

## Double-stranded RNA-dependent Protein Kinase Mediates *c-Myc* Suppression Induced by Type I Interferons\*

(Received for publication, January 29, 1996, and in revised form, July 1, 1996)

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**The antiproliferative functions of interferons result from specific effects that these cytokines exert on several cell cycle-controlling genes. The possible coupling between the interferon-responsive genes that are directly transactivated by the interferon signaling and the genes that constitute the basic machinery of the cell cycle is not clear yet. We report in this work that interferon-induced double-stranded RNA-activated protein kinase (PKR) is one of the specific mediators of the antiproliferative effects of the cytokine. Transfections of M1 myeloid leukemia cells with two catalytically inactive mutant forms of PKR abrogated the ability of interferon to suppress *c-Myc* without interfering with the pRB/cyclin D responses. As a consequence, these genetically manipulated cells displayed a small but significant reduction in their growth sensitivity to interferons, a phenotype that characterizes a single pathway disruption. Transfection of the parental M1 cells with the functional wild-type human PKR restricted their proliferation in the absence of interferons. This PKR-mediated growth inhibition could be efficiently rescued by the ectopic expression of deregulated *c-myc*. Taken together these results prove the existence of direct or indirect links between PKR and *c-Myc* suppression, thereby placing this gene along one of the complementary growth suppressive pathways that are triggered by interferons.**

Growth inhibitory cytokines exert their antiproliferative effects on cells by interacting with specific cell surface receptors and initiating complementary cascades of intracellular biochemical events that affect the expression or activity of cell cycle controlling genes. Interferons (IFNs)<sup>1</sup> are among the well known cytokines that function as potent growth inhibitors. A few components from the basic cell cycle machinery have been shown to be the downstream target genes for IFN signaling (1). The *c-myc* gene is a key target whose mRNA and protein expression is selectively suppressed by the different members of the IFN family (2–4). Disruption of *c-Myc* suppression by the ectopic expression of deregulated *c-myc* caused partial relaxation of the proliferative responses to IFNs (4) and further suggested the existence of additional complementary molecular

pathways in the system. The pRB protein was then identified as a second independent target for the IFN signaling. Activation of pRB by IFNs through the suppression of its phosphorylation complemented the outcome of *c-myc* inhibition (5). The pRB responses to IFNs were recently found to result from inhibition of the pRB kinases through the suppression of cyclin D and *cdc25A* phosphatase expression (6).

A major question is how IFN signaling impinges on the aforementioned components of the cell cycle machinery and which of the transcriptionally activated IFN-responsive genes may couple these events. Among the well known immediate-early genes, the IFN-induced double-stranded RNA (dsRNA)-activated protein kinase (PKR) (7) was chosen in this work as a possible candidate for study because of its unique functional features. PKR is a cytoplasmic serine/threonine kinase that is largely ribosomal associated. It is expressed constitutively at low levels in a large variety of mammalian cells and is induced severalfold in response to IFNs (8). PKR is latent unless activated by dsRNA, single-stranded RNA with double-stranded regions, or some polyanions (*e.g.* heparin, dextran sulfate) (9). The dsRNA binds to basic regions in the regulatory N-terminal portion of the protein, and this binding leads to the activation of the protein kinase domain located at the C-terminal portion of the protein. Upon activation, PKR is autophosphorylated at multiple sites (7, 10, 11), and then it phosphorylates the  $\alpha$  subunit of the eukaryotic peptide chain initiation factor eIF-2. The phosphorylated eIF-2- $\alpha$  sequesters the guanine nucleotide exchange factor eIF-2B. This in turn prevents the recycling of eIF-2-GDP to eIF-2-GTP and thereby blocks initiation of translation (12).

PKR was first identified as an important component of the host antiviral defense mechanism (13–15). Subsequently it was found that this kinase exhibits features characteristic of a tumor suppressor gene. Overexpression of wild-type PKR was deleterious to the growth of certain mammalian cells (16) and slowed the proliferation of yeasts (17). Moreover, transfections of NIH 3T3 cells with the catalytically inactive PKR mutants, which functioned in a dominant-negative fashion, induced malignant transformation (16, 18). In light of these growth suppressive functions we studied in this work whether PKR couples the IFN signaling to the basic cell cycle machinery. We report here that the ectopic expression of two different catalytically inactive PKR mutants abrogated the IFN-induced suppression of *c-Myc*. In contrast, cyclin D/pRB responses were not interrupted. Moreover, the introduction of wild-type PKR into cells restricted their proliferation in the absence of IFNs, a process that could be rescued by the ectopic expression of deregulated *c-myc*. These results functionally place PKR upstream of *c-Myc* along one of the molecular pathways that mediate the antiproliferative effects of IFNs.

