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Immunology

Truncation of the class II β-chain cytoplasmic domain influences the level of class II/invariant chain-derived peptide complexes

(major histocompatibility complex/antigen presentation)

STEPHEN T. SMILEY*, ALEXANDER Y. RUDENSKY†, LAURIE H. GLIMCHER*‡, AND MICHAEL J. GRUSBY*‡§

*Department of Cancer Biology, Harvard School of Public Health, Boston, MA 02115; †Department of Immunology, Howard Hughes Medical Institute, University of Washington, Seattle, WA 98195; and ‡Department of Medicine, Harvard Medical School, Boston, MA 02115

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ABSTRACT **Previous studies have established that antigen presenting cells (APC) expressing major histocompatibility complex class II β chains with truncated cytoplasmic domains are impaired in their capacity to activate T cells. While it had been widely accepted that this impairment is due to a defect in class II cytoplasmic domain-dependent signal transduction, we recently generated transgenic mice expressing only truncated class II β chains, and functional analyses of APC from these mice revealed signaling-independent defects in antigen presentation. Here, we demonstrate that T cells primed on such transgenic APC respond better to stimulation by APC expressing truncated β chains than by wild-type APC. This finding suggests that APC expressing truncated class II β chains are not inherently defective in their antigen presenting capacity but, rather, may differ from wild-type APC in the peptide antigens that they present. Indeed, analysis of the peptides bound to class II molecules isolated from normal and transgenic spleen cells revealed clear differences. Most notably, the level of class II-associated invariant chain-derived peptides (CLIP) is significantly reduced in cells expressing only truncated β chains. Prior studies have established that CLIP and antigenic peptides compete for binding to class II molecules. Thus, our results suggest that the cytoplasmic domain of the class II β chain affects antigen presentation by influencing the level of CLIP/class II complexes.**

Major histocompatibility complex class II molecules are α/β chain heterodimers that function in the binding and presentation of antigenic peptides to $CD4^+$ T cells. By analyzing cell lines transfected with truncated class II β chains, previous studies have established that the β-chain cytoplasmic domain functions in the process of antigen presentation. For example, T-cell hybridomas respond poorly to such antigen presenting cells (APC) (1–6), and tumor cells transfected with full-length, but not truncated, class II molecules are rejected upon injection into mice (7, 8). In addition, B-cell differentiation can be specifically induced after transfection of full-length, but not truncated, class II β chains (9). Mechanistic studies have generally evaluated the signaling capacity of the β-chain cytoplasmic domain and have concluded that truncated class II molecules fail to induce the production of cAMP (2–5, 9).

Recently, we generated transgenic mice that express only class II molecules lacking most of the β-chain cytoplasmic domain (10). Consistent with previous studies of cell lines transfected with similarly truncated class II molecules (1–6), APC from mice expressing only truncated class II β chains are impaired in their capacity to present antigen (10). However, in contrast to those studies, the antigen presenting defect of physiological APC expressing only truncated class II molecules did not appear to correlate with impaired class II-mediated signal transduction. Here, we examine an alternative explanation for the differential antigen presenting capacities of truncated and full-length class II molecules—namely, that such molecules may differ with regard to the peptides they present.

Class II molecules acquire antigenic peptides during their biosynthesis. Upon cotranslational translocation of the class II α and β chains into the endoplasmic reticulum lumen, they associate with a third molecule, the invariant chain (Ii) (11, 12), which aids in the formation of the α/β heterodimers (13, 14) and also prevents class II molecules from prematurely binding peptides present in the endoplasmic reticulum (15, 16). In addition, the cytoplasmic domain of Ii contains a sequence that directs Ii/class II complexes away from the default secretory pathway and into organelles of the endocytic pathway (17–20). There, class II molecules accumulate in recently characterized acidic, lysozome-like organelles (MIIC and CIIV), and Ii is proteolyzed—thus enabling class II molecules to bind peptide fragments of endocytosed antigens (21–27). Mature class II/peptide complexes are then transported to the cell surface.

The sequence of Ii that interacts with class II molecules has been mapped (28) and corresponds to that of the class II-associated Ii-derived peptides (CLIP) frequently isolated from purified α/β heterodimers (29–31). Class II molecules cannot simultaneously bind CLIP and antigenic peptides (32–34). Indeed, mutant APC have been described in which surface class II molecules lack antigenic peptides and, instead, remain largely CLIP-associated (35–37). The genetic defects of these APC have been mapped to the HLA-DM genes (38–40), which encode class II-like molecules that also localize to MIIC/CIIV (41) and apparently function in the removal of CLIP from nascent class II heterodimers. These DM mutant APC present native protein antigens poorly (42–44), establishing an important role for CLIP removal in antigen presentation.

