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Loss of Interferon-γ Inducibility of TAP1 and LMP2 in a Renal Cell Carcinoma Cell Line[1]

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ABSTRACT

The inadequate ability of cancer cells to present antigen on the cell surface via MHC class I molecules is one mechanism by which tumor cells evade antitumor-associated antigen immunity. In many cases, such as in renal cell carcinoma (RCC), the lack of MHC class I antigen presentation can be attributed to the down-regulation of genes needed for antigen processing, such as the transporters associated with antigen processing (TAP)1 and TAP2, and the proteasomal components low molecular weight proteins (LMP)2 and LMP7. The *TAP1* and *LMP2* genes are transcribed from a shared bidirectional promoter containing an IFN response factor element that confers IFN-γ inducibility. Here, we investigate the differential responsiveness to IFN-γ of RCC cell lines, Caki-1 and Caki-2, which have been reported to have abnormally low expressions of TAP1 and LMP2. We now demonstrate that the Caki-2 cell line is defective in the IFN-γ signaling pathway. The effects of IFN-γ on TAP1 and LMP2 expression revealed a loss of up-regulation in Caki-2 cells, but not in Caki-1 cells. *In vivo* DNA footprinting shows a specific loss of occupancy at the IFN response factor element site in Caki-2 cells, whereas Caki-1 cells show full promoter occupancy. Furthermore, *in vitro* DNA-binding studies indicated that Caki-2 cells do not have IFN-regulatory factor 1- or signal transducer and activator of transcription 1 (Stat1)-binding activity after IFN-γ stimulation. Examination of Stat1, Jak1, and Jak2 proteins demonstrated that the proteins were expressed, however, not phosphorylated, upon IFN-γ treatment in Caki-2 cells. Also, this cell line expressed both IFN-γ receptor chains. IFN-γ inducibility could not be rescued by introduction of normal Jak1 and/or Jak2 proteins. However, overexpression of Jak1 did increase TAP1 and LMP2 expression independent of IFN-γ, indicating that the Stat1 and IFN-regulatory factor 1 proteins present in Caki-2 can be activated. These findings suggest that the loss of TAP1 and LMP2 induction is a defect in the earliest steps of the IFN-γ signaling pathway resulting in the inability of Caki-2 cells to up-regulate the MHC class I antigen-processing pathway. Because immunotherapy may be one of the most promising approaches for treating RCC, understanding the mechanisms by which these tumors circumvent cytokine signaling, thereby evading antitumor-specific-antigen immunity, would greatly aid the efficacy of such therapy.

INTRODUCTION

The ability of tumor cells to present tumor-associated antigens on the cell surface via MHC class I molecules is necessary for the generation of an effective antitumor-specific-antigen CTL response. Unfortunately, many tumor cells have lost this ability, thereby evading CTL-mediated immune surveillance and elimination (1, 2). The lack of MHC class I surface expression in many cases can be attributed to the down-regulation of genes needed for antigen presentation such TAP1[3] and TAP2, and the proteasomal components LMP2 and LMP7 (3–6). Reduced expression and function of TAP1 and TAP2 and/or LMP2 and LMP7 have been found in several distinct tumor types, such as SCLC, cervical carcinoma, prostate cancer, melanoma, Burkitt's lymphoma, breast carcinoma, prostate cancer, basophilic leukemia, and RCC (reviewed in Ref. 6).

TAP1, TAP2, LMP2, and LMP7 are encoded in the MHC class II region (7–11). LMP2 and LMP7 are reported to modify the specificity of the proteasome complex by enhancing the production of peptides cleaved after hydrophobic and basic residues (12–14), which are often preferred by the MHC class I molecules (15–17). TAP1 and TAP2 heterodimers then mediate transport of the antigenic peptides into the lumen of the endoplasmic reticulum (18–19). The TAP1 and TAP2 heterodimer is important for MHC class I function because mutant cells that lack TAP1 or TAP2 have a selective inability to present intracellular antigens on MHC class I complexes on the cell surface (7, 9, 20–23). In TAP1- or TAP2-deficient cells, only a limited set of peptides derived from signal sequences is expressed on MHC class I molecules through a TAP-independent pathway (24).

The *TAP1* and *LMP2* genes are transcribed from a shared bidirectional promoter with only 596 bp separating their ATG translation initiation codons (25). The promoter is regulated by three known sequences located at the *TAP1* proximal region: (*a*) an IRF-E; (*b*) a nuclear factor κB binding site; and (*c*) a SP-1 binding site (25, 26). Previously, the IRF-1 protein has been shown to mediate the IFN-γ induction of TAP1 and LMP2 by binding at the IRF-E (26). More recently, Stat1 has been implicated in the regulation of these genes as well (27, 28).

IFN-γ strongly induces transcription of the *IRF-1* gene (29) through binding of a GAS element in the *IRF-1* promoter by activated Stat1α (30). The ligation of IFN-γ to its receptor composed of IFN-γR1 and IFN-γR2 (31) results in the activation and phosphorylation of the associated Janus kinases Jak1 and Jak2 (32). Phosphorylation of the receptor then generates a docking site on IFN-γR1 for latent Stat1α (31). Subsequently, Stat1α is phosphorylated by Jak1 and Jak2 to form the active Stat1α homodimers that can bind the GAS elements in the promoters of IFN-γ-responsive genes such as *IRF-1* (32). Stat1 and IRF-1 both act as transcriptional activators (33, 34), whereas the constitutively expressed IRF-2 generally acts as a transcriptional repressor (35). IRF-1 is expressed at low levels prior to IFN-γ stimulation, and transcription and translation are required for its induction (36).

Down-regulation of *TAP1/LMP2* genes has been demonstrated in multiple tumor types including RCCs (5, 6) and is associated with malignant transformation and disease progression. Pro-inflammatory cytokines, such as IFN-γ, are potent inducers of MHC class I antigens, TAP1/TAP2, and LMP2/LMP7 (37, 38). The TAP expression and function induced by IFN-γ may be involved in augmenting MHC class I-restricted tumor associated antigen-specific CTL recognition of several tumor types such as melanoma, prostate carcinoma, SCLC, and RCC (4, 5, 38, 39). These data suggest that IFN-γ−induced

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[3] The abbreviations used are: TAP, transporters associated with antigen processing; LMP, low molecular weight proteins; RCC, renal cell carcinoma; IRF-E, IFN response factor element; IRF-1, IFN-regulatory factor 1; Stat1, signal transducer and activator of transcription 1; RPA, RNase Protection Assay; DMS, dimethyl sulfate; wt, wild type; mt, mutant; EMSA, electrophoretic mobility shift assays; SIE, serum inducible element; GAS, IFN-γ-activated sequence; SCLC, small cell lung carcinoma.

restoration of antigen-processing machinery such as TAP1 and LMP2 may improve antitumor-specific-antigen CTL recognition in some patients, thus approaches to activate this pathway may be of benefit to patients with TAP and/or LMP deficiencies.

