Developmental Extinction of Major Histocompatibility Complex Class II Gene Expression in Plasmocytes Is Mediated by Silencing of the Transactivator Gene CIITA

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Summary

Constitutive major histocompatibility complex (MHC) class II gene expression is tightly restricted to antigen presenting cells and is under developmental control. Cells of the B cell lineage acquire the capacity to express MHC class II genes early during ontogeny and lose this property during terminal differentiation into plasma cells. Cell fusion experiments have suggested that the extinction of MHC class II expression in plasma cells is due to a dominant repression, but the underlying mechanisms are not understood. CIITA was recently identified as an MHC class II transactivator that is essential for MHC class II expression in B lymphocytes. We show here that inactivation of MHC class II genes in plasmocytes is associated with silencing of the CIITA gene. Moreover, experimentally induced expression of CIITA in plasmocytes leads to reexpression of MHC class II molecules to the same level as that observed on B lymphocytes. We therefore conclude that the loss of MHC class II expression observed upon terminal differentiation of B lymphocytes into plasmocytes results from silencing of the transactivator gene CIITA.

MHC class II molecules play a fundamental role as restriction molecules for antigen presentation to the TCR of CD4+ cells (1, 2). Surface expression of MHC class II products is restricted to APCs including B lymphocytes, macrophages, dendritic cells, thymic epithelium, and activated T cells (3-5). MHC class II expression is regulated throughout B cell development. Class II genes are silent in murine pro-B cells (6-9) and subsequently turned on in pre-B cells (8, 9). In humans, the expression of MHC class II molecules coincides with some of the earliest determinants of B cells development, such as CD10 and CD34 (10, 11). The capacity to express class II molecules is maintained throughout the life of B lymphocytes until their terminal differentiation into plasma cells, when class II genes are shut off (12).

MHC class II expression on B lymphocytes is functionally relevant for an efficient humoral response against T cell-dependent antigens (13). Antigens in their native conformation are captured by surface Ig molecules, internalized, processed, and presented to T cells in a MHC-restricted fashion (1, 2). Although constitutive expression of class II genes is restricted to professional APCs, many cell types can be induced to express these molecules by external stimuli, in particular by IFN-γ and IL-4 (4, 14-18). Constitutive as well as inducible expression of MHC class II genes is controlled via cis-acting DNA sequences that are present in all MHC class II promoters (19, 20). These cis-acting sequences are known as the class II consensus boxes: W, X1, X2, and Y.

Several proteins that bind to these sequences have been identified and some of them cloned. In addition, a non-DNA-binding MHC class II transactivator (CIITA)1 protein has been cloned by genetic complementation of a MHC class II regulatory mutant (21). CIITA is the first gene shown to be affected and mutated in primary MHC class II deficiency, and expression of CIITA is absolutely required for MHC class II gene expression in B lymphocytes (21). The tissue distribution of CIITA mRNA correlates tightly with that of MHC class II genes. CIITA gene activation is also involved in MHC class II induction by IFN-γ and TNF-α (21a, and Silacci, P., A. Mottet, V. Steimle, and B. Mach, manuscript in preparation).

Although both constitutive and inducible expression of MHC class II genes have been studied intensively, little is known about the extinction of MHC class II gene expression during differentiation of mature B cells into plasma cells. Previous work (22, 23) involving cell fusion experiments suggested that the lack of MHC class II gene expression in plasmocytes is due to a dominant repression mechanism. It is also known that class II promoters are unoccupied in vivo class II-negative murine plasmacytoma cells (24).

1 Abbreviations used in this paper: CAT, chloramphenicol acetyl transferase; CIITA, class II transactivator; TBP, TATA-binding protein; X2BP, X2 binding protein.
An important question is whether this developmental extinction is operating directly at the level of MHC class II gene transcription or rather at the level of the regulation of a positive trans-acting factor involved in class II gene regulation. Results presented here show that repression of MHC class II expression in plasma cells correlates with the loss of expression of the MHC class II transactivator CIITA. More importantly, experimentally induced expression of CIITA in both human and mouse plasmocytes restores MHC class II transcription to levels comparable to that observed in mature B cells. We conclude therefore, that the extinction of MHC class II expression observed in the terminal differentiation of B lymphocytes into plasmocytes results from silencing of the MHC class II transactivator gene CIITA.

