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pRB is required for interferon-γ-induction of the MHC class II Aβ gene

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pRB is required for IFN-γ-induction of MHC class II in human tumor cell lines, providing a potential link between tumor suppressors and the immune system. However, other genes, such as cyclin D1, show pRB-dependency only in tumor cells, so by analogy, pRB may not be necessary for cII-regulation in normal cells. Here, we demonstrate that induction of the mouse MHC class II I-A heterodimer is normal in $RB^{+/+}$ mouse embryonic fibroblasts (MEFs), but deficient in $RB^{-/-}$ MEFs. Inducibility is restored in $RB^{-/-}$ MEFs stably transfected with wild type *RB* cDNA or infected with an adenovirus expressing pRB. Thus, involvement of pRB in MHC class II expression is conserved in the mouse and is not an aberrant feature of tumorigenic, aneuploid, human tumor cells. Although cII genes are generally induced in a coordinate fashion, suggesting a common mechanism, we found that pRB was specifically required for induction of the Aβ, but not Aα or other MHC cII genes including Eβ, Ii and H2-Mα. Finally, IFN-γ-induction of class II transactivator (CIITA), was pRB-independent, suggesting that pRB works downstream of this master-regulator of MHC class II expression.

Keywords: retinoblastoma; MHC class II; interferon-γ; transcription; CIITA

Introduction

The retinoblastoma gene (*RB*) is mutated in a number of human tumors, and is part of a regulatory cascade which includes several oncogenes and tumor suppressors (Weinberg, 1995; Zacksenhaus *et al*., 1993). It encodes a nuclear protein, pRB, which regulates gene expression both positively and negatively. Most attention has focused on the inhibitory transcriptional effects of pRB, with particular emphasis on negative regulation of the E2F family of activators (Dyson, 1998; White, 1997). However, the effect of several other activators, including Sp1 (Chen *et al*., 1994; Kim *et al*., 1992a; Udvadia *et al*., 1993), ATF-2 (Kim *et al*., 1992b) the glucocorticoid receptor (GR) (Singh *et al*., 1995), C/EBP (Chen *et al*., 1996), and c-Jun (Nead *et al*., 1998) is potentiated by pRB. Various pRB-induced genes have been identified, although the mechanism underlying most of these effects is unclear (Rohde *et al*., 1996). A particularly intriguing example of positive regulation by pRB is its role in the up-regulation of major histocompatibility complex (MHC) genes (Lu *et al*., 1994b).

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The MHC expresses two major classes of membrane molecules, termed class I (cI) and class II (cII). cI molecules are heterodimers of a polymorphic heavy chain and an invariant β_2-microglobulin chain, while cII molecules are heterodimers of an α and β chain (Robinson and Kindt, 1989). Their role in the immune system is to present processed antigens to T-cells and activate the cell mediated immune response. Peptides expressed on cI molecules are derived from intracellular (e.g viral) proteins, whereas peptides presented by cII molecules are derived mainly from extracellular proteins which are digested and presented through the endosomal pathway (Kuby, 1994). cII/peptide complexes activate the helper arm of the immune system, which consists of CD4+ T-helper cells (Doyle and Strominger, 1987). These cells provide immunological memory and secrete cytokines which activate killer T-cells (Robinson and Kindt, 1989).

cII molecules are encoded by multiple genes at the HLA-D or H2-I loci on human or mouse chromosomes 6 and 17, respectively (Robinson and Kindt, 1989). There are three main subregions in humans; DR, DQ and DP, while in mice there are two; I-A and I-E. Each subregion encodes both α and β chains. Aα and Aβ are most closely related to DQ genes, while Eα and Eβ are related to DR (Robinson and Kindt, 1989). In inbred strains of mice, each set of MHC alleles (haplotype) has an arbitrary superscript e.g $H\text{-}2^a$, $H\text{-}2^b$, which are often included when referring to a particular region, eg $I\text{-}A^b$, or in the case of a single gene $A\alpha^b$. cII molecules are constitutively expressed on antigen presenting cells (APCs), which include B cells, and macrophages. However, cII expression can be induced in many cell types by various stimuli (Glimcher and Kara, 1992), one of the most potent of which is IFN-γ (Basham and Merigan, 1983; King and Jones, 1983; Pober *et al*., 1983; Wong *et al*., 1984).