Because the importance and involvement of the IFN-γ-induced transactivators, Stat1 and IRF-1, in the transcriptional regulation of the *TAP1/LMP2* promoter have been established, we wanted to determine whether the loss of TAP1 and LMP2 expression may be attributable to deficiencies of these up-regulatory factors in RCC. Discerning TAP1 and LMP2 transcriptional regulation in tumor cells will provide essential information for the development of genetic and immunotherapeutic strategies to enhance MHC class I-antigen expression to combat cancer.

MATERIALS AND METHODS

Cell Lines and Media. The RCC cell lines, Caki-1 (HTB-46) and Caki-2 (HTB-47), were purchased from ATCC (Manassas, VA) and maintained in McCoy's media (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and nonessential amino acids. HeLa cells were maintained in Eagle's MEM supplemented with 10% fetal bovine serum, L-glutamine (Life Technologies, Inc., Baltimore, MD) and nonessential amino acids. Cell viability was measured using a Cell Titer 96 Aqueous One Solution Assay (Promega, Madison, WI).

RPA. Cells were untreated or treated with 300 units/ml IFN-γ (BioSource International, Camarillo, CA) 18 h before RNA harvest. Total RNA was prepared from $6–10 \times 10^6$ cells using RNeasy kit (Qiagen, Inc., Santa Clarita, CA) following the manufacturer's protocol. The TAP1, LMP2, and GAPDH probes each protect a 120-bp, 195-bp, and 146-bp (respectively) internal fragment of their corresponding mRNAs. Total RNA (10 μg) was hybridized to [α^{32} P]CTP-labeled TAP1, LMP2, or GAPDH probe for 16 h at 42°C and then digested with 2 units of RNaseONE (Promega, Madison, WI) for 30 min at 25°C. RPA proceeded following manufacturer's recommendation and resolved on a 6% polyacrylamide/7.75 M urea gel and quantitated with a storage phosphor screen (PhoshorImager, Molecular Dynamics, Sunnyvale, CA).

***In Vivo* DNA Footprinting.** Caki-1 and Caki-2 cells were untreated or treated with 300 units/ml IFN-γ for 18 h prior to harvesting. *In vivo* methylation and genomic DNA preparation were performed as described previously (40). Ligation-mediated PCR-amplified *in vivo* genomic footprinting was performed as described previously (41) with the primer set R412E (26).

Reporter Constructs and Transfections. The TAP1 (593-1) and LMP2 (1-593) wt and IRF-E mt luciferase constructs were generated by PCR amplification of the promoter region from LMP2-HGH/TAP1-CAT and IRF-E mt bidirectional reporter constructs (26) and cloned into pGL3-Basic (Promega) in both orientations. Plasmid DNA (6 μg total) was introduced into Caki-1 and Caki-2 cells by lipofectin (Life Technologies, Inc.) according to the manufacture's recommendation. To minimize sample variability, quadruplicate lipofectin and DNA amounts were used then divided between the IFN-γ-treated and -untreated cells. All transfections contained 200 ng of TK-RL (Promega) as an internal transfection efficiency control. IFN-γ (final concentration of 300 units/ml; BioSource International) was added 24 h after transfection and the cells incubated for an additional 24 h. Cells were harvested and assayed in Dual Luciferase Passive Lysis Buffer (Promega). Jak1 and Jak2 cotransfections were performed by using the indicated luciferase constructs with 2 μg of the indicated expression constructs (a kind gift from Dr. George R. Stark, Cleveland Clinic Research Foundation, Cleveland, OH) or vector control.

Nuclear Extracts and EMSA. Nuclear extracts were prepared following the method of Dignam *et al.* (42). IFN-γ-induced extracts were prepared from cells incubated with 300 units/ml IFN-γ for 15 min and 2 h for Caki-1 and Caki-2 cells, or 15 min and 5 h for HeLa cells. The gel shift analysis was performed as described previously in Wright *et al.* (25) and used 4 μg of nuclear extract in each reaction. The oligonucleotides used in the EMSA were described previously: guanylate binding protein-GAS (43), T1wtIRF-E and T1mtIRF-E (26), and T1mtGAS and TImtICS1 (27), high-affinity SIE probe (44). The IRF-1 (C-20), IRF-2 (C-19), and Stat1 p84/p91 (E23) antibodies are all rabbit polyclonal antibodies purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). RFX5 antibody was a polyclonal rabbit antiserum specific for the 75-kDa component of the RFX complex on MHC class II and related genes (kindly provided by Dr. J. Ting, University of North Carolina, Chapel Hill, NC; Ref. 45).

Immunoprecipitation and Western Blots. Whole cell extracts from $3–5 \times 10^6$ cells were lysed in buffer containing 50 mM Tris-HCl, 0.1 mM EDTA, 200 mM NaCl, 10% glycerol, 0.5% NP-40, 1 mM sodium orthovanadate, complete protease inhibitor (Roche Diagnostics, Indianapolis, IN), 0.5 mM phenylmethylsulfonyl fluoride, 1 mM DTT. Total cell lysate was precleared with normal rabbit serum (Sigma-Aldrich, St. Louis, MO) and 20 μl of protein-A agarose beads (Pierce Chemical Co., Rockford, IL), then immunoprecipitated with 2 μg of either Jak1 or Jak2 specific antibodies (Santa Cruz Biotechnologies). Samples were resolved on a 10% SDS-polyacrylamide gel, and transferred onto Immobilon-P (Millipore Corp., Bedford, MA). The blots were visualized with a phosphotyrosine antibody (Santa Cruz), and chemiluminescence (ECL-Plus, Amersham-Pharmacia Biotech.). Blots were stripped and reprobed with their respective Jak1 or Jak2 antibodies.

For detection of Stat1, IFN-γR1 chain, and IFN-γR2 chain, 100 μg of whole cell lysates were resolved as above. The blots were incubated with rabbit polyclonal antityrosine phosphorylated-Stat1 (Zymed Laboratories, Inc., S. San Francisco, CA), Stat1 (Santa-Cruz Biotechnologies), IFN-γR1 chain, or IFN-γR2 chain (PBL Biomedical Laboratories, New Brunswick, NJ) antibodies and detected as described above. Detection of IRF-1 and IRF-2 was performed with 50 μg of nuclear extracts and anti-IRF-1 and anti-IRF-2 antibodies (Santa-Cruz Biotechnologies).

Flow Cytometry. Cells were analyzed using a Becton Dickinson FACScan flow cytometer (San Jose, CA) using standard protocols. The anti-IFN-γR1 antibody was the same as used in the Western blots and was labeled with a FITC-conjugated goat antimurine IgG antibody (Sigma-Aldrich).

