Inhibition of Double-Stranded RNA- and Tumor Necrosis Factor Alpha-Mediated Apoptosis by Tetratricopeptide Repeat Protein and Cochaperone P58[IPK]

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P58[IPK] is a tetratricopeptide repeat-containing cochaperone that is involved in stress-activated cellular pathways and that inhibits the activity of protein kinase PKR, a primary mediator of the antiviral and antiproliferative properties of interferon. To gain better insight into the molecular actions of P58[IPK], we generated NIH 3T3 cell lines expressing either wild-type P58[IPK] or a P58[IPK] deletion mutant, ΔTPR6, that does not bind to or inhibit PKR. When treated with double-stranded RNA (dsRNA), ΔTPR6-expressing cells exhibited a significant increase in eukaryotic initiation factor 2α phosphorylation and NF-κB activation, indicating a functional PKR. In contrast, both of these PKR-dependent events were blocked by the overexpression of wild-type P58[IPK]. In addition, the P58[IPK] cell line, but not the ΔTPR6 cell line, was resistant to dsRNA-induced apoptosis. Together, these findings demonstrate that P58[IPK] regulates dsRNA signaling pathways by inhibiting multiple PKR-dependent functions. In contrast, both the P58[IPK] and ΔTPR6 cell lines were resistant to tumor necrosis factor alpha-induced apoptosis, suggesting that P58[IPK] may function as a more general suppressor of programmed cell death independently of its PKR-inhibitory properties. In accordance with this hypothesis, although PKR remained active in ΔTPR6-expressing cells, the ΔTPR6 cell line displayed a transformed phenotype and was tumorigenic in nude mice. Thus, the antiapoptotic function of P58[IPK] may be an important factor in its ability to malignantly transform cells.

P58[IPK], a member of the tetratricopeptide repeat (TPR) family of proteins (31), is a cellular inhibitor of the interferon-induced protein kinase PKR. This property of P58[IPK] has been exploited by influenza virus, which recruits P58[IPK] to repress PKR-mediated eukaryotic initiation factor 2α (eIF-2α) phosphorylation (32, 33), thereby enabling influenza virus to evade the host antiviral response by maintaining a high level of protein synthesis. P58[IPK] also has growth-regulatory properties in the absence of virus infection, and the overexpression of P58[IPK] results in malignant transformation (5). Although the mechanism by which this occurs has not been defined, one possibility is that P58[IPK] transforms cells by interfering with PKR-regulated pathways. There is a well-established correlation among PKR inhibition, reduced eIF-2α phosphorylation, and malignant transformation (4, 6, 13, 27, 37). The stimulation of mRNA translation initiation rates, which occurs in response to decreased eIF-2α phosphorylation, may result in an increase in the translation of normally poorly translated mRNAs, such as those encoding growth factors or oncogenes. In addition, decreased eIF-2α phosphorylation may contribute to the suppression of apoptosis (44), consistent with numerous reports that have implicated PKR as a mediator of programmed cell death pathways (3, 12, 30, 56, 57).

Studies examining the regulatory pathways governing P58[IPK] function have yielded additional insight into the cellular activities of this protein. Under "normal" physiological conditions, P58[IPK] is present in an inactive complex with one or more regulatory proteins. These proteins include the molecular chaperone Hsp40 (35) and a novel protein referred to as P52[rIPK] (for regulator of the inhibitor of protein kinase) (17). Several types of stimuli, including influenza virus infection and heat shock, promote the disruption of the Hsp40–P58[IPK] complex and the activation of P58[IPK] (36). The same stimuli trigger an association between P58[IPK] and the ATPase domain of Hsp70. P58[IPK] shares structural similarities with other Hsp70-interactive proteins, such as the cochaperones Hip, Hop, and Cyp40, all of which contain TPR domains that are required for stable Hsp70 interaction (42). The carboxyl terminus of P58[IPK] contains a J domain, and like other J-domain proteins, such as Hsp40, Hdj-2, and auxilin (8), P58[IPK] stimulates the ATPase activity of Hsp70. Thus, P58[IPK] also functions as a cochaperone protein. Interestingly, the second regulator of P58[IPK], P52[rIPK], contains homology to a segment of the molecular chaperone Hsp90, which is essential for the function of a variety of steroid hormone receptors, transcription factors, and protein kinases (39). P58[IPK], through its interaction with Hsp40, Hsp70, and an Hsp90-related protein, therefore appears to be intimately tied to the molecular chaperone machinery and to cellular stress response pathways.

Given the emerging relationship between the molecular chaperone machinery and apoptosis (23, 24, 45), and the numerous reports of the apoptotic functions of PKR (reviewed in reference 49), we sought to examine the role of P58[IPK] as an inhibitor of the programmed cell death response and to determine whether this property could contribute to the ability of P58[IPK] to malignantly transform cells. To this end, we generated NIH 3T3 cell lines expressing either wild-type P58[IPK] or a P58[IPK] deletion mutant, ΔTPR6, that does not bind to PKR or inhibit its ability to phosphorylate eIF-2α (18, 50), thus

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enabling us to evaluate whether P58^{IPK} also acts outside PKR-dependent pathways. We found that overexpression of wild-type P58^{IPK} inhibited the double-stranded RNA (dsRNA)-induced phosphorylation of eIF-2α and the activation of NF-κB, whereas these PKR-dependent functions remained active in ΔTPR6-expressing cells. Moreover, the P58^{IPK} cell line, but not the ΔTPR6 cell line, was resistant to dsRNA-induced apoptosis. In contrast, both the P58^{IPK} and ΔTPR6 cell lines were resistant to apoptosis induced by tumor necrosis factor alpha (TNF-α). Significantly, both cell lines exhibited a transformed phenotype and induced tumor formation in nude mice. Thus, our findings suggest that inhibition of PKR is not a requirement for P58^{IPK}-induced transformation and that suppression of apoptosis may be a primary mechanism by which P58^{IPK} malignantly transforms cells. A model depicting the role of P58^{IPK} as a regulator of apoptosis is presented.