The expression of all cII genes, whether constitutive or induced, is coordinately regulated by class II transactivator (CIITA) (Mach *et al*., 1996). CIITA induces cII expression when ectopically expressed in cII-negative cells, mediates cII-induction by IFN-γ, and is defective in a subset of patients with bare lymphocyte syndrome, which is characterized by the absence of cII molecules (Chang and Flavell, 1995; Chang *et al*., 1994; Mach *et al*., 1996; Steimle *et al*., 1993, 1994). Mice lacking CIITA show defects in both constitutive and IFN-γ-inducible cII expression (Chang *et al*., 1996), although there is detectable cII expression in several cell types (Williams *et al*., 1998). Although CIITA itself is not a DNA-binding protein, its effects are mediated through sequences common to all cII promoters, the S, X, X2 and Y motifs (reviewed in Mach *et al*., 1996). Numerous ubiquitous transcription factors have been identified that bind these motifs

(Mach *et al*., 1996), but site-occupancy is only observed in cII-positive B cells or IFN-γ-treated cells (Brown *et al*., 1993; Kara and Glimcher, 1991, 1993; Wright and Ting, 1992). Forced CIITA expression in cII-negative cells results in transcription factor binding at cII promoters (Wright *et al*., 1998). CIITA apparently facilitates promoter occupancy through interactions with TBP-associated factor, the B-cell coactivator Bob1, X-box-binding factor RFX5, and/or CBP (Fontes *et al*., 1996, 1997; Kretsovali *et al*., 1998; Scholl *et al*., 1997).

In the RB-deficient human breast carcinoma cell line S4, IFN-γ-induction of all cII loci analysed (DRα, DRβ, and DPα) was found to be pRB-dependent to some degree (Lu *et al*., 1994b). Drα promoter occupancy was also shown to be pRB-dependent in this line (Osborne *et al*., 1997). In the RB-deficient human non small cell lung carcinoma (non-SCLC) line H2009, IFN-γ-induction of DRβ, and to a lesser extent DPα and DPβ, was rescued by reconstitution of with wild type RB (Lu *et al*., 1996). DRα expression was not rescued in this line, suggesting that an additional component is defective in these cells.

The role of pRB in cII-regulation could represent an important link between tumor suppressors and the immune system. However, this phenomenon has only been observed in tumorigenic cells, and could be an aberrant consequence of aneuploidy. The importance of this issue is underscored by the fact that although cyclin D1 expression correlates with the presence of pRB in tumor lines (Lukas *et al*., 1994; Muller *et al*., 1994), its expression is pRB-independent in normal cells (Lukas *et al*., 1995; Marhin *et al*., 1996). Furthermore, Chen *et al*. (1993) showed that constitutive expression of cII molecules is unaffected in mouse B lymphocytes derived from $RB^{-/-}$ stem cells, suggesting that pRB may not be important for cII expression in normal cells. To examine the biological significance of cII-regulation by pRB, we studied IFN-γ-treated, non-transformed $RB^{+/+}$ and $RB^{-/-}$ mouse embryo fibroblasts (MEFs). Our results confirm the importance of pRB for cII-induction, and also reveal a highly specific role for this tumor suppressor in regulating the mouse cII Aβ gene. This finding, coupled with data from Chen *et al*. (1993) also suggests that while IFN-γ-induced CIITA requires pRB to induce Aβ, constitutively expressed CIITA (e.g. in B cells) acts in a pRB-independent fashion. This paradox suggests that there may be a pRB-dependent, IFN-γ-induced isoform of CIITA, and/or an IFN-γ-induced CIITA-inhibitor which is blocked by pRB.

Results

pRB-dependent induction of cII expression in mouse embryo fibroblasts

The human lines used to study cII regulation by pRB were tumorigenic and highly aneuploid (Lu *et al*., 1994b, 1996; Muncaster *et al*., 1992). We studied cII regulation in mouse embryonic fibroblasts (MEFs), derived from $RB^{-/-}$ embryos prior to their death on embryonic day 14/15, to determine if pRB is required for cII regulation in normal cells. These MEFs were derived from mice which have a 129/Sv background (Jacks *et al*., 1992). These mice have the $H\text{-}2^b$ haplotype, and do not express a functional I-E heterodimer because of an Eα promoter deletion (Mathis *et al*., 1983). We treated these MEFs with recombinant IFN-γ and assessed surface expression by flow cytometry. $I\text{-}A^b$ was induced in two $RB^{+/+}$ MEF lines, but not in two $RB^{-/-}$ MEF lines (Figure 1). Thus, cII regulation by pRB is not only a feature of tumorigenic, aneuploid, human tumor lines. Furthermore, these results establish that this function is conserved in mice and humans.

pRB-dependent induction of Aβ, but not Aα or Eβ

Lack of $I\text{-}A^b$ induction by IFN-γ in MEFs could be due to defective expression of Aα, Aβ or both genes. To address this issue, RNA from untreated or IFN-γ-treated $RB^{+/+}$ and $RB^{-/-}$ MEFs was reverse transcribed with random hexanucleotides and amplified with specific primers to Aα or Aβ. In agreement with the FACS data (Figure 1), both Aα and Aβ were induced following IFN-γ-treatment of the $RB^{+/+}$ cell lines (Figure 2, lanes 4 and 6). In contrast, while Aα was induced normally in $RB^{-/-}$ MEFs, the Aβ gene

