Class II Transactivator (CIITA) Is Sufficient for the Inducible Expression of Major Histocompatibility Complex Class II Genes

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Summary

The class II transactivator (CIITA) has been shown to be required for major histocompatibility complex (MHC) class II gene expression in B cells and its deficiency is responsible for a hereditary MHC class II deficiency. Here we show that CIITA is also involved in the inducible expression of class II genes upon interferon γ (IFN-γ) treatment. The expression of CIITA is also inducible with IFN-γ before the induction of MHC class II mRNA. In addition, CIITA mRNA expression does not require new protein synthesis, although new protein synthesis is necessary for the transcription of class II. This suggests that synthesis of new CIITA protein may be essential to induce class II gene expression. We also showed that the JAK1 protein tyrosine kinase activity is required to induce the expression of CIITA upon IFN-γ stimulation. This finding indicates that CIITA is part of the signaling cascade from the IFN-γ receptor to the activation of class II genes. In addition, the expression of CIITA is sufficient to activate class II genes in the absence of IFN-γ stimulation suggesting that CIITA is the major regulatory factor for the inducible expression of class II genes. Together, these data suggest that CIITA is the IFN-inducible cycloheximide sensitive factor previously shown to be required for the induction of MHC class II gene expression.

Major histocompatibility complex (MHC)-encoded class II antigens are heterodimeric cell surface molecules that present antigens to CD4 positive lymphocytes and thus play a important role in the regulation of immune responses. The expression of MHC class II molecules is developmentally regulated, and only limited cell types normally express class II antigen, primarily antigen-presenting cells such as macrophages, dendritic cells, and B cells. Class II expression can be induced on human T cells by mitogens or antigens (1-3) and on macrophages, fibroblasts, and endothelial cells by IFN-γ (4).

The molecular mechanisms responsible for the regulation of MHC class II gene expression in B cells has been extensively studied (reviewed in 5). Many cis regulatory elements have been identified and DNA-binding proteins that could potentially be involved in the regulation of class II gene expression have been characterized. However, relatively little is known about the molecular mechanisms of IFN-γ induction of class II genes. The IFN-γ induction of cell surface MHC class II antigens is mediated by an increase in steady state mRNA. Previous work showed that this increase in steady state mRNA is due, at least in part, to an increased rate of transcription (6-8). In HeLa cells, macrophages, and a human osteosarcoma cell line this induction can be blocked by cycloheximide, implying that de novo synthesis of an intermediate factor(s) is required (6-8).

IFNs regulate gene expression by tyrosine phosphorylation of several transcriptional factors that share the 91-kD protein (termed Stat91 for signal transducer and activator of transcription) of IFN-stimulated gene factor 3 (ISGF3) as a common component (9-15). ISGF3 binds to a specific DNA sequences, IFN-stimulated response element (ISRE), and directs IFN-dependent gene transcription. Stat91 is also known as the IFN-γ-activated factor (GAF) that binds the IFN-γ activation sequence (GAS) in the promoter of the guanylate-binding protein (GBP) gene (9, 15). Although analysis of the nuclear response of class II genes to IFN-γ has defined the DNA sequences required for the IFN response to a minimal promoter region from -136 to +10 bp of HLA-DRA and this promoter sequence is sufficient for both IFN-γ induction and basal expression on a transient assay (16, 17), neither ISRE nor GAS sequences have been found in this region.

1 Abbreviations used in this paper: CIITA, class II transactivator; GAS, INF-γ activation sequence.
gion. In addition no inducible DNA-binding protein has been isolated which is responsible for the IFN-inducible expression of class II genes.

Although the expression of MHC class II genes in fibroblasts can usually be induced with IFN-γ treatment, we found that fibrosarcoma LTK- cells are not able to express either IE or IA class II molecules. In addition it has been shown that LTK- cells are unable to mount an antiviral response and to transcribe IFN-β-inducible genes to IFN-β treatment (18–20). However, the expression of MHC class I and invariant chain genes are inducible upon IFN-γ treatment (Chang, C.-H., unpublished data). We, therefore, undertook a genetic approach to find the regulatory factor(s) that can activate class II genes in LTK- cells. Here we report that the class II transactivator (CIITA) is able to activate the expression of class II genes in LTK- cells and suggest a model in which CIITA is the key IFN-inducible factor required for MHC class II gene expression.