RESULTS

TAP1 and LMP2 Are Not Induced by IFN-γ in the Renal Cell Line Caki-2. Several human tumors have been shown to express low levels of TAP1 and LMP2 resulting in deficiencies in MHC class I antigens on the cell surface (3, 5, 6, 38, 39). IFN-γ has been established as a potent inducer of MHC class I as well as TAP1 and LMP2 (37, 38). Recently, the RCC cell lines Caki-1 and Caki-2 have been shown to express low levels of TAP1 and LMP2 (5), although their response to IFN-γ had not been investigated. The effects of IFN-γ on TAP1 and LMP2 expression were examined by RPA (Fig. 1). TAP1 and LMP2 message was not induced by IFN-γ treatment in Caki-2 cells (*Lane 5 versus Lane 6*), although Caki-1 cells (*Lane 3 versus Lane 4*) had similar induction when compared with HeLa cells (*Lane 1 versus Lane 2*). This amount of IFN-γ was sufficient to maximally induce the TAP1 promoter in both Caki-1 and HeLa cells (see below) and increasing IFN-γ to 500 units/ml did not induce expression in Caki-2 cells. Both TAP1 and LMP2 displayed two protected bands very similar in size. Both are specific for the mRNA because neither is observed in the y-tRNA control lane (*Lane 7*), and the undigested probe runs much higher in the gel (not shown). The bands likely arise from incomplete digestion and so both were quantitated together. Thus it appeared that Caki-2 cells had lost their ability to up-regulate TAP1 and LMP2 message by IFN-γ.

Previously, *in vivo* DNA footprinting has shown that IFN-γ is able to induce protein/DNA contacts at the IRF-E in the TAP1/LMP2 bidirectional promoter (26). The IRF-E is located upstream of the nuclear factor κB site and GC1 box as diagramed in Fig. 3. We investigated the *in vivo* protein-DNA contacts at these sites. *In vivo* DNA footprinting revealed protection at the IRF-E in Caki-1 cells (Fig. 2, *Lane 2*) and enhancement with IFN-γ treatment for 2 h (*Lane 3, open arrows*). However, Caki-2 cells did not show occupancy at the IRF-E (*Lane 5*) and no change upon IFN-γ treatment (*Lane 6*). These results show differential *in vivo* protein-DNA interactions between the Caki-1 and Caki-2 cell lines at the IRF-E, and the loss of IFN-γ up-regulation of TAP1 and LMP2 in Caki-2 cells may be attributable

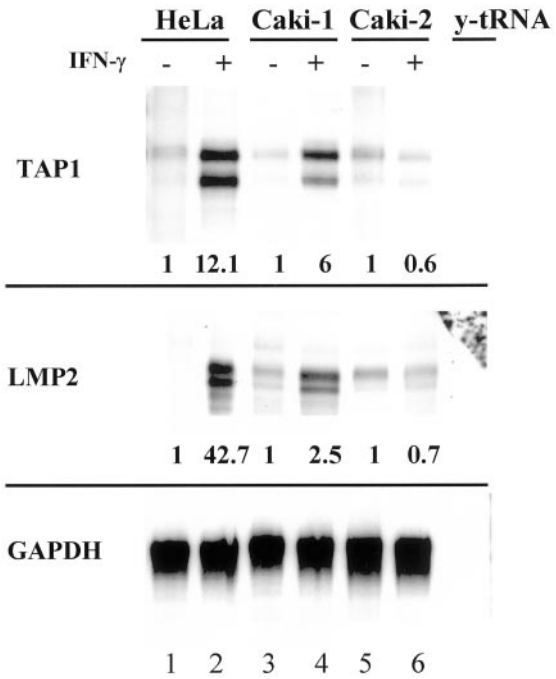

Fig. 1. Lack of TAP1 and LMP2 induction by IFN-γ in the renal carcinoma cell line Caki-2. Ten μg of total RNA from untreated (−) or 18-h IFN-γ (300 units/ml)-treated (+) HeLa, Caki-1, and Caki-2 cells were analyzed by RPA as described in "Materials and Methods." The two bands for TAP1 and LMP2 both are specific for the mRNA and are quantitated together. GAPDH probe served as a control for gel-loading and the yeast tRNA (*y-tRNA*) as a control for nonspecific binding. Quantitation of the bands was performed by scanning the gels with a Phosphor Imager and the values corrected *versus* GAPDH. Values represent fold induction for each cell line.

to the loss of this interaction. A similar pattern of protections at two GC boxes adjacent to the IRF-E was observed in both Caki-1 and Caki-2 cell lines. This indicates that the loss of IRF-E binding is specific and is not attibutable to a general inaccessibility of the promoter in Caki-2 cells.

Loss of IFN-γ-induced TAP1 and LMP2 Promoter Activity in Caki-2. Previously, the IRF-E was shown to be required for TAP1 and LMP2 up-regulation by IFN-γ (26). To determine whether IFN-γ could activate the TAP1/LMP2 promoter in the renal cell lines, Caki-1 cells and Caki-2 cells were transiently transfected with TAP1 and LMP2 wt and IRF-E mt promoter-luciferase constructs. IFN-γ elicited a three-fold induction of TAP1 promoter activity (Fig. 3) and a two-fold induction of LMP2 in Caki-1 cells consistent with previous findings in HeLa cells (26). However, no induction was observed in Caki-2 cells. Mutation of the IRF-E abolished the ability of IFN-γ to induce TAP1 and LMP2 expression in Caki-1 cells. These data are consistent with the endogenous mRNA levels showing coordinate activation of TAP1 and LMP2 in Caki-1 cells, but not in Caki-2 cells. Interestingly, mutation of the IRF-E site reduced the basal expression levels in both cell lines indicating that this site also plays a role in constitutive expression. These findings indicate that the TAP1/LMP2 promoter is IFN-γ-inducible in Caki-1 cells, but not in Caki-2 cells.

Caki-2 Cells Lack IRF-1 DNA-Binding Activity. The identity of the proteins that interact with the IRF-E in Caki-1 and Caki-2 cells was characterized *in vitro*. EMSA combined with antibody reactivity were performed with nuclear extracts from Caki-1 and Caki-2 cells unstimulated or stimulated with IFN-γ for 15 min and 2 h and incubated with a TAP1/LMP2 IRF-E oligonucleotide. Two closely migrating specific complexes (indicated as IRF-2) were observed with the uninduced extracts in both Caki-1 cells (Fig. 4*A*, *Lane 1*) and Caki-2 cells (Fig. 4*B*, *Lane 1*). Nuclear extracts from 2 h IFN-γ induced Caki-1 cells produced an additional prominent complex (indicated as IRF-1) and a minor more slowly migrating complex (indicated with an *arrowhead*; Fig. 4*A*, *Lane 1 versus Lane 11*). No change was observed with Caki-2 extracts (Fig. 4*B*, *Lane 1 versus Lane 11*). Antibody-blocking results indicated that both cell lines had constitutive IRF-2-binding in both untreated and IFN-γ-treated extracts (Fig. 4*A* and *B*, *Lanes 3*, *8*, *13*). Preincubation of 15 min induced Caki-1 nuclear extracts (Fig. 4*A*, *Lane 9*), but not Caki-2 extracts (Fig. 4*B*, *Lane 9*), with anti-Stat1 antibody produced a slow mobility band, which may be the result of the antibody's ability to stabilize the weak association of Stat1 to the probe. Incubation of anti-IRF-1 antibody to the 2 h induced extracts resulted in a shift of both the induced complexes from Caki-1 cells (Fig. 4*A*, *Lane 12*), but not Caki-2 cells (Fig. 4*B*, *Lane 12*). The irrelevant antiserum, anti-RFX5, did not effect the formation of the complexes (Fig. 4*A* and *B*, *Lanes 5*, *10*, and *15*). The specificity of the induced complex for binding to the TAP1/LMP2 IRF-E site *in vitro* was assessed by oligonucleotide competition assays using nuclear extracts from Caki-1 cells induced with IFN-γ for 2 h (Fig. 4*C*). The one prominent inducible protein/DNA complex previously indicated as IRF-1 was clearly visible (*Lane 1 versus Lane 2*). This complex was specifically abolished by competition with 100-fold molar excess of unlabeled wt oligonucleotide (T1wtIRF-E; *Lane 2 versus Lane 4*). Competition with oligonucleotides containing mutations in the IRF-E consensus sequence (T1mtIRF-E and T1mtICS, *Lanes 5* and *7*) did not significantly compete the complex, indicating that the IRF-1 complex requires this consensus sequence. A mutation outside of the IRF-E consensus (*Lane 6*) was still competent to bind IRF-1. The consensus Stat1