\* This work was supported in part by a grant from the Pasteur-Weizmann Joint Research Program (to A. K. and A. G. H.) and in part by the Gesellschaft für Biotechnologische Forschung mbH foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>1</sup> The abbreviations used are: IFN, interferon; PKR, interferon-induced double-stranded RNA-activated protein kinase; dsRNA, double-stranded RNA; IL-6, interleukin-6.

## EXPERIMENTAL PROCEDURES

**Cell Lines and Culture Conditions**—M1 mouse myeloid cells (19) and Daudi human Burkitt lymphoma cell lines (20) were grown in RPMI medium (BioLab) supplemented with 10% heat-inactivated fetal calf serum (BioLab), 4 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. For the experiments described in this work, cells were cultured at an initial cell density of  $2-4 \times 10^5$  cells/ml and were always harvested at densities below  $10^6$  cells/ml. The characterization of the *myc*-transfected M1 clones was described previously in details (Ref. 4; we used in these studies a mixture of clones 5 and 9). Cells were counted in a hemocytometer and viability was determined by trypan blue exclusion. The GP + E ecotropic retrovirus packaging cells (21) were grown in Dulbecco's modified Eagle's medium (BioLab) supplemented with 10% fetal calf serum (BioLab), 4 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Murine IFNs ( $\alpha + \beta$ ),  $1.2 \times 10^7$  U/ml, were purchased from Lee Biomolecular Inc. Human IFN- $\alpha$  ( $4 \times 10^6$  U/ml) was purified by affinity chromatography with monoclonal antibodies as described (3). Human recombinant IL-6, purified to  $3 \times 10^7$  U/ml, was a kind gift from Interpharm Co.

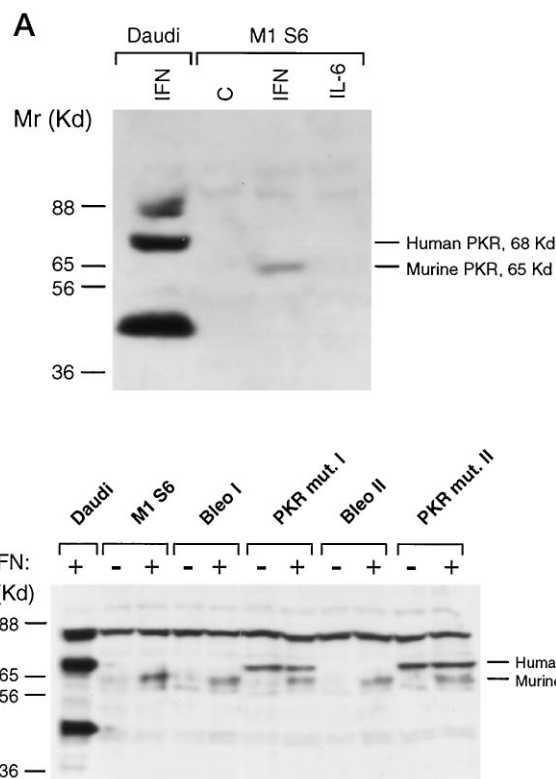
**Transfection and Infection Procedures**—The retroviral vector-based plasmids were transfected into the retrovirus packaging cell line, GP + E, by CaPO<sub>4</sub>/DNA coprecipitation method as described (22). Polyclonal populations of transfectants were selected and propagated in selection medium containing the appropriate drugs, G418 (Sigma), 1 mg/ml, or bleomycin (Phleomycin, Life Technologies, Inc.), 25  $\mu$ g/ml.

M1 cells were infected with recombinant retroviruses by cocultivation with retrovirus producer cells. Subconfluent cultures of programmed GP + E cells were treated with 25  $\mu$ g/ml Mitomycin C (Sigma) for 2 h. Cells were then washed twice with phosphate-buffered saline, trypsinized, centrifuged for 5 min at 1000 rpm, and recultured in RPMI medium containing 2  $\mu$ g/ml polybrene and no selective drug. Recipient M1 cells, at a final concentration of  $2 \times 10^5$  cells/ml, were cocultivated with the producer cells. After 48 h, M1 cells were collected and transferred into selective medium containing the appropriate drug, G418 (500  $\mu$ g/ml) or bleomycin (15  $\mu$ g/ml).

**RNA Analysis**—Total cellular RNA was isolated using Tri-Reagent (Molecular Research Center, Inc.). Northern blot analysis was performed with 25  $\mu$ g of RNA as described (2). Messenger RNA levels were assessed by hybridization to the <sup>32</sup>P-labeled DNA fragments, the 2.4-kilobase *Xba*I-*Xho*I genomic fragment containing the second and third exons of murine *c-myc* (23) and the 1.3-kilobase *Pst*I-*Pst*I cDNA fragment of rat glyceraldehyde-3-phosphate dehydrogenase (24).

