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The 58-kilodalton inhibitor of the interferon-induced double-stranded RNA-activated protein kinase is a tetratricopeptide repeat protein with oncogenic properties

(translation/eIF-2/oncogene)

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ABSTRACT **The interferon-induced RNA-dependent protein kinase (PKR) is considered to play an important role in the cellular defense against viral infection and, in addition, has been suggested to be a tumor suppressor gene because of its growth-suppressive properties. Activation of PKR by double-stranded RNAs leads to the phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF-2α) and a resultant block to protein synthesis initiation. To avoid the consequences of kinase activation, many viruses have developed strategies to down-regulate PKR. Recently, we reported on the purification and characterization of a cellular inhibitor of PKR (referred to as p58), which is activated during influenza virus infection. Subsequent cloning and sequencing has revealed that p58 is a member of the tetratricopeptide repeat (TPR) family of proteins. To further examine the physiological role of this PKR inhibitor, we stably transfected NIH 3T3 cells with a eukaryotic expression plasmid containing p58 cDNA under control of the cytomegalovirus early promoter. By taking advantage of a recently characterized p58 species-specific monoclonal antibody, we isolated cell lines that overexpressed p58. These cells exhibited a transformed phenotype, growing at faster rates and higher saturation densities and exhibiting anchorage-independent growth. Most importantly, inoculation of nude mice with p58-overexpressing cells gave rise to the production of tumors. Finally, murine PKR activity and endogenous levels of eIF-2α phosphorylation were reduced in the p58-expressing cell lines compared with control cells. These data, taken together, suggest that p58 functions as an oncogene and that one mechanism by which the protein induces malignant transformation is through the down-regulation of PKR and subsequent deregulation of protein synthesis.**

PKR (protein kinase, RNA dependent) is an interferon-induced protein kinase that plays a key role in the cellular defense against viral infection, the regulation of cellular gene expression, and control of cell growth and proliferation (1–6). Activation by double-stranded RNAs (dsRNAs) or polyanions induces PKR autophosphorylation and, in turn, catalyzes phosphorylation of its natural substrate, the α subunit of the eukaryotic protein synthesis initiation factor, eIF-2α. These events lead to an often dramatic inhibition of protein synthesis initiation (1–3, 7). The activation of PKR can result from interactions with viral-specific RNAs (reviewed in refs. 1–3). However, certain viruses down-regulate PKR, presumably to avoid the harmful consequences that activation of this enzyme would cause to their replication (1–4). Several years ago we observed that PKR activity was suppressed in cells infected by influenza virus (8). Subsequently, we identified and purified the PKR inhibitor from influenza-infected cells, which we termed p58 [from its apparent molecular mass of 58 kDa (9, 10)]. Unexpectedly, this inhibitor was determined to be a cellular and not a viral protein, thus inferring that p58 could be an intrinsic regulator of PKR even in the absence of virus infection (9). Cloning and sequencing analysis has revealed that the 58-kDa inhibitor is a member of the TPR (tetratricopeptide repeat) family of proteins (11). TPR proteins are characterized by containing several internal 34-amino acid repeats, which are thought to play a role in protein–protein interactions. Many of the TPR proteins, which include CDC23, CDC16, and BimA, are thought to play an important role in mitosis and the regulation of the cell cycle (12–14). None of the members of this family, with the exception of p58, has yet been assigned a precise biochemical function.

To further explore the physiological role of p58, we generated NIH 3T3 cell lines that overexpressed the p58 protein. We reasoned that p58, as an inhibitor of PKR, may have dramatic effects on cell growth and proliferation. This premise primarily was based on our previous demonstration that expression of catalytically inactive PKR molecules in NIH 3T3 cells caused their malignant transformation, likely by transdominant inactivation of the endogenous wild-type PKR (5, 6). We can now report that p58-expressing cell lines also exhibit a transformed phenotype, showing altered morphology, faster doubling times, and growth in soft agar. Further, these cell lines caused tumors in nude mice within 4 weeks after inoculation. Analysis of murine PKR activity in p58-expressing cell lines shows that one mechanism underlying the ability of p58 to induce malignant transformation is through reduced activity of the endogenous protein kinase.