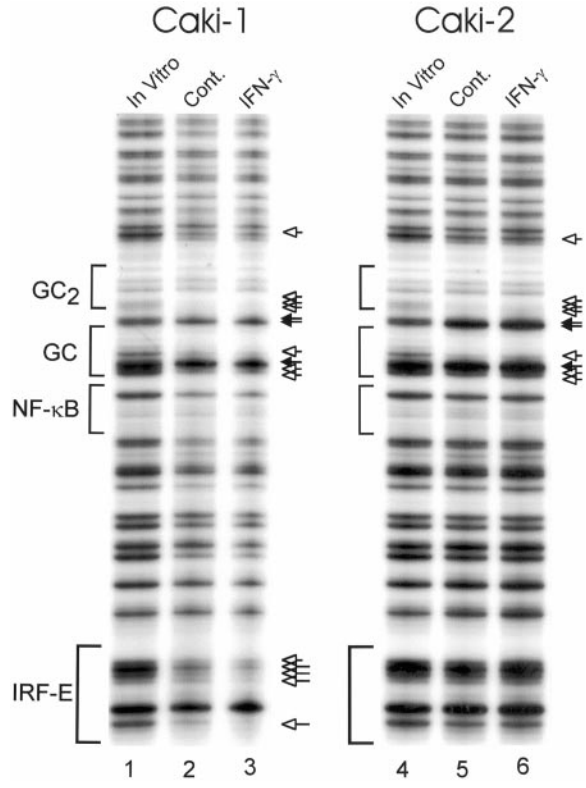

Fig. 2. Differential occupancy at the IRF-E of the TAP1/LMP2 bidirectional promoter in Caki-1 and Caki-2 cells as analyzed by *in vivo* footprinting. Cells were untreated (*Lanes 2* and *5*) or treated with 300 units/ml IFN-γ for 18 h (*Lanes 3* and *6*). Cells were incubated *in vivo* with DMS, which methylates the unprotected G residues, and harvested. *In vitro* treated DNA (*Lanes 1* and *4*) was incubated with DMS following deproteination. Ligation-mediated PCR-amplified genomic footprinting was performed with a primer set that reveals the promoter region surrounding the IRF-E site. The *open arrows* indicate guanine residues protected from DMS methylation by protein/DNA interactions in the live cell. *Closed arrows* indicate enhanced DMS reactivity protein binding. The transcription factor binding sites are indicated on the *left* of each panel marked with a bracket and the name.

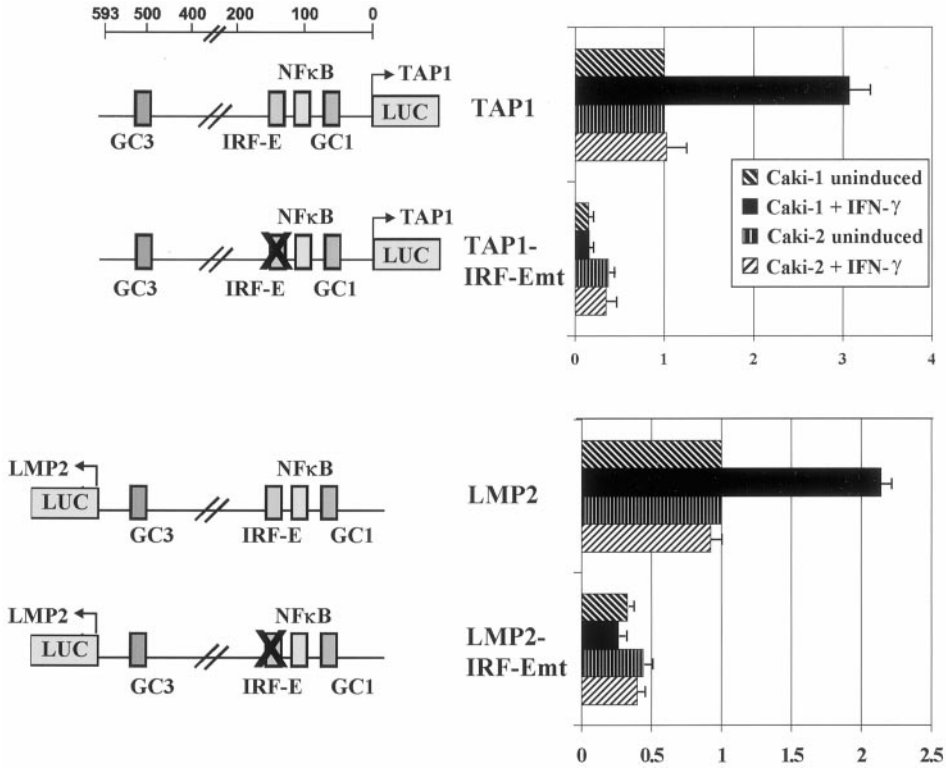

Fig. 3. Differential activity of IFN-γ induced TAP1 and LMP2 wt and IRF-E mt promoter in Caki-1 and Caki-2 cells. Diagrams of the TAP1 and LMP2 wt (TAP1 593-1/pGL3 and LMP2 1-593/pGL3) and their respective IRF-E mt promoter constructs are shown. The Caki-1 or Caki-2 cells were transfected, IFN-γ was added 24 h later and the cells incubated an additional 24 h prior to harvesting. Cotransfections with TK-RL were performed to normalize transfection efficiency. Results are shown as relative TAP1 (*upper graph*) or LMP2 (*lower graph*) activity, normalized to constitutive TAP1 or LMP2 luciferase activity determined separately for Caki-1 and Caki-2 cells. Results shown are the average of three independent experiments, with the error bars representing the SE.

binding site, guanylate binding protein-GAS (*Lane 3*) also was an ineffective competitor when compared with the wt competitor. These results are consistent with the *in vivo* DNA footprinting data showing a lack of occupancy at the IRF-E in Caki-2 cells (Fig. 2, *Lane 6*). Thus, apparently the inability of Caki-2 cells to induce TAP1 and LMP2 expression may be attributed to a loss of IRF-1-binding and potentially the loss of the weak Stat1-binding.