**Protein Analysis**—Cells were lysed in extraction buffer (10 mM Tris, pH 7.2, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% Na deoxycholate, and 5 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride, 50  $\mu$ g/ml aprotinin, and 50  $\mu$ g/ml leupeptin. Samples (40  $\mu$ g) were analyzed by Western blotting as described previously (5). The following antibodies were used: anti-human PKR monoclonal antibodies (71/10) (25); mouse polyclonal antibodies prepared against purified human PKR (7); anti-c-Myc monoclonal antibodies raised against a highly conserved peptide that corresponds to the amino acids 173–188 in human c-Myc (MYC1–3C7) (26); anti-RB monoclonal antibodies (G3–245, Pharmingen); and anti-cyclin D polyclonal antiserum that recognizes both D1 and D2 cyclins (27). Detection of monoclonal (or mouse polyclonal) antibodies was done with horseradish peroxidase conjugated to goat anti-mouse antibodies (IgG (H + L chains), Jackson ImmunoResearch Laboratories, Inc.) at a 1:10,000 dilution. Detection of rabbit polyclonal antibodies was done with protein A conjugated to horseradish peroxidase (Amersham Corp.) at a 1:10,000 dilution.

**Construction of Recombinant Plasmids**—The *Hind*III-*Eco*RI 2.4-kilobase fragments of the two PKR cDNAs, wild type and a mutant (kinase-defective) version (*Lys*<sub>296</sub> → *Arg*) (18) were subcloned into Bluescript KS polylinker (*Hind*III-*Bam*HI sites) in order to delete undesired destabilization signals in the 3'-untranslated region (a 600-base pair *Pst*I-*Pst*I fragment). The expression of the resulting 1.8-kilobase cDNA was then tested in an *in vitro* transcription-translation system (Promega). The PKR cDNA inserts were excised from Bluescript by *Hind*III-*Spe*I digestion. Cohesive ends were filled in using recombinant Klenow enzyme (Boehringer Mannheim). The retroviral vector pBabe-Bleo (22) was cut with *Bam*HI and *Sal*I (in its polylinker), and perturbing ends were filled in as described above and dephosphorylated by calf intestinal alkaline phosphatase (CIP, Boehringer). The PKR  $\Delta 6$  mutant (16) was in a retroviral vector, pMV7 (28), under the control of MoMuLV 5' LTR and with a neomycin selectable marker. The same system of GP + E retrovirus producer cells was utilized for infection of M1 cells.



**FIG. 1. Selective induction of PKR by IFNs in parental and infected M1 cells.** A, M1-S6 cells were seeded at  $2 \times 10^5$  cells/ml with or without the indicated cytokine (murine IFNs ( $\alpha + \beta$ ), 1000 U/ml; IL-6, 300 U/ml) and incubated for 24 h. C, control. Daudi cells treated with human IFN- $\alpha$  (300 U/ml, 24 h) were used as a positive control. Equal amounts of protein extract (40  $\mu$ g) were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting with anti-PKR polyclonal antibodies raised against the human PKR which cross-react with the murine homolog. In human cells, these antibodies also cross-react with an unidentified 80-kDa protein and an interferon-induced 48-kDa protein (41). B, Western blot analysis of ectopically expressed (human PKR) and endogenous PKR (murine PKR) detected by the anti-PKR polyclonal antibodies in IFN-treated and untreated cells as indicated. Extracts derived from parental, M1-S6 cells; Bleo I, II, two polyclonal populations of M1 cells infected with the vector pBabe-Bleo; PKR mut. I, II, two polyclonal populations of M1 cells infected with pBabe-Bleo-PKR/K296R. Daudi cells were treated with 300 U/ml human IFN- $\alpha$ , and M1 cells were exposed to 1000 U/ml murine IFNs ( $\alpha + \beta$ ) for 18 h.

## RESULTS

**Construction of M1 Cell Lines Expressing a Catalytically Inactive PKR Mutant**—Exposure of the mouse M1 myelomonocytic cell line, M1-S6, either to IFNs type I ( $\alpha + \beta$ ) or to IL-6 induces a potent proliferation arrest at the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle (4). We found that the 65-kDa murine PKR protein was induced by IFNs ( $\alpha + \beta$ ) but not by IL-6 (Fig. 1A). As expected, the elevation by IFNs of the protein levels was due to the induction of transcription as previously reported (7). To study the possible coupling of PKR to molecular events that mediate growth arrest, we used two dominant-negative mutant versions of PKR which were shown to cause malignant transformation (16, 18). The cDNAs corresponding to mutant PKR were expressed in M1 cells to investigate the responses to IFNs. IL-6 was used as an internal reference.

