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# IFN-γ-Mediated Transcriptional Induction of the IDO (Indolamine 2,3-Dioxygenase) Gene Requires Activity of p68/PKR Protein Kinase

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**Abstract:** We investigated whether the activity of double-stranded RNA activated kinase (p68/PKR) is necessary for induction of IDO (indolamine 2,3 –dioxygenase) gene by IFN-γ. For this purpose, we planned to abrogate the function of cellular wt-PKR gene. Clones of ME180 cells expressing the dominant negative form of PKR were selected in the presence of neomycin. Then these cells were treated with IFN-γ and accumulation of IDO protein was determined by Western blot analysis. We found that clones expressing the dominant negative form of the PKR gene were not only resistant to IFN-γ-mediated killing, but were also incapable of inducing accumulation of IDO protein after IFN-γ treatment.

**Key Words:** IFN, p68/PKR, IDO, transcription

## Introduction

Interferon was discovered as an antiviral agent during studies on viral interference. Viral infection of eucaryotic cells causes early and transient expression of various cytokine and chemokine genes that constitute a crucial step in stimulation of interferon (IFN) genes and the subsequent activation of other genes stimulated by IFNs. There are two groups of IFNs. Type I IFNs are IFN-α and –β, type II interferon is IFN-γ. Type I IFNs are produced by leucocyte and fibroblast, respectively, in response to virus infection, whereas type II IFN is produced by natural killer cells and T-cells in response to mitogenic or antigenic stimuli (1,2).

Released interferons show their effects by binding to specific receptors on the cell surface. While IFN-α and -β bind to the same receptor and induce activation of similar sets of genes, IFN-γ binds to different receptors and induces activation of different sets of genes. IFN-γ receptor is composed of two subunits, IFNGR1, 2. IFN-γ binding to its receptor induces heterodimerization of the receptor (3,4). JAK1 and JAK2 tyrosine kinases bind to heterodimerized receptors and subsequently phosphorylate latent cytoplasmic transcription factors, STATs. Phosphorylated forms of STATs translocate to the nucleus and bind to cis-acting elements, designated the gamma-activated sequence, GAS, that is commonly found in IFN-γ-inducible genes, such as IDO (Indolamine 2,3-dioxygenase), GBP (Guanylate Binding protein), MHC-I,II (Major Histocompability Complex) and IRF-1 (Interferon Regulatory Factor-1) (5-10).

PKR is an IFN-inducible, dsRNA-dependent protein kinase (11,12). In IFN-treated cells, PKR is found predominantly in the cytoplasm and is associated with ribosomes; however, small amounts of PKR have also been shown in the nucleus (13). PKR is activated by autophosphorylation after binding to dsRNA, heparin, chondrotin sulfate and poly I: C, all of which exhibit an anionic character. Following activation, PKR catalyzes the intermolecular phosphorylation of at least six protein substrates: the PKR protein itself, the α-subunit of eIF-2, the IkB, the Tat protein of HIV, the NFAT, and MPP4 (14). Protein synthesis initiation factor 2, factor eIF-2, is the best characterized PKR substrate so far. Phosphorylation of eIF-2 by PKR leads to an inhibition of translation by impairing the eIF-2B-catalyzed guanine nucleotide exchange reaction (15). In addition to playing a role in IFN's action, PKR is also implicated in the control of cell proliferation (16,17).

Indoleamin-2,3-dioxygenase is one the best known IFN-γ inducible genes. The product of IDO gene degrades cellular tryptophan into kynurenin and formylkynurenin and this causes cell death because of tryptophan starvation. This is one of the best known mechanisms explaining the antiviral and antiproliferative effects of IFN-γ (10,18,19).

Several studies have established that changes in protein phosphorylation mediated by PKR play an important role in the antiviral action as well as the inhibition of cell proliferation mediated by IFNs. In this regard, we have previously shown that IFN-γ induces phosphorylation of this kinase. We have also shown that IFN-γ-mediated induction of IDO transcription was suppressed in the presence of PKR inhibitor, 2-aminopurine (10).

In this study, we used a genetic approach to further investigate the involvement of PKR activity in IFN-γ-mediated induction of IDO gene transcription as well as in IFN-γ-mediated cytotoxicity. We used an eucaryotic expression vector expressing a dominant negative allele of PKR, which was previously used and claimed to have tumor suppressor function (16,17). We found that both activities of IFN-γ can be ablated by overexpression of the dominant negative form of PKR in the IFN-γ sensitive human cervical carcinoma cell line, ME180.