Here, we demonstrate that the number of CLIP/class II complexes is markedly reduced in cells expressing only truncated class II β chains. The reduced CLIP levels correlate with an altered capacity to present antigens, thus providing further evidence that CLIP levels regulate antigen presentation and indicating a role for the cytoplasmic domain of the class II β chain in the exchange of CLIP for antigenic peptides.

MATERIALS AND METHODS

Mice. The generation of the mice used has been described (10). Briefly, C57BL/6 mice expressing a truncated I-A β-chain transgene ($A_{\beta tr}$) were crossed to class II-deficient $A_\beta^{-/-}$ mice (45) that had been C57BL/6-back-crossed six generations. The nomenclature and genotypes of the mice used

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Abbreviations: APC, antigen presenting cells; wt, wild-type; tg, transgenic; CLIP, class II-associated invariant chain-derived peptides; Ii, invariant chain; A_β, I-A β-chain; A_{Btr}, truncated I-A β-chain transgene; het, $A_\beta^{+/-}$; wt, $A_\beta^{+/+}$; null-tg, $A_\beta^{-/-}A_{\beta tr}^{+}$; het-tg, $A_\beta^{+/-}A_{Btr}^{+}$; mAb, monoclonal antibody; BIns, bovine insulin.

§To whom reprint requests should be sent at the * address.

in this study are as follows: null, $A_\beta^{-/-}$; het, $A_\beta^{+/-}$; wt, $A_\beta^{+/+}$; null-tg, $A_\beta^{-/-}A_{\beta tr}^{+}$; het-tg, $A_\beta^{+/-}A_{\beta tr}^{+}$.

Peptide Analyses. A^b class II molecules were purified from splenic cells of 400 wt or 150 null-tg mice. Cells were lysed, and proteins were purified as described (46, 47). Briefly, cell pellets were homogenized; isolated membranes were solubilized with Nonidet P-40 and applied to a series of five columns containing 3 ml each of protein A-agarose, protein A-agarose, mouse IgG2a-conjugated protein A-agarose, Y3 (anti-K^b)-conjugated protein A-agarose and Y3P (anti-A^b)-conjugated protein A-agarose. The columns were then separately washed, and bound protein eluted with 50 mM glycine (pH 11.5)/0.1% deoxycholate, neutralized with 2 M glycine (pH 2), dialyzed against 10 mM Tris (pH 8)/0.1% deoxycholate, concentrated (Centriplus 30, Amicon) and analyzed by SDS/PAGE. Class II molecules were quantitated by comparison to Bio-Rad molecular weight standards and concentrated in a Centricon 10 (Amicon). Bound peptides were eluted with 10% acetic acid and heating at 70°C for 15 min, collected in the Centricon 10 flow-through, concentrated by vacuum centrifugation, and analyzed. Reverse-phase HPLC and peptide sequencing analyses were conducted by the Harvard Microchemistry Facility as described (47).

Flow Cytometry. Splenic cells were sequentially treated with Fc receptor-blocking monoclonal antibody (mAb) 24G2 (Pharmingen), followed by phycoerythrin-conjugated CD45 receptor-specific mAb B220 (Pharmingen) in conjunction with fluorescein isothiocyanate-conjugated A^b-specific mAb AF6120.1 (Pharmingen), or biotin-conjugated A^b/CLIP-specific mAb 30-2 (A.Y.R., unpublished work) and then phycoerythrin-conjugated CD45 receptor-specific mAb B220 in conjunction with fluorescein isothiocyanate-conjugated streptavidin (GIBCO/BRL). After being washed, cells were analyzed on a Beckton Dickinson FACScan. Viable cells were gated by propidium iodide staining. B cells were gated by forward/side light scatter and CD45 receptor staining.

RESULTS AND DISCUSSION

To define the importance of the β-chain cytoplasmic domain in antigen presentation by physiological APC, we recently generated $A_\beta^{-/-}A_{\beta tr}^{+}$ transgenic mice (designated null-tg) which express only truncated class II molecules (10). As shown in Fig. 1, T cells from bovine insulin (BIns)-immunized $A_\beta^{+/+}$ wild-type (wt) mice respond better when stimulated *in vitro* with wt APC than with null-tg APC. Strikingly, however, T cells from null-tg mice respond poorly when stimulated with wt APC, although their response to null-tg APC is comparable to the response of wt T cells to wt APC (Fig. 1 and data not shown). Thus, wt and null-tg T cells each respond preferentially to APC expressing the same type of class II molecules they were initially primed upon. These results suggest that APC expressing truncated class II β chains are not inherently defective in their antigen presenting capacity but, rather, may differ from wild-type APC in the peptide antigens that they present.

