

Repression of Major Histocompatibility Complex IA Expression by Glucocorticoids: The Glucocorticoid Receptor Inhibits the DNA Binding of the X Box DNA Binding Protein

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Summary

Glucocorticoids are effective repressors of major histocompatibility complex (MHC) class II gene expression. The repression occurs in B cells, which constitutively express MHC class II, as well as in macrophages, which only express MHC class II after the cells are treated with interferon γ . For the MHC class II gene IA β , this negative regulation has been linked to the X box DNA sequence, located with the IA β promoter. The addition of the glucocorticoid receptor was shown to inhibit the DNA binding of the X box DNA binding protein to the X box. The DNA binding of two other DNA binding proteins that recognize elements within this promoter was unaffected by the addition of glucocorticoid receptor. It is likely that the repression of IA β gene expression by glucocorticoids occurs because the X box DNA binding protein is prevented from binding to the DNA and activating transcription.

The MHC class II proteins play a central role in the development and maintenance of the immune system (1). They participate in the generation of the T cell repertoire in the thymus and are required for antigen presentation to T lymphocytes. Class II proteins are normally expressed on a limited number of cell types, which include B, thymic epithelial, dendritic, and glial cells, as well as activated macrophages (2). The aberrant expression of class II proteins has been implicated in immune dysfunction. The lack of class II expression leads to a severe combined immunodeficiency (3), while the abnormal expression may be linked to the development of autoimmune diseases (4, 5). Upstream of all MHC class II genes are at least three *cis*-acting elements that are essential for the transcriptional regulation of these genes (6). The elements have been referred to as W, X, and Y, and nuclear factors have been shown to bind each element (6).

Corticosteroids are important suppressors of the immune system, and one of the targets affected by corticosteroids is the expression of the MHC class II proteins. Corticosteroids have been found to repress MHC IA expression on B cells (8, 9) and the IFN- γ -induced expression of the MHC IA on macrophages (10, 11). The downregulation of MHC class II gene expression by corticosteroids appears to be at the level of transcription (12, 13).

In the present report, we have examined the ability of glucocorticoids to negatively regulate the expression of the MHC class II gene IA β . Our results indicate that the mechanism by which glucocorticoids inhibit IA β gene expression is by interfering with the DNA binding of the X box DNA binding protein.

Materials and Methods

Cells. In these studies, the cell lines used included: A20-2J (mouse B lymphocyte), EL4 (mouse T lymphocyte), and WR19 M.1 (mouse macrophage). Mouse bone marrow-derived macrophages (BMM) were produced in vitro using L cell-conditioned medium as described (14). After 6 d in culture, BMM were washed and incubated in the absence of L cell-conditioned medium for 4 d. For IFN stimulation studies, 300 international reference units (IRU)/ml of murine IFN- γ (a gift from Genentech, Inc., South San Francisco, CA) was added to the medium for 24 h. Under these conditions, IFN- γ induces the expression of mRNA for IA β and IA surface expression (15). The treatment of cells with dexamethasone (Sigma Chemical Co., St. Louis, MO) (10^{-6} M) was for 24 or 48 h depending on the assay. RU 486 was a generous gift from Roussel Uclaf (Romanville, France). The RU 486 was dissolved in DMSO and used at a concentration of 10^{-6} M. Cells were treated with RU 486 for 24 h.

Nuclear Extract Preparation. Nuclear extracts were prepared as described previously (16). In brief, cells were lysed in a homogenizer (Dounce). The crude nuclei were extracted at 4°C with a buffer containing 0.4 M NaCl for 30 min with continuous stirring followed by centrifugation at 10^5 g for 1 h. The supernatant was dialyzed, and extracts were cleared by centrifugation at 10^4 g for 10 min. The supernatant (protein concentration of 1–5 mg/ml) was frozen in aliquots and stored at -70°C .

RNA Protection Assay. The RNA protection assay was performed as described (14, 15). For the analysis of IA β gene expression, a 1.5-kb SacI-HindIII fragment containing the β 1 exon of the IA β gene was subcloned into the vector, pGEM1 (Promega Biotec, Madison, WI). In the RNA protection assay, this 1.5-kb probe protected a fragment of 273 bases. Samples were electrophoretically separated on a 6% polyacrylamide-urea gel. As a control,

we followed the expression of a ribosomal protein gene, L32. A 1.6-kb *SacI* fragment containing this gene was subcloned into the vector, pGEM1. This probe was made from the genomic clone RPL32-4A, kindly provided by Dr. R. P. Perry (Fox Chase Cancer Center, Philadelphia, PA) (17). The L32-4A plasmid was linearized at an *AvaII* site within the coding region giving a 0.7-kb probe in the RNA transcription system. This probe protected a 410-base fragment in the RNA protection assay.