MATERIALS AND METHODS

Construction of P58^{IPK} and ΔTPR6 cell lines. Murine NIH 3T3 fibroblasts (American Type Culture Collection, Manassas, Va.) were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS). To generate stable cell lines, plasmid DNA consisting of wild-type P58^{IPK}/pcDNAI/NEO (31), ΔTPR6/pcDNAI/NEO (50), or pcDNAI/NEO (Invitrogen, Carlsbad, Calif.), was introduced into monolayer cell cultures (20 μg of DNA/1.3 $\times$ 10^6 cells) by calcium phosphate transfection (2). Following a 20-h transfection period, the DNA mix was removed and replaced with fresh medium. This medium was removed after an additional 24-h incubation and replaced by medium supplemented with 600 μg of G418 (Geneticin; Life Technologies, Inc., Gaithersburg, Md.) per ml. After 9 days of drug selection, G418-resistant cells were trypsinized and individual clones were harvested. Clonal cell lines were maintained in medium containing 400 μg of G418 per ml.

Preparation of polyclonal antiserum to P58^{IPK}. Purification of glutathione S-transferase (GST)-P58^{IPK} fusion protein was performed as described previously (31). For the generation of a polyclonal antiserum to P58^{IPK}, New Zealand White rabbits were immunized with 100 μg of GST-P58^{IPK} in 5 ml of incomplete Freund's adjuvant containing 100 μg of N-acetylmuramyl-L-alanyl-D-isoglutamine. Two subsequent boosts of 100 μg of GST-P58^{IPK} in incomplete Freund's adjuvant were administered at monthly intervals.

Immunoblot analysis. Monolayer cell lines or dissociated tumor cells were washed twice with ice-cold Hanks' balanced salt solution and lysed in Triton lysis buffer (10 mM Tris hydrochloride [pH 7.5], 50 mM KCl, 1 mM dithiothreitol, 2 mM MgCl$_2$, aprotinin [100 μg/ml], 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100). After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, polypeptides were transferred to nitrocellulose membranes (54) and detected with the P58^{IPK} monoclonal antibody 2F8 (5) or with the P58^{IPK} polyclonal antibody described above.

eIF-2α phosphorylation analysis. The state of eIF-2α phosphorylation in cultured cells was determined by vertical-slab isoelectric focusing and immunoblotting. Cell lysates were prepared essentially as described previously (40), but without BPA-1000 treatment. The 10,000 $\times$ g supernatant (20 μg of protein) was subjected directly to vertical-slab isoelectric focusing (without prior immunoprecipitation of eIF-2α), and the resolved proteins were transferred to nitrocellulose membranes. After transfer, blots were blocked in phosphate-buffered saline (PBS) containing 10% (wt/vol) nonfat dried milk and 0.2% Tween 20. All incubations with antibody against eIF-2α were performed in PBS containing 0.1 to 0.2% Tween 20. Autoradiographs were analyzed by scanning laser densitometry to determine the ratio of phosphorylated to unphosphorylated eIF-2α in each sample. A change in the ratio in response to poly(I · C) is considered indicative of PKR activity.

Electrophoretic mobility shift assay (EMSA) for NF-κB activation. Cells (80% confluent in 10-cm-diameter dishes) were incubated in serum-free medium for 18 h, after which they were stimulated with either poly(rI · rC) (100 μg/ml) or TNF-α (10 ng/ml; Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Whole-cell extracts were prepared as described previously (19), with the exception that the 15,000 $\times$ g supernatant material was not subjected to dialysis. To assay for activation of NF-κB, 5 μg of cell extract was incubated with 0.2 ng of a ^{32}P-labeled oligonucleotide encoding positive regulatory domain II (PRDII) in binding buffer [20 mM HEPES, 50 mM NaCl, 5 mM MgCl$_2$, 10% glycerol, 1 mM dithiothreitol, 0.6 mM EDTA, 1.0 μg of poly(dI · dC) per ml] for 20 min at room temperature. Protein-DNA complexes were resolved by native 5% polyacrylamide gel electrophoresis in 0.5$\times$ TBE (1$\times$ TBE is 90 mM Tris, 90 mM boric acid, and 2 mM EDTA). The gels were dried, and NF-κB–DNA complexes were visualized by autoradiography.

Apoptosis assay. Cells (5 $\times$ 10^5/sample) were treated with poly(I · C) (1, 10, or 100 μg/ml) together with actinomycin D (50 ng/ml) for 16 h. Apoptosis-induced DNA fragmentation was detected by the in situ labeling of DNA strand breaks with fluorescein dUTP and terminal deoxynucleotidyltransferase as described by
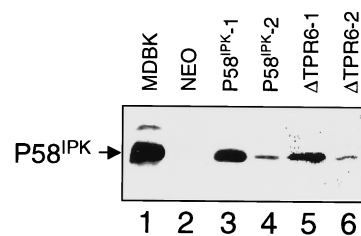

FIG. 1. Bovine P58^{IPK} or ΔTPR6 is efficiently produced in stably transfected NIH 3T3 cell lines. Immunoblot analysis of P58^{IPK} and ΔTPR6 protein production was performed. Protein extracts (100 μg) from wild-type P58^{IPK} (P58^{IPK}-1 and P58^{IPK}-2) and ΔTPR6 (ΔTPR6-1 and ΔTPR6-2) cell lines were analyzed with a polyclonal antibody that recognizes bovine P58^{IPK} (lane 1, Madin-Darby bovine kidney cells) but not the endogenous P58^{IPK} protein present in murine cells (lane 2, NEO). Since the deletion in ΔTPR6 is small, the ΔTPR6 protein migrates at approximately the same position as P58^{IPK} in the 12% acrylamide gel shown. Gradient gels (10 to 20% acrylamide) were therefore used to distinguish between the 58-kDa wild-type P58^{IPK} and the 54-kDa ΔTPR6 protein (not shown).

the manufacturer (Boehringer Mannheim). Fluorescein incorporation was analyzed with a FACStar flow cytometer (Immunocytometry Systems; Becton Dickinson, Mountain View, Calif.). Alternatively, cells were treated with TNF-α (0.2 to 1.0 ng/ml) together with actinomycin D (50 ng/ml) for 16 h and analyzed for cell viability by trypan blue dye exclusion or for apoptosis-induced DNA laddering as described previously (12).

Cell growth assays. The growth rate of exponential-phase cells was measured by seeding 2 $\times$ 10^4 cells in DMEM supplemented with 10% FBS and 400 μg of G418 per ml. The culture medium was changed every 3 days, and the doubling time was determined by counting cells at 2-day intervals. To determine cloning efficiency, 10^4 cells were suspended in medium containing 0.35% agarose and overlaid onto medium containing 0.5% agarose in 35-mm-diameter plates. Colonies were then counted 2 to 4 weeks after plating. Percent cloning efficiency is defined as the number of colonies present 2 to 4 weeks after plating, divided by the number of cells plated, multiplied by 100.