MATERIALS AND METHODS

Plasmid Construction. The cloning and sequencing of the bovine p58 gene was performed as described by Lee *et al.* (11). A 1.7-kb *Eco*RV–*Xba* I fragment representing the entire coding region for p58 was subsequently subcloned into pcDNAI/NEO, as described earlier, so that the p58 cDNA was under control of the cytomegalovirus early promoter [pc58-NEO] (11).

Construction of p58-Expressing Cell Lines. Murine NIH 3T3 cells (plated in 100-mm dishes, 5×10^4 cells per dish) were transfected with 1–3 μg of pc58-NEO per ml (15). After 18 hr of incubation, the DNA mix was removed from the cells, and Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) fetal calf serum and antibiotics was added to the plates. After 24 hr, this medium was removed,

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Abbreviations: TPR, tetratricopeptide repeat; PKR, protein kinase, RNA dependent; dsRNA, double-stranded RNA; eIF-2α, eukaryotic initiation factor 2 α subunit; mAb, monoclonal antibody.

and complete medium containing 600 μg of G418 (Genetecin, GIBCO) per ml was added. Surviving cells were collected by trypsinization, and individual clones were harvested from 24-well plates.

Monoclonal Antibody (mAb) to p58. p58 cDNA was inserted into pET15b (Novagen) and expressed and purified as a histidine-tagged fusion protein (His-p58) in *Escherichia coli* as described (11). Purified protein (10 μg) was administered monthly for 3 months with 200 μg of poly(A)·poly(U) as adjuvant (16). Mice were sacrificed, and mAbs were prepared as described (17). p58-specific mAbs were screened by using purified His-p58 and ELISA and subsequently by immunoblotting against cell extracts from several species.

Analysis of p58 Levels in Cell Lines. Cells were rinsed in PBS and disrupted in lysis buffer (10 mM Tris·HCl, pH 7.5/50 mM KCl/1 mM dithiothreitol/2 mM EDTA/1 mM $MgCl_2$/0.2 mM phenylmethanesulfonyl fluoride/100 units of aprotinin per ml/1% Triton X-100). Extracts were electrophoresed on SDS/10% polyacrylamide gels and transferred to nitrocellulose for immunoblot analysis using p58-specific mAbs and the Amersham enhanced chemiluminescence detection method (11).

Analysis of PKR Activity and eIF-2α Phosphorylation Levels. Murine PKR was immunoprecipitated from NIH 3T3 cell lines by using a polyclonal rabbit antiserum raised against human PKR expressed in insect cells (18). PKR activity assays were performed as described (8–10, 19) with 0.5 μg of purified eIF-2α per ml as substrate. The state of eIF-2α phosphorylation was determined by vertical-slab gel isoelectric focusing followed by immunoblotting as described (20) with the mAb developed by the Henshaw laboratory.

Nude Mice. Athymic nude mice (*nu/nu*, 4–6 weeks old; Seimenson Laboratories, Seattle) on a BALB/c background were maintained in a pathogen-free environment. Mice were injected subcutaneously with 2×10^6 cells in 500 μl of DMEM.

Culture and Analysis of Tumors. Tumors were removed under sterile conditions, washed in PBS, and diced into small pieces. Tumor fragments were added to a solution of 0.5% collagenase/Dispase (Sigma), homogenized with a Dounce vessel, and incubated for 2 hr at 37°C. Cells were washed once with complete DMEM and seeded into plates for the generation of tumor cell lines.

RESULTS

Characterization and Species Specificity of p58 mAbs. As a prerequisite to generating NIH 3T3 cell lines overexpressing p58, it was essential to prepare high-affinity mAbs to the cellular PKR inhibitor. To accomplish this and assist us in determining the biological function of p58, we generated mouse mAbs to highly purified recombinant bovine p58, expressed as a histidine fusion protein in *E. coli* (11). Two mAbs, 2F8-58 and 9F10-58, were first positively identified by using recombinant p58 protein as antigen in an ELISA assay (data not shown). To further determine the specificity of these mAbs, we performed immunoblot (Western blot) analysis on p58 levels in extracts prepared from Madin–Darby bovine kidney (MDBK) cells, 293 or HeLa human cells, COS-1 monkey cells, and NIH 3T3 mouse cells. The 9F10-58 mAb recognized a single protein corresponding in size to the 58-kDa PKR inhibitor in all species tested, confirming that p58 is highly conserved (Fig. 1*A*). mAb 2F8-58 recognized the p58 protein in all species of cells tested except the murine NIH 3T3 cells (Fig. 1*B*).