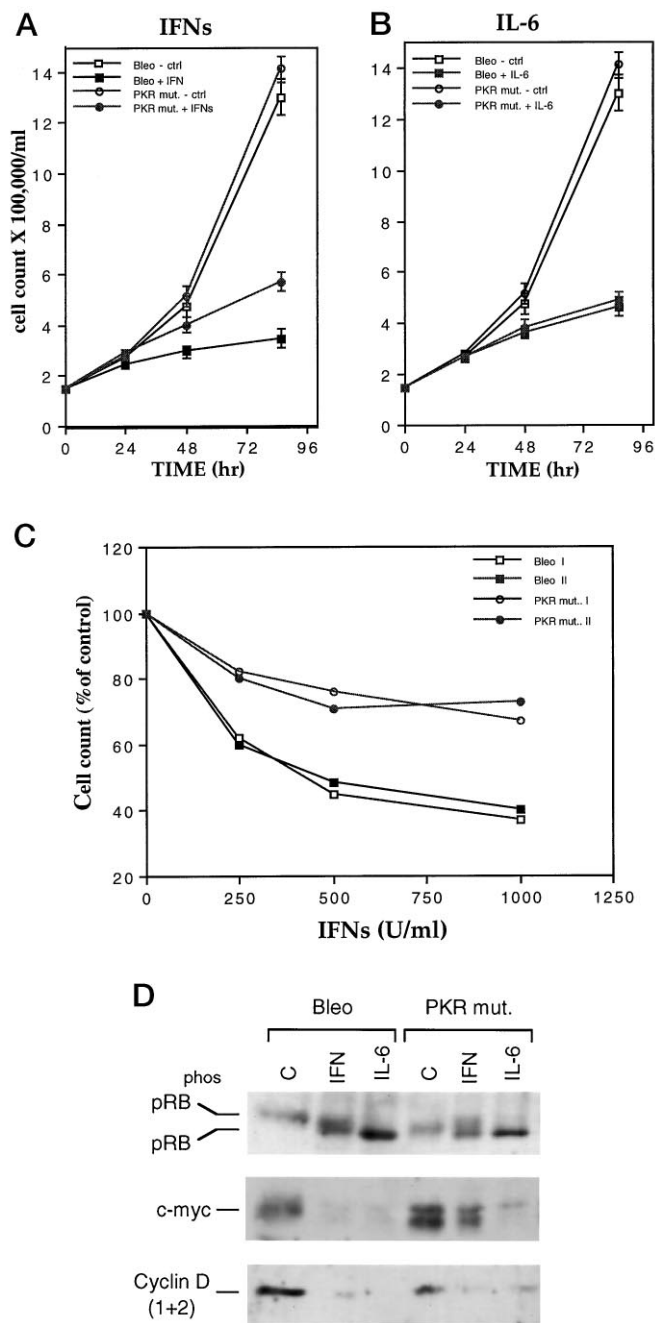
First, we generated M1-S6 cells that expressed the catalytically inactive mutant form of human PKR, K296R (15), previously shown to act in a transdominant mode (29, 30). The kinase inactivation was achieved by the conversion of the conserved lysine at position 296 to arginine. The cDNA coding for the mutant K296R was subcloned into a bleomycin selectable retroviral expression vector, pBabe-Bleo, downstream of the

MoMuLV 5' LTR promoter. This construct, as well as the original retroviral vector pBabe-Bleo, were both transfected into the retrovirus producer cell line GP + E, and stable polyclonal populations were selected. M1-S6 cells were then infected with the recombinant retroviruses by cocultivation with these producer cells. Four polyclonal cell populations of M1 infectants were generated, two carrying the control pBabe-Bleo virus and a second pair carrying the pBabe-Bleo-PKR K296R virus. Each polyclonal cell population originated from a pool of a few hundred stable clones.