Injection of nude mice and propagation of tumor cell lines. Four- to six-week-old athymic mice (BALB/c nu/nu; Taconic Farms, Germantown, N.Y.), housed in a specific-pathogen-free environment, were injected subcutaneously in the right inguinal area with 2 $\times$ 10^6 cells in 500 μl of DMEM. Tumors were extracted from euthanized mice under aseptic conditions, washed four times in PBS to remove excess blood, fat, and necrotic tissue, and minced. The tumor pieces were then added to DMEM containing 0.5% collagenase-dispase (Sigma Chemical Co., St. Louis, Mo.) and were homogenized with a Dounce vessel, and the homogenate was incubated for 2 h at 37°C with gentle stirring. Dissociated cells were washed once with complete DMEM and seeded into plates for the generation of tumor cell lines.

RESULTS

A P58^{IPK} protein that lacks the sixth TPR motif does not inhibit phosphorylation of eIF-2α. We reported previously on the construction and characterization of P58^{IPK} deletion mutants to determine the regions of P58^{IPK} required to inhibit PKR activity (50). Using this approach, we demonstrated that a P58^{IPK} mutant lacking the sixth TPR motif, ΔTPR6, was unable to bind to PKR (18) or to inhibit PKR-mediated eIF-2α phosphorylation in vitro (50). This characteristic of ΔTPR6 enabled us to generate a cell line that would allow us to evaluate the ability of P58^{IPK} to function outside PKR-dependent pathways. To generate stable cell lines, NIH 3T3 cells were transfected with a pcDNAI/NEO expression construct encoding the ΔTPR6 protein. Control cell lines were generated in a similar fashion by using a pcDNAI/NEO construct encoding the wild-type bovine P58^{IPK} (31) or by using the pcDNAI/NEO vector alone (NEO). After selection for G418 resistance, multiple clones from each transfection were analyzed by immunoblotting for ΔTPR6 or P58^{IPK} protein production. Using a P58^{IPK} polyclonal antibody that detects bovine P58^{IPK} but does not recognize the endogenous mouse protein, we confirmed that the bovine P58^{IPK} or ΔTPR6 protein was produced in each of the clones examined (Fig. 1).
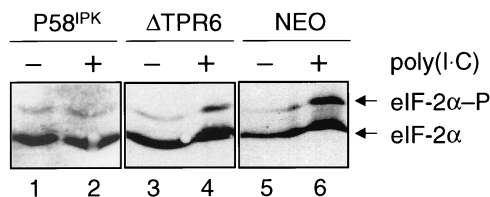
FIG. 2. PKR phosphorylates eIF-2α in ΔTPR6-expressing cells. An analysis of the steady-state level of eIF-2α phosphorylation in cultured cell lines was performed. Extracts were prepared from P58^{IPK}-1, ΔTPR6-1, or NEO cells in mid-log phase, and 20 μg of each extract was subjected to vertical-slab isoelectric focusing. The resolved proteins were transferred to a nitrocellulose membrane, and the blot was probed with an eIF-2α monoclonal antibody. The ratio of phosphorylated to unphosphorylated eIF-2α in each lane was determined by scanning laser densitometry. A change in the ratio in response to treatment with poly(I · C) is indicative of PKR activity. The ratio of phosphorylated to unphosphorylated eIF-2α increased 1.3-fold in the P58^{IPK} cell line and 4.5-fold in the ΔTPR6 and NEO cell lines. Phosphorylated (P) and unphosphorylated forms of eIF-2α are indicated by arrows.

Using a variety of biochemical and genetic techniques, we have previously demonstrated that ΔTPR6 fails to interact with PKR (18), disrupt the formation of PKR dimers (48), or inhibit PKR activity (50). To confirm these results in stable cell lines, we used isoelectric focusing to resolve the phosphorylated and unphosphorylated forms of eIF-2α in the wild-type P58^{IPK}, ΔTPR6, and NEO cell lines. In this assay, a change in the ratio of phosphorylated to unphosphorylated eIF-2α in response to poly(I · C) is considered indicative of PKR activity. When treated with poly(I · C) to activate PKR, the P58^{IPK} cell line exhibited only a 1.3-fold increase in the ratio of phosphorylated to unphosphorylated eIF-2α (Fig. 2; compare lane 2 to lane 1), indicating that PKR activity was inhibited by the overexpression of wild-type P58^{IPK}. In contrast, the ΔTPR6 cell line exhibited a 4.5-fold increase in the ratio of phosphorylated to unphosphorylated eIF-2α (Fig. 2; compare lane 4 to lane 3), demonstrating that PKR is active in ΔTPR6-expressing cells. A 4.5-fold change in the ratio of phosphorylated to unphosphorylated eIF-2α was also observed in NEO cells. Thus, consistent with our earlier in vitro studies, a P58^{IPK} mutant that lacks the sixth TPR motif is also unable to block PKR-mediated eIF-2α phosphorylation in vivo.

P58^{IPK}, but not ΔTPR6, inhibits the dsRNA-induced activation of NF-κB. Given the multiple functions of PKR, we next sought to evaluate the ability of P58^{IPK} to inhibit additional PKR-dependent pathways. PKR is essential for the dsRNA-induced activation of the transcriptional regulator NF-κB (28, 34, 55). We therefore began by examining whether the ability of dsRNA to induce activation of NF-κB was altered in the P58^{IPK} or ΔTPR6 cell line. An EMSA was used to detect NF-κB DNA-binding activity as a measure of NF-κB activation. We found that when NEO cells were treated with poly (I · C), there was an increase in NF-κB DNA-binding activity (Fig. 3; compare lanes 1 and 2). Similarly, in ΔTPR6-expressing cells, poly(I · C) treatment also resulted in activation of NF-κB, but to an even greater extent than that observed in NEO cells, indicating a functional dsRNA signaling pathway (Fig. 3, lanes 7 and 8). In contrast, poly(I · C) treatment did not appear to increase NF-κB activation in P58^{IPK}-overexpressing cells (Fig. 3, lanes 4 and 5). This observation suggests that P58^{IPK} not only inhibits PKR's ability to phosphorylate eIF-2α but also can block the ability of PKR to activate NF-κB. It should be noted, however, that the effect of poly(I · C) on NF-κB activation in P58^{IPK}-overexpressing cells may be somewhat obscured, since P58^{IPK}-overexpressing cells exhibited a high basal level of NF-κB activation even in the absence of poly

(I · C) (Fig. 3; compare lane 1 to lane 4). This effect was observed in multiple P58^{IPK} cell lines (data not shown) and may be similar to the increase in NF-κB-dependent gene expression that also occurs in response to the expression of oncogenic forms of Ras, Raf-1, or Neu (ErbB-2/HER2) (14–16).