Stat1 Is Not Activated by IFN-γ in Caki-2. The lack of the IRF-1-binding at the IRF-E and loss of IFN-γ-mediated IRF-1 induction led us to investigate where in the pathway the defect may reside. Because IRF-1 is transcriptionally up-regulated by IFN-γ through Stat1, we examined the IFN-γ-induced activity of Stat1 by EMSAs and supershifts. Because HeLa cells were used originally for elucidating the regulation of the TAP1/LMP2 bidirectional promoter by IFN-γ (26), they were used as a positive control. Nuclear extracts were prepared from Caki-1 and Caki-2 cells treated with 300 units/ml IFN-γ for 15 min and 2 h, and incubated with a radiolabeled SIE probe derived from the *c-fos* gene that contains a high-affinity binding site for Stat1α homodimers (46). HeLa (Fig. 5, *Lane 1 versus Lane 4*) and Caki-1 (*Lane 7 versus Lane 10*) extracts produced a single IFN-γ-induced complex at 15 min, whereas Caki-2 (*Lane 13 versus Lane 16*) extracts had no complex formation. To specifically identify the protein in the complex, supershifts were performed. Preincubation with an anti-Stat1 antibody with the HeLa and Caki-1 IFN-γ induced nuclear extracts resulted in a completely shifted complex (*Lane 4 versus Lane 5* and *Lane 10 versus Lane 11*, respectively), the irrelevant antiserum, anti-RFX5, had no effect on the formation of the complex (*Lane 4 versus Lane 6*, and *Lane 10 versus Lane 12*). Nuclear extracts from induced Caki-2 cells had no complex to shift with anti-Stat1 antibody, indicating that this protein is absent (*Lane 16 versus Lane 17*). Thus Caki-2 cells lack functional IFN-γ-induced Stat1 activity, which may account for its deficiency.

Lack of IFN-γ-mediated IRF-1 Induction or Stat1 Phosphorylation in Caki-2 Cell Line. The loss of IRF-1-binding activity in Caki-2 cells suggested that IRF-1 protein may be absent in this cell line. Therefore Western blots were used to assess the levels of this protein. Caki-1 cells show low basal expression of IRF-1, yet significant IFN-γ-mediated induction of IRF-1 expression (Fig. 6*A*, *Lane 1 versus Lane 2*, *upper panel*). In comparison, Caki-2 cells demonstrate complete loss of both constitutive and stimulated IRF-1 (*Lanes 3* and *4*, *upper panel*). Nevertheless, IRF-2 is expressed in both cell lines (*Lanes 1-4*, *lower panel*). These findings are consistent with the antibody blocking experiments (Fig. 4*A* and *B*).

To determine whether the loss of IRF-1 induction and Stat1-binding activity was attributable to the lack of Stat1 phosphorylation or the absence of Stat1 protein, Western blots were used. Cell lysates from IFN-γ treated HeLa, Caki-1, and Caki-2 cells were run on a SDS-PAGE and blotted with anti-phospho-Stat1 antibody. As expected, IFN-γ was able to induce phospho-Stat1 levels in HeLa cells (Fig. 6*B*, *Lane 1 versus Lane 2*, *upper panel*) and Caki-1 cells (*Lane 3 versus Lane 4*, *upper panel*). In contrast, Caki-2 phospho-Stat1 levels remained unchanged compared with uninduced levels (*Lane 5 versus Lane 6*, *upper panel*). A similar level of phospho-Stat1 in uninduced Caki-1 and Caki-2 cells was detected (*Lanes 3* and *5*, *upper panel*). In addition, the total amount of Stat1 protein in Caki-1 and Caki-2 cells was similar (*Lanes 3* and *5*, *lower panel*). IFN-γ treatment did not change expression of Stat1 protein in any of the cell lines examined. Although the levels of constitutive phospho-Stat1 in Caki-1 and Caki-2 cells were similar to induced levels in HeLa cells (*Lanes 3* and *5 versus Lane 2*), no Stat1 DNA binding activity was observed with uninduced nuclear extracts (Fig. 5, *Lanes 7* and *13*). The use of whole cell lysates for the Western blots and nuclear extracts for the DNA binding activity may explain this discrepancy. Potentially Stat1 may also be activated by other growth factors or cytokines and be associated in other transcription complexes. The loss of responsiveness of Caki-2 cells to IFN-γ, therefore, may not be attributed to a deleted or absent Stat1, but rather may lie upstream of the pathway in Jak1 or Jak2 or in the IFN-γ receptor.