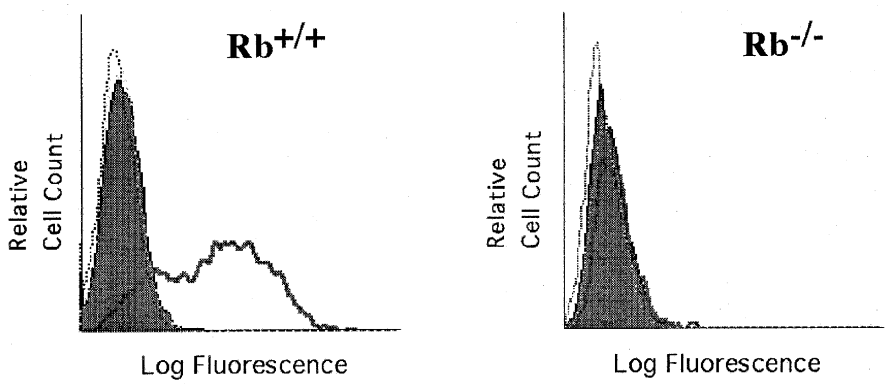

Figure 1 IFN-γ-induction of I-A is pRB-dependent. Untreated and IFN-γ-treated $RB^{+/+}$ and $RB^{-/-}$ MEFs were analysed for I-A expression by flow cytometry. The plot for untreated cells is shaded. The dotted line represents treated cells analysed using an isotype control antibody, and the solid line represents treated cells analysed with the anti-I-A antibody. Four $RB^{-/-}$ MEF lines were tested; a representative outcome is shown

remained silent (Figure 2, lanes 8 and 10). The Eβ locus, which is normal in b haplotype mice, was induced in a pRB-independent fashion (data not shown). The absence of Aβ message in the treated $RB^{-/-}$ MEFs was not due to failure of the RT–PCR reaction, since β-actin message was amplified in every sample (Figure 2, lanes 4–11), nor was it caused by a defect in the Jak-Stat IFN-γ-signaling pathway in these cells, since GBP was induced in all lines after exposure to IFN-γ (Figure 2, lanes 4, 6, 8 and 10). Thus, only Aβ, and not Aα or Eβ, induction by IFN-γ is pRB-dependent.

IFN-γ-induction of Ii and H2-M is pRB-independent

In addition to MHC class II molecules, exogenous antigen presentation requires a number of other factors. The invariant chain (Ii) is required for assembly of class II molecules (Anderson and Miller, 1992; Peterson and Miller, 1990), and transport of the class II heterodimer to the appropriate endosomal compartment (the MIIC) (Bakke and Dobberstein, 1990; Lamb and Cresswell, 1992; Lotteau *et al*., 1990; Neefjes *et al*., 1990; Peters *et al*., 1991). An Ii proteolytic fragment, the class II-associated Ii peptide (CLIP), occupies the peptide binding site until it is subsequently replaced by antigenic peptides in the MIIC. The latter step is facilitated by products of the HLA-DM locus. DM associates with class II molecules in the MIIC and facilitates the exchange of CLIP with antigenic peptides (Weber *et al*., 1996). The mouse version of the HLA-DM locus is termed H2-M.

Many of the classic features associated with MHC class II gene regulation are shared by the Ii and HLA-DM loci. Expression of all these genes is constitutive in APC cells, and can be induced by IFN-γ in other cell types (Barr and Saunders, 1991; Brown *et al*., 1993; Chang and Flavell, 1995; Collins *et al*., 1984; Kern *et al*., 1995). At the molecular level, Ii and HLA-DM promoters have the S-X-Y motifs found upstream of class II genes that are essential for both constitutive and IFN-γ-induced expression (Brown *et al*., 1991, 1993; Radley *et al*., 1994; Westerheide *et al*., 1997), and activation of these promoters is mediated by the class II master regulator, CIITA (Chang and Flavell, 1995; Kern *et al*., 1995). To determine if IFN-γ-induction of the mouse Ii and H2-Mα loci requires pRB, expression of these genes was assessed in IFN-γ-treated $RB^{+/+}$ and $RB^{-/-}$ MEFs by RT–PCR. Induction of both Ii and H2-Mα was detected in the presence or absence of a functional RB gene (Figure 3). Thus, in mouse cells, IFN-γ-induction of the Aβ locus is RB-dependent, but other genes required for presentation of antigens to $CD4^+$ T-cells are induced by an RB-independent mechanism.