As an alternative method of activating NF-κB, we treated each of the cell lines with TNF-α. In contrast to the effect observed with poly(I · C), treatment with TNF-α resulted in a marked increase in NF-κB DNA-binding activity in both the ΔTPR6 and P58^{IPK} cell lines (Fig. 3). This result demonstrates that activation of NF-κB, in response to an inducer other than dsRNA, is not impaired in P58^{IPK}-overexpressing cells. Moreover, the ability of P58^{IPK} to inhibit NF-κB activation is specific for dsRNA signaling pathways. These findings are consistent with those of previous studies, which found that the elimination of PKR (through gene knockout, targeted mRNA ablation, or expression of a transdominant negative PKR mutant) impairs the ability of poly(I · C) to induce NF-κB activation but has no effect on the ability of TNF-α to activate NF-κB (12, 34, 44, 55). Again, this effect was observed in multiple P58^{IPK} and ΔTPR6 cell lines (data not shown).

P58^{IPK}, but not ΔTPR6, mediates resistance to dsRNA-induced apoptosis. Since PKR has been implicated as a mediator of the apoptotic pathway that is activated in cells upon treatment with dsRNA (3, 12, 44), we examined the ability of dsRNA to induce apoptosis in the NEO, ΔTPR6, and P58^{IPK} cell lines. For these experiments, cells were treated with poly (I · C), and apoptosis-induced DNA fragmentation was detected by the in situ labeling of DNA strand breaks with fluorescein dUTP and terminal deoxynucleotidyltransferase. We found that treatment with poly(I · C) induced apoptosis in a dose-dependent manner in both the NEO and ΔTPR6 cell lines. When ΔTPR6-expressing cells were treated with poly (I · C) at a concentration of 10 μg/ml, 77% of the cells underwent apoptosis, and 85% of the cells became apoptotic at a poly(I · C) concentration of 100 μg/ml (Fig. 4). Thus, ΔTPR6 does not inhibit the ability of PKR to mediate dsRNA-induced apoptosis. In contrast, only 9% of P58^{IPK}-overexpressing cells underwent apoptosis when treated with poly(I · C) at a concentration of 10 μg/ml. Even at a 10-fold higher concentration of poly(I · C), only 35% of P58^{IPK}-overexpressing cells underwent apoptosis. These observations were consistent in multiple clonal cell lines and further extend the role of P58^{IPK} as a regulator of multiple PKR-dependent functions.
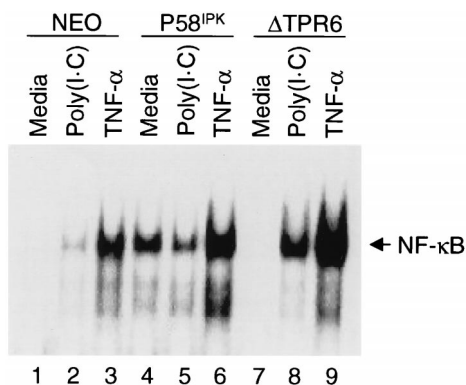

FIG. 3. P58^{IPK}, but not ΔTPR6, inhibits the dsRNA-induced activation of NF-κB. NF-κB DNA-binding activities in NEO, P58^{IPK}-1, and ΔTPR6-1 cell lines are shown. Cells were treated with medium alone, medium containing poly (I · C) (100 μg/ml), or medium containing TNF-α (10 ng/ml). NF-κB activation was detected in an EMSA using a ^{32}P-labeled PRDII oligonucleotide probe. NF-κB–PRDII complexes are indicated by the arrow.
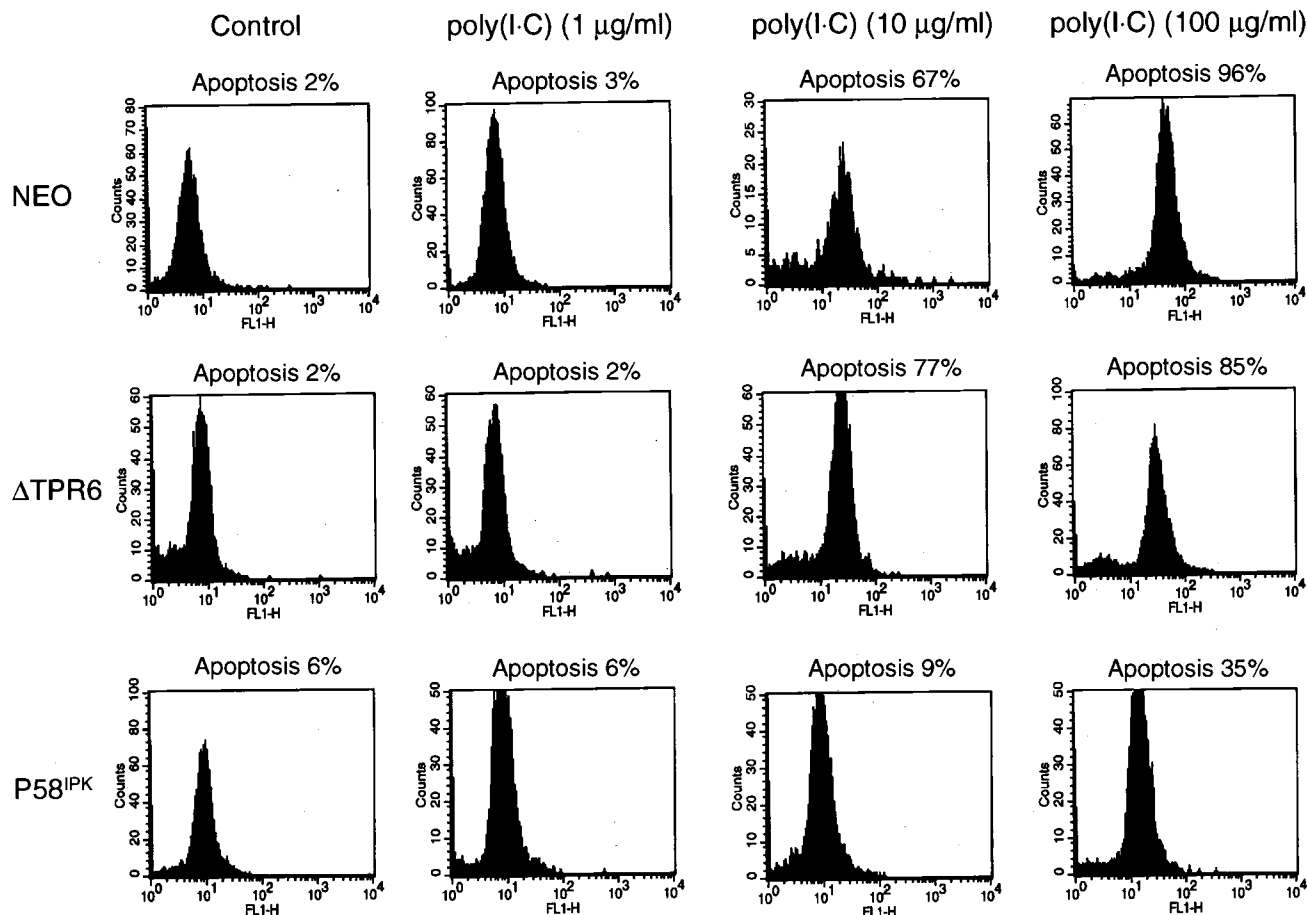
FIG. 4. P58[IPK], but not ΔTPR6, mediates resistance to dsRNA-induced apoptosis. An analysis of dsRNA-induced apoptosis in NEO, ΔTPR6-1, and P58[IPK]-1 cell lines was performed. Cells were treated with poly(I · C) (1, 10, or 100 μg/ml) for 16 h. Apoptosis-induced DNA fragmentation was detected by the labeling of DNA strand breaks with fluorescein dUTP and deoxynucleotidyltransferase. The percentage of apoptotic cells was quantified by flow cytometry as described in Materials and Methods.