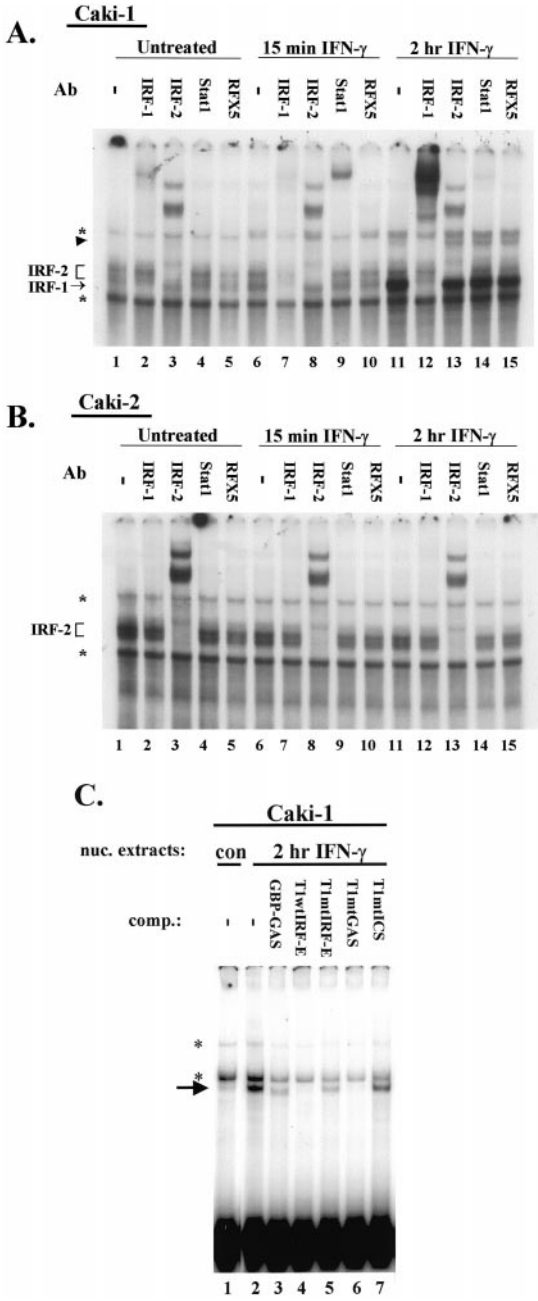

Fig. 4. Caki-2 cells lack IRF-1 binding at the TAP1/LMP2 IRF-E. Caki-1 (*A*) and Caki-2 (*B*) nuclear extracts were tested for *in vitro* DNA-binding activity with the TAP1/LMP2 IRF-E probe by electrophoretic mobility shift assays and the complexes identified with specific antibodies. Nuclear extracts were prepared from cells untreated or treated with 300 units/ml IFN-γ for 15 min or 2 h and incubated with or without the indicated antibodies for 2 h at 4°C prior to addition of probe. IRF-1- and IRF-2-containing complexes are indicated; in addition, the minor complex reactive with the IRF-1 antibody is demarcated with a *solid arrowhead*. *Asterisks* identify nonspecific complexes. Anti-RFX5 antibody was used as an irrelevant antibody to control for nonspecific binding. *C,* specific competition of the IRF-1 protein bound to the TAP1/LMP2 IRF-E oligonucleotide. A 100-fold excess of the indicated competitors was coincubated for 30 min with the probe and 4 μg of nuclear extracts prepared from untreated Caki-1 cells (*Lane 1*) or 2 h with IFN-γ-treated Caki-1 (*Lanes 2-7*) and resolved by EMSA as in *A.*

Loss of IFN-γ-induced Jak1 and Jak2 Phosphorylation in Caki-2 Cells. Because the tyrosine phosphorylation of Jak1 and Jak2 induces Stat1 activation via the IFN-γ signaling pathway, we examined the presence and phosphorylation status of these proteins. Thus, immunoprecipitation of whole cell lysates from 15-min IFN-γ-stimulated cells was performed with either Jak1 or Jak2 specific antibodies and probed with an antiphosphotyrosine antibody. Caki-1 cells showed induced tyrosine phosphorylation of Jak1 (Fig. 7, *Lane 1 versus Lane 2, upper panel*) and Jak2 *(Lane 1 versus Lane 2, lower panel*), whereas no phosphorylation was evident from Caki-2 lysates (*Lane 3 versus Lane 4, upper panel*) and (*Lane 3 versus Lane 4, lower panel*). These results also confirm the differential ability of these RCC cell lines to respond to IFN-γ. The blots were stripped and reprobed with their respective anti-Jak1 or anti-Jak2 antibodies to detect the presence of protein. Although Caki-2 cells showed a lack of Jak1 and Jak2 tyrosine phosphorylation, reprobing the blots revealed expression of 135-kDa- and 130-kDa-sized proteins of Jak1 (Fig. 7, *upper panel*) and Jak2 (*lower panel*), respectively. IFN-γ stimulation did not alter the levels of Jak1 or Jak2 in either cell line.

Although we have shown both Jak1 and Jak2 proteins are expressed in the Caki-2 cell line, and that they have molecular weights indistinguishable from those in the Caki-1 cell line, that does not exclude a possible mutation that may leave them dysfunctional. Shown previously, IFN-γ responsiveness was restored in mt human cell lines

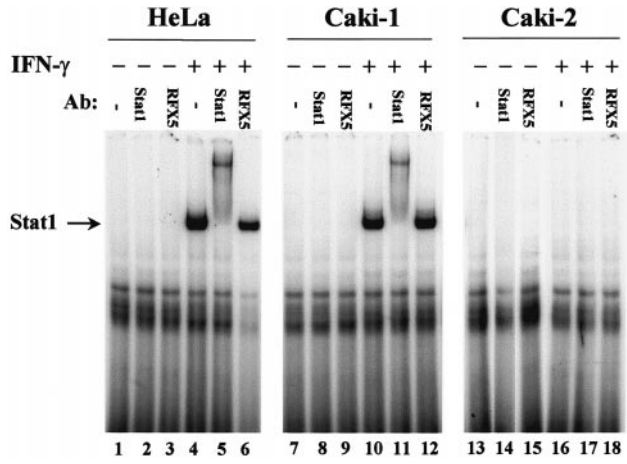

Fig. 5. Loss of Stat1-binding to the SIE probe in Caki-2 cells. HeLa, Caki-1, and Caki-2 nuclear extracts from unstimulated (−) and 15-min-IFN-γ-stimulated (+) cells were used in an EMSA assay with a SIE probe, which is a high-affinity binding site for Stat1 homodimers. Extracts were incubated with the indicated antibodies for 2 h at 4°C prior to addition of probe and resolved by PAGE.

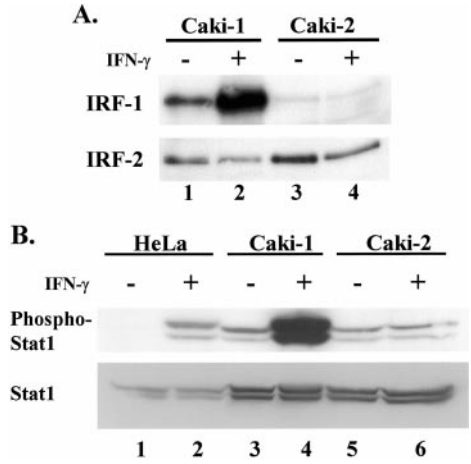

Fig. 6. Lack of IRF-1 protein and Stat1 phosphorylation in the Caki-2 cell line. *A,* nuclear extracts were prepared from uninduced (−) or 2-h-IFN-γ-induced (+) Caki-1 and Caki-2 cells, and 50 μg were resolved by 10% SDS-PAGE. The levels of IRF-1 and IRF-2 were assayed by Western blot analysis. The blots were probed with an anti-IRF-1-specific antibody (*upper panel*) and then reprobed with an anti-IRF-2-specific antibody (*lower panel*). *B,* whole cell lysates (100 μg) prepared from HeLa, Caki-1, and Caki-2 cells untreated (−) or stimulated (+) for 15 min with IFN-γ (300 units/ml) were resolved on a 10% SDS-PAGE and Western blotted with a phospho-Stat1-specific antibody (*upper panel*). The blot was stripped and reprobed with a Stat1-specific antibody (*lower panel*).