Materials and Methods

Cell Culture. All cell lines are maintained in DMEM supplemented with 10% FCS and 2 mM glutamine. Murine IFN-γ (GIBCO BRL, Gaithersburg, MD) was used at 100 U/ml and human IFN-γ (gift from Biogen, Cambridge, MA) was used at concentration of 500 U/ml.

Transfection. For genomic transfection, 80 µg of genomic DNA with 1 µg of linearized hygromycin DNA were coprecipitated into LTK- cells using calcium phosphate. 2 d after transfection, cells were split 1:2 into medium containing 400 µg/ml of hygromycin (Calbiochem-Novabiochem Corp., San Diego, CA). The same procedure was employed for the transient transfection except that 25 µg of DNA was used and cells were harvested 2 d after transfection.

Construction of CIITA cDNA. The CIITA cDNA, which has Sall sites at each end in the cloning linkers, was subcloned into the Sall site of pUC19. An oligonucleotide, coding for the 8 amino acid flu-tag on the 5′ end, as well as the first 19 amino acids of CIITA was subcloned into the EcoRI site of pUC19 and the unique DraIII site of CIITA. The flu-CIITA (from EcoRI to Sall) was then moved from pUC19 into pCDNA/ampl (Invitrogen, San Diego, CA) into the EcoRI and XhoI sites.

Flow Cytometry Analysis. Cells were stained for IE surface expression with 14.4.4S (PharMingen, San Diego, CA), for IA expression with 10-3.6.2 (21) and secondary staining was performed with FITC-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL). The stained cells were analyzed using FACStar® (Becton Dickinson & Co., Mountain View, CA).

PCR. RNA was prepared as described (22). First strand cDNA was synthesized by reverse transcriptase (Seikagaku America, Inc., Rockville, MD) using 10 ng of oligo dT as a primer and 2 µg of cytoplasmic RNA in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 1 mM dithiothreitol, and 200 mM deoxynucleotides. PCR amplification was performed using 1/10 of synthesized first strand cDNA, 5 U of Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT) in the reaction condition same as for first strand synthesis. 30 cycles were run at 1 min at 94°C, 1 min at 55°C, and 3 min at 72°C. After the PCR amplification reaction, one half of PCR products were analyzed by ethidium bromide staining in agarose gels using standard techniques (23). The sequences of oligonucleotides for primers used in PCR amplification are as follows: Eox sense, CCAGAAAGTC- ATGGGCTATCA in exon 1; Eox antisense, GGTCTCATTGCGCGTCTCA in exon 4; DRα sense, CGAGTTCATCTGAAA- TCCTG in exon 2; DRα antisense, GTTCCTGCTGACATTGC- TTTTGC in exon 4; CIITA sense, CAAGTCCCTGAAGGTG- GGA; CIITA antisense, AGTCATCCACCGGAGGGAC; actin sense, CACCTGTCGTGCTCACCCGAGGC; actin antisense, CCACACAGACATTTGCCTCAGG.

Results

Genomic Transfection. There are several lines of evidence suggesting that the positive regulatory factor(s) required for MHC class II gene expression is not present in LTK- cells. First, we found that both the IE and IA MHC class II genes are not induced in LTK- cells upon murine IFN-γ treatment although a normal response of class I expression is observed (Chang, C.-H., unpublished data). Second, when LTK- cells were fused with MHC class II positive human B cells, the endogenous mouse class II gene was reexpressed (Chang, C.-H., unpublished data). We, therefore, chose LTK- cells to study the regulatory factors that mediate the expression of class II genes.

To clone a gene capable of complementing the defect in LTK- cells, genomic DNA prepared from human cells was cotransfected with the hygromycin marker gene. Hygromycin resistant colonies were pooled from each plate of cells, induced with mouse IFN-γ, and tested for class II expression by PCR. One pool (no. 9) was identified by PCR as positive for Eox mRNA and class II positive cells were sorted using flow cytometry after staining with the antibody to IE. Eight clones were isolated after a limiting dilution of the sorted population of IE positive cells. They showed a similar pattern of class II expression on cell surface (data not shown). One clone, 9.18A4, which shows inducible expression of both IE and IA upon IFN-γ treatment (Fig. 1A) was further characterized and used for the subsequent experiments. To determine whether the gene of interest was linked to the hygromycin marker gene, secondary transfection was performed. Genomic DNA prepared from 9.18A4 was transfected back into LTK- cells without the addition of the hygromycin plasmid. Out of 19 hygromycin resistant colonies, 6 class II positive clones were isolated, suggesting that the gene of interest was physically linked to the marker gene. A third round of transfection was carried out using the genomic DNA from either class II positive or negative secondary transfectants. Tertiary transfectant, class II positive clones were isolated from hygromycin resistant clones but only from the DNA prepared from class II positive transfectants, not from negative transfectants (Fig. 1, B and C).