Transfection Assays. A20-2J (mouse B lymphocytes) cells were transfected in suspension in 1 ml using the DEAE-dextran method (18). 15 μ g of the experimental DNA and 3 μ g of the plasmid, pCH110, a β -galactosidase expression vector used to measure transfection efficiency and 30 μ g of DEAE-dextran, were added to each plate. In the cotransfection experiments with the glucocorticoid receptor or retinoic acid receptor, 2 μ g of the indicated receptor was used. When dexamethasone was added in the cotransfection assay, it was added to the media after the DMSO shock treatment. Chloramphenicol acetyl transferase (CAT) assays were performed using a standard protocol (19). Briefly, cells were isolated 48 h after the DNA was added and subjected to three freeze-thaw cycles in dry ice/ethanol and 37°C. The extracts (10–60 μ l standardized by β -galactosidase activity) were incubated with [¹⁴C]chloramphenicol and acetyl coenzyme A for 60 min at 37°C, heated 7 min at 60°C, followed by extraction with ethyl acetate. The samples were dried and resuspended in 20 μ l of ethyl acetate for TLC. The quantitation of acetylation was performed using a radioanalytic imaging system (AMBIS, Inc., San Diego, CA). Each experiment was done at least three times, and the result of a representative experiment is shown in each figure.

Plasmid Constructions. The CAT reporter constructions were made using the KS⁺-SV2 CAT vector (20) in which the SV40 enhancer region (*SphI* to *PstI*) was removed. This modified vector contains the SV40 promoter, but lacks the enhancer region. The different constructions, KS1-WXY (-124 to -26), KS1-X (-105 to -77), KS1-Y (-74 to -44), KS1-W (-137 to -108), and KS1 mutants (mutations in the W, X, or Y box as indicated in Fig. 5), containing different elements of the IA β promoter, were generated using specific oligonucleotides or by PCR using the plasmid 12.33.2, which contains the IA β promoter (16). The DNA fragments were ligated directionally into *XbaI*-*SmaI*-digested KS1 vector. DNA fragments used for the gel electrophoresis DNA binding assay were generated by cloning. Double-stranded oligonucleotides were synthesized on a DNA synthesizer (380 A; Applied Biosystems Inc., Foster City, CA) and cloned into the *Bam*HI site of the vector, pGEM1. Plasmids containing the genes for either the retinoic acid receptor (pECE RAR β) or the glucocorticoid receptor (OB10-7) were obtained from Dr. M. Pfahl (La Jolla Cancer Research Foundation) (21, 22). The pECE vector contains the SV40 promoter and enhancer (23). In vitro transcription/translation of the glucocorticoid receptor was performed as described by the manufacturer (Promega Biotec). RNA was transcribed using T₃ RNA polymerase. The RNA template (2 μ g) was added to a rabbit reticulocyte lysate (Promega Biotec) to generate in vitro [³⁵S]methionine-labeled protein. Protein production was confirmed by SDS-PAGE and autoradiography. Gel electrophoresis DNA binding assays were performed directly with the in vitro translated product. The gels were dried and exposed to two sheets of Kodak XAR film to distinguish the ³⁵S label from the ³²P label.

Gel Electrophoresis DNA-binding Assay. The plasmids containing the synthesized oligonucleotides were digested with *Hind*III, labeled by filling in the ends with α [³²P]-labeled dNTPs and Klenow polymerase, and digested with *Eco*RI. A 70-bp fragment containing the desired protein binding site was then isolated from

a 6% polyacrylamide gel. For gel electrophoresis DNA binding assays, binding was performed in a total volume of 20 μ l in 12 mM Hepes (pH 7.9), 60 mM KCl, 5 mM MgCl₂, 0.12 mM EDTA, 0.3 mM PMSF, 0.3 mM DTT, 10,000 cpm of ³²P-labeled DNA (0.1 ng), 2 μ g of poly(dI-dC), 12% glycerol, and the indicated amounts of nuclear extract. Binding was allowed to proceed for 30 min at 20°C (16). Samples were then applied to a 6% polyacrylamide gel (acrylamide/bisacrylamide, 30:1), 0.25 \times TBE (1 \times TBE = 89 mM Tris, 89 mM boric acid, 2 mM EDTA) containing 5% glycerol, and electrophoresis was carried out for 1 h at 4°C at 250 V (10 V/cm). After electrophoresis, the gels were dried and exposed to X-ray film (XAR5; Kodak).