To directly address this, class II molecules were purified from wt or null-tg splenic cells, and bound endogenous peptides were acid-extracted and analyzed. HPLC chromatographs of the low-molecular-weight material isolated from full-length and truncated class II molecules are shown in Fig. 2. Clear differences in the chromatographs are evident, indicating that peptide acquisition is affected by the β-chain cytoplasmic domain. Sequence analysis established that the major class II-specific peak contained amino acids 85–100 of mouse Ii (peak 2), which corresponds to the proteolytic fragment of Ii designated CLIP. Notably, CLIP was significantly under-represented in the material isolated from truncated class II molecules.

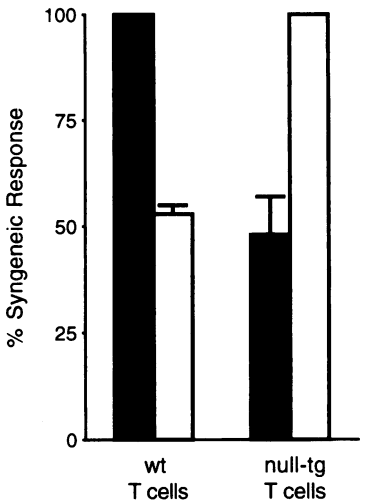

FIG. 1. Response of purified, *in vivo*-primed T cells to APC expressing full-length or truncated class II molecules. wt or null-tg mice were immunized with 1 μg of BIns in complete Freund's adjuvant. Eight days later lymph node T cells were purified, and 1×10^5 T cells were restimulated *in vitro* with BIns at 250 μg/ml and 5×10^5 irradiated splenic cells from naive animals: wt APC, solid bars; null-tg APC, open bars. Proliferation was determined by [^{3}H]thymidine incorporation after 72 hr of culture. Results are presented as the percentage of response (+SE) to syngeneic APC, as derived from four independent experiments. As shown, preferential stimulation by syngeneic APC is observed when mice are immunized with 1 μg of BIns. Actual values for syngeneic responses during these low-dose immunizations ranged from 1000 to 9000 cpm, and responses in the absence of BIns or irradiated APC were <10% of those obtained in their presence. If larger dosages of immunogen are used, higher cpm values are obtained, but preferential stimulation by syngeneic APC is no longer observed.

Using a mAb that specifically recognizes CLIP/class II complexes (A.Y.R., unpublished work), we directly analyzed their surface expression. As shown in Fig. 3, null cells ($A_\beta^{-/-}$),

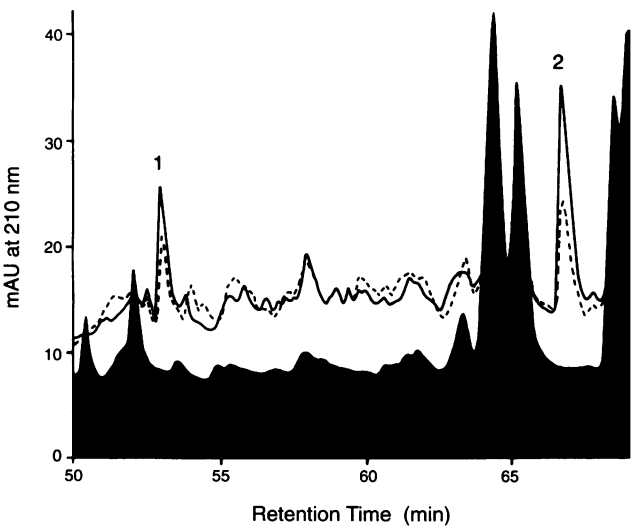

FIG. 2. Peptides eluted from full-length and truncated class II molecules. HPLC chromatographs of material isolated from affinity-purified class II molecules of wt (solid) or null-tg (dashed) splenic cells. A chromatograph of nonspecific material isolated from a mock affinity column containing an isotype-matched control antibody is shown in black. Peptide sequence analyses established that peaks 1 and 2 contain amino acids 375–391 of mouse immunoglobulin μ heavy chain (LPQEKYVTSAPMPEPGA) and amino acids 85–100 of mouse Ii (KPVSQMRMATPLLMRP), respectively. mAU, milli-absorbance units.

het cells ($A_\beta^{+/-}$), and wt cells ($A_\beta^{+/+}$) each express surface levels of CLIP/class II complexes that correlate with their expression of class II molecules. However, CLIP staining on null-tg cells was lower than that on wt cells, despite the significantly higher levels of class II expression on null-tg cells. Thus, these flow cytometric analyses confirm that CLIP/class II complexes are under-represented in cells expressing only truncated class II molecules. As CLIP/class II complexes are normal intermediates in the formation of peptide antigen/class II complexes (40, 48), these data suggest an over-exchange of CLIP for other peptides in null-tg cells. Thus, both CLIP levels and peptide acquisition are affected by truncation of the β-chain cytoplasmic domain.