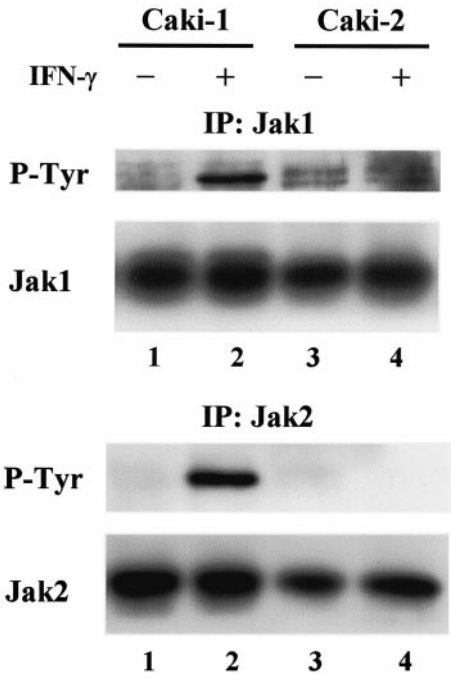

Fig. 7. IFN-γ does not activate Jak1 or Jak2 in Caki-2. Cells were treated for 15 min with 300 units/ml IFN-γ (+) or left untreated (−) prior to harvesting cell lysates. Lysates were immunoprecipitated with Jak1- or Jak2-specific antibodies and Western blotted with an antiphosphotyrosine-specific antibody. The blots were stripped and reprobed with their respective Jak-specific antibodies.

with defective Jak1 and Jak2 by transfection of murine *Jak1* and *Jak2* cDNA expression constructs (47, 48). To evaluate whether the transcriptional response of IFN-γ could be rescued in Caki-2 cells, murine *Jak1* and/or *Jak2* expression vectors were transiently cotransfected with TAP1 and LMP2 luciferase reporter constructs. Exogenous Jak1 and/or Jak2 were unable to restore IFN-γ inducibility of the TAP1 or LMP2 promoter as detected by luciferase activity (Fig. 8). However, Jak1 was able to increase constitutive TAP1 and LMP2 promoter activity compared with pCDNA control. Overexpression of murine Jak1 and Jak2 by transient transfection has been shown previously to lead to tyrosine autophosphorylation of Jak1 and Jak2 and to activate Stat1α, as determined by DNA binding (49). Thus, the downstream IFN-γ signaling factors, Stat1 and IRF-1, are functional. Therefore, the inability of Caki-2 cells to respond to IFN-γ stimulation may not be the result of an absence or defect of these signaling factors.

Caki-2 Cells Express IFN-γR1 Chain and IFN-γR2 Chain. To establish whether lack of IFN-γR1 or IFN-γR2 chain expression may be responsible for the absence of IFN-γ-mediated signal transduction pathway, Western blots were performed. The anti-IFN-γR1-specific antibody and the anti-IFN-γR2-specific antibody detected an identical 90-kDa protein (Fig. 9*A*, *upper panel*) and 65-kDa protein (*lower panel*), respectively, in each of the cell lines consistent with their expected molecular weights. IFN-γ stimulation had no effect on expression levels compared with lysates from unstimulated cells. The blots revealed that both receptor components were expressed in Caki-2 cells at similar levels and with similar molecular weights to those in Caki-1 and HeLa cells.

To examine the expression of IFN-γR1 on the cell surface FACS analysis was used with an anti-IFN-γR1 antibody. Caki-1 cells showed greater cell surface expression of the IFN-γR1 chain than either HeLa or Caki-2 cells (Fig. 9*B*). Caki-2 and HeLa cells had similar levels of surface expression of the IFN-γR1 chain. Because Caki-2 cells had relatively comparable surface expression of the IFN-γR1 chain to HeLa cells, the loss of IFN-γ responsiveness in the Caki-2 cell line may not be attributable to inadequate surface expression of this IFN-γ signaling component.

DISCUSSION

TAP1 and LMP2 serve important roles for the cell-surface expression of peptides on HLA class I molecules. Several tumors have deficiencies of TAP1 and LMP2, suggesting a mechanism for the reduction of MHC class I surface expression (3, 5, 6, 38, 39). The down-regulation of *TAP1* and *LMP2* genes may be one of the mechanisms tumor cells use to evade immunosurveillance. Here, we show that a RCC cell line, Caki-2, has lost its ability to induce TAP1 and LMP2 expression by IFN-γ, which may be attributed to a defect in the IFN-γ signaling pathway. This defect has been localized upstream of Jak1 and Jak2 because transfections with their expression vectors could not restore TAP1 or LMP2 induction by IFN-γ (Fig. 8). Western blots revealed that Caki-2 cells were able to express both chains of the IFN-γR, and FACS analysis showed that the IFN-γR1 chain was expressed on the cell surface. However, these data do not exclude the possibility of a mutation in or aberrant expression of the IFN-γR1 chain or the IFN-γR2 chain in Caki-2 cells leading to the lack of IFN-γ sensitivity. Abnormalities in the IFN-γ receptor components could prevent the transactivation of IRF-1 by Stat1α and the subsequent up-regulation of TAP1 and LMP2 in Caki-2 cells.

Defects in the IFN-γ signaling pathway, either naturally occurring or artificially created, have demonstrated the essential role of each signaling component in host resistance to microbial pathogens; however, less is known about the consequences in antitumor-specific antigen immunity. In patients, IFN-γR1 mutations and IFN-γR2 mutations lead to severe disseminated infections with nontuberculous *Mycobacterium* (50–53). IFN-γ has been shown to function to aug-

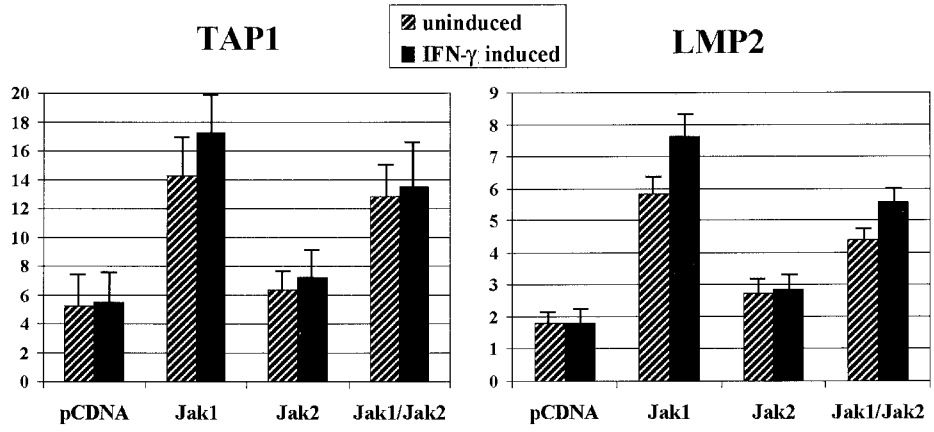

Fig. 8. Overexpression of Jak1 or Jak2 does not restore induction of TAP1 and LMP2 expression by IFN-γ. Caki-2 were cotransfected with either of pCDNA control (2 μg), murine Jak1 (2 μg), Jak2 (2 μg), or the combination of both Jak1 and Jak2 (1 μg each) expression vectors as well as TAP1 (593-1) or LMP2 (1-593) luciferase reporter constructs (4 μg). IFN-γ was added 24 h after transfection and the cells incubated an additional 24 h prior to harvesting. Cotransfections with TK-RL (200 ng) were performed to normalize transfection efficiency. Results are shown as relative luciferase units, and are the average of three experiments. *Bars*, SE.