Results

The effect of dexamethasone treatment on the expression of the MHC class II gene IA β was tested using various cell lines and normal bone marrow macrophages. The steady-state level of IA β RNA isolated from these cells was measured using an RNA protection assay (Fig. 1). A probe for the ribosomal protein gene L32, whose RNA level does not change after treating the cells with IFN- γ or dexamethasone, was included as a control for each cell type. The B cell A20-2J constitutively expresses IA β mRNA, which resulted in a fragment of 273 bases being protected in the RNA protection assay. The quantity of this protected fragment did not change significantly when the cells were treated with IFN- γ for 24 h before the RNA was isolated. When dexamethasone was added for 24 h before the isolation of RNA, however, the level of IA β mRNA was reduced considerably. In bone marrow macrophages or the macrophage-like cell line WR19 M.1, both of which do not express MHC class II genes unless treated with IFN- γ , a background level of IA β mRNA was observed. After the cells were treated with IFN- γ for 24 h, the level of the IA β mRNA increased \sim 25-fold. When RNA was assayed from cells treated with both IFN- γ and dexamethasone, no increase in the level of IA β mRNA above the background level was observed. The T cell line in EL4 does not normally express MHC class II mRNA and, therefore, the level of IA β mRNA is at a background level as measured by the RNA protection assay. The treatment of these cells with either IFN- γ or dexamethasone for 24 h did not have any significant effect on the level of IA β mRNA. The results indicated that in those cells that express IA β mRNA, either constitutively as in B cells or after IFN- γ treatment as in macrophages, dexamethasone treatment lowered the level of IA β mRNA.

To examine the basis for the repression of IA β expression by dexamethasone, we tested the possibility that transcription from the IA β promoter may be affected by dexamethasone treatment. Using the IA β promoter linked to the CAT gene, we observed a low CAT activity when this construction was transfected into the B cell line, A20-2J. Because we were interested in the cell type-specific enhancing activity of the IA β promoter, we linked a 124-bp fragment of the IA β promoter containing the W, X, and Y boxes to the SV40 promoter (Fig. 2), which then gave us a better signal in the CAT assay. This type of construction has been used by others to obtain more efficient expression of other MHC class II

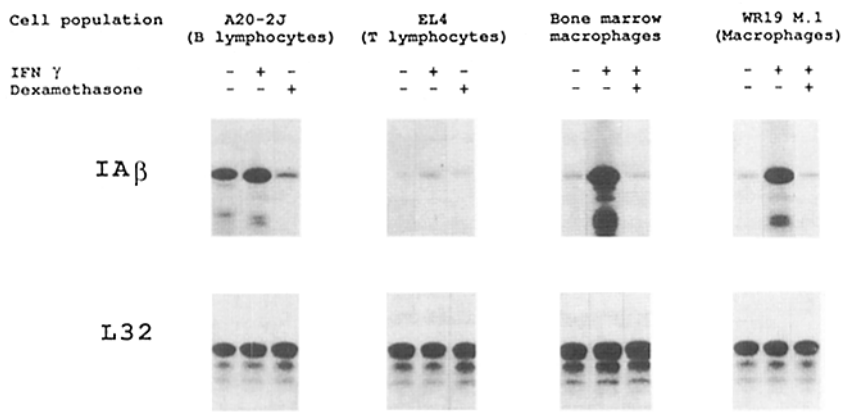


Figure 1. Inhibition of IA β mRNA expression by dexamethasone. Cells were incubated for 24 h with IFN- γ (300 IRU/ml) and/or dexamethasone (10^{-6} M) as indicated, total RNA was isolated, and 20 μ g was analyzed using an RNA protection assay, as described in Materials and Methods.