The cytoplasmic domain of the β chain could influence peptide acquisition by directing the trafficking of class II molecules through organelles where CLIP and antigen are exchanged. Indeed, the deleted portion of the truncated β chain contains a dileucine motif analogous to those implicated in the endosomal trafficking of a variety of molecules (49–52). Impaired trafficking of truncated class II molecules might cause over-sampling of peptides present in some organelles and under-sampling in others, thereby affecting the levels and/or types of peptides presented by null-tg APC. Pulse-chase immunoprecipitation analyses have not revealed any gross differences in the biosynthesis of full-length and truncated class II molecules with regard to their rates of passage through the endoplasmic reticulum to the Golgi apparatus, nor in their rates of dissociation from the p31 and p41 forms of Ii, nor in their rate of peptide acquisition (data not shown). However, altered class II transport through peptide-loading compartments would only be evident in such kinetic studies if this represents a rate-limiting step in class II biosynthesis. Moreover, ultrastructural studies have revealed the class II-rich MIIC/CIIV compartments to be multilamellar organelles (21, 23, 27). Thus, the β-chain cytoplasmic domain could influence trafficking between specific regions of these structures. Immunoelectronmicroscopic and recently described cell-fractionation techniques should allow more detailed studies of the subcellular distribution of truncated and full-length class II molecules.

Analyses of cells from het-tg mice ($A_\beta^{+/-}A_{\beta tr}^{+}$), which express a mixture of full-length and truncated class II molecules, provide further evidence that the β-chain cytoplasmic domain affects antigen presentation via its influence on CLIP levels. CLIP levels on het-tg cells were not diminished but rather correlated with surface class II levels (Fig. 3) and, in contrast to null-tg APC, het-tg APC present antigens well to wt T cells (10). Thus, coexpression of full-length class II molecules rescues both the CLIP deficiency and the altered antigen-presenting capacity of truncated class II molecules.

That the coexpression of full-length and truncated β chains in het-tg cells leads to increased levels of CLIP/class II complexes relative to null-tg cells expressing only truncated molecules suggests that the β-chain cytoplasmic domain influences the trafficking of class II molecules while they are part of larger, multimeric class II complexes. Indeed, multimeric class II complexes have been identified both during class II biosynthesis and after surface expression (53, 54). In het-tg cells, multimeric complexes containing a mixture of both truncated and full-length class II molecules might traffic normally, as the presence of a single β-chain cytoplasmic domain in a complex could direct appropriate transport of any associated truncated molecules.

Previous reports have implicated defective class II-mediated signal transduction in the impaired function of APC expressing truncated class II β chains (1–5, 9). However, null-tg APC remain deficient in their capacity to present peptides even after chemical fixation (10) and are not impaired in their capacity to activate null-tg T cells (Fig. 1 and ref. 10), suggesting that alternative mechanisms also exist. Indeed, our findings clearly demonstrate that the altered antigen presenting capacity of null-tg APC correlates with the presence of reduced levels of CLIP/class II complexes in these cells. As CLIP and antigenic peptides bind to class II molecules in a mutually exclusive manner, these results suggest that the cytoplasmic domain of the class II β chain affects antigen presentation by influencing the level of CLIP/class II complexes.

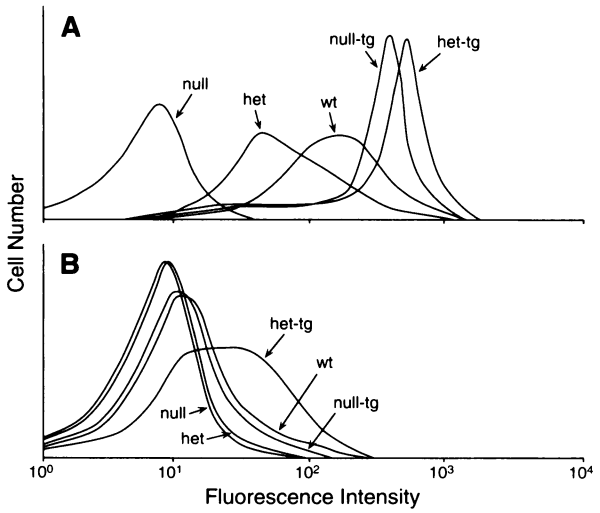

FIG. 3. Surface expression of class II/CLIP complexes on B cells expressing full-length or truncated class II β chains. Splenic B cells were analyzed by flow cytometry for surface expression of total class II (*A*) or class II/CLIP (*B*) complexes. Null, class II deficient $A_\beta^{-/-}$; het, $A_\beta^{+/-}$; wt, $A_\beta^{+/+}$; null-tg, $A_\beta^{-/-}A_{\beta tr}^{+}$; het-tg, $A_\beta^{+/-}A_{\beta tr}^{+}$.

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