

Adenoviral Transfer of the Melanoma Differentiation-associated Gene 7 (*mda7*) Induces Apoptosis of Lung Cancer Cells via Up-Regulation of the Double-Stranded RNA-dependent Protein Kinase (PKR)¹

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Abstract

Adenoviral-mediated overexpression of the melanoma differentiation-associated gene 7 (*Ad-mda7*) induces apoptosis in a wide range of cancer cells, although the mechanism is not well understood. We report that *Ad-mda7* induces and activates the double-stranded RNA-dependent protein kinase (PKR), which leads to phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF-2 α) and the induction of apoptosis in lung cancer cells. Treatment with 2-aminopurine (2-AP), a serine/threonine kinase inhibitor, inhibits PKR activation, eIF2 α phosphorylation, and apoptosis induction by *Ad-mda7*. Additionally, PKR null but not wild-type fibroblasts are resistant to *Ad-mda7*-induced apoptosis. These results suggest that the activation of PKR and its downstream targets may be a critical pathway for *Ad-mda7*-mediated apoptosis.

Introduction

*mda-7*⁴ is a novel tumor suppressor gene that was first identified by subtraction hybridization of a human melanoma cell line (HO-1) induced to terminally differentiate by IFN- β and mezerin (1). The *mda-7* cDNA encodes a novel evolutionarily conserved protein of 206 amino acids with a predicted size of M_r 23,800 and limited homology to the pleiotropic homodimeric cytokine IL-10 (2–4). Several groups have demonstrated that *Ad-mda7* (using *Ad-mda7*) induces apoptosis in a wide range of cancer cells (breast, colorectal, cervical, sarcoma, glioblastoma, prostate, and lung; Refs. 3, 5, 6). Although clearly tumor suppressive, the mechanism by which *Ad-mda7* induces apoptosis is not known. In this paper, we evaluate the role of the IFN-induced, dsRNA-activated serine/threonine kinase (PKR) in *Ad-mda7*-induced apoptosis because of PKR's known role as mediator of

antiviral and antitumor responses. Two dsRNA-binding domains reside in the NH₂ terminus, and interaction with dsRNA or other activators modifies the conformation of PKR allowing it to undergo autophosphorylation and activation (7–9). Once activated, PKR is able to phosphorylate a variety of substrate targets, the most well characterized being eIF-2 α , which can lead to the inhibition of protein synthesis, growth suppression, and apoptosis induction (10–12). We report in this study that transduction of lung cancer cells with *Ad-mda7* results in PKR activation, eIF-2 α phosphorylation, and apoptosis induction. Pretreatment of the cells with 2-AP (a serine/threonine kinase inhibitor) inhibits PKR activation, eIF-2 α phosphorylation, and apoptosis. Additionally, PKR null but not wild-type fibroblasts are resistant to *Ad-mda7*-induced apoptosis. These results suggest a novel role for PKR as a critical mediator of *Ad-mda7* apoptosis induction in lung cancer cells.

Materials and Methods

Cell Lines and Reagents. The human lung cancer cell lines A549 (wt p53) and H1299 (p53 null) were obtained from the American Type Culture Collection. PKR^{+/+} and PKR^{-/-} MEF cells were obtained from Dr. Glen Barber (University of Miami School of Medicine, Miami, FL; Ref. 13). MEF cells were maintained in DMEM containing 10% fetal bovine serum, 10 mM glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin (Life Technologies, Inc., Grand Island, NY) in a 5% CO₂ atmosphere at 37°C. 2-AP was obtained from Sigma Chemical Co. (St. Louis, MO).

Adenovirus Production. Construction of the *Ad-mda7*, AdBak, and Ad-Luc vectors have been previously reported (3, 14). The transduction efficiencies of adenoviral vectors in various cancer cell lines were determined by infecting cells with Ad-LacZ and then determining the titers needed to transduce at least 70% of the cells.

Flow Cytometry Analysis and XTT assay. We measured apoptotic cells by propidium iodide staining and FACS analysis. Cells were harvested, pelleted by centrifugation, and resuspended in PBS containing 50 μ g/ml propidium iodide, 0.1% Triton X-100, and 0.1% sodium citrate. Samples were stored at 4°C for 16 h and vortexed before FACS analysis (Becton-Dickenson FACScan, Mountain View, CA; FL-3 channel). Cellular viability was assessed with the XTT assay by growing cells in 96-well plates at 100- μ L volume/well. After treatment with *Ad-mda7* or control vectors, cells were incubated with the tetrazolium salt XTT according to the Roche protocol (Roche Diagnostics, Mannheim, Germany). Viability was assessed spectrophotometrically on an ELISA plate reader.

