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

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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 1 (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 nonamer 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,
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thus allowing the transport of (αββ)3 containing this isoform of li out of the ER (17, 18). The transport of MHC class II-li nonamers containing exclusively li 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-li complexes also differs between those containing only li p33 and those containing at least one molecule of li p35 (12, 17). The ER retention signal of li 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 li 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 li 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 li, is faster in cells expressing exclusively the p33 isoform of li, and is faster still in cells lacking both li 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 twin pairs discordant for autoimmune type 1 diabetes. 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 li. Stable transfection of cells from diabetic twins with a vector encoding li p35 corrected these defects in the assembly of and peptide presentation by MHC class II molecules. The reduced production of a chaperone protein (li 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 from the same family, all twin sets were sex-matched and the twin sets were typed for HLA class II loci that are clustered into three major subregions: HLA-DR, -DQ, and -DP. The anti-DR antibody 2H7 (American Type Culture Collection), 9.3F10 (Coulter) recognizes all HLA-DR, -DP, and -DQ; L243 recognizes predominantly DR molecules, 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 × 105 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 (5000 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 li (American Type Culture Collection), 9.3F10 (Coulter) recognizes all HLA class II proteins (DR, DP, and DQ), I-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 DRβ and DR dimers. mAb PIN.1 was generated in response to a peptide corresponding to aa 12–28 of the p33 isoform of human li, but detects the cytoplasmic NH2-terminal regions of both li p33 and li p35. The mAb CerCLIP.1 recognizes human CLIP in association with MHC class II. Rabbit polyclonal Abs to li p35 (R.Ip35N) and R.Ip41 (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 × 10^6) 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 × 10^6) were labeled with 2 µCi 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 6.5), 0.5% SDS, and 0.1% NaDodSO4) by heating at 100°C for 5 min, and the eluted proteins were then incubated for 16 h at 37°C in the absence or the presence of 2 µl of EndoH. The reaction mixtures were then analyzed by SDS-PAGE on a 12.5% gel and autoradiography. For detection of stable MHC class II complexes, the immunoprecipitates prepared with mAb 9.3F10 or DA6.147 were dissolved in SDS sample buffer (100 mM Tris-HCl (pH 7.4), 50% sucrose, 10% glycerol, 0.5% sodium dodecyl sulfate, 1.5% 2-mercaptoethanol, and 0.001% bromophenol blue) in a volume of 100 µl containing 30 µg of pBabe-II 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.

Immunoblot analysis

Cells (2 × 10^6) 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 DNA (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 × 10^5) were incubated for 5 min on ice in a volume of 100 µl containing 30 µg of pBabe/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

<table>
<thead>
<tr>
<th></th>
<th>Ab</th>
<th>Diabetic</th>
<th>Nondiabetic</th>
<th>p Value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Control</th>
<th>p Value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-class II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L243</td>
<td>1.23 (n = 12)</td>
<td>17.02 ± 0.32</td>
<td>17.02 ± 0.18</td>
<td>0.915</td>
<td>17.05 ± 0.05</td>
<td>1.000</td>
</tr>
<tr>
<td>I-2</td>
<td>1.2 (n = 12)</td>
<td>15.71 ± 0.86</td>
<td>16.09 ± 1.09</td>
<td>0.079</td>
<td>16.25 ± 0.35</td>
<td>0.967</td>
</tr>
<tr>
<td>9.3F10</td>
<td>1.2 (n = 12)</td>
<td>25.2 ± 0.78</td>
<td>20.3 ± 0.825</td>
<td>0.001</td>
<td>21.89 ± 0.18</td>
<td>0.009</td>
</tr>
<tr>
<td>I-3</td>
<td>1.2 (n = 12)</td>
<td>12.30 ± 1.30</td>
<td>13.40 ± 0.66</td>
<td>0.470</td>
<td>12.50 ± 0.01</td>
<td>0.100</td>
</tr>
<tr>
<td>Anti-CLIP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CerCLIP</td>
<td>1 (n = 8)</td>
<td>6.80 ± 2.20</td>
<td>8.80 ± 3.20</td>
<td>0.062</td>
<td>7.10 ± 1.5</td>
<td>0.060</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data are the means ± SD for the indicated number (n) of cell lines examined.

<sup>b</sup> Comparison by Student's t test between diabetic and nondiabetic twins.

<sup>c</sup> 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.1. 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 antigentic peptides (HA307–319 and IgCβ2–35) 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 IgCβ2–35 (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 indicated that the protein was still located in the ER at the time of sampling.