Reconstitution of $RB^{-/-}$ MEFs with RB restores cII IFN-γ-responsiveness

The lack of Aβ induction following IFN-γ-treatment of $RB^{-/-}$ MEFs could be due to second site mutations in the cell lines we tested. Therefore, we tested whether stable introduction of wild type RB cDNA into $RB^{-/-}$ MEFs could rescue IFN-γ-induction of I-A in these cells. The RB expression vector was cotransfected with pBABEpuro plasmid, which confers resistance to puromycin. Two stable,

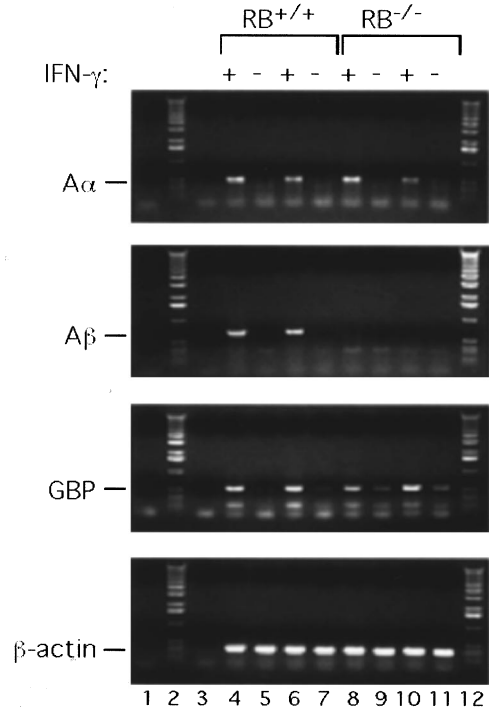

Figure 2 IFN-γ-induction of Aβ, but not Aα mRNA is pRB-dependent. RNA from untreated (lanes 5, 7, 9 and 11) or IFN-γ-treated (lanes 4, 6, 8 and 10) $RB^{+/+}$ (lanes 4–7) and $RB^{-/-}$ (lanes 8–11) MEFs was analysed by RT–PCR using primers for the indicated genes (Aα, Aβ, GBP and β-actin; the small difference in background levels of GBP in $RB^{-/-}$ versus $RB^{+/+}$ MEFS was not reproducible). Lanes 2 and 12 contain 1 kb ladder. Lanes 1 and 3 are negative controls in which PCR was carried out in the absence of cDNA, or in which the RT reaction was carried out with no RNA, respectively. Samples were analysed after 30 cycles, when amplification was still linear (data not shown)

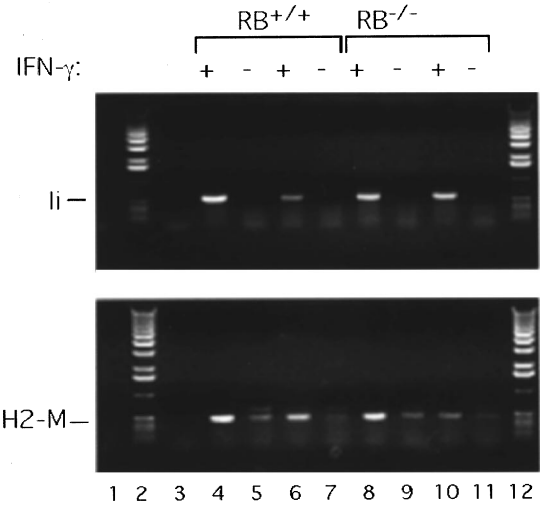

Figure 3 IFN-γ-induction of Ii and H2-Mα is pRB-independent. RNA from untreated or IFN-γ-treated cells was analysed by RT–PCR using primers for the indicated genes. Lanes are as in Figure 2. The background level of H2-Mα was variable between cell lines, but in each case induction was observed independent of pRB

puromycin-resistant clones (mg9 and mg10) were obtained which expressed pRB as detected by Western blot (Figure 4a). Treatment of these clones with IFN-γ followed by flow cytometric analysis showed that I-A^b-inducibility had been restored (Figure 4b). RT–PCR analysis of RNA from these clones confirmed that Aβ-induction had been rescued (Figure 4c). No Aβ message was induced in a clone which had been transfected with a luciferase expression vector (Figure 4c), indicating that restoration of IFN-γ responsiveness in pRB-positive clones was not an artifact of the cloning procedure. Aα and GBP RNAs were induced in all clones independent of their RB status (data not shown).