Immunoblot Assays. Forty-eight h after transfection, cell extracts were prepared for immunoblot assays as described previously (14). The following antibodies were used: for PKR (K-17); eIF-2 α , β -actin, and p38 (A-12); phosphospecific p38 (D-8); stat1 (C-136); phosphospecific stat1 (A-2); stat3 (F-2); phosphospecific stat3 (B-7); anti-Tyk2 (C-20) and phosphospecific anti-Tyk (PY-99; Santa Cruz Biotechnology, Santa Cruz, CA); caspase 3, caspase-9, caspase-8, and Bid (PharMingen, San Diego, CA), and the phosphospecific PKR (pT451) and eIF-2 α (pS51; BioSource International, Ca-

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⁴ The abbreviations used are: *mda-7*, melanoma differentiation-associated-7 (gene); 2-AP, 2-aminopurine; dsRNA, double-stranded RNA; MEF, mouse embryo fibroblast; eIF-2 α , α subunit of eukaryotic translation initiation factor 2; ds, double-stranded; PKR, dsRNA-dependent protein kinase; IL, interleukin; *Ad-mda7*, adenoviral-mediated overexpression of *mda-7*; FACS, fluorescence-activated cell sorting; XTT, 2,3-bis[2-methoxy-4-nitro-5-sulfonylphenyl]-2H-tetrazolium-5-carboxanilide inner salt; PARP, poly(ADP-ribose) polymerase.

marillo, CA). The polyclonal and monoclonal antibodies to Mda-7 were obtained from Introgen Therapeutics, Inc. (Houston, TX).

Measurement of Cellular Protein Synthesis. Cells were treated with Ad-mda7 or Ad-Luc for 48 h. Where indicated in the experiment, cells were treated with 1 mM 2-AP for 48 h. Measurement of protein synthesis was performed as described previously (15). After treatment, $\sim 1 \times 10^6$ cell equivalents were incubated with L-[U- 14 C] amino acid mixture (Amersham, Piscataway, NJ) at 2 μ Ci/ml for 10 min at 37°C. The reaction was terminated by the addition of 20% (wt/vol) trichloroacetic acid, and the radioactivity in the acid-precipitable fraction was measured in a scintillation counter.

Statistical Analysis. The data reported in Figs. 1, 3, and 4 represent the mean of three or more independent experiments and the bars show the SD. ANOVA and two-tailed Student's *t* test were used for statistical analysis of multiple groups and pair-wise comparison, respectively, with *P* < 0.05 considered significant.

Results

Ad-mda7 Induces PKR Expression and Apoptosis in Lung Cancer Cells. Flow cytometric analysis of apoptosis was performed on the A549 (wt p53) and H1299 (null p53) lung cancer cells 72 h after infection with Ad-mda7, Ad-Luc, or PBS. Only Ad-mda7 trans-