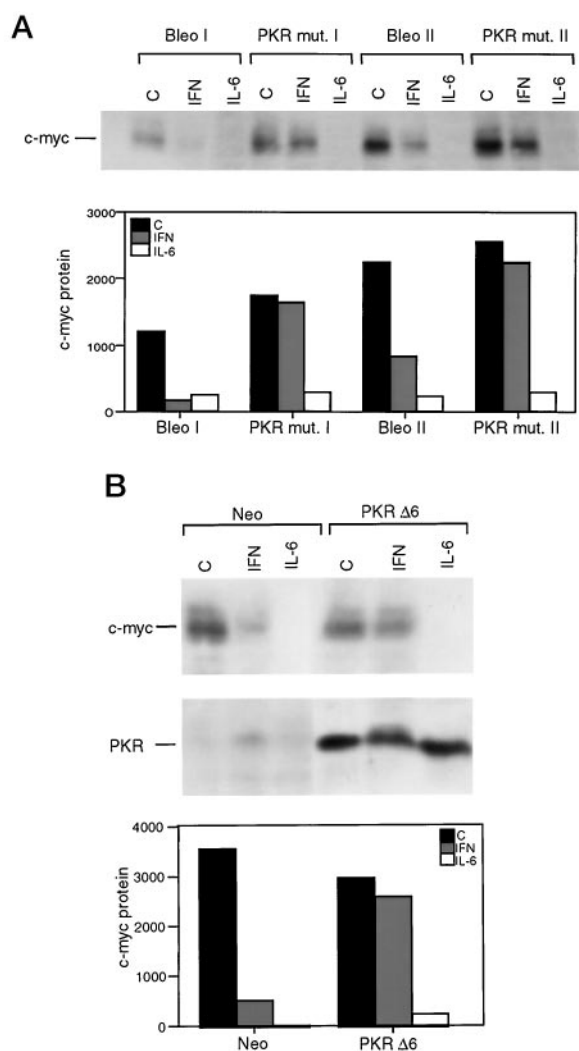
The infected M1 cells were analyzed for PKR protein expression. In order to detect simultaneously both the exogenous (human 68-kDa protein) and the endogenous (murine 65-kDa protein) kinases, we used polyclonal antibodies that were raised against human PKR. These antibodies cross-react with the murine PKR homolog (7) that displays 61% sequence identity to the human kinase (31). Immunoblot analysis indicated that the human PKR was strongly expressed in the two polyclonal populations that were independently infected with the K296R mutant PKR (Fig. 1B). Following treatment with IFNs ( $\alpha + \beta$ , 1000 U/ml), the endogenous murine PKR was induced in all the infectant cell populations (the two Bleo controls and the two cell lines carrying the mutant PKR) to an extent that was similar to the induction in the parental M1 cultures. In contrast, the exogenous human PKR protein levels were not affected by IFNs and remained constitutively high (Fig. 1B).

**Expression of Mutant PKR Interferes with the Reduction of *c-Myc* Protein by IFNs**—The growth kinetics of M1 cells in 10% fetal calf serum were not changed by the expression of the mutant PKR protein. This was determined by measuring the doubling times of the infectants (Figs. 2, A and B) and their cell cycle distribution during the logarithmic phase of growth (data not shown). In contrast, the infected M1 cells differed in their growth sensitivity to IFNs. Detailed cell counts indicated that the extent of growth arrest by IFNs was consistently reduced in the mutant PKR expressing cells, *i.e.* they reached higher cell densities in the presence of IFNs as compared to the corresponding Bleo-infected cells (Fig. 2A). When the same cells were treated with IL-6, no difference was observed in the extent of growth arrest between the control and mutant PKR-expressing cells (Fig. 2B). To further quantitate the reduced growth susceptibility to IFNs, a detailed dose-response assay was performed on each of the above-mentioned four polyclonal cell populations. The dose-response curves (ranging between 250 and 1000 U/ml IFNs) clearly indicated that the ectopic expression of mutant PKR rendered the cells less susceptible to the antiproliferative effects of IFNs (Fig. 2C). The finding that mutant PKR did not change the growth sensitivity to IL-6 was consistent with the failure of this cytokine to induce the endogenous PKR in these cells. The interference with the negative growth signaling of IFN was incomplete, since the cells failed to reach saturation cell densities in the continuous presence of IFNs, suggesting that some but definitely not all of the growth inhibitory pathways were affected, as shown below.

We next determined which of the molecular mechanisms that mediate the antiproliferative effects were interrupted by the ectopic expression of mutant PKR. We focused on a few components of the basic machinery of the cell cycle, previously shown to be the downstream targets for the IFN signaling. These included the *c-myc*, cyclin D, and pRB genes. After exposing the control Bleo-infected cells to IFNs or to IL-6, the protein levels of *c-Myc* and cyclin D (D1 and D2) sharply declined, and the pRB protein was converted into the rapidly migrating underphosphorylated forms (Fig. 2D). These molecular responses to IFNs characterize the behavior of the parental M1-S6 cells (5). Interestingly, the IFN-induced suppression