Materials and Methods

Plasmids Construction and Oligonucleotides. The chloramphenicol acetyl transferase (CAT) expression vectors used in this study were derived from pOCAT (25) and pBLCAT3 (26). A HaelIII-SacI fragment containing the ~150 to +6 bp of the Dra I promoter was inserted upstream of the CAT gene into pOCAT, to obtain the pDRACAT plasmid. The SV40 early promoter was obtained by a SalI-BamHI digestion of pSG5 (Stratagene, La Jolla, CA) and inserted in pBLCAT3.

The riboprobes used to detect HLA-DRA, CIITA, and TATA-binding proteins (TBP) mRNA have all been described previously (21, 27). The plasmids used for the transfection were EBO-Sf/CIITA (21) and EBO-76PL, which was generated from EBO-Sf (21) by deleting the Sf-CATSf cassette. The WXY oligonucleotide is the HindIII-BglII fragment derived from pDRsyn (28). The WX2 oligonucleotide has been described previously (29). Cell Culture and Transfections. The B lymphoma cell lines RAJI and RJ2.2.5 were grown in RPMI-1640 medium supplemented with 2 mM l-glutamine, 10% FCS, 10 U/ml penicillin, and 10 μs/ml streptomycin. The SKO human myeloma cell line (ATCC #98133, American Type Culture Collection, Rockville, MD) was maintained constant, 400 μs/ml hygromycin (Calbiochem, San Diego, CA). The antibodies used were 2.06 (30) specific for HLA-DR, Tu22 (31) for HLA-DQ, B7/21 (32) for DP, and w6/32 for MHC class I. Cells were incubated with the antibodies in a reaction mixture containing 1x PBS, 10% nonstimulated rabbit serum (NRS), and 1% BSA for 30 min on ice. The cells were then washed and incubated with FITC-labeled anti-mouse IgG (Serotec Ltd., Oxford, UK) for 15 min on ice and subsequently washed twice before the FACScan analysis.

CAT Assays. Half of the harvested cells were used for placental alkaline phosphatase (PAP) assay (33) and the rest were used for CAT assay essentially as previously described (34). The results were quantified by excision of the acetylated and nonacetylated 14C-chloramphenicol forms from the chromatograms and direct scintillation counting. The results obtained were corrected for transfection efficiency on the basis of the PAP assay. The actual percent acetylation obtained with the SV40 early promoter was 83.2% (Raji cells) and 79.0% (SKO cells) for 30 μs μs of protein. All results with the Dra promoter are expressed as percent activity obtained with the SV40 promoter.

In Vivo Footprint Analysis, Nuclear Extract Preparation, and Gel Retardation Assays. In vivo footprint analyses were performed as previously described (35), using Vent DNA Polymerase (New England Biolabs, Beverly, MA) for all the steps of the ligation-mediated PCR reaction (LMPCR). The sequences of the oligonucleotides used and the incubation temperatures for LMPCR reactions were the same as those used by Kara and Glimcher (36).

Nuclear extracts from RAJI and SKO cell lines were prepared from 1.5-2 x 10^6 cells as described previously (37).

For electrophoretic mobility shift assays, binding was performed in a final volume of 20 μI containing 12% glycerol, 12 mM Hepes, pH 7.9, 60 mM KCl, 5 mM MgCl_2, 0.12 mM EDTA, 0.3 mM PMSF, 0.3 mM dithiothreitol, 1 μI poly(dI-dC)-poly(dI-dC), ~20,000 cpm 32P-labeled probe, and 8 μg nuclear protein. Competitors were added at the same time as the labeled probe. Reaction mixtures were incubated for 30 min on ice or at 20°C and the DNA-protein complexes were resolved on a 4% polyacrylamide 0.25x Tris-borate/EDTA (TBE) gels (1x TBE = 89 mM Tris, 89 mM boric acid, and 2 mM EDTA).