Additional proof that pRB is required for Aβ induction was obtained from an experiment in which we infected $RB^{-/-}$ MEFs with an adenovirus vector expressing constitutively hypophosphorylated pRB (Chang *et al*., 1995b). Virus was titered on 293 cells and used to infect MEFs at 30 infectious particles per cell. At this concentration, approximately 30% of cells expressed pRB, as assessed by immunostaining (data not shown). Significantly, I-A induction was detected

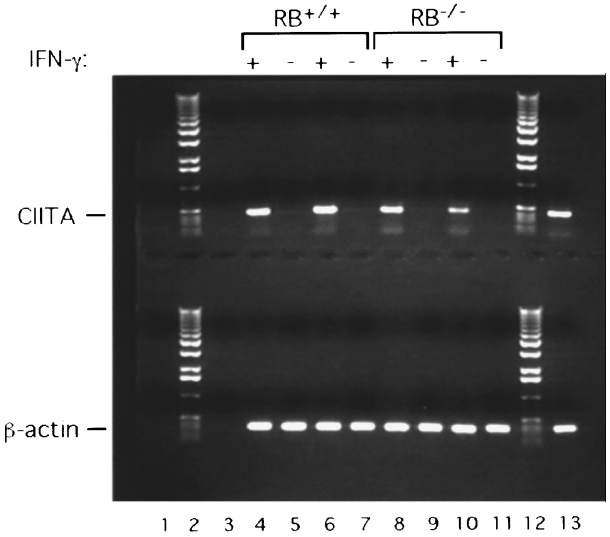

Figure 5 CIITA-induction is pRB-independent. RNA from untreated or IFN-γ-treated cells was analysed by RT–PCR using primers for CIITA or β-actin. Lanes 1–12 are as in Figure 2. RNA from a B cell line, which constitutively expresses CIITA, was also analysed (lane 13)