The Expression of CIITA in Class II Positive Transfectants. Recently, a new regulatory factor, CIITA, for the expression of MHC class II genes was isolated and characterized (24). To determine whether this factor was responsible for the phenotype observed in LTK- transfectants, CIITA expression was tested. Since the message of CIITA is reported to be low (24), reverse transcriptase–PCR analysis was employed instead of Northern analysis. As shown in Fig. 2, all class
II positive transfectants show signals for both class II and CIITA, whereas all class II negative cells show neither. As a positive control, Raji human B lymphoma cells, were used to show normal class II and CIITA. Because we observed the same PCR product for CIITA using both mouse and human (data not shown), it was necessary to determine whether the PCR products from the transfectants were of human or mouse origin. To identify the origin of CIITA the PCR products from transfectants were isolated and sequenced. Sequence analysis showed that the PCR product from the transfectants have the identical sequence to human CIITA not mouse suggesting that CIITA expressed in transfectants is derived from the transfected human gene (data not shown).

Although the levels of class II and CIITA are increased after the induction with IFN-γ, both genes were expressed at low level even before the IFN-γ treatment (compare lanes of uninduced and induced with IFN-γ) as is commonly observed in inducible cell lines.

**The Expression of CIITA Is Sufficient to Activate the Expression of Class II Genes.** Although we showed that there is a very good correlation between the presence of CIITA and the IFN-inducible expression of class II genes in LTK− transfectants, it still does not prove the requirement of CIITA for MHC class II gene expression. We therefore transfected the cDNA for CIITA into LTK− cells to test whether class II genes can be activated. As shown in Fig. 3, Eo mRNA is present in LTK− cells transfected with CIITA cDNA but not with control DNA. In addition cells transfected with CIITA express IE on cell surface (data not shown). It should be noted that Eo mRNA is present at the same level with or without the IFN-γ treatment (Fig. 3, lane 3 and 4) suggesting that the overexpression of CIITA which is directed by a constitutive, non-IFN-inducible promoter seems to be sufficient to activate class II genes without IFN-γ induction.

**IFN-γ Induction of CIITA.** The data from the LTK− transfectants suggested a model in which the endogenous CIITA expression is induced by IFN-γ treatment and that this is responsible for MHC class II induction (see Fig. 2). Therefore, the expression of CIITA was tested at different time points after the addition of IFN-γ to the medium. CIITA expression is detectable without IFN-γ but the level is gradu-

**Figure 1.** Cell surface expression of class II molecules, IE and IA, on transfectants. (A) Primary transfectants (9.18A4) and parent LTK− cells were stained. Cells were stained with either IE or IA antibodies. Cells were stained with secondary antibody alone (—), class II antibodies followed by the secondary antibody before the IFN-γ induction (-- - -), and class II antibodies after IFN-γ induction (---). Results are expressed as relative cell number (y-axis) vs. log fluorescence intensity (x-axis). (B) Tertiary transfectants with DNA from class II positive secondary transfectants. Solid line represents the class II staining without IFN-γ induction and broken line represents after IFN-γ induction. (C) Tertiary transfectants with DNA from class II negative secondary transfectants.

**Figure 2.** Expression of CIITA and class II gene in transfectants. PCR analysis was performed using primers for both CIITA and class II genes. Bottom shows actin as a control. Lanes marked with + are cells treated with murine IFN-γ (100 U/ml) for 2 d. The data are representative of three independent analyses.

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Figure 3. Class II expression of LTK- cells with the transfection of CIITA cDNA. LTK- cells were transfected transiently with either control DNA or CIITA cDNA. Cells were harvested 2 d after the transfection and RNA was analyzed by reverse transcriptase-PCR. -/+ represents the absence/presence of mouse IFN-γ. 9.18 is the clone which is class II positive LTK- transfectant with human genomic DNA as described in the text.