RNSase Protection Assay. Total RNA was extracted from frozen cell pellets by using guanidium isothiocyanate (38). The plasmids containing CIITA and TBP cDNA fragments were linearized and transcribed with T3 RNA polymerase and SP6 polymerase, respectively (Boehringer Mannheim, Mannheim, Germany) in order to synthesize the 32P-labeled riboprobes complementary to CIITA and TBP mRNAs. Reactions were performed at conditions described (18). Hybridization of 2 x 10^6 cpm of the riboprobe with 5 or 10 μg total RNA was done in 80% formamide, 40 mM Pipes, pH 6.4, 0.4 M NaCl, and 1 mM EDTA at 50°C overnight. The samples were then digested in a volume of 300 μI, with 10 μg RNase A and 1 U RNase T1 at 25°C for 30 min. After subsequent treatment with 50 μg proteinase K in the presence of 1% SDS for 15 min at 37°C, the protected RNA fragments were extracted twice with phenol-chloroform, ethanol precipitated, and resolved on denaturing 6% polyacrylamide-8 M urea gels.

Results

MHC Class II Promoters Are Silent in Plasmaocyte Cells. Flow cytotoxicometric analysis, Northern blots, and RNAscope protection experiments have demonstrated a lack of expression of HLA class II molecules (HLA-DR, -DQ, and -DP) and mRNAs in the plasmacytoma cell line SKO (data not shown). To determine whether this lack of expression is controlled at the level of transcription, transient transfection assays were performed using a CAT expression vector in which ex-
pression of the CAT gene is under the control of the first 150 bp of the HLA-DRA promoter. As positive control, an SV40 early promoter-CAT construct was used. Transfections were performed in parallel in a B cell line (RAJI) and in a plasmocytoma cell line (SKO). DRA-driven CAT activity is high in the class II-positive RAJI cell line (23% of that obtained using the SV40 early promoter) (Fig. 1, columns 1 and 2) whereas it is undetectable in the class II-negative SKO plasmocyte cell line (1.5% of the SV40 control) (Fig. 1, columns 3 and 4). The DRA promoter is thus inactive in plasmocytes, indicating that the mechanism responsible for extinction of MHC class II genes operates at the transcriptional level. Moreover, these data demonstrate that extinction of MHC class II genes is mediated by the 150-bp promoter proximal region. This segment of DNA contains all the cis-acting elements previously defined to be important for tissue specificity and inducibility of MHC class II gene transcription.

In general, the state of occupancy of MHC class II promoters in vivo correlates well with the activity of these promoters (24, 36). In vivo DMS (dimethylsulfate) footprinting experiments were performed and occupation of the W, X1, X2, and Y cis-acting elements were analyzed. In MHC class II-positive RAJI cells, a general occupation of the DRA promoter region is observed (36, and Fig. 2, lane 1 and 2). Particularly evident is the protection at the guanosine residues -104 (X1 box), -93 (X2 box), -68, -67 (Y box) -48 (octamer [O] sequence), and -53. Weaker protection is also observed for positions -118 and -122 (W box), -98 (X1 box), and -71 (Y box). In the plasmocytoma cell line SKO, on the other hand, none of the above positions is protected, indicating that the promoter is bare (Fig. 2, lanes 3 and 4). This is in agreement with the situation observed in mouse plasmocytoma cells (24). We therefore conclude that MHC class II promoters are not occupied and are inactive in plasmocytes.