P58[IPK] and ΔTPR6 cell lines are resistant to TNF-α-induced apoptosis. To extend these observations, and to determine if P58[IPK] might function as a more general apoptotic inhibitor, we examined the ability of TNF-α to induce apoptosis in the P58[IPK] and ΔTPR6 cell lines. Microscopic examination revealed that in response to TNF-α, only 20% of NEO cells remained viable, whereas the P58[IPK] and ΔTPR6 cell lines each maintained 75% cell viability (Fig. 5A). The loss of viability in NEO cells was confirmed to be due to apoptosis by the presence of extensive DNA laddering (Fig. 5B), a characteristic apoptotic signature of DNA cleavage into oligonucleosome-sized fragments. In contrast, even at a TNF-α concentration of 1.0 ng/ml, DNA laddering was not detected in either the P58[IPK] or the ΔTPR6 cell line. It should be noted that since both cell lines showed activation of NF-κB in response to TNF-α (Fig. 3), resistance to TNF-α-induced apoptosis did not reflect an inability to respond to this cytokine. Moreover, the recent report that PKR[0/0] cells exhibit no defect in the apoptotic response to TNF-α (1) indicates that the ability of P58[IPK] (and ΔTPR6) to mediate resistance to TNF-α-induced apoptosis is not mediated through inhibition of PKR. Rather, it appears that P58[IPK] may function to prevent cells from undergoing programmed cell death in response to a variety of inducers, establishing a novel role for P58[IPK] as an inhibitor of apoptosis.

ΔTPR6-expressing cells exhibit a transformed phenotype and are tumorigenic in nude mice. Our observation that ΔTPR6 was unable to inhibit PKR-dependent functions but acted as a suppressor of TNF-α-induced apoptosis prompted us to examine whether ΔTPR6 retained additional growth-regulatory properties. Microscopic examination revealed that, like the P58[IPK] cell line, ΔTPR6-expressing cells exhibited spindle-shaped morphology and increased refractivity (Fig. 6B and C). Consistent with these morphological changes, transformed foci readily formed on top of ΔTPR6 cell monolayers (Fig. 6F), and ΔTPR6-expressing cells grew faster and to a higher saturation density than the NEO cell line (Table 1). Most significantly, the ΔTPR6 cell line formed colonies in soft agar, indicating anchorage-independent growth (Fig. 6I). We noted, however, that ΔTPR6-expressing cells were approximately twofold less efficient at forming colonies in soft agar than cells overexpressing wild-type P58[IPK] (Table 1). Thus, although ΔTPR6-expressing cells exhibit the characteristics of a transformed phenotype, this phenotype is less pronounced than that observed in the wild-type P58[IPK] cell line.

As a further test of the transforming properties of ΔTPR6, we examined whether ΔTPR6-expressing cells could form tumors in nude mice. For these studies, BALB/c nu/nu mice were injected in the right inguinal area with 2×10^6 ΔTPR6-expressing cells per animal. As controls, additional animals were injected with P58[IPK] or NEO cells. As observed previously (5), mice injected with P58[IPK]-overexpressing cells developed tumors approximately 2 weeks after injection (Table 1). In ac-
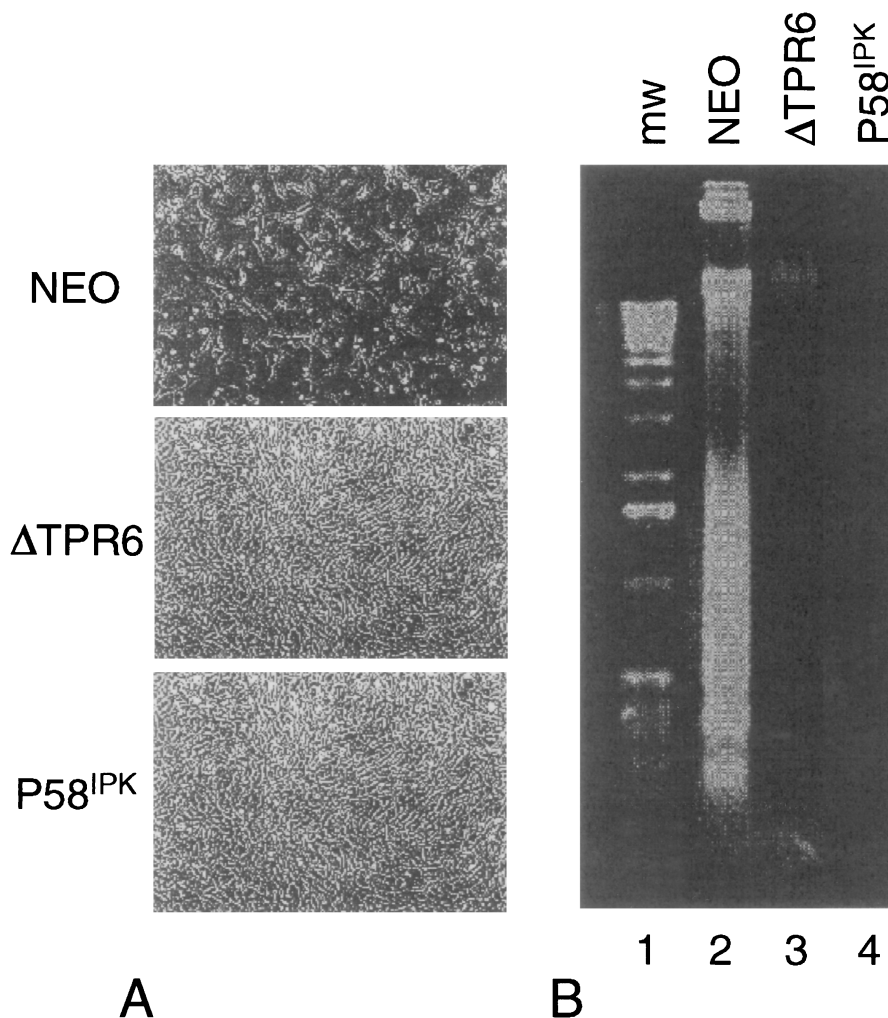
FIG. 5. P58^{IPK} and ΔTPR6 mediate resistance to TNF-α-induced apoptosis. The NEO, ΔTPR6-1, and P58^{IPK}-1 cell lines were treated with TNF-α for 16 h and examined for cell viability and apoptosis-induced DNA fragmentation as described in Materials and Methods. (A) Morphologic characteristics of cell monolayers 16 h post-TNF-α treatment. (B) Electrophoretic analysis of DNA prepared from TNF-α-treated cells.