NIH 3T3 Cell Lines Expressing the Bovine p58 Inhibitor Exhibit a Transformed Phenotype and Are Tumorigenic. To examine the effects of p58 overexpression on cellular growth and proliferation, NIH 3T3 cells were stably transfected with pcDNAI/NEO plasmids containing the cDNA encoding the

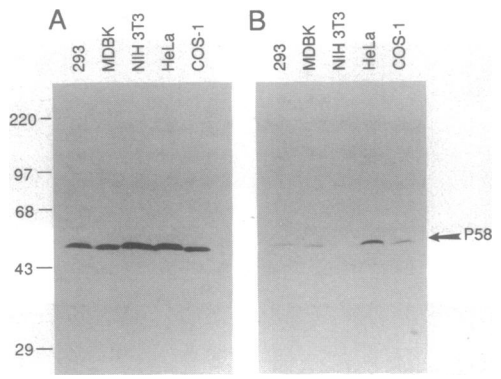

FIG. 1. Specificity of mAbs prepared against p58. Western blot analysis was performed on extracts from cells indicated at the top of the lanes. Cytoplasmic extracts were fractionated by SDS/PAGE on 10% gels, blotted onto nitrocellulose, and probed with either 9F10-58 (*A*) or 2F8-58 (*B*) p58-specific mAbs. The position of p58 is indicated on the right. Molecular mass markers are shown on the left in kDa. Proteins in all Western blots were visualized by using the Amersham enhanced chemiluminescence detection system.

bovine 58-kDa protein (11). Control transfections were carried out by using the pcDNAI/NEO vector alone (NEO cells). After selection for neomycin resistance, we isolated ≈20 independent clones. Several representative NIH 3T3 clones that expressed various levels of recombinant bovine p58 were analyzed by Western blot (Fig. 2). Although NIH 3T3 cells normally express the murine p58 (Fig. 1*A*), mAb 2F8-58 enabled us to distinguish the endogenous p58 from the exogenously introduced bovine p58 expressed from the pcDNAI/NEO vector. It also is important to note that the size of the bovine p58 expressed in the NIH 3T3 cells is identical to the endogenous p58 present in the MDBK cells. Microscopic examination of cell lines expressing p58 (p58-9) revealed that they displayed a transformed phenotype, possessing spindle-shaped morphology and increased refractility (Fig. 3*B*) compared with control NEO cells that had a flat morphology (Fig. 3*A*). When p58-expressing cells were grown to confluence, transformed foci formed on the monolayer (Fig. 3*D*), making them distinct from control NEO cells (Fig. 3*C*). We also examined the growth properties of the transformed clones (Table 1). p58-overexpressing cell lines grew faster than the NEO control cells and to a higher saturation cell density. Consistent with their transformed

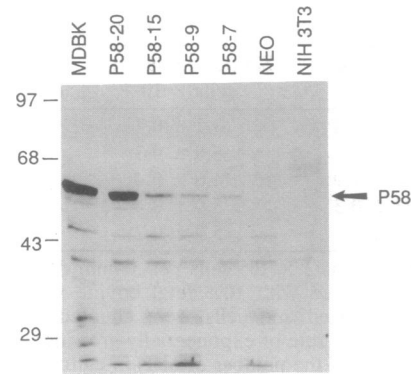

FIG. 2. Expression of bovine p58 in stably transfected NIH 3T3 cells. Cytoplasmic extracts prepared from four independent bovine p58-expressing cell lines (p58-7, -9, -15, and -20) were analyzed by Western blot with the p58 mAb 2F8-58. The bovine MDBK cells were used as a source of endogenous p58 protein and served as a positive control. Extracts prepared from an NIH 3T3 cell clone transfected with pcDNAI/NEO alone (NEO) and the parental NIH 3T3 cell line were analyzed in parallel. Molecular mass markers are shown in kDa.