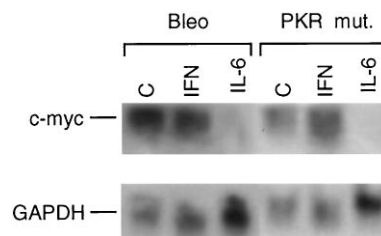


**FIG. 2. Expression of K296R mutant PKR causes partial relaxation of growth responses to IFNs and complete abrogation of *c-Myc* protein suppression.** Cells were seeded at a density of  $1.5 \times 10^5$  cells/ml in the absence or presence of cytokines as indicated. In A and B, viable cells were counted at 24, 48, and 80 h. Every experiment was performed with the two Bleo and two PKR mutant cell populations. The values are the average of the two cell populations from each type, each tested in four independent experiments. A, cell exposed to murine IFNs ( $\alpha + \beta$ ) (1000 U/ml); B, cells exposed to IL-6 (300 U/ml); C, untreated cells. C, cells were seeded at a density of  $1.5 \times 10^5$  cells/ml in the presence of IFNs ( $\alpha + \beta$ ) at the indicated final doses. Viable cells were counted after 48 h. Experiments were performed with the two Bleo and two PKR mutant cell populations. The graph presents average values obtained from three independent experiments. D, immunoblot analysis of pRB, *c-Myc*, and cyclin D1 from cells that were treated with either murine IFNs ( $\alpha + \beta$ ) (1000 U/ml) or IL-6 (300 U/ml) for 48 h. Protein extracts were prepared from M1 cells that were infected with pBabe-Bleo (Bleo I + II, a mixture of the two polyclonal cell populations) and a cell population that was infected with pBabe-Bleo/PKR K296R. Western blotting was performed with antibodies directed against pRB, *c-Myc*, or cyclin D (1 + 2) proteins. The same blot was reacted with the different antibodies. *pRB*<sup>phos</sup>, slow-migrating phosphorylated pRB; C, control.



**FIG. 3. Quantitations on immunoblots of the relaxed regulation of *c-Myc* caused by K296R mutant PKR and by PKR  $\Delta 6$  mutant.** A, cells were treated with murine IFNs ( $\alpha + \beta$ ) (500 U/ml) or IL-6 (300 U/ml) for 48 h. C, control. Protein extracts were prepared from polyclonal populations of M1 cells that were infected with pBabe-Bleo (Bleo I, II) or with pBabe-Bleo/K296R PKR, and immunoblots were reacted with anti *c-Myc* antibodies. The histograms represent densitometric values obtained for the corresponding Western blot analysis. B, Western blot analysis of *c-Myc* and PKR proteins in control clones and PKR  $\Delta 6$  mutant. Human PKR was detected with specific monoclonal antibodies. Proteins were extracted from polyclonal populations of infectant M1-S6 cells. Neo, cells that were infected with the vector carrying the selectable marker neomycin; PKR  $\Delta 6$ , infected with the retroviral vector pMV7 carrying the cDNA of the mutant PKR  $\Delta 6$ . Cells were treated with murine IFNs ( $\alpha + \beta$ ) (1000 U/ml) or IL-6 (300 U/ml) for 48 h. C, control untreated cells. The histogram represents densitometric values obtained for the corresponding Western blot analysis.

of *c-Myc* protein was greatly diminished in M1 cells expressing the mutant K296R PKR. In contrast, the pRB and cyclin D responses to IFNs were not impaired at all by the mutant PKR. The shift in pRB migration and the suppression of cyclin D proteins by IFNs were both indistinguishable between the control and the mutant PKR-infected cell populations (Fig. 2D). Similarly, there was no difference in the extent of cyclin A protein reduction between these cell populations (not shown). These results were reproduced several times. Detailed quantitation of *c-Myc* protein levels revealed that while in the control cultures the extent of *c-Myc* reduction ranged between 65 and 95%, it dropped to less than 10% in the mutant PKR-infected cells (Fig. 3A). It is noteworthy that the IL-6-induced *c-Myc* suppression was not interfered with at all in these PKR-in-



**FIG. 4. Effect of IFNs and IL-6 on *c-myc* mRNA levels.** Cells were treated with the indicated cytokines as described in Fig. 3A. Total RNA preparations were analyzed by Northern blotting and hybridization to labeled *c-myc* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes.

fect cells (Figs. 2D and 3A), which again stressed the specificity of the mutant PKR effects toward systems where the wild-type PKR is transactivated as part of the receptor-generated signaling.

To further establish this important link between PKR and *c-myc*, another catalytically inactive form of PKR, the deletion mutant PKR  $\Delta 6$ , was introduced into the M1-S6 cells. This mutant PKR lacks six invariant amino acids (residues 361–366) between the kinase subdomains V and VI (17) and has been also reported to function in a dominant-negative fashion (16). It was cloned in the G418 selectable retroviral vector, pMV7 (28) downstream from the MoMuLV 5' LTR promoter and the resulting viruses were used for the infection of M1-S6 cells. The expression levels of the PKR  $\Delta 6$  protein in the infected M1 cells are shown in the immunoblot in Fig. 3B. Analysis of *c-Myc* protein levels after IFN treatment revealed again that the ectopic expression of the PKR  $\Delta 6$  deletion mutant interfered with the suppression of *c-Myc* protein by IFNs. PKR  $\Delta 6$  had no effect on *c-Myc* suppression by IL-6, in spite of the high constitutive levels of its expression during IL-6 treatment (Fig. 3B).