genes, including the IE α and IA α genes (24–26). In addition, previous published work has demonstrated that neither the SV40 enhancer nor promoter are influenced by glucocorticoids (27). Each CAT construction was cotransfected with the β -galactosidase expression plasmid, pCH110, into the B cell line, A20–2J. All CAT values were then normalized to the level of β -galactosidase expression to correct for any differences in transfection efficiency. The constructions containing the individual W, X, and Y sequences or the IA β promoter, each gave a higher level of CAT activity than the vector alone. The KS1 vector gave a level of CAT activity of <1%, while KS1 containing the W, X, and Y boxes gave a CAT activity of 18.5% (Fig. 2). When a mutation of either the W, X, or Y box was made in the context of all three boxes, CAT activity was lowered to 10.5% for the W box mutant, and 2.0 and 7.0% for the X and Y box mutants, respectively. Each mutation was designed to disrupt the binding of a nuclear factor to the respective box as measured by a gel electrophoresis DNA binding assay (data not shown). For the constructions containing individual W, X, or Y elements, the CAT activities were 4.2, 10.3, and 6.5%, respectively.

The addition of dexamethasone to the transfected cells resulted in a decrease in CAT activity in cells transfected with the KS1 constructions containing the X box sequence either alone or together with the W and Y boxes. In cells transfected with the KS1-WXY construction, dexamethasone treatment resulted in a decreased CAT activity from 18.5 to 3.2%. Dexamethasone treatment also resulted in a decreased CAT activity from 10.5 to 3.6% in cells transfected with the CAT construction, KS1-mutant W, in which the W box has been mutated. When the Y box was mutated (KS1-mutant Y), CAT activity dropped from 7.0 to 3.2% when dexamethasone was added to the cells. The KS1-mutant X construct resulted in a CAT activity of only 2.0%, making it difficult to interpret the CAT activity (1.5%) when dexamethasone was also included. Dexamethasone had no effect on the CAT activity from cells transfected with the KS1-W or KS1-Y constructions, but lowered the CAT activity from 10.3 to 2.8% in cells transfected with the KS1-X construction. As a control, transfected cells were also treated with retinoic acid, which did not adversely affect the level of CAT activity compared to the control for any of the constructions tested. Thus, the

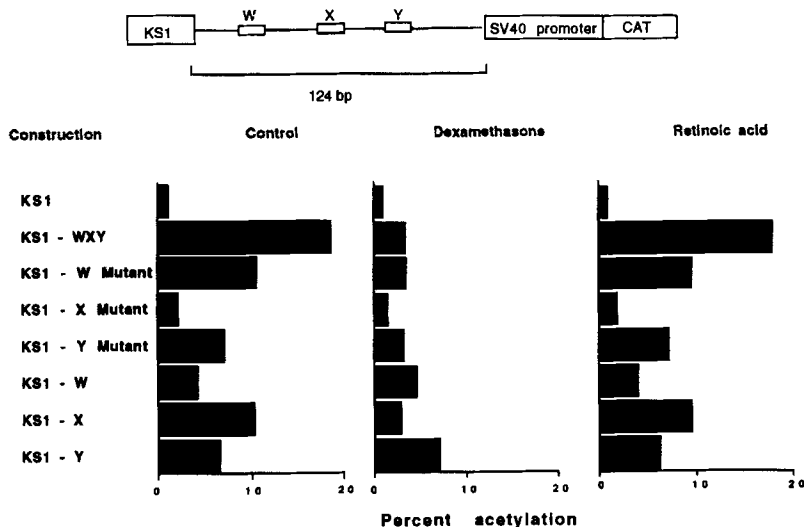


Figure 2. Repression of IA β promoter expression by glucocorticoids. A reporter plasmid (15 μ g) (top) containing fragments of the IA β promoter (WXY -124 to -26; W -137 to -108; X -105 to -77; or Y -74 to -44) was transfected into A20–2J cells, together with the β -galactosidase expression plasmid. After the DMSO shock the cells were incubated in DME plus 5% FCS in the presence or absence of 10^{-6} M dexamethasone or 10^{-6} M retinoic acid for 48 h. The amount of cell extract used for CAT assays was normalized according to the level of β -galactosidase expression. The CAT enzymatic activity was quantitated using an imaging system (AMBIS, Inc.).

effect of adding dexamethasone seemed to be specific and to affect the level of CAT activity from those constructions containing the X box sequence.