cord with their transformed phenotype, mice injected with ΔTPR6-expressing cells also developed tumors, although with a longer latency period. No tumors were observed in mice injected with NEO cells. To verify that the tumors produced either ΔTPR6 or the wild-type bovine P58^{IPK} protein, cellular extracts were prepared from tumor homogenates and analyzed by immunoblotting. In each case, a high level of ΔTPR6 or P58^{IPK} protein production was observed (Fig. 7, left panel).

To confirm that P58^{IPK} or ΔTPR6 was responsible for inducing tumor formation, we generated tumor-derived cell lines by culturing excised tumors and selecting for G418-resistant cells. When analyzed by immunoblotting, cell lines derived from P58^{IPK} tumors showed a high level of bovine P58^{IPK} protein production. However, a comparatively low level of the ΔTPR6 protein was produced in cell lines derived from ΔTPR6 tumors (Fig. 7, right panel). This low level of ΔTPR6 may reflect an instability of the ΔTPR6 protein or a downregulation of ΔTPR6 mRNA expression during cell culture. Nevertheless, cell lines derived from ΔTPR6 tumors were capable of inducing rapid tumor formation when injected into a second round of nude mice (Table 1). Together, these results demonstrate that cells expressing a P58^{IPK} mutant that lacks the ability to inhibit PKR are tumorigenic in nude mice, indicating that

inhibition of PKR is not an absolute requirement for P58^{IPK}-induced malignant transformation. Rather, it appears that P58^{IPK} also functions in a PKR-independent manner to malignantly transform cells.

DISCUSSION

In the present report, we have established that P58^{IPK}, originally identified as a cellular inhibitor of PKR, clearly has biological properties independent of the PKR pathway. We have found that P58^{IPK} is an antiapoptotic protein, able to mediate inhibition of programmed cell death in response to both TNF-α and dsRNA signaling. Utilization of cell lines expressing the P58^{IPK} deletion mutant ΔTPR6 has allowed us to further elucidate the complex molecular pathways involving the P58^{IPK} cochaperone. In our model, we suggest that P58^{IPK} functions through at least two separate pathways to mediate resistance to apoptosis (Fig. 8). In response to dsRNA, wild-type P58^{IPK}, but not ΔTPR6, can inhibit PKR-mediated NF-κB activation and eIF-2α phosphorylation. These events lead to a repression of apoptosis through pathways that may involve death effector caspases (3) or deregulation of the translation of mRNAs encoding proapoptotic proteins (44). In con-
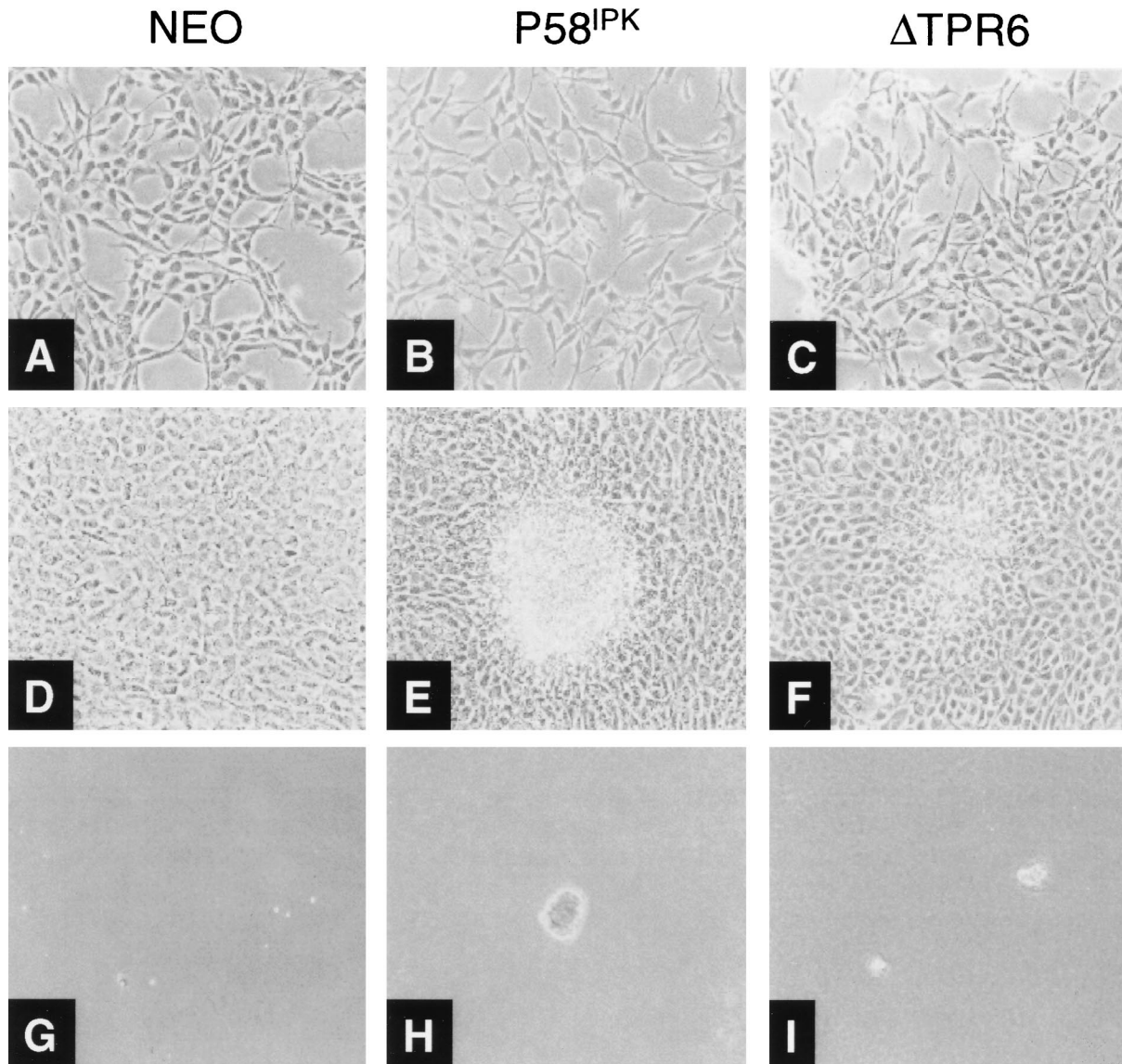
NEO   P58^{IPK}   ΔTPR6