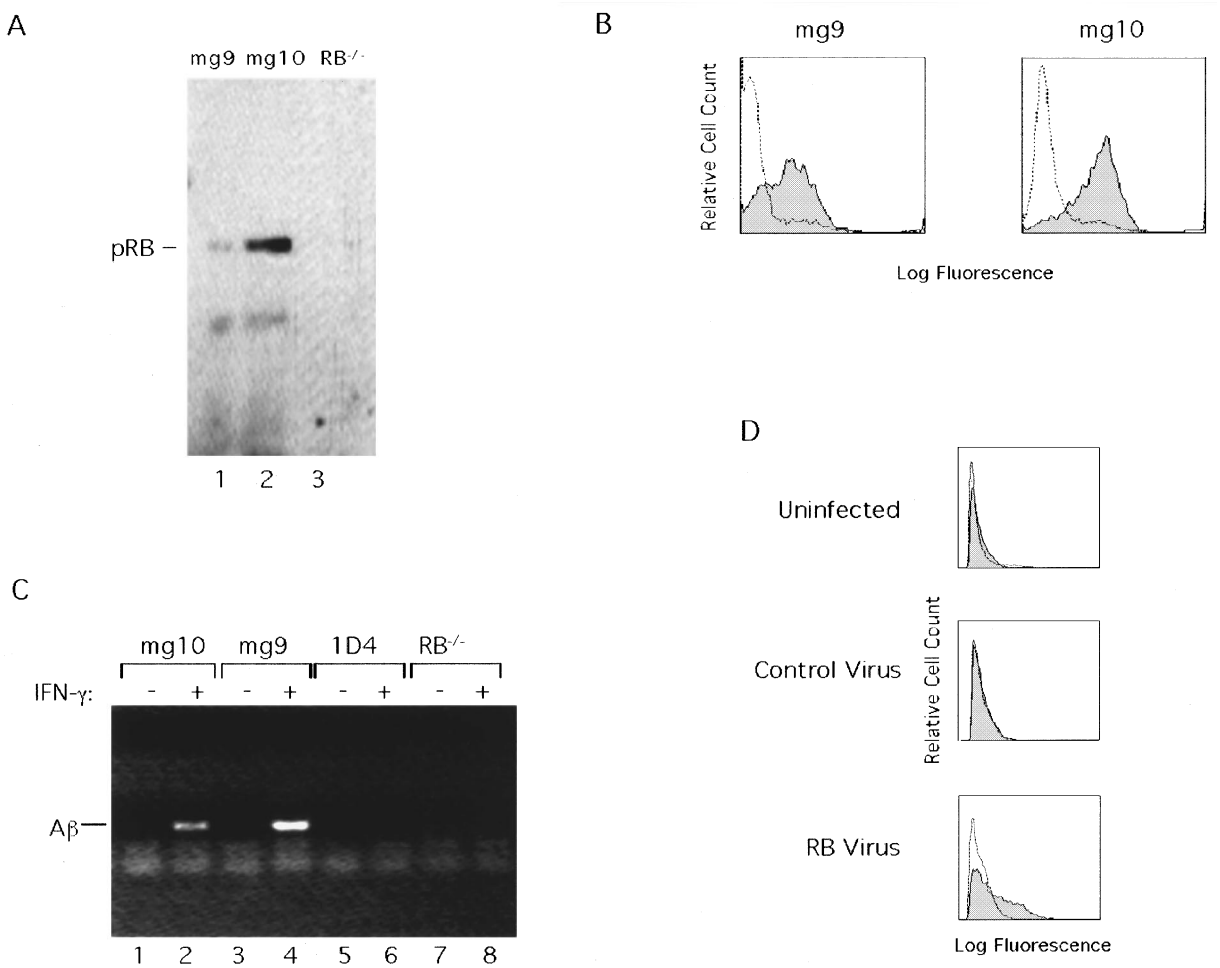

Figure 4 Reconstitution of $RB^{-/-}$ MEFs with wild type RB restores IFN-γ-induction of I-A. (**a**) Western blot demonstrating expression of pRB in mg9 (lane 1) and mg10 (lane 2) clones, and the absence of pRB in the parental line (lane 3). (**b**) Untreated, or IFN-γ-treated mg9 and mg10 clones were analysed for I-A surface expression by flow cytometry. The plot for treated cells is shaded. (**c**) RT–PCR analysis of untreated (lanes 1, 3, 5 and 7) or IFN-γ-treated (lanes 2, 4, 6 and 8) mg10 (lanes 1 and 2), mg9 (lanes 3 and 4), 1D4 (lanes 6 and 7; 1D4 is a clone stably transfected with a control plasmid), and the parental RB-deficient MEF cells (lanes 7 and 8). (**d**) $RB^{-/-}$ MEFs were left uninfected or infected with control or RB virus as indicated. Untreated, or IFN-γ-treated cells were then analysed for I-A surface expression by flow cytometry

in cells which received the pRB virus, but not in cells that received control virus (Figure 4d).

CIITA induces class II expression in RB-*deficient cells*

CIITA mediates IFN-γ-induction of all cII genes (Chang and Flavell, 1995). The fact that pRB is required specifically for the induction of Aβ, but not other cII genes, suggests that it acts in cooperation with, or downstream of, CIITA. If this was the case, induction of CIITA should be pRB-independent. Indeed, analysis of RNA from untreated or IFN-γ-treated MEFs confirmed that CIITA was up-regulated in both $RB^{+/+}$ and $RB^{-/-}$ cell lines (Figure 5).

Discussion

We have demonstrated that I-A induction in fibroblasts derived from $RB^{-/-}$ embryos is defective, but can be restored following stable transfection of a wild type RB cDNA expression vector, or following infection with an adenoviral vector expressing a constitutively hypophosphorylated version of pRB. These observations show that, unlike positive regulation of cyclin D1 (Lukas *et al*., 1995; Marhin *et al*., 1996), pRB regulates cII-induction in normal, as well as tumorigenic cells. Our results also show that cII-regulation by pRB is conserved in mice and humans. Therefore, in addition to its roles in controlling the cell cycle, apoptosis, and differentiation, pRB also plays a part in regulating the immune response. Interestingly, tumor cells can be transformed into effective vaccines by ectopic expression of cII molecules (Armstrong *et al*., 1997; Ostrand-Rosenberg, 1994). Thus, mutation of RB may help tumor cells avoid immune surveillance, as well as negative growth signals.

Analysis of the transcripts encoding the I-A heterodimer revealed, surprisingly, that pRB is required for induction of Aβ, but not Aα, expression. Furthermore, we showed that other cII loci, including Eβ, Ii and H2-Mα, were all induced in a pRB-independent fashion. Thus, the Aβ locus appears to be uniquely sensitive to the absence of pRB. The same degree of specificity is not seen in human tumor lines, where pRB affects IFN-γ-induction of DRα, DRβ and DPα. (Lu *et al*., 1994b, 1996), although it is not required for induction of the invariant chain (Lu *et al*., 1994a).

The coordinate expression of multiple cII genes, orchestrated by CIITA, is a well-established phenomenon (Mach *et al*., 1996). In addition to CIITA, several ubiquitously expressed factors are thought to have key roles at all cII promoters (Mach *et al*., 1996). Despite these unifying features, however, cII genes also have regulatory controls that influence individual genes. Our results, indicating a pRB-specific effect on Aβ-induction, are one such example. Reconstitution of the RB-deficient H2009 human SCLC line with wild type RB restores IFN-γ-responsiveness at the DRβ locus, but not at the DRα locus, indicating the existence of a specific factor required for induction of DRα (Lu *et al*., 1996). Indeed, several transcription factors have been shown to specifically modulate one or a subset of cII genes (Borras *et al*., 1995; Lloberas *et al*., 1995; Scholl *et al*., 1996). However, other than the current study on RB, no genetic experiments have been carried out to determine if these factors are essential for normal cII-regulation. There are also many examples showing absence or under-representation of DQ despite the expression of other cII molecules (Ameglio *et al*., 1983; Anichini *et al*., 1988; Burmester *et al*., 1987; Gonwa and Stobo, 1984; Lucey *et al*., 1989; Manyak *et al*., 1988; Muller *et al*., 1985; Symington *et al*., 1985). It would be interesting to investigate if pRB is involved in some of these cases, given that the I-A locus is the mouse equivalent of DQ. The numerous examples of non-coordinate and coordinate cII-regulation suggest that the cII genes are equipped to respond, either separately or together, to a variety of stimuli.