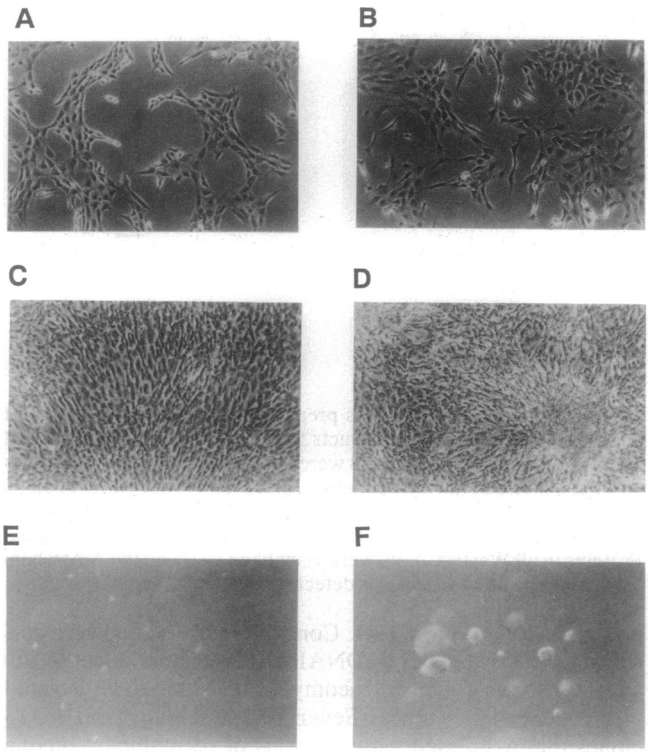

FIG. 3. Morphological characteristics of NIH 3T3 cells expressing p58. Cells from control NIH 3T3/NEO (*A*, *C*, and *E*) and NIH 3T3/p58-9 (*B*, *D*, and *F*) clones were plated at 1×10^5 cells per 100-mm dish in DMEM supplemented with 10% heat-inactivated fetal calf serum and G418. (*A* and *B*) Cells in exponential growth. (*C* and *D*) Cells maintained in culture 4 days after reaching confluency. (*E* and *F*) Anchorage-independent growth of clones maintained in soft agar. (×50.)

phenotype, all of the p58-expressing cell lines displayed anchorage-independent growth. The cells formed colonies in soft agar, whereas no colony formation was observed with control cells (in Fig. 3, compare *E* and *F*). These data thus far indicate that NIH 3T3 cells expressing p58 exhibit the *in vitro* characteristics of transformed cell lines, similar to NIH 3T3 cells stably expressing catalytically inactive PKR molecules (5, 6).

To further evaluate the tumorigenic properties of the p58-expressing cell lines, we subcutaneously injected athymic nude mice with several NIH 3T3 cell clones (2×10^6 cells per mouse). All nude mice ($n = 26$) injected with four independent p58-expressing cell lines developed tumors within 30 days (Table 2). Animals injected with NEO control clones did not develop tumors within the 6- to 8-week observation period. To confirm that the established tumors expressed the bovine p58, they were homogenized, and protein extracts were analyzed by Western blot using the p58-specific mAb 2F8-58. High-level expression of bovine p58 protein could be detected in all tumors tested, with three examples shown in Fig. 4*A*. However, one unexpected observation was the complex pattern of the bovine p58 expression in the tumors. For example, the major p58 species migrated slightly faster than the endogenous p58 in MDBK cells. Further, in all tumors examined there were smaller p58-specific products, ≈22–25 kDa in size. The significance of these observations is unclear; a trivial explanation is that these are nonspecific proteolytic products of the intact p58.

As additional confirmation that p58 was responsible for malignant transformation and tumor induction, we examined the levels of p58 expression in cell lines derived from selected p58 tumors. Tumors were treated as described in *Materials and Methods*, and cells were derived from tumors selected for their ability to grow in medium containing G418. As predicted, most clones obtained from tumors showed a continued capacity to grow under neomycin selection. Further, immunoblot analysis of representative tumor cell lines revealed that these cells expressed bovine p58 protein (Fig. 4*B*). Interestingly, relative to p58 levels in MDBK cells, p58 levels appeared to be higher in certain cell lines derived from the tumors than in the primary cell lines that were injected into the nude mice. For example, the levels of p58 in the p58-9 tumor cell line are essentially equivalent to the levels of p58 in MDBK cells, whereas p58 levels in the original p58-9 cell line were considerably lower than the MDBK p58 level (see Fig. 2). It is tempting to speculate that there has been *in vivo* selection for enhanced expression of p58 in the tumors and subsequently in the tumor-derived cell lines. Consistent with this hypothesis, we found that cell lines derived from tumors themselves induced tumors in nude mice with a considerably shorter latent period (7–9 days) than the original cell lines (Table 2). Similarly, it has been observed that there is *in vivo* selection in nude mice for enhanced levels of functionally inactive PKR molecules (6). Finally, it is noteworthy that more than one species of p58 are present in the tumor cell lines, migrating around 58 kDa. Since we have preliminary evidence that p58 is a phosphoprotein (M. Melville, N. Tang, and M.G.K., unpublished data), it is possible that p58 molecules in the tumor cell lines (and the tumors) are differentially phosphorylated.