To determine if the effect of dexamethasone on IA β expression was mediated by the glucocorticoid receptor, we cotransfected a receptor expression plasmid (OB10-7) into A20-2J cells together with the CAT constructions. No change in the level of CAT expression was observed when cells were cotransfected with the pECE vector alone (Fig. 3). In cells cotransfected with the glucocorticoid receptor expression plasmid, however, there was a diminution of CAT activity in those cells transfected with CAT constructions containing the X box sequence. The CAT activity dropped from 18.0 to 4.2% for the KS1-WXY construction. When either the W or Y box were mutated, CAT activity fell from 11.1 to 4.2% for the W box mutant and 7.0 to 4.1% for the Y box mutant. The X box mutant only gave a CAT activity of \sim 2.0%, so when the glucocorticoid receptor expression plasmid was cotransfected, the drop in CAT activity was negligible. When the construction KS1-X was tested, CAT activity went from 10.8% in the absence of glucocorticoid receptor to 2.5% when the glucocorticoid receptor construction was cotransfected. In contrast, there was no reduction in CAT activity when the glucocorticoid receptor construction was cotransfected with the KS1-W or KS1-Y constructions. Cotransfection of a construction able to express the retinoic acid receptor together with any of the IA β constructions had no effect on CAT activity. Thus, the glucocorticoid receptor was able to repress CAT activity in only those constructions containing the X box sequence.

Additional support for the involvement of the glucocorticoid receptor in the repression of IA β expression was obtained by using the glucocorticoid receptor antagonist RU 486. The binding of RU 486 by the receptor results in an inactive receptor in most cases. We found that when RU 486

was added together with dexamethasone to cells transfected with the KS1-WXY construction, the repressive effect of dexamethasone was blocked (Fig. 4). Cells treated with both RU 486 and dexamethasone had a level of CAT activity (16.0%) that was comparable to the control cells (16.3%). In cells treated with dexamethasone alone, the CAT activity was lowered to 4.6%. RU 486 by itself had no effect on CAT activity when compared to the control.

The blocking effect of RU 486 was also observed when RU 486 was added to cells cotransfected with a glucocorticoid receptor expression construct (Fig. 4). In this experiment, the presence of the glucocorticoid receptor reduced CAT activity from 16.3 to 3.8%. When RU 486 was added, the repression by the glucocorticoid receptor was blocked and CAT activity was 15.8%. The addition of dexamethasone to cells cotransfected with KS1-WXY and the glucocorticoid receptor expression construct resulted in a CAT activity of 1.8%. This repression was completely reversed by the addition of RU 486. These results emphasize that it is the glucocorticoid receptor that causes the repression of CAT activity in our assays and that this activity can be blocked by the drug RU 486.

Recently, it has been demonstrated that the transcriptional interference between c-Jun and the glucocorticoid receptor is due to a mutual inhibition of DNA binding due to the direct protein-protein interaction between c-Jun, c-Fos, and the glucocorticoid receptor (27-31). To investigate if a similar mechanism could account for the transcriptional repression of the IA β gene by the glucocorticoid receptor, we tested the possibility that the glucocorticoid receptor may inhibit the interaction between a DNA binding protein and a DNA sequence within the IA β promoter. Using footprinting and competition assays, we showed previously that specific nuclear factors bind to the Y box (16, 32), the X box (33), and the W box (Celada, A., and R. Maki, manuscript submitted for publication) of the IA β gene. Using nuclear extracts prepared from the B cell line A20-2J and cloned oligonucleotide probes covering the W, X, and Y boxes, a gel electrophoresis DNA binding assay was performed (Fig. 5). Each probe

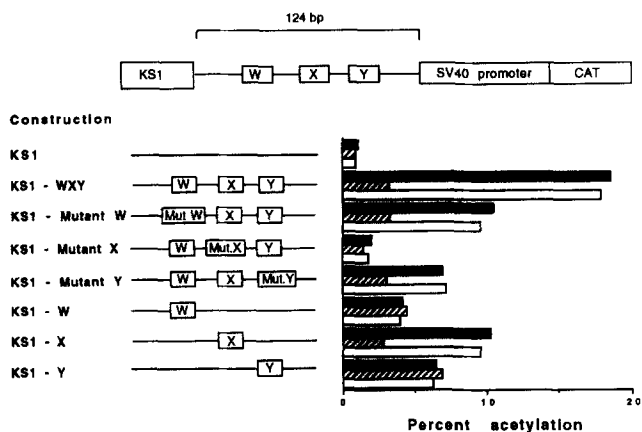


Figure 3. The glucocorticoid receptor mediates the repression of IA β expression. A20-2J cells were transfected with 15 μ g of the KS1-CAT plasmid containing different elements of the IA β promoter as described in Fig. 2, together with 2 μ g of the pECE vector (■), the glucocorticoid receptor expression plasmid (▨), or the retinoic acid receptor expression plasmid (□). CAT enzymatic activity was quantitated using an imaging system (AMBIS, Inc.).