FIG. 6. ΔTPR6-expressing cells exhibit a transformed phenotype. Morphologic and growth characteristics of the NEO, P58^{IPK}-1, and ΔTPR6-1 cell lines are shown. Cell lines were plated at 2×10^4 cells per 100-mm-diameter dish in DMEM containing 10% FBS and 400 μg of G418 per ml. (A through C) Morphologic characteristics of mid-log-phase cells. In contrast to NEO cells, P58^{IPK} and ΔTPR6 cell lines exhibited spindle-shaped morphology and increased refractivity. (D through F) Cells maintained in culture 4 days after they reached confluency, demonstrating transformed foci on P58^{IPK} and ΔTPR6 cell monolayers. (G through I) Growth in soft agar. Anchorage-independent growth was observed in P58^{IPK} and ΔTPR6 cell lines. Magnification, ×100.

trast to dsRNA-mediated events, both wild-type P58^{IPK} and the ΔTPR6 mutant can inhibit apoptosis in response to TNF-α. Based on studies with PKR-null mice (1, 12) and the work presented in this report, it is probable that TNF-α signals primarily through PKR-independent pathways, although one cannot completely rule out a minor PKR involvement. It is tempting to speculate, therefore, that P58^{IPK} and ΔTPR6 suppress apoptosis by blocking an event upstream of PKR. For example, P58^{IPK} may disrupt the recruitment or activation of an upstream caspase, such as caspase 8 (FLICE), which is at the apex of the TNF-α-mediated apoptotic cascade (11). This step in the apoptotic pathway is also a key point for inhibition by a variety of viral antiapoptotic proteins, collectively referred to as FLIPs (FLICE-inhibitory proteins) (7, 53). Together, our results indicate that the ability of P58^{IPK} to suppress apoptosis may be a primary factor in P58^{IPK}-induced malignant transfor-

mation. In addition, the ability of wild-type P58^{IPK} to also inhibit PKR may account for the more-pronounced transformed phenotype observed in P58^{IPK}-overexpressing cells. Compared with the ΔTPR6 cell lines, P58^{IPK}-overexpressing cells exhibited a higher cloning efficiency and faster tumor formation in nude mice.

The ability of P58^{IPK} to suppress apoptosis suggests that there may be parallels between P58^{IPK} and other antiapoptotic proto-oncogenes, such as Bcl-2. Interestingly, Bcl-2 blocks both influenza virus-induced and PKR-mediated apoptosis (22, 30), but in contrast to P58^{IPK}, it does not affect the ability of PKR to inhibit translation. Thus, Bcl-2 is most likely exerting its action downstream of PKR, at or before the point where the PKR-dependent and -independent pathways converge into a common (or core) cell death pathway. Intriguingly, the recently described cochaperone activities of P58^{IPK} may provide

TABLE 1. Growth properties and tumorigenicity of
P58[IPK] and ΔTPR6 cell lines

Clone	Growth property[a]			Tumorigenicity	
	Doubling time (h)	Saturation density (10^6 cells)	Cloning efficiency (%)	Tumor formation[b]	Latency (days)[c]
NEO	32.7 ± 0.4	1.4 ± 0.1	0	0/5	
P58[IPK]-1	21.9 ± 0.2	4.9 ± 0.3	15.2 ± 3.8	5/5	14–16
P58[IPK]-2	23.3 ± 1.3	3.1 ± 0.4	13.3 ± 5.3	2/2	14–16
ΔTPR6-1	22.8 ± 0.9	4.8 ± 0.2	3.1 ± 0.4	5/5	29–35
ΔTPR6-2	25.7 ± 0.2	3.3 ± 0.4	7.0 ± 0.1	5/5	29–51
ΔTPR6-1 (TCL)[d]	ND	ND	ND	6/6	4
ΔTPR6-2 (TCL)	ND	ND	ND	6/6	4

[a] Assays to determine growth properties of P58[IPK] and ΔTPR6 cell lines are described in Materials and Methods. Saturation density is the number of cells present 4 days after cells become confluent. Cloning efficiency (%) is the number of colonies present in soft agar 2 to 4 weeks after plating, divided by the number of cells plated, multiplied by 100. Each value represents the average from two experiments. ND, not determined.
[b] Number of animals with tumors of ≥2 mm per number of animals inoculated.
[c] Number of days required to produce tumors of ≥2 mm.
[d] TCL, tumor cell line.

additional insight into the mechanism by which P58[IPK] suppresses apoptosis. The ability of P58[IPK] to bind to and regulate Hsp70 may provide a link between P58[IPK], the cellular chaperone machinery, and the programmed cell death response. This may be analogous to the link that has been proposed for the antiapoptotic protein BAG-1. BAG-1 interacts with and enhances the activity of Bcl-2, and like P58[IPK], BAG-1 interacts with the ATPase domain of Hsp70 (23, 24, 45). Thus, the cochaperone activities of P58[IPK] may facilitate the interaction of P58[IPK], and possibly Hsp70, with one or more essential components of the apoptotic pathway.