Table 1. Growth properties of cells expressing p58

Clone	Doubling time,* hr	Saturation density,† cells × 10^{-6}	Cloning efficiency,‡ %
NEO	28 ± 1.4	3.1 ± 0.3	0
p58-7	20 ± 0.5	6.0 ± 0.3	15.1
p58-9	24 ± 1.9	4.6 ± 0.4	8.1
p58-15	23 ± 0.3	5.0 ± 0.4	8.8
p58-20	23 ± 3.5	5.5 ± 0.7	10.2

*Cells were seeded at 2×10^4 cells per 35-mm dish and grown in DMEM supplemented with 10% fetal bovine serum and G418. Medium was changed and cells were counted every 48 hr to establish the growth rate of exponentially growing cells.

†Saturation density is the number of cells in culture 3–4 days after reaching confluency. Numbers show the average of two experiments and the range of values.

‡Cells (1×10^4) were suspended in 0.35% agar solution in DMEM containing 20% fetal bovine serum and superimposed onto a 0.5% agar solution in the same medium. The cells in turn were overlaid with the 0.5% agar/medium solution prior to addition of 2 ml of DMEM/10% FBS medium. Colonies were scored 3 wk after plating. Each experiment was done in triplicate. Cloning efficiency is the number of colonies times 100 divided by the number of cells plated.

Table 2. Tumorigenicity of cells expressing p58

Clone	Animals with tumors/ animals injected	Latency, days
NEO	0/9	—
p58-7	4/4	15–21
p58-9	9/9	15–21
p58-15	4/4	26–30
p58-20	9/9	15–18
p58-7 (TCL)	2/2	7–9
p58-9 (TCL)	2/2	7–9
p58-15 (TCL)	2/2	7–9
p58-20 (TCL)	2/2	7–9

Nude mice (4 to 6 wk old; BALB/c nu^-/nu^-) were injected subcutaneously with 2×10^6 cells resuspended in 500 μl of serum-free DMEM. The time required to produce tumors of at least 5 mm was considered the latency period. Mice that did not produce tumors were examined for 6 wk. Cell lines derived from p58-producing tumors (TCL) were injected in a similar manner.

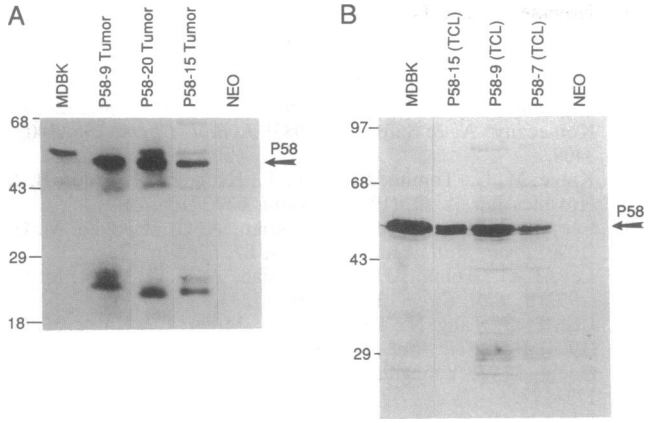

FIG. 4. Expression of p58 in tumors and in cell lines derived from tumors. (*A*) Tumors from nude mice injected with cells expressing p58 (p58-9, -15, and -20) were retrieved and homogenized in SDS disruption lysis buffer. Approximately 100 μg of protein was analyzed by immunoblot with the mAb 2F8-58. Cell extracts from NEO control cells and MDBK cells were analyzed in parallel as negative and positive controls, respectively. (*B*) Cell lines (TCL) were prepared from p58-7, -9, and -15 tumors and cultured in DMEM containing 10% fetal calf serum and G418. Cell extracts were prepared and analyzed by immunoblot with mAb 2F8-58. Extracts from cells expressing pcDNAI/NEO alone (NEO) were used as negative controls, and MDBK cell extracts served as a source of endogenous p58 protein. Molecular mass is shown in kDa.