**Normal Pattern of Protein Binding to MHC Class II Promoters in Plasmocytes.** Because of the inactivity and lack of occupancy of MHC class II promoters in plasmocytes, we studied the presence or absence of known class II promoter-binding proteins by electrophoretic mobility shift assays. Nuclear extracts from the B cell line Raji were used as a positive control. Three major specific bands are obtained with WXY oligo, which spans all the relevant promoter elements (Fig. 3, lane 1). Competition experiments using unlabeled oligonucleotides allowed us to determine that these three bands are due to NF-Y, RF-X, and to the known cooperative binding between NF-Y and RF-X (39) (data not shown). The binding pattern observed is identical between nuclear extracts derived from the plasmocytoma cell line SKO (Fig. 3, lane 4) and the RAJI cell line, indicating that RF-X and NF-Y are present normally in the class II-negative plasmocytes.

**Figure 1.** MHC class II expression extinction involves a transcriptional mechanism acting via the proximal promoter of these genes. RAJI (columns 1 and 2) and SKO (columns 3 and 4) cell lines were transfected with pSVpCAT (columns 2 and 4), and pDRA150 (columns 1 and 3) plasmids, by electroporation. 48 h after transfection, the CAT activity present in the transfected cells was determined. The CAT activity obtained with the DRA promoter is expressed as a percentage of the activity obtained with the SV40 early promoter.

**Figure 2.** In vivo footprint analysis reveals a bare HLA-DRA promoter in plasmocytoma cells. RAJI (columns 1 and 2) and SKO (columns 3 and 4) cells were incubated with DMS. After purification, the DNA was cleaved by piperidine treatment, at positions corresponding to modified guanosine. Cleaved fragments spanning the DRA promoter region were amplified by LMPCR and resolved on a 6% polyacrylamide-8 M urea gel. Purified DNAs from RAJI (column 1) and SKO (column 3) were also treated with DMS, cleaved with piperidine, and then amplified by LMPCR, in order to have the complete guanosine pattern corresponding to the upper strand of DRA promoter region. Positions of the W, X1, X2, and Y elements and the protected positions observed in RAJI are indicated.
Figure 3. Binding of nuclear proteins to the class II consensus boxes of the DRA promoter is not affected in nuclear extracts from plasmacytoma cells. Nuclear extract prepared from RAJI (lanes 1, 3, and 5) and SKO (lanes 2, 4, and 6) cells were used in gel retardation assays with the WXY oligonucleotide containing all the class II consensus boxes (lanes 1 and 2), and with an oligonucleotide WX2, spanning the W, X1, and X2 boxes. With the WX2 oligonucleotide, incubation temperatures used were 0°C (lanes 3 and 4) and 20°C (lanes 5 and 6).

To analyze X2 binding proteins (X2BP), we used conditions that have allowed us to demonstrate cooperativity in binding between RF-X and X2BP (40, 40a). With an oligonucleotide that spans the W, X1, and X2 boxes, a major specific band formed by ILF-X is observed when the binding reaction is performed at 0°C whereas at 20°C an additional band consisting of RF-X bound together with X2BP is formed (Fig. 3, lanes 3 and 5). Again the RAJI and SKO extracts give identical binding patterns (Fig. 3, compare lanes 3, 4 and lanes 5, 6), indicating that X2BP is also normally present.

These results thus demonstrate that, although MHC class II promoters are inactive and are not occupied in plasmocytes, the different nuclear transcription factors which can bind to MHC class II promoters in vitro are all present in these class II-negative cells. Therefore no impairment in the DNA binding ability can be invoked to account for the inactivity of MHC class II promoters.

Plasmocytes Do Not Express the MHC Class II Transactivator CIITA. In addition to DNA-binding proteins such as RF-X, NF-Y, and X2BP, which are expressed in a ubiquitous fashion, expression of class II genes requires the MHC class II transactivator CIITA (21). Expression of CIITA is known to be tightly regulated (21, 21a, and Steimle, V., A. Mottet and B. Mach, unpublished observations). To explore the possibility of a role of CIITA in the negative control of MHC class II expression in plasmocytes, we first analyzed its expression in these cells. The expression of CIITA gene was assayed by RNase protection experiments using a probe that protects 225 nucleotides of CIITA mRNA in MHC class II-positive B cells (Fig. 4, lane 1). With RNA derived from plasmacytoma cells however, no protected fragment is observed (Fig. 4, lane 2), even after prolonged exposure. We therefore conclude that the MHC class II transactivator CIITA is not expressed in plasmocytes and that this might be responsible for the lack of expression of MHC class II genes in these cells.