The mechanism through which pRB regulates cII-expression is unknown. CIITA-induction is pRB-independent in both mouse (this study) and human cells (Tschickardt *et al*., 1995), indicating that pRB acts in parallel or downstream of this activator. Constitutive expression of mouse cII genes is pRB-independent, as shown by studies using chimeric mice in which B cells are derived purely from $RB^{-/-}$ ES cells injected into $RAG^{-/-}$ blastocysts (Chen *et al*., 1993). This data shows that constitutively expressed CIITA induces Aβ expression in a pRB-independent fashion, while our work shows that IFN-γ-induced CIITA requires pRB. One explanation for this paradox is that constitutively expressed CIITA and IFN-γ-induced CIITA differ in some way that renders the latter isoform pRB-dependent. Interestingly, the human CIITA gene contains four alternative first exons (I–IV), each driven by distinct promoters (Muhlethaler-Mottet *et al*., 1997). Three of these exons (I, III and IV), are conserved in the mouse. Type IV CIITA is the major IFN-γ-induced isoform. Types I and III are constitutively expressed, but type I is confined mainly to dendritic cells, while type III is abundant in all APCs (Muhlethaler-Mottet *et al*., 1997). Different usage of AUG codons is thought to generate constitutively expressed CIITA proteins that are longer than the IFN-γ-induced isoform. This structural difference may render type IV CIITA pRB-dependent at the Aβ promoter. Alternatively, post-translational modifications of CIITA may also generate a pRB-dependent isoform. Testing this hypothesis may not be trivial since we have found that CIITA is insufficient to induce endogenous cII in MEFs (data not shown). It may be possible to use RB-deficient human tumor lines to determine if type IV CIITA induces cII in a pRB-dependent or independent fashion.

Another possibility is that IFN-γ, in addition to inducing CIITA, also stimulates the synthesis of an inhibitory factor that blocks the action of CIITA at the Aβ promoter. In this model, pRB would be required to antagonize the inhibitor. One protein that could fulfill this role is p202; it is induced by IFNs, acts as a transcriptional repressor, and interacts directly with pRB (Choubey and Lengyel, 1995; Choubey *et al*., 1996; Min *et al*., 1996). The inhibitor could also be a protein like PU.1, which specifically represses Aβ expression by binding an element next to the Y box, and also interacts with pRB (Borras *et al*., 1995; Hagemeier *et al*., 1993). However, PU.1 is not expressed in fibroblasts, and binding to pRB has not

been demonstrated *in vivo*. Nevertheless, a related factor could be present in IFN-γ induced fibroblasts. We are currently investigating these 'isoform' and 'inhibitor' models, which, it should be noted, are not mutually exclusive.

One approach to establish how pRB regulates Aβ expression would be to map the pRB-responsive element (RbRE) in the Aβ promoter. We have used Aα and Aβ reporter constructs to investigate IFN-γ-inducibility in $RB^{+/+}$ or $RB^{-/-}$ MEFs. These vectors contain the highly conserved S, X and Y boxes that are essential for expression of all cII genes (Mach *et al*., 1996). Surprisingly, Aα and Aβ reporters were not induced in IFN-γ-treated MEFs, either in an episomal form following transient transfection, or integrated into the genome following stable transfection (data not shown). Others have shown that cII reporter plasmids are IFN-γ-inducible in primary macrophages (Johnson and Adams, 1995), macrophage cell lines, and certain non-lymphoid cells such as epithelial lines (e.g. HeLa and MCT) (Albert *et al*., 1994; Tsang *et al*., 1990). However, in some studies, investigators have resorted to using hybrid promoters in order to generate detectable levels of IFN-γ-inducible promoter activity (Celada *et al*., 1996). These observations suggest that under certain circumstances, possibly due to cell-specific repressors of cII, the classical core cII promoter (S-X-Y boxes) is insufficient to mediate IFN-γ-induction. We are currently delineating the minimal Aβ region required for IFN-γ-induction in MEF cells, which will allow us to map the RbRE.

In summary, we have shown that MHC gene regulation is a *bona fide* function of pRB. This role is more selective in mouse versus human cells, since pRB is specifically required for regulation of Aβ, but not other cII loci. Elucidating the mechanism behind this effect should provide some intriguing links between tumor suppression and immune function.