Down-Regulation of the Murine dsRNA-Activated PKR in p58-Expressing Cell Lines. One possible mechanism by which p58 induced malignant transformation may be through down-regulation of the murine dsRNA-activated PKR. Previously, we presented evidence that catalytically inactive PKRs caused transformation through the transdominant down-regulation of the murine PKR in NIH 3T3 cells (5). Murine PKR activity was assayed after immunoprecipitation of the kinase from p58-expressing cell lines by using antisera raised against purified human PKR protein, which cross-reacts with the mouse PKR (18). Kinase activity was measured by addition of purified exogenous eIF-2α in the presence of [γ-^{32}P]ATP. Importantly, murine PKR physical levels were analyzed in parallel and were identical in all cell lines (data not shown). Results of a representative murine PKR assay, which includes the necessary quantitation of eIF-2α phosphorylation, are shown in Fig. 5 *A* and *B*. Murine PKR activity was reduced 55–75% in p58-expressing lines compared with the control cell line (NEO). Consistent with these data, there was a slight reduction in the autophosphorylation of the murine PKR in the p58 cell lines. However, these levels are dependent on the phosphorylation state of PKR when the cells are harvested. We have previously found that these levels do not consistently correlate with eIF-2α phosphorylation and are not a reliable measure of kinase activity (19, 20). To more directly analyze PKR activity in p58-expressing cell lines, we examined endogenous levels of eIF-2α phosphorylation by isoelectric focusing and immunoblot analysis (20). In corroboration of the activity assays described above, we found a several-fold reduction in endogenous eIF-2α phosphorylation levels in both p58-9 and p58-20 cell lines compared with NEO control cells (Fig. 5*C*). Indeed phosphorylation levels were barely detectable in the p58 cell lines. Taken together, these data provide evidence that one potential mechanism leading to p58-induced malignant transformation is through reduced activity of the murine PKR.

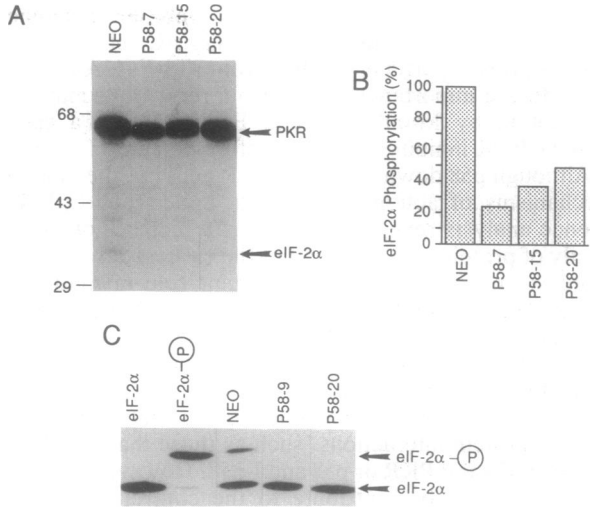

FIG. 5. Analysis of murine PKR activity in NIH 3T3 cells expressing p58. (*A*) Murine PKR was immunoprecipitated from p58-expressing cell lines (p58-7, -15, and -20) by using anti-PKR polyclonal antisera, and activity was measured after incubation with 0.5 μg of purified eIF-2 as described in *Materials and Methods*. The radiolabeled products were analyzed by SDS/PAGE with 10% gels. The positions of the murine PKR and eIF-2α are shown on the right, and the positions of molecular mass markers in kDa are on the left. (*B*) The levels of eIF-2α phosphorylation in *A* were quantitated by laser densitometry with the levels in NEO control cells arbitrarily given the value of 100%. (*C*) Isoelectric focusing (IEF) gel electrophoresis of endogenous eIF-2α phosphorylation levels in p58-expressing cells. Extracts were prepared, fractionated by IEF, transferred to Immobilon-P, and subjected to immunoautography as described (20). Lanes marked eIF-2 and eIF-2α-P are standards of purified eIF-2 incubated in the absence or presence of the heme eIF-2α kinase to demonstrate the position of the phosphorylated and nonphosphorylated forms of eIF-2α.