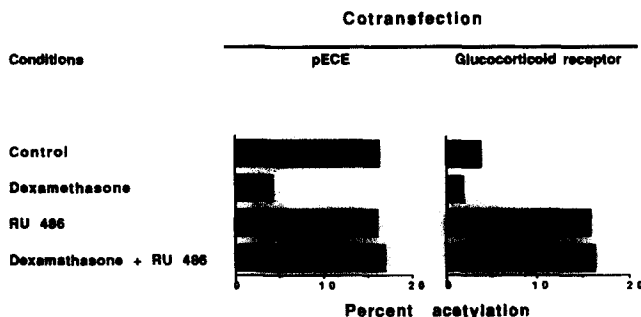


Figure 4. Repression of IA β by the glucocorticoid receptor is blocked by the drug RU 486. A20-2J cells were transfected with 15 μ g of the KS1-WXY construction and cotransfected with 2 μ g of pECE or an expression construct containing the glucocorticoid receptor. Cells were treated with dexamethasone, RU 486, or a combination of both. CAT enzymatic activity was quantitated using an imaging system (AMBIS, Inc.).

an AP-1 recognition site. The transcription factor recognizing the AP-1 site is a protein complex composed of the c-Jun and c-Fos proteins. It appears that the glucocorticoid receptor inhibits the expression of the collagenase genes by binding to the c-Jun/c-Fos complex and preventing it from binding to the AP-1 recognition site on the DNA (27-31, 44).

The inhibition of IA β gene expression by the glucocorticoid receptor does not seem to be mediated by competition between the glucocorticoid receptor and the X box binding factor for binding to the DNA. This conclusion was reached based on the findings that first, there is no consensus binding site for the glucocorticoid receptor in the X box sequence, and second, we did not see any retarded band in the gel electrophoresis DNA binding assay when the glucocorticoid receptor was mixed with the labeled X box-containing DNA fragment. A more likely possibility is that the glucocorticoid receptor is interacting directly with the X box binding protein preventing the X box binding protein from binding to the DNA. Recently, c-Fos has been shown to associate with hXBP-1, an X box DNA binding protein that binds to the X box of the HLA-DRA and HLA-DP MHC genes (45, 46). One possible model for the repression of MHC class II gene expression would involve the binding of the glucocorticoid receptor to the c-Fos protein, leading to an inhibition of MHC class II transcription. It is unclear if c-Fos is necessary for the expression of the mouse MHC class II gene, IA β . Experiments are in progress to test this possibility.

The retinoic acid receptor has also been shown to inhibit AP-1-regulated transcription (47-49). As with the glucocorticoid receptor, the retinoic acid receptor interacts directly with the c-Jun/c-Fos complex in vitro (49). Since expression of the retinoic acid receptor in our cotransfection experiments

had no effect on the expression of the IA β gene, c-Jun or c-Fos may not be involved in the expression of IA β .

The inhibitory effect of the glucocorticoid receptor on MHC class II gene expression may in part explain the clinical effects of glucocorticoids on the immune system. Macrophages play a key role in inflammatory processes and require the expression of class II molecules on the cell surface in order to present antigens to T cells (50). In humans, glucocorticoid treatment is an established method for treating autoimmune diseases, and part of the effect could be related to the inhibition of class II expression. Murine models of autoimmune diseases in which animals were treated with anti-IA mAbs resulted in an increased survival rate from 10 to >90% (51).

Class II antigens are not only expressed on the cell surface of immunoreactive cells; other cell types, such as endothelial cells, fibroblasts, and epithelial cells, can express class II antigens (1, 2). Moreover, the induction of class II antigens on these nonimmunoreactive cells plays an essential role in the augmentation of the immune response during autoimmune disease and graft rejection (52, 53). The inhibition of class II antigen expression on renal tubular cells has been associated with the absence of graft rejection in rat renal allografts despite the presence of activated T cells within the graft (54). Finally, steroid therapy has been shown to reduce class II expression on diseased tissues (54, 55).

Although, at present, we do not know the precise mechanism of glucocorticoid receptor interaction with the X box binding protein, these results may help in the design of new therapeutic approaches for the treatment of immunological diseases.

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