Finally, it should be emphasized that all the above-mentioned experiments were performed in a subline of M1-S6 that upon treatment with IFNs failed to reduce the *c-myc* mRNA levels. It is shown in Fig. 4 that the infectants derived from these parental cells, including both Bleo control and mutant PKR expressing cultures, displayed constitutive *c-myc* mRNA levels in response to IFNs. In contrast, IL-6 reduced very efficiently the *c-myc* mRNA levels. This indicates that the IFN-induced negative regulation of *c-Myc* protein, shown in Figs. 2D and 3, occurred post-transcriptionally at the protein translation or turnover levels.

***myc*-Transfected Cells Are More Permissive to Ectopic Expression of Wild-type PKR**—The introduction of wild-type PKR suppressed the growth of M1 cells. When  $10^7$  M1 cells were infected with either pBabe or pMV7 retroviral vectors that carried the wild-type human PKR cDNA, dense drug-resistant cultures appeared only after a prolonged selection time (a few weeks; data not shown). This stood in contrast to the rapid outgrowth of drug resistant cultures after identical infections with the vector alone or with the mutant PKR-carrying viruses. It suggested that the wild-type PKR imposed growth inhibition on the majority of the M1 cells, resulting in an extremely low frequency of permissive stable clones.

We next tested whether the introduction of deregulated *myc*, that is refractory to the negative control of IFNs, could rescue the growth inhibitory effects exerted by wild-type PKR. For this purpose M1 cells that express *c-Myc* ectopically were infected with wild-type PKR. In these *myc*-transfected cells that were previously generated and characterized in our laboratory, the introduced construct includes only the two coding exons of *c-myc* driven by the SV40 early promoter, and the ectopic RNA and protein expression is unresponsive to IFNs (4). If PKR

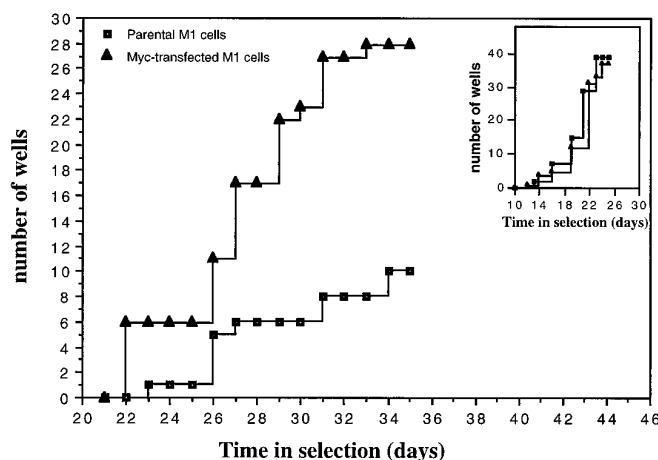


FIG. 5. *c-myc* transfected cells are permissive to introduction of functional PKR. The curves represent the outgrowth in individual wells of cells that were infected with pBabe-Bleo/PKR wild type. Parental and *c-myc* transfected M1 cells are compared. The graph in the inset represents the outgrowth of the same target cells that were infected under identical conditions with the vector alone.

mediates at least part of the inhibitory effects of IFNs on *c-myc*, then these *myc*-transfected cells should be less susceptible to the antiproliferative effects of the wild-type PKR. M1-S6 parental cells and *myc*-transfected M1 cells were infected at the same time and under identical conditions with the retroviral vector, pBabe-Bleo wild-type PKR. The infectants were then seeded into 12-well plates at an initial density of  $10^5$  cells/well and selected with bleomycin. The *myc*-transfected M1 cells were also selected with G418 for maintaining the ectopic expression of *c-myc*. The appearance of drug-resistant populations was monitored daily under a phase microscope, and a well was scored as positive when it reached a cell density of about  $10^5$  cells/ml. A remarkably higher outgrowth rate of drug resistant cultures was observed in the *myc*-transfected cells. The curve in Fig. 5 illustrates the follow-up of culture outgrowth in individual wells. It is clear that when M1 cells expressing the deregulated *c-myc* were infected with wild-type PKR, positive wells appeared earlier and much more frequently than in the parental infected M1 cell populations. This difference was not observed when the two cell populations were infected with the control empty vector, thus indicating that it did not result from a possible higher infectability of the *myc*-transfected cells (Fig. 5, inset; also notice the rapid outgrowth of wells in both cell lines with the control vector as opposed to wild-type PKR). Thus, in contrast to naive M1 cells that were inhibited by overexpression of wild-type PKR, *myc*-transfected cells could tolerate excess amounts of this protein kinase at a much higher frequency.