Figure 4. Effect of IFN-γ on CIITA and class II (DRα) gene transcription. HeLa cells were incubated with medium containing human IFN-γ (500 U/ml), and cells were harvested and RNA was prepared at given time points. Reverse transcriptase-PCR analysis was performed using primers for both CIITA and DRα genes. The data are representative of three independent analyses. The level of CIITA mRNA is not affected by cycloheximide, unlike MHC class II, the induction of CIITA gene expression does not require new protein synthesis.

Figure 5. Effect of cycloheximide on the expression of CIITA. HeLa cells were exposed to human IFN-γ (500 U/ml) or cycloheximide (5 μg/ml) alone, or to a combination of IFN-γ/CHX for 12, 18, and 24 h. Reverse transcriptase-PCR analysis is described in the legend to Figure 3. The data are representative of three independent analyses.
JAK1-transfected cells after IFN-γ treatment but not in cells transfected with JAK2, a related protein tyrosine kinase (Fig. 6, lanes 3–4). The data suggest that protein tyrosine kinase activity is required to induce CIITA expression.

We also tested whether the expression of CIITA can bypass the IFN-γ signaling defect in these mutants. Interestingly, the mutant cells transfected with CIITA cDNA express class II genes even before IFN-γ induction, suggesting that the overexpression of CIITA is sufficient to activate class II genes without IFN-γ induction (Figs. 3 and 6).

**Discussion**

Although the mechanism of class II expression has been studied extensively over the years, it is still not clear how class II genes are regulated by IFN; many DNA binding proteins have been isolated and characterized, but their functional significance is in many cases unclear. The reverse genetic approach that we have taken has led us the demonstration that CIITA, first identified as a gene responsible for the BLS (24) affecting class II expression on human B cells, is a regulatory factor for IFN-γ inducible expression of class II genes. The study demonstrates first that, the regulatory factor, CIITA, can participate in the activation of the IFN-γ–mediated expression of class II genes and that it is itself also inducible with IFN-γ (Figs. 1–4). Second, the expression of CIITA is sufficient for the inducible expression of class II genes (Figs. 3 and 6). Third, the induction of CIITA requires protein tyrosine kinase activity (Fig. 6). Although our transfected LTK− cells are derived from a clone (9.18A4), after subsequent growth there is a bimodal distribution of class II positive population (Fig. 1, A and B). This may due to the segregation and loss of the CIITA gene during passaging of cells which suggests that the CIITA gene is integrated at a significant distance from the hygromycin gene. This is supported by the fact that genomic cloning, and “walking” experiments covering more than 60 kb of the DNA flanking the hygromycin gene has not revealed the CIITA gene.

Both IE and IA expression are induced by CIITA in the complemented LTK− cells implying that CIITA is involved in the regulation of both genes. Since the expression of the MHC class I and invariant genes are induced by IFN in LTK− cells without functional CIITA protein, CIITA is presumably not involved in the regulation of these genes by IFN-γ stimulation. In addition, BLS-2 cells have normal levels of class I and invariant chain supporting the idea that CIITA is not the regulatory factor that directs the expression of these genes in B cells (Chang, C.-H., unpublished data). It is not clear at this point whether CIITA is responsible for the expression of other IFN-γ-inducible genes.

CIITA is upregulated upon IFN stimulation before class II induction. The comparison of the kinetics of CIITA and class II indicates that the increase of the transcription of CIITA precedes MHC class II transcription which is consistent with the hypothesis that CIITA may be required for the expression of class II genes. It should be noted that CIITA is expressed at a low level, even before IFN-γ treatment, but MHC class II expression is undetectable which suggests that this low basal level of CIITA may not be sufficient to activate class II expression to detectable levels, or that CIITA, or other proteins needs to be activated by IFN-γ. We consider the latter possibility unlikely, however, since transfection of CIITA into our mutant cell line that cannot respond to IFN-γ is sufficient for MHC class II gene expression. Alternatively, the uninduced level of CIITA transcripts detected by PCR may not be physiologically significant. It is interesting that the class II positive LTK− transfectants show basal levels of both CIITA and class II mRNA which increase substantially upon IFN-γ treatment although they do not exhibit cell surface expression of class II protein before IFN induction (compare Figs. 1 and 2). Basal expression of transfected, inducible genes is commonly seen and may be due to the fact that CIITA gene has integrated into a chromosomal environment in LTK− cells such that the transfected CIITA shows a low basal level of transcription. It is, therefore, possible that the level of CIITA transcripts could be the limiting factor for the induction of the class II gene transcription. In addition, the level of class II mRNA detected by PCR may be still below the threshold required for detectable cell surface expression by flow cytometry.