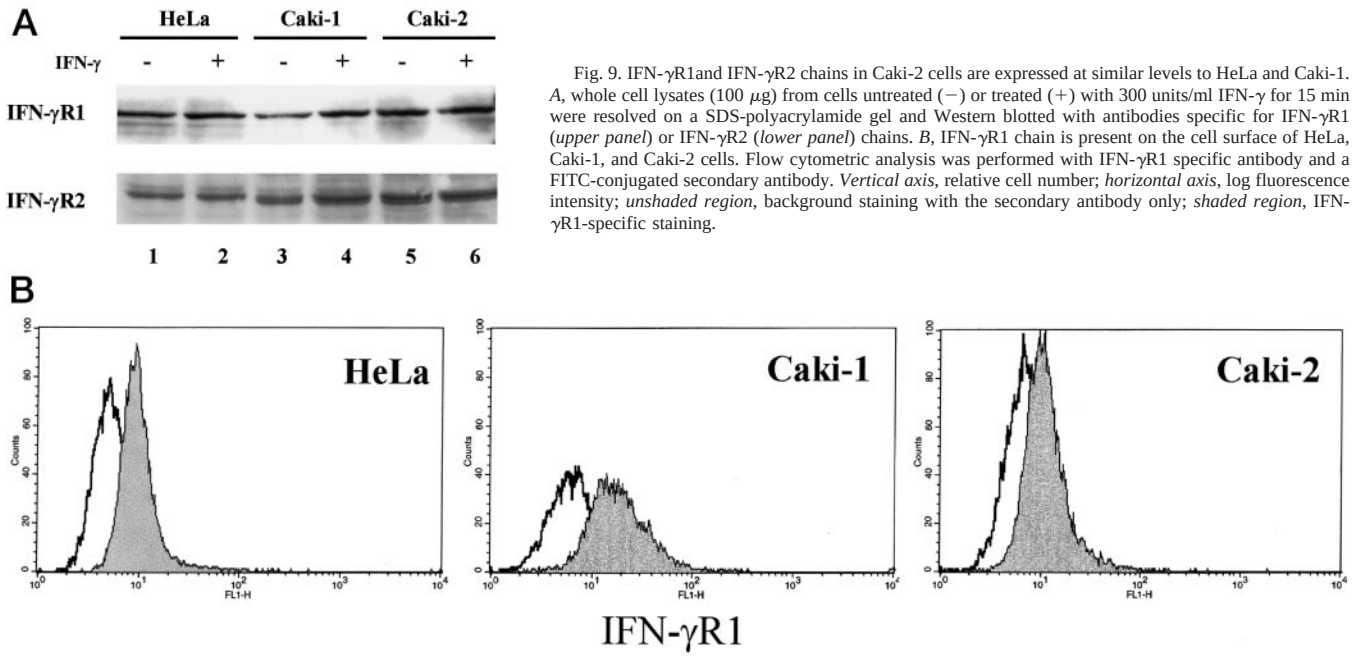

Fig. 9. IFN-γR1and IFN-γR2 chains in Caki-2 cells are expressed at similar levels to HeLa and Caki-1. *A*, whole cell lysates (100 μg) from cells untreated (−) or treated (+) with 300 units/ml IFN-γ for 15 min were resolved on a SDS-polyacrylamide gel and Western blotted with antibodies specific for IFN-γR1 (*upper panel*) or IFN-γR2 (*lower panel*) chains. *B*, IFN-γR1 chain is present on the cell surface of HeLa, Caki-1, and Caki-2 cells. Flow cytometric analysis was performed with IFN-γR1 specific antibody and a FITC-conjugated secondary antibody. *Vertical axis*, relative cell number; *horizontal axis*, log fluorescence intensity; *unshaded region*, background staining with the secondary antibody only; *shaded region*, IFN-γR1-specific staining.

ment the immunogenicity of certain tumor cells (54). IFN-γ sensitivity by the tumor was shown to be required for the enhancement of tumor immunogenicity, thereby promoting development of tumor-specific immune response. Although the possibility may exist that the loss of IFN-γ sensitivity of the Caki-2 RCC cell line may have occurred during its time in culture, no selective pressures *in vitro* have been placed on this cell line from culturing it with IFN-γ. This finding can be further supported by the identification of other cell lines with IFN-γ insensitivity. Recently the incidence of IFN-γ unresponsiveness in human tumors was examined in several melanoma and lung tumor cell lines, which revealed that approximately 33% of each group exhibited a reduction in IFN-γ sensitivity (55). Four other lung carcinoma cell lines demonstrated a complete inability to develop a biological response to IFN-γ, as well as a loss of IFN-γ signaling that could be attributed to an absence or abnormality of expression of one of the IFN-γ-signaling components: IFN-γR1, Jak1, or Jak2 (55). Thus, tumors that develop from IFN-γ-unresponsive tissues may be able to circumvent detection and rejection by the host immune system.

This study is consistent with the original examination in which IRF-1 binding to the IRF-E is essential for TAP1 and LMP2 up-regulation by IFN-γ (26). The absence of TAP1 and LMP2 induction in Caki-2 cells clearly demonstrates the necessity of IRF-1 in the transactivation of their promoter. Another interesting finding is the involvement of the IRF-E in basal expression of these genes as well. Mutation of the IRF-E greatly diminished the constitutive expression of both TAP1 and LMP2 as indicated by the transfection experiments (Fig. 3). Stat1 binding to a GAS element overlapping the IRF-E site has been reported (27, 28), however our findings in RCC suggest this interaction is weak. Incubation of an oligonucleotide that contained both the TAP1/LMP2 IRF-E as well as the putative GAS site with extracts from 15-min IFN-γ-induced Caki-1 cells did not produce a specific band (Fig. 4*A*, *Lane 6*). Only with the addition of an anti-Stat1-specific antibody was a complex detectable, possibly a weak but potentially important, Stat1/DNA interaction that was stabilized by the antibody *in vitro* (Fig. 6).

RCC is a devastating disease with half of the patients who die from RCC having advanced incurable disease at the time of diagnosis (56). Immunotherapy is currently the most promising treatment for RCC. Several cytokines with growth-inhibitory and immunomodulatory properties such as IFN-α and IFN-γ have been widely tested in clinical trials with response rates of 15% (56). IFNs have antitumor activities, which may be mediated by a direct cytotoxic effect on tumor cells or activation of T lymphocytes and natural killer cells, and through augmentation of tumor immunogenicity by up-regulation of MHC complexes, antigen processing machinery, and tumor-associated antigens. However, the molecular and clinical characteristics of the responses of RCC to IFN therapies have yet to be defined. The IFN-γ-induced expression of TAP has been demonstrated to enhance tumor-specific, MHC class I restricted CTL recognition of melanoma, SCLC, prostate carcinoma, and RCC (4–6, 38, 39). The up-regulation of MHC class I antigen processing machinery, including TAP1 and LMP2, may be one of the strategies in which these tumors enhance antineoplastic immunity. Recently, gene transfer of *TAP1* was shown to induce immunogenicity of a human RCC cell line (57). The loss of IFN-γ sensitivity as a result of a defect in the signaling pathway would obviously compromise the efficacy of IFN-γ therapy. This is a potentially important mechanism, which tumors may use to evade and escape the antineoplastic immune response. Thus, discerning TAP1 and LMP2 regulation and understanding the mechanisms by which tumors circumvent cytokine signaling may provide essential information for the development of genetic and immunotherapeutic strategies to enhance MHC class I expression to combat cancer.

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