Experimentally Induced Expression of CIITA Can Restore MHC Class II Gene Expression in Plasmocytes. To test this hypothesis, we transfected EBO-76PL (control vector) and EBO-S6/CISTA (full-length CIITA cDNA in an expression vector) (21) into the class II-negative plasmacytoma cell line SKO and analyzed the effect of CIITA expression on (a) the activity of an MHC class II promoter in transient transfection experiments, and (b) the activity of the endogenous MHC class II genes in stably transfected cells. Transient cotransfection experiments using the CIITA cDNA and DRA promoter-CAT constructs demonstrate that an inactive MHC class II promoter can be reactivated in plasmocytes by expression of CIITA (Fig. 5 A). The level attained by this trans-activation is comparable to that observed in the class II-negative B cell regulatory mutant line R2J.2.5 (data not shown).

SKO cells were transfected with the CIITA expression vector and stably transfected cells were isolated. In these transfectants, expression of all three HLA class II isotypes, HLA-DR, -DQ, and -DP, is reactivated (Fig. 5 B, panels 5–7). The level of class II expression in these CIITA-transfected plasmocytes is comparable to that observed in the B lymphocyte line RAJI. Stable transfectants obtained after transfection with the expression vector alone (EBO-76PL) do not show any effect on the phenotype of SKO cells (Fig. 5, B, panels 1–3) demonstrating that the effect of the CIITA (EBO-S6/CISTA) is indeed specific. This effect of CIITA is not limited to human plasmocytes, since transfection of the class II-negative mouse plasmacytoma line P3U1 (kindly provided by R. Accolla, Institute of Immunology, Verona, Italy) by human CIITA cDNA leads to expression of both IA and IE molecules (data not shown).

Figure 4. CIITA gene is silent in plasmacytoma cells. Total RNA was hybridized to a CIITA riboprobe complementary to nucleotides 1824–2049. After hybridization, RNAs were digested with RNase A and T1. Protected fragments of 225 nt were purified and resolved on a denaturing 6% polyacrylamide-8 M Urea gel. Lane 1: RAJI; lane 2: SKO; and lane 3: undigested probe. The quantity and quality of the RNA were controlled by hybridization to a TBP specific probe (data not shown).
Enforced expression of CIITA restores MHC class II expression in plasmocytoma cells. (A) 20 μg of the pDRACAT plasmid was cotransfected with increasing amounts of the CIITA expression vector EBO-Sfi/CIITA (column 1: none; column 2: 5 μg; column 3: 10 μg, and column 4: 20 μg). CAT activity was determined 48 h after transfection. (B) MHC class II gene expression was analyzed by flow cytometry in stable transfectants obtained by transfection of SKO with EBO-76PL (panels 1-4) and EBO-Sfi/CIITA (panels 5-8). mAbs used in these experiments were: 2.06 (HLA-DK, panels 1 and 5), T922 (HLA-DQ, panels 2 and 6), B7/21 (HLA-DP, panels 3 and 7), W6.32 (MHC class I, panels 4 and 8).

We conclude that in plasmocytes MHC class II genes are silent, because the CIITA gene is not expressed. Experimentally induced reexpression of CIITA in these cells is sufficient to restore normal levels of MHC class II gene expression.

Discussion

Extinction of MHC class II gene expression represents an interesting model for the study of the developmental control of gene regulation. The ontogeny of B cells is characterized by profound phenotypic changes, associated with extensive modifications of B lymphocyte function. MHC class II expression is modulated during this process. Class II molecules are primarily responsible for restricted B-T cell interactions, required to trigger an immune response directed against a foreign antigen (1, 2). During B cell maturation, MHC class II-restricted interaction with T lymphocytes also allows T cells and their cytokines to play an important role in this developmental process (41).