It has been shown that the transcription of class II requires new protein synthesis (6–8). Data from our cycloheximide study suggests that the CIITA translation may be necessary for class II expression because cycloheximide treatment inhibits the synthesis of CIITA protein without affecting its transcription. Consequently, we suggest that in the absence of CIITA protein the class II genes can not be inhibited. Interestingly, the level of CIITA transcripts was slightly enhanced with cycloheximide. The mechanism of the induction by cycloheximide treatment is not clear.

It has been suggested by Steinle et al. (24) that CIITA does not bind to DNA directly and that instead it may act as a coactivator through protein–protein interactions. The promoter of DR–α in BLS-2 cells, which lack CIITA, shows the same binding pattern seen in normal cells (28, 29) although BLS-2 cells do not express class II genes because of
the defect in CIITA (24). Gel shift mobility assays using class II negative cells also demonstrated that there is no significant difference in the pattern of DNA binding proteins (Chang, C.-H., unpublished data). In addition, in vivo footprinting studies showed that no IFN-γ-specific DNA binding site was induced after IFN-γ induction (30) suggesting that IFN-γ-mediated transcription is not the result of the induction of a unique IFN-γ-responsive DNA binding protein that activates transcription. These interesting results indicate that DNA binding proteins already occupy the class II promoter in BLS-2 without CIITA or possibly LTK− cells but that this promoter is not transcriptionally active.

It is interesting that JAK1 tyrosine kinase activity is necessary for the induction of CIITA. Tyrosine phosphorylation has emerged as a major component of the signal transduction mechanism for both IFN-α (11, 31–33) and IFN-γ (11, 14, 15, 31, 33–36). TYK2 is involved in the signaling pathway for the type I IFNs (33) and JAK2 is required for the IFN-γ pathway (36, see Fig. 7); JAK1 is involved in the signaling of both interferons (26, 27). Although our data using the mutant cells, E1C3, show that JAK1 is required for the induction of class II gene expression by interferon, the intermediate molecules between the IFN-γ receptor and the class II gene had not been identified. The data presented here show a clear cause and effect relationship between the presence of CIITA and the expression of class II. The mutant cells that are defective in JAK1 protein tyrosine kinase activity do not show any induction of class II or CIITA mRNA but the transfection of functional JAK1 into these cells restores the expression of both. Moreover, the transfection of CIITA cDNA into mutant cells also induces the expression of class II gene suggesting that CIITA is the key transcriptional factor for IFN-γ-inducible expression of class II genes. We do not know the mechanism whereby JAK1 mediates its effects nor how many steps are involved. It seems that the induction of endogenous CIITA requires a phosphoprotein (see below) which has to be phosphorylated by a tyrosine kinase.

The induction of most genes by IFN-γ is mediated by the phosphorylation of the transcription factor Stat91 (9, 15, 37). Stat91 is a phosphoprotein and is one of the components of the well-characterized ISGF3 transcription factor complex (10–14). Although the components of the ISGF3 complex are phosphorylated by IFN-α/β, Stat91 is the only protein of this complex that can be phosphorylated by both IFN-α/β and -γ (9–11, 13, 15, 32). The expression of MHC class II is only inducible upon IFN-γ not by -α/β. There is, however, no evidence for a direct involvement of Stat91 in class II gene expression. In addition, CIITA mRNA is not induced by IFN-α (data not shown). It is, nonetheless, reasonable to postulate that the promoter of CIITA may contain GAS, the sequence that can be recognized by Stat91 upon IFN-γ induction and that the binding of Stat91 activates the CIITA gene resulting in the induction of the class II genes (Fig. 7). This hypothesis is further supported by our observation that JAK1− mutant HeLa cells transfected with CIITA cDNA driven by the CMV viral promoter express class II genes in the absence of IFN-γ induction.

Figure 7. Schematic model of IFN-γ pathway for the activation of class II gene. Ligand binding to the receptor triggers the phosphorylation of JAK1 and JAK2 kinase, which then activates CIITA by either directly or through an intermediate such as Stat91.

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