By functioning as a regulator of apoptosis, P58[IPK] may be important not only in cellular antiproliferative pathways but also in the cellular response to influenza virus infection. We demonstrated previously that influenza virus recruits P58[IPK] to downregulate PKR, thereby ensuring the efficient synthesis of viral proteins. Intriguingly, influenza virus infection induces apoptosis (22, 46), and there is evidence for the involvement of
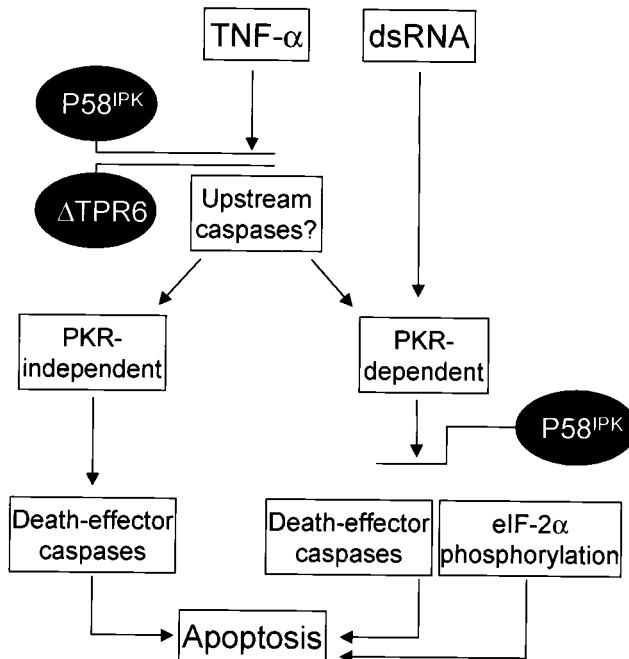

FIG. 7. Bovine P58[IPK] or ΔTPR6 is produced in tumors and tumor cell lines. Immunoblot analysis of P58[IPK] and ΔTPR6 protein production was performed. (Left) Four tumors from mice injected with ΔTPR6 cell lines (two from mice injected with a ΔTPR6-1-expressing cells, designated ΔTPR6-1.1 and ΔTPR6-1.2, and two from mice injected with ΔTPR6-2-expressing cells, designated ΔTPR6-2.1 and ΔTPR6-2.2) and one tumor from a mouse injected with the P58[IPK]-1 cell line (P58[IPK]-1) were analyzed by using the anti-P58[IPK] monoclonal antibody, 2F8 (5). (Right) Cell lines derived from ΔTPR6-expressing and P58[IPK]-overexpressing tumors were analyzed by immunoblotting using the P58[IPK] polyclonal antibody. In each panel, cell extracts prepared from MDBK cells were used as a source of bovine P58[IPK] protein to serve as a positive control (lane 1). Cell extracts prepared from the NEO cell line were analyzed in parallel as a negative control (lane 2).

FIG. 8. Model of P58[IPK] suppression of apoptosis. PKR has been implicated as an essential component of the apoptotic pathway that is induced by dsRNA, and inhibition of PKR by overexpression of P58[IPK] results in resistance to dsRNA-induced apoptosis. Consistent with the inability of ΔTPR6 to inhibit PKR-dependent functions, ΔTPR6-expressing cells undergo apoptosis in response to dsRNA treatment. In contrast, both the P58[IPK] and ΔTPR6 cell lines were resistant to TNF-α-induced apoptosis. We propose that P58[IPK] (and ΔTPR6) can also act in a PKR-independent fashion to regulate the TNF-α pathway. This regulation most likely occurs at a point in the pathway that is upstream of PKR. Suppression of apoptosis may be a primary mechanism by which overexpression of P58[IPK] induces malignant transformation. See Discussion for additional details.
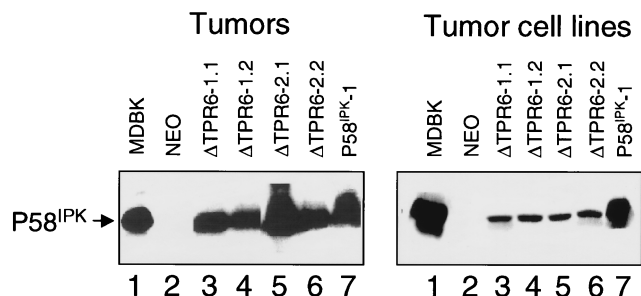
PKR in this process (47). Therefore, the activation of P58[IPK] by influenza virus may also be a mechanism to inhibit or delay PKR-mediated apoptosis, thereby providing sufficient time for viral replication to occur. The ability to inhibit apoptosis is proving to be a general theme among many viruses, and a variety of viral antiapoptotic proteins have been identified (reviewed in references 20, 38, and 51). In addition to the inhibition of FLICE, discussed above, these viral proteins may either mimic the function of cellular antiapoptotic proteins or target various activators of apoptosis for inhibition. For instance, the E1B protein of adenovirus (25) and the KSbcl-2 protein of human herpesvirus 8 (9) are functional homologues of the cellular antiapoptotic protein Bcl-2. Alternatively, the papillomavirus E6 protein blocks apoptosis by targeting p53 for proteolysis (41), and the cowpox virus CrmA protein is a specific inhibitor of caspases (43, 52). The relationship between viral inhibition of PKR and apoptosis is also becoming apparent (49). Infection with a vaccinia virus mutant that lacks the *E3L* gene, which encodes a dsRNA-binding protein that is a potent inhibitor of PKR activation, triggers an apoptotic response that is not observed in cells infected with the wild-type virus (26, 29). There is also evidence that herpes simplex virus may utilize its $\gamma_1 34.5$ gene product to counteract both the translational regulatory and apoptotic activities of PKR. It appears that the $\gamma_1 34.5$ gene product interacts with and directs a cellular type-1 protein phosphatase to reverse PKR-mediated eIF-2α phosphorylation (21). This prevents the shutoff of protein synthesis, an event that is associated with neuronal apo-

ptosis (10). Influenza virus, rather than encoding a viral gene product to inhibit apoptosis, may instead recruit P58^{IPK}. Thus, P58^{IPK} may represent a novel example of a cellular antiapoptotic protein being recruited by a virus to avoid the host's programmed cell death response.

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