DISCUSSION

The majority of work on PKR has focused on its participation in the interferon inhibitory response to virus infection and viral strategies to avoid these events (1–4). In fact, we first identified p58 in influenza virus-infected cells. However, recent data show that PKR, which is constitutively expressed in eukaryotic cells, plays a pivotal role in the regulation of protein synthesis and gene expression even in uninfected cells (21–24). For example, there is evidence that, because of its inhibitory effects on protein synthesis (21–23), PKR is growth-suppressive in yeast (22, 25) and is toxic in insect and mouse cells as well (5, 18). PKR also has been suggested to regulate the transcriptional induction of select protooncogenes and growth factors (26, 27). Further, PKR may be involved with adipocyte differentiation (28) and signal transduction pathways (29). Potentially the most dramatic evidence for a PKR role in cell regulation was provided by our demonstration that the gene encoding PKR may be a tumor suppressor gene (refs. 5 and 6; reviewed in ref. 24). As earlier stated, introduction of catalytically inactive PKR mutants into NIH 3T3 cells induced their malignant transformation (5, 6). We provided evidence that this occurred, at least in part, through the transdominant inhibition of the wild-type endogenous PKR by the nonfunctional mutants (5). On the basis of these observations, it was reasonable to assume that an inhibitor of PKR, such as p58, also could play an important cell regulatory role. The results presented in this report suggest that p58, like the PKR mutants, has oncogenic properties resulting from the down-regulation of PKR in NIH 3T3 cells. Thus, these data provide an additional link between the PKR regulatory pathway and malignant transformation. The regulation of p58 and PKR is likely to be even more complex: we now have evidence that p58 is itself regulated by another protein, which we term "I-p58" for inhibitor of p58 (10). Other less-well-understood cellular regulators of PKR

also have been identified (reviewed in ref. 30), including an inhibitor partially purified from human FL cells (31) and a 15-kDa protein partially purified from 3T3-F442A cells (32). Of particular importance based on our recent data may be the description by Mundschau and Faller (33) of a specific cellular PKR inhibitor in *ras*-transformed cells.

Although p58 down-regulates PKR activity, the molecular mechanisms of action still are not understood. DNA sequence analysis revealed that p58 is a member of the TPR family of proteins (11). Several of the earliest described TPR proteins appear to play an important role in mitosis and cell-cycle regulation (reviewed in refs. 12 and 13). Curiously, considerable homology exists between p58 and IEF SSP 3521, which is a human TPR protein up-regulated in simian virus 40 transformed fibroblasts (34). It is speculated that TPR repeats form amphipathic helices that could direct protein–protein interactions, such as those that could occur between p58 and PKR or p58 and I-p58. However, how these properties directly contribute to the control of cell-cycle regulation or in the case of p58, cell proliferation, remains to be elucidated. A high degree of homology also exists between regions of p58 and the conserved J region of the DnaJ heat shock family of proteins (35). Again these regions of homology are speculated to direct protein–protein interactions. p58 also shows limited homology to the PKR natural substrate, eIF-2α, suggesting that the inhibitor may function as a pseudo-substrate (11).

Finally, despite our demonstration that p58-induced malignant transformation may occur, at least in part, through down-regulation of PKR, we still do not understand the molecular events linking protein synthesis and growth control. The deregulation of protein synthesis resulting from increased amounts of functional eIF-2 may nonspecifically up-regulate all mRNA translation. However, several protooncogenes and growth factors have multiple upstream AUGs, open reading frames, and/or 5′ untranslated regions that appear to be translationally regulated (reviewed in ref. 36). Thus, reduced PKR function may induce the enhanced translation of these select mRNAs, which are normally programmed to be translated at low levels. In closing, it is relevant to note two other translationally important proteins that also up-regulate cell proliferation and tumorigenesis. Overexpression of the protein synthesis initiation factor eIF-4α (4E) causes malignant transformation as judged by several criteria, including tumor formation in nude mice (37, 38). It also was found that constitutive expression of protein synthesis elongation factor 1 α caused cells to become highly susceptible to transformation by 3-methylcholanthrene and ultraviolet light (39). The growing list of translational regulatory proteins involved in tumorigenesis suggests an increasingly important and widening role for the protein synthetic pathway in the regulation of cellular gene expression.

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