## Materials and Methods

### Cell Culture and Transfection

Cell line, ME180 (human cervical carcinoma cell line), plasmid carrying the dominant negative form of PKR and antibodies to IDO and GAPDH were provided by Milton W. Taylor (Indiana University, Bloomington, Indiana/USA). ECL and PVDF membrane were purchased from Amersham, USA. All the chemicals used for electrophoresis were purchased from Sigma. Cells were grown in DMEM (D-5280, Sigma) medium supplemented with 10% FCS (fetal calf serum) and 1% Antibiotic-Antimycotic solution, at 37 °C in 5% $CO_2$ medium. Plasmid was transfected into ME180 cells by calcium-phosphate transfection and neomycin-resistant clones were selected in medium containing 400μg/ml of neomycin.

### Cytotoxicity Assay

ME180 cells were grown until they completely covered the bottom of 96-well plates. Cells were treated with various doses of IFN-γ. Treated cells were incubated for 72 hours at 37 °C in humidified 5% $CO_2$ atmosphere. Following incubation, floating cells were removed and attached cells were stained with crystal violet (0.5% in methanol) and dye absorbed by live cells was extracted with sodium citrate (0.1 M) in 50% ethanol. Absorbance was read at 600 nm. Absorbance values of samples were normalized to that of untreated cells to obtain percentage cytotoxicity.

### Western Blot Analysis

Cell extract was prepared in Triton X-100 buffer (50 mM Hepes pH: 7.0, 150 mM NaCl, 10% glycerol, 1 mM $MgCl_2$, 1 mM EDTA, 1.2% Triton X-100). Amount of protein was determined and electrophoresis was performed by loading a 100 μg protein sample into each well. Proteins were then blotted onto nitrocellulose membrane in transfer buffer (3.75 g glycin, 7.25 g Tris base, 2.5 ml methanol was dissolved in total 2.5 liters) at 4 °C. The membranes were incubated in 5% fat-free milk solution prepared in PBST buffer (1X PBS pH: 7.0, 1 ml Tween-20/liter) at room temperature for 2 hours and then primary antibodies were added over an additional hour in 1: 5000 dilution for IDO and 1: 50000 for GAPDH. Once the primary antibody labeling was performed, the blots were washed with PBST for an hour at room temperature. Secondary antibody labeling was performed at room temperature for one hour at 1: 5000 dilution in 5% fat-free milk in PBST. After one hour of washing at room temperature with PBST, ECL reagents were used to visualize proteins.

## Results

### Expression of the Dominant Negative Form of PKR Gene Inhibits IFN-γ-Mediated Cytotoxicity

Cells expressing the dominant negative form of PKR gene were selected in the presence of neomycin, and then these cells were tested for IFN-γ sensitivity along with wild type ME180 cells. As shown in Figure 1, normal ME180 cells treated with IFN-γ displayed very high death rates with increasing IFN-γ concentrations, while the clones expressing the dominant negative form of PKR showed very little sensitivity to IFN-γ, indicating the fact that activity of PKR is necessary for IFN-γ to show its cytotoxic effects on these cells.

### Dominant Negative Form of PKR Inhibits IFN-γ-Mediated Accumulation of IDO Protein

After determining the requirement of PKR activity for IFN-γ-mediated killing of ME180 cells, we wanted to determine whether PKR activity might be doing this by affecting the induction of IDO protein accumulation. As shown in Figure 2 (upper), wild type ME180 cells treated

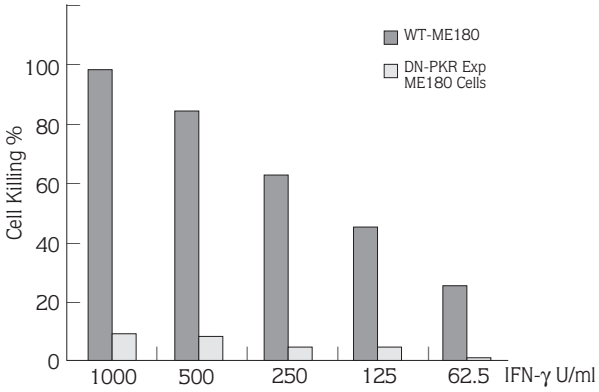


Figure 1. Expression of the dominant negative mutant of PKR protects ME180 cells from IFN-γ-mediated cytotoxicity. ME180 cells were plated a in 96-well plate and incubated in serum containing DMEM until they covered the bottom of the wells. Then IFN-γ was added at different concentrations and cells were further incubated in this medium for an additional 72 hours. At the end of this incubation period, dead cells were removed and attached cells were stained with crystal violet for 1 hour, then cell-absorbed dye was eluted with sodium citrate buffer and absorbance was read at 600 nm. Absorbance of treated cells were divided by that of untreated control cells to calculate percentage killing.

