich, Germany), and K. Guy (Medical Research Council Human Genetics Units, Edinburgh, U.K.) for reagents and cDNA constructs.

References
1. Cresswell, P. 1994. Assembly, transport, and function of MHC class II molecules. Annu. Rev. Immunol. 12:259.
2. Roche, P. A., M. S. Marks, and P. Cresswell. 1991. Formation of a nine-subunit complex by HLA class II glycoproteins and the invariant chain. Nature 354:392.
3. Lamb, C. A., and P. Cresswell. 1992. Assembly and transport properties of invariant chain trimers and HLA-DR invariant chain complexes. J. Immunol. 148: 2478.
4. Roche, P. A., and P. Cresswell. 1990. Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding. Nature 345:615.
5. Newcomb, J. R., and P. Cresswell. 1993. Characterization of endogenous peptides bound to purified HLA-DR molecules and their absence from invariant chain-associated dQ dimers. J. Immunol. 150:499.
6. Bakke, O., and B. Dobberstein. 1990. MHC Class II-associated invariant chain contains a sorting signal for endosomal compartments. Cell 63:707.
7. Lotteau, V., L. Teyton, A. Pelleux, T. Nilson, L. Karlsson, S. L. Schmid, V. Quaranta, and P. A. Peterson. 1990. Intracellular transport of class II MHC molecules directed by invariant chain. Nature 348:600.
8. Lamb, C. A., J. W. Yewdell, J. R. Bennink, and P. Cresswell. 1991. Invariant chain targets HLA class II molecules to acidic endosomes containing internalized influenza virus. Proc. Natl. Acad. Sci. USA 88:5998.
9. Sette, A., S. Southwood, J. Miller, and E. Appella. 1995. Binding of major histocompatibility complex class II to the invariant chain-derived peptide, CLIP, is regulated by allelic polymorphism in class II. J. Exp. Med. 181:677.

10. Buckner, J. W. W. Kwok, B. Nepom, and G. T. Nepom. 1996. Modulation of HLA-DQ binding properties by differences in class II dimer stability and pH-dependent peptide interactions. J. Immunol. 157:4940.

11. Strubin, M., E. O. Long, and B. Mach. 1986. Two forms of the Ia antigen-associated invariant chain result from alternative initiations at two in-phase AUGs. Cell 47:619.

12. Anderson, H. A., D. T. Bergstralh, T. Kawamura, A. Blauvelt, and P. A. Roche. 1999. Phosphorylation of the invariant chain by protein kinase C regulates MHC class II trafficking to antigen-processing compartments. J. Immunol. 163:5435.

13. Warmerdam, P. A., E. O. Long, and P. A. Roche. 1996. Isoforms of the invariant chain regulate transport of MHC class II molecules to antigen processing compartments. J. Cell Biol. 133:281.

14. Saudrais, C., D. Spehner, H. de la Salle, A. Bohbot, J. P. Cazenave, B. Goud, D. Hanau, and J. Salamero. 1998. Intracellular pathway for the generation of functional MHC class II peptide complexes in immature human dendritic cells. J. Immunol. 160:2597.

15. Roche, P. A., C. L. Teletski, E. Stang, O. Bakke, and E. O. Long. 1993. Cell surface HLA-DR-invariant chain complexes are targeted to endosomes by rapid internalization. Proc. Natl. Acad. Sci. USA 90:8581.

16. Wang, K., P. A. Peterson, and L. Karlsson. 1997. Decreased endosomal delivery of major histocompatibility complex class II-invariant chain complexes in dynamin-deficient cells. J. Biol. Chem. 272:17035.

17. Kuwana, T., P. A. Peterson, and L. Karlsson. 1998. Exit of major histocompatibility complex class II-invariant chain p35 complexes from the endoplasmic reticulum is modulated by phosphorylation. Proc. Natl. Acad. Sci. USA 95:1056.

18. Arunachalam, B., C. A. Lamb, and P. Cresswell. 1994. Transport properties of free and MHC class II-associated oligomers containing different isoforms of human invariant chain. Int. Immunol. 6:439.

19. Manoury, B., E. W. Hewitt, N. Morrice, P. M. Dando, A. J. Barrett, and C. Watts. 1998. An asparaginyl endopeptidase processes a microbial antigen for class II MHC presentation. Nature 396:695.

20. Bikoff, E. K., L. Y. Huang, V. Episkopou, J. van Meerwijk, R. N. Germain, and E. J. Robertson. 1993. Defective major histocompatibility complex class II assembly, transport, peptide acquisition, and CD4+ T cell selection in mice lacking invariant chain expression. J. Exp. Med. 177:1699.

21. Rowe, R. E., and R. D. Leslie. 1995. Twin studies in insulin dependent diabetes and other autoimmune diseases. Diabetes Metab. Rev. 11:121.

22. Busch, R., and J. B. Rothbard. 1990. Detection of peptide-MHC class II complexes on the surface of intact cells. J. Immunol. Methods 134:1.

23. Van Voorhis, W. C., J. Valinsky, E. Hoffman, J. Luban, L. S. Hair, and R. M. Steinman. 1983. Relative efficacy of human monocytes and dendritic cells as accessory cells for T cell replication. J. Exp. Med. 158:174.

24. Stebbins, C. C., M. E. Petersen, W. M. Suh, and A. J. Sant. 1996. DM-mediated release of a naturally occurring invariant chain degradation intermediate from MHC class II molecules. J. Immunol. 157:4892.

25. Harris, P. E., A. Maffei, A. I. Colovai, J. Kinne, S. Tugulea, and N. Suciu-Foca. 1996. Predominant HLA-class II bound self-peptides of a hematopoietic progenitor cell line are derived from intracellular proteins. Blood 87:5104.

26. Elliott, E. A., J. R. Drake, S. Amigorena, J. Elsemore, P. Webster, I. Mellman, and R. A. Flavell. 1994. The invariant chain is required for intracellular transport and function of major histocompatibility complex class II molecules. J. Exp. Med. 179:681.

27. Varki, A., W. Sherman, and S. Kornfeld. 1983. Demonstration of the enzymatic mechanisms of α-N-acetyl-α-glucosamine-1-phosphodiester α-N-acetylglucosaminidase (formerly called α-N-acetylglucosaminylphosphodiesterase) and lysosomal α-N-acetylglucosaminidase. Arch. Biochem. Biophys. 222:145.

28. Li, F., J. Guo, Y. Fu, G. Yan, and D. Faustman. 1994. Abnormal class I assembly and peptide presentation in the diabetic NOD mouse. Proc. Natl. Acad. Sci., USA 91:11128.

29. Sadegh-Nasseri, S., and R. N. Germain. 1991. A role for peptide in determining MHC class II structure. Nature 353:167.

30. Ashton-Rickardt, P. G., and S. Tonegawa. 1994. A differential-avidity model for T-cell selection. Immunol. Today 15:362.