Pulse-chase data for B cells derived from one discordant twin pair are shown in Fig. 2A (similar results were obtained with cells from three different twin sets). At 0 or 30 min into the chase incubation, the MHC class II-Ii complexes in cells from both the diabetic and nondiabetic twins exhibited EndoH sensitivity, indicating that they had not yet traversed the Golgi complex. However, at time zero of the chase, MHC class II molecular complexes of the diabetic twin, compared with those of the nondiabetic twin, appeared to be associated to a reduced extent with the p35 isoform of Ii (Fig. 2A, arrow). Furthermore, after EndoH treatment at this time point, MHC class II-Ii from the diabetic twin, compared with that from the nondiabetic twin, exhibited a reduced extent of band heterogeneity. This reduced electrophoresis heterogeneity was reflective of the results of published pulse-chase studies of MHC class II in transfected cells lacking Ii(p35, but expressing Ii(p33 (7, 18).

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 Ii(p35.

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 Ii(p35 (7).

As a control protein assembled in the ER, the rate of intracellular transport of MHC class I to the Golgi was estimated by the rate of sialylation of MHC class I lysates from two sets of discordant identical twins. Exit of MHC class I exit to the Golgi results...
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 nondiabetic 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 IgCκ residues 47–59, 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
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. 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 Ii p35. The amount of Ii p35 in each diabetic cell line was reduced by 30–60% compared with that in the cell line derived from the corresponding nondiabetic twin (Fig. 3B). In contrast, the abundance of the p41 and p33 isoforms of Ii in B cell lines did not differ between diabetic and nondiabetic twins. Furthermore, as a control protein, the amounts of the proteasome subunit HC3 were similar in the diabetic and nondiabetic twin cells. Given that both p35 and p33 isoforms of Ii are produced from the same mRNA, these data suggested that translation of the p35 isoform is specifically reduced in diabetic B cells; alternatively, the degradation of Ii p35 may be increased in diabetic B cells. The unlikely possibility that the Ii gene of the diabetic twins contained a somatic mutation was eliminated by sequencing of the promoter, early introns, and coding regions of this gene from all four twin pairs studied and from one random control individual. The gene sequences were identical for all individuals (data not shown) and were in complete agreement with the previously determined sequence.

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 cells. Cells were thus incubated for 4 h at 4°C with the biotinylated HA107–319 peptide and then analyzed by flow cytochemistry. 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 or transfected 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 twin 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.
and of their subsequent turnover. Transfected and untransfected cells were
of arrival of newly synthesized MHC class II molecules at the cell surface
in Fig. 1
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abnormal MHC class II processing apparent in human diabetic
impair interaction of APC with CD4
molecules at the cell surface of diabetic cells might be expected to
reduced functional abundance of the p35 isoform of Ii, a human-
coding Ii p35.
twins were reversible by stable transfection with a vector en-
and the transport of these molecules from the ER to endosomes-
somes-lysosomes and thereby presumably increasing the opportu-
rate delivery of newly synthesized MHC class II proteins to the
MHC class II molecules that were devoid of endogenous peptides,
the ER residence time of Ii p33 is 3 h, whereas that of Ii p35 is 6–9
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 endo-
somes-lysosomes and thereby presumably increasing the opportu-
nity 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, Ii3p5, that possibly explains the selective influence of at-
risk MHC class II genes to confer altered Ag presentation in only
diabetic twins with disease.

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Discussion
We have characterized biochemically MHC class II processing in
EBV-immortalized B cell lines derived from human identical
twins discordant for type 1 diabetes. Our study design thus cir-
cumvents the possible confounding effects of differences in MHC
class II alleles between individuals and allows the detection of
changes in class II function associated with disease. The B cells
from diabetic twins exhibited an increased proportion of surface
MHC class II molecules that were devoid of endogenous peptides,
A reduced surface density of MHC class II molecules loaded with antigenic peptides and increased rate of turnover of these
molecules at the cell surface of diabetic cells might be expected to
impair interaction of APC with CD4+ T cells and thereby influ-
ence T cell education. It remains to be determined whether the
abnormal MHC class II processing apparent in human diabetic
cells is a direct cause of disease or whether it represents a pheno-
type potentially useful for tracking disease expression. Each paired
twin set of affected and nonaffected twin blood cells was trans-
formed 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 num-
ber 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 com-
pared 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 Ii3p5 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 endo-
somes-lysosomes and thereby presumably increasing the opportu-
nity for their interaction with antigenic peptides (18).

FIGURE 4. Effects of stable transfection of B cell lines derived from
diabetic and nondiabetic twins with an Ii p35 vector. A, Immunoblot anal-
ysis of Ii p35 abundance in paired diabetic and nondiabetic twin B cells
before (DM and T, respectively) and after (DMp35 and Tp35) stable trans-
fection with an Ii p35 vector. Control B cells were also analyzed, and all
cell lysates were also probed with control Abs to HC3. B, Binding of
biotinylated peptide HA307–319 to the surface of transfected and untrans-
fected cells. Cells were incubated for 4 h at 4°C with the labeled peptide
and then were analyzed by flow cytometry for peptide binding as described
in Fig. 1A. Background fluorescence for all cell lines was <4%. C, Rates
of arrival of newly synthesized MHC class II molecules at the cell surface
and of their subsequent turnover. Transfected and untransfected cells
were analyzed as described in Fig. 2B.