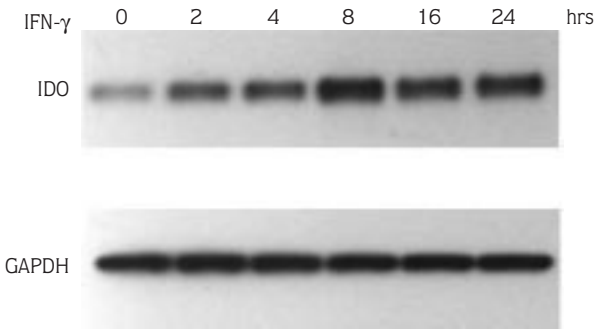


Figure 2. IFN-γ induces the accumulation of IDO protein in a time-dependent manner in MT-ME180 cells. ME 180 cells were treated with 500 Units/ml of IFN-γ for different time periods and cell lysates were collected in lysis buffer, and 100 µg of total proteins were used for Western blotting. The blot was labeled with anti-IDO antibody (upper), then stripped off and labeled with anti-GAPDH antibody to show that each lane contained equal amounts of protein (lower).

with 500 Unit/ml of IFN-γ showed significant accumulation of IDO protein in a time-dependent manner. However, the same amount of IFN-γ for the same time period did not induce any IDO protein accumulation in ME180 cells overexpressing the dominant negative form of PKR (Figure 3 [upper]). However, overexpression of the dominant negative PKR did not affect the activity of

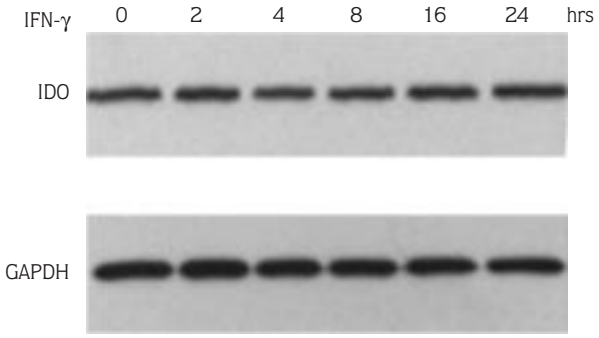


Figure 3. IFN-γ-induced accumulation of IDO protein is impaired in cells expressing the dominant negative mutant of PKR. ME180 cells overexpressing the dominant negative mutant of PKR were selected as described in the Materials and Methods. Then the cells were treated with 500 Units/ml of IFN-γ for different time periods and cell lysates were collected in lysis buffer, and 100 µg of total proteins were used for Western blotting. The blot was labeled with anti-IDO antibody (upper), then stripped off and labeled with anti-GAPDH antibody to show that each lane contained equal amounts of protein (lower).

the basal transcription complex. These results clearly indicate that the enzyme activity of PKR not only is necessary for delivering the cytotoxic effect of IFN-γ, but is also necessary for induction of accumulation of IDO protein after IFN-γ treatment in ME180 cells.

## Discussion

In this study, we used dominant negative mutant form PKR to investigate whether PKR activity is required for IFN-γ-mediated induction of IDO gene. The results indicate that enzymatic activity of the kinase is necessary for IFN-γ to induce cytotoxicity as well as IDO induction in the human cervical carcinoma cell line ME180.

Several lines of publications support the notion that PKR may function through STAT transcription factors (20). PKR is specifically associated with Stat 3 in several cell types in response to PDGF, and PDGF-induced Stat 3 DNA-binding activity is deficient in cells derived from PKR knock-out mice. It has been also shown that PKR is upstream of MAP kinases, p38 and ERK 1/2 (21,22) and these kinases were shown to be involved in the phosphorylation of Serin 727 in Stat 1. Serin phosphorylation is absolutely necessary for the transactivation function of Stat 1. In addition to its requirement for Stat 1, 3 and MAP kinase activation, PKR was also shown to be necessary for the activation of

transcription factor NF-kB by different stimuli (23,24).

In terms of the transcriptional induction of IDO gene by IFN-γ, there are only two transcription factors claimed to have roles in this process. We have shown that interferon regulatory factor 1 (IRF1) is involved in this process (18,25). Chon et al. claimed that both IRF1 and Stat 1 cooperatively bind to the promoter of IDO gene after IFN-γ treatment (26). No data providing evidence of phosphorylation of these transcription factors by PKR has been shown yet, but it is very likely that either transcription factors can be a direct target of PKR. However, given the fact that the activation of NF-kB and Stat transcription factors requires the activity of PKR and the activity of both transcription factors is essential for transcriptional upregulation of IRF1 gene (14), we strongly think that PKR activity is necessary for the accumulation of IRF1 protein after IFN-γ treatment. In fact, during preparation of this manuscript, Blair et al. have shown that poly I: C and IFN-γ-mediated accumulation of IRF1 is completely inhibited in PKR-/- mouse cells (20).

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