#### DISCUSSION

PKR has been identified as one of the immediate-early genes that are transactivated by IFNs through the ISRE enhancer element (32). Previous findings indicated that this dsRNA-dependent serine/threonine kinase mediates some of the antiviral effects of IFNs. Yet, the possibility that it may function as a positive mediator of the antiproliferative effects of IFNs has not been addressed directly. This study presents two independent lines of evidence that firmly support an intriguing possibility that PKR may function as a mediator of the inhibitory effects of IFNs on *c-myc* expression and thereby on cell growth.

The first line of experiments which suggested a functional coupling of PKR to *c-myc* was based on the ectopic expression of two dominant-negative mutants of PKR. These PKR mutants could act in a transdominant mode either by forming het-

erodimers with the endogenous kinase (33, 34) or by sequestering the putative dsRNA activator of the kinase in the cells (30). The ectopic expression of the two catalytically inactive mutant versions interfered with the IFN-induced suppression of *c-Myc* protein. No detectable relaxation in the IL-6-induced *c-Myc* suppression or cell growth inhibition took place, thus supporting the notion that the mutant kinase interferes with the normal function of the endogenous kinase that is exclusively turned on by IFNs. Other molecular responses to IFNs, such as the conversion of pRB into the underphosphorylated protein and the decline in cyclin D and cyclin A protein levels, were not affected by the mutant PKR. The inhibitory effects of IFNs on pRB phosphorylation, achieved through the suppression of cyclin D and *cdc25A* expression, constitute another growth inhibitory pathway that functions in parallel to the *c-myc* path (5, 6). Since this pathway was not interrupted by the mutant PKR, it was not surprising that the outcome of the partial relaxation in the molecular responses was a mild yet consistent reduction in the growth sensitivity to IFNs.

The second line of experiments that coupled PKR to *c-myc* was based on infections of M1 cells with retroviruses carrying the wild-type PKR. It was found that only a very small fraction of the M1 cell population was permissive to the ectopic expression of wild-type PKR. This was consistent with previous studies in which PKR was inhibitory to NIH 3T3 cells (16) and in the yeasts (17). Very interestingly, the ectopic expression of a deregulated version of *c-myc* rescued to some extent the antiproliferative effects of the wild-type kinase as deduced by the increased frequency of stable drug-resistant infectants that emerged from *myc*-transfected cells. PKR therefore functions upstream of *c-Myc*. Moreover, this line of experiments provided a second independent support for the possibility that PKR functions as a mediator of the IFN inhibitory effects on *c-Myc* protein expression, since the same deregulated construct was also refractory to the inhibitory effects of IFNs (4).

The mechanism by which PKR may suppress *c-Myc* protein expression remains to be determined. PKR could directly inhibit *c-myc* translation in light of its well established function as an inhibitor of peptide chain initiation by phosphorylating the eIF-2 initiation factor (8). Alternatively, PKR could have indirect effects. The parental M1-S6 subclone that was chosen for the infections displayed reduction of *c-Myc* protein by IFNs in the absence of any detectable fluctuations in the *c-myc* mRNA levels. This may be critical if the involvement of PKR is direct, e.g. involvement in the translational suppression of *c-Myc* (35). In that case specificity could be conferred by the stem-loop secondary structure predicted to be formed in the *c-myc* mRNA between complementary regions in the first and second exons (36). Interestingly, the version of deregulated *c-myc* which was refractory to both IFN and PKR lacked the first non-coding exon. Alternatively, the linkage between PKR and *c-myc* could be indirect, i.e. the mutant PKR could interfere with upstream IFN-induced afferent activities that are evoked by the wild-type PKR and lead to *c-Myc* suppression. The latter is consistent with some of the previous reports that showed that in NIH 3T3 cells that were transfected with mutant PKR the extent of eIF-2 phosphorylation upon viral infection was not diminished (15). Similarly, in the yeast model system, the PKR K296R mutant was found to be recessive, i.e. it did not interfere with the action of the wild-type PKR for the eIF2 phosphorylation (37). It is conceivable that PKR may phosphorylate additional substrates besides eIF-2 and therefore may be involved in other processes. At least two additional proteins were reported to serve as substrates for PKR, including a 90-kDa reticulocyte protein (38) and the I $\kappa$ B inhibitor (39, 40). Taken together, our results link the IFN-induced PKR to the

negative control of the nuclear proto-oncogene *c-myc* and thereby place this well characterized dsRNA-dependent serine/threonine kinase on one of the multiple growth suppressive pathways that are initiated by IFNs.

**Acknowledgments**—We thank Elena Feinstein for critical reading of the manuscript and Michael B. Mathews for helpful discussions.

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