Materials and methods

Cells and culture conditions

Mouse embryonic fibroblasts (MEFs) were derived from embryonic day 13 $RB^{-/-}$ or $RB^{+/+}$ embryos prior to their death one or 2 days later. MEFs were grown in alpha-MEM medium supplemented with glutamine, 10% FBS, and antibiotics. For IFN-γ induction of cII in MEFs, cells were treated with recombinant murine IFN-γ (Biosource International Canada) at 300 U/ml for 48–72 h. Two hundred and ninety-three cells (human embryo kidney cells, from Microbix Biosystems Inc., Canada) were grown in Iscove's medium supplemented with glutamine and 10% heat-inactivated FBS.

Flow cytometry analysis

MEF cells (each sample of 10^6 cells) were stained for I-A surface expression with FITC-conjugated anti mouse I-Aβ (Pharmingen, USA). The stained cells were analysed by flow cytometry on a FACScan analyser (Becton Dickinson Immunocytometry System), 5000 cells were analysed for each determination.

RT–PCR

RNA was extracted from IFN-γ induced and uninduced MEFs by phenol(PH4)/chloroform extraction. First strand cDNA was synthesized using 200 ng random hexanucleotides and 400 ng of RNA in a 20 μl reaction containing 50 mM Tris-HCL (pH 8.3), 75 mM KCL, 3 mM $MgCl_2$, 10 mM DTT, 1 mM dNTP, and 200 units of M-MLV reverse transcriptase (Canadian Life Technologies Inc.). 1/10 of first strand cDNA was amplified for 30 cycles using the following primer pairs: Aα sense: TCCTGGAGACATTGGCCAGT, Aα antisense: TCTCAGGTTCCCAGTGTTTC; Aβ sense: GGCATTTC-GTGTACCAGTTC, Aβ antisense: AGGTCCTTTCTGA-CTCCTGT; Eβ sense: GGAGAGTTGAGCCTACGGTGA, Eβ antisense: AGACTGTCCTTTCTGGTT-CCT; H2Ma sense: GACCCACAGAACCACACATTC, H2Ma antisense: TTCTCTAGGAGATCCGAAGGC; Ii sense: CTTCCGAA-ATCTGCCAAACCT, Ii antisense: TTCCCTTTAGATG-CGGTGCCA; GBP sense: ACTAGTGATCAACCAGGA-GGC, GBP antisense: AGCCAGTGAAGTCTCCAGGTA; β-actin sense: TGGAATCCTGTGGCATCCATGAAAC, β-actin antisense: TAAAACGCAGCTCAGTAACAGTCCG; CIITA sense: ACCTGGACCTGGACTCACTTA; CIITA antisense: CATCTCAGACTGATCCTGGCA. These primer pairs generate fragments of the following sizes: Aα: 499 bp; Aβ: 670 bp; Eβ: 407 bp; H2M: 578 bp; Ii: 622 bp; GBP: 556 bp; CIITA: 430 bp.

Generation of stable cell lines

$RB^{-/-}$ MEFs were cotransfected using lipofectamine with pBABEpuro, which confers puromycin-resistance, and mgRblox, which contains the Rb1 mouse promoter region driving expression of RB cDNA (gift from E Zacksenhaus) (Zacksenhaus *et al*., 1996), or SVLuc, a luciferase expression vector (Bremner *et al*., 1995). Forty-eight hours after transfection, the cells were detached by trypsin, diluted 1 : 10 or 1 : 200 and replated on 10 cm dishes. Cells were cultured in medium with puromycin for 3 weeks. Cells isolated from single colonies were maintained in puromycin-containing medium. Western analysis of pRB-expression was described (Bremner *et al*., 1995).

Adenovirus propagation and infection

AdΔRB encodes a hemagluttinin (HA)-tagged version of pRB that is constitutively hypophosphorylated (Chang *et al*., 1995a; Hamel *et al*., 1992). AdBgl is a replication defective control adenovirus that does not encode a recombinant protein (Barr *et al*., 1994). High titer stocks were obtained by infecting 293 cells at 90% confluence in 100 mm dishes with virus at 1–10 plaque forming units (p.f.u.) per cell. New virus was titered by plaque assay and stored at $-70^{\circ}C$ in small aliquots. $Rb^{-/-}$ MEF cells were grown in a 24-well plate at 6×10^4 per well, infected with 50 PFU of virus per cell in 200 μl of serum-free medium. After 1 h, 1 ml of growth medium was added and infection continued overnight. Cells were washed, and fresh medium added either with or without IFN-γ (300 U/ml). After 48 h, the extent of infection was determined by immunostaining with an anti-pRB antibody, and cII expression was determined by immunostaining and/or flow cytometry analysis.

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