During the last step of B cell ontogeny, mature B lymphocytes disengage from T cells, acquire a highly developed secretory machinery, and differentiate into plasma cells, whose major function is to secrete Ig molecules. This change in function is also characterized by a loss in the expression of numerous genes, including MHC class II genes (12, 22, 23). It has been suggested from the results of cell fusion experiments, that extinction of class II genes in plasma cells results from a dominant mechanism. In hybridomas obtained by fusion between a MHC class II-positive human B cell line and a mouse plasmocytoma cell line, expression of the MHC class II genes is turned off, suggesting the existence of a repressor activity in the plasmocytoma cells (22, 23).

To clarify the molecular mechanism responsible for the extinction of MHC class II genes during the terminal differentiation of B cells into plasmocytes, we have extended the analysis of class II promoters and, more importantly, have explored the possible role of the newly discovered MHC class II trans-activator CIITA. We first confirmed that the defect in MHC class II genes is transcriptional and identified the minimal sequence requirements, within MHC class II promoters, that confer the MHC class II-negative phenotype to plasmocytes. The first 150 bp of the DRA promoter, active in RAJI cells, are silent in SKO cells (Fig. 1). This region of the promoter includes all the conserved class II consensus boxes previously described by sequence comparisons and functional assays (3-5, 20). These sequences are unoccupied in class II-negative plasmocytes, although all factors known to bind to these class II promoter motifs in B cells are present normally in nuclear extracts from plasmocytes and are capable of binding in vitro to their target sites in the promoter. We conclude that these DNA binding factors are not missing but that they cannot form a stable transcriptional complex in intact cells.

It was then possible to ask whether the observed developmental extinction of MHC class II genes results from a direct effect on the class II gene promoter or whether it is controlled at the level of a positive trans-acting factor required for MHC class II gene expression. The lack of expression of CIITA in plasmocytes suggested a role for CIITA in MHC class II gene extinction. This was confirmed by showing directly that experimentally induced expression of CIITA in plasmocytes restores full expression of MHC class II genes. In these cells, expression of CIITA also leads to reoccupation of the class II promoter elements (data not shown). The level
of HLA-DR, -DQ, and -DP molecules expressed at the surface of these transfected plasmocytes is comparable to that of B cell lines. Expression of IA and IE is also restored by CIITA in the mouse plasmacytoma cell line P3U1, demonstrating that the results obtained here are not restricted to the SKO cell line.

Based on these results, we propose a model for MHC class II gene extinction in plasma cell that is based on the activation of a repressor function during the differentiation of B cells to plasma cells. This repression is acting on the expression of the CIITA gene, a transcription factor whose presence is essential for MHC class II transcription, rather than directly on the MHC class II promoter itself (Fig. 6). The model presented is also consistent with recent observations (42) which showed in transient heterokaryons obtained by cell fusion experiments between a mouse plasmacytoma cell line (P3U1) and human B cell line (RAJI), a reactivation of the murine MHC class II gene expression 48–72 h after fusion. We propose that in these heterokaryons, transcription of the murine MHC class II genes is due to the presence and the effect of CIITA provided from the RAJI cells. The apparent discordance between these observations and earlier cell fusion experiments, involving stable hybridomas (22, 23), can thus be explained by the half-life of the CIITA regulatory protein.

These findings open the way for future studies on the mode of extinction of the CIITA gene itself in differentiated plasmocytes. Factors responsible for CIITA extinction could even be cloned by direct expression of plasmocyte cDNA libraries in a normal B cell line and by selection for loss of MHC class II expression. The mechanism of action of CIITA on MHC class II transcription is still unclear. The CIITA protein itself does not bind to MHC class II promoters and it has been suggested that it functions by interacting with other proteins bound to the promoter (21). In transfected plasmocytes, CIITA is capable of inducing occupancy of MHC class II promoters by the various transcription factors that bind to the class II consensus boxes. CIITA might well favor and reinforce the cooperative binding described recently between RF-X, X2BP, and NF-Y (40, 40a) above a certain functionally relevant threshold and thus allow promoter occupancy and transcription to take place.

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