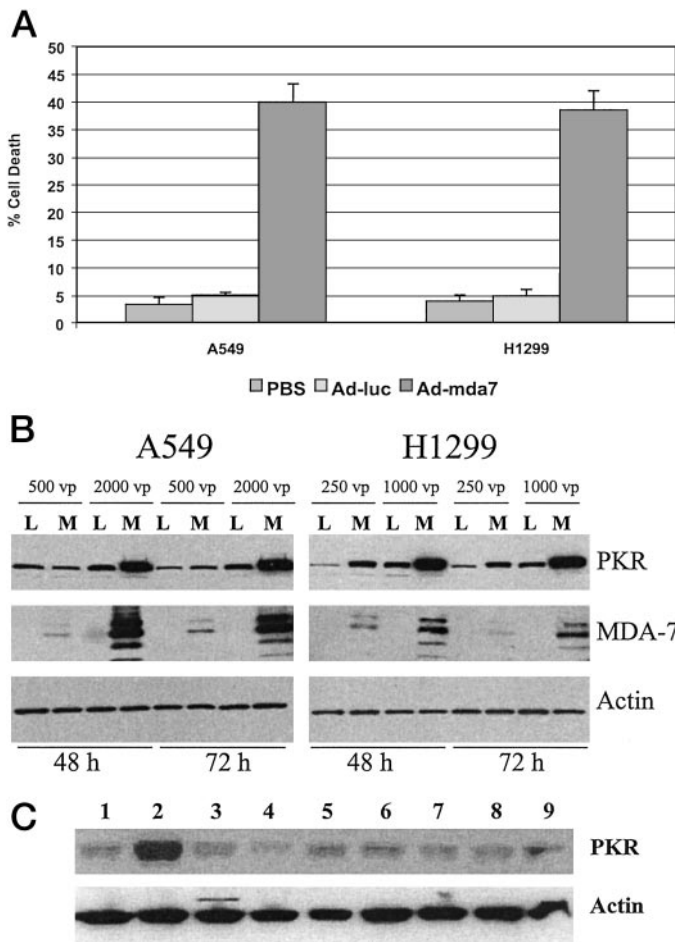


Fig. 1. Ad-mda-7 induces cell death and increased PKR expression. **A**, percentage of cell death in A549 (wild-type p53) and H1299 (null p53) cells after treatment with PBS, Ad-Luc (2500 viral particles [vp]), or Ad-mda7 (2500 vp). The cells were analyzed by flow cytometry 72 h after transduction. Triplicate experiments were performed for each cell line, and data are represented as mean \pm SD. **B**, Western blot analysis of the dose-dependent expression of PKR after Ad-mda7 transduction. H1299 and A549 cells were treated with increasing doses of Ad-mda7 (M) and Ad-Luc (L) and assayed 48 or 72 h after transduction. The expression level of actin was used as a control. **C**, Western blot analysis of the specificity of Ad-mda7 up-regulation of PKR in A549 cell lysates assayed 48 h after treatment with PBS (Lane 1), Ad-mda7 (Lane 2), Ad-Bak (Lane 3), Ad-Bax (Lane 4), Ad-LacZ (Lane 5), Ad-GFP (Lane 6), Ad-p53 (Lane 7), staurosporine (Lane 8) and Ad-Luc (Lane 9). The expression level of actin was used as a control.

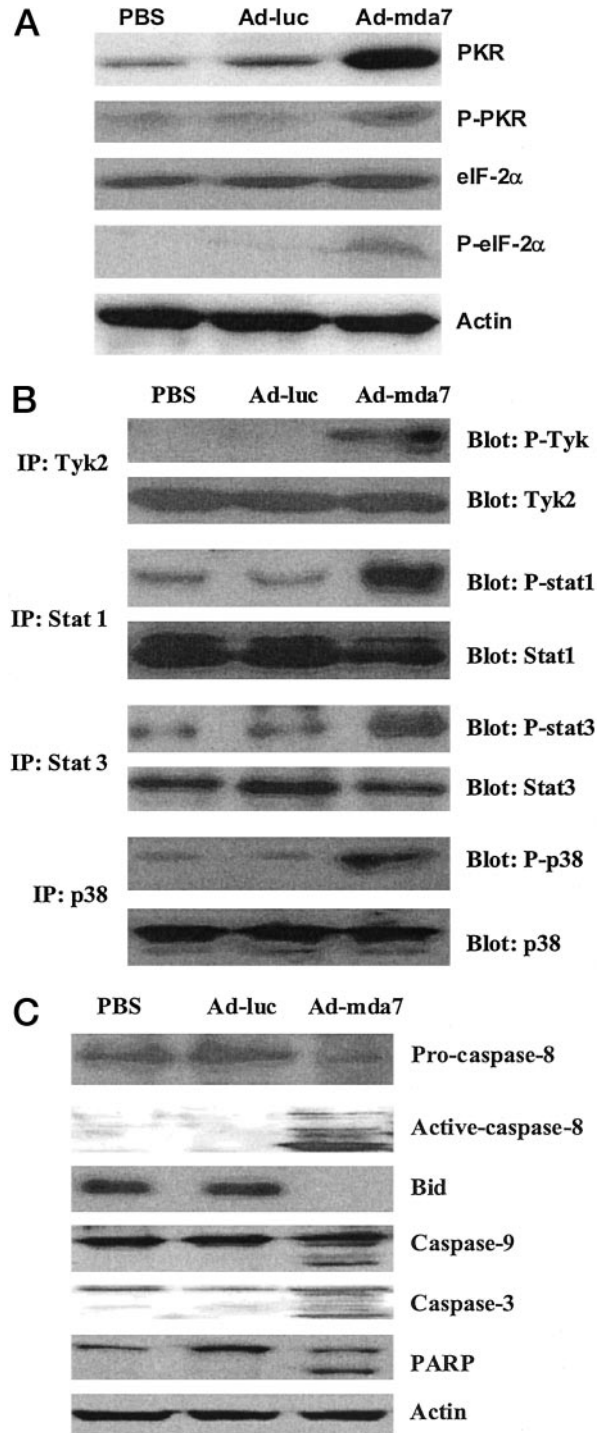


Fig. 2. Ad-mda7 induces phosphorylation of PKR, eIF-2 α , and other PKR substrate targets with resulting Bid, PARP, and caspase cleavage. **A**, Western blot analysis of the expression of PKR, phospho-PKR, eIF-2 α , and phospho-eIF-2 α in A549 cell lysates 48 h after treatment with PBS, Ad-Luc, or Ad-mda7. The expression level of actin was used as a control. **B**, protein fractions, obtained from A549 cells, treated with PBS, Ad-Luc, or Ad-mda7 for 48 h were immunoprecipitated with antihuman Tyk2, Stat1, Stat3, or p38. **C**, Western blot analysis of the expression of Bid, PARP, caspase-3, caspase-8, and caspase-9 cleavage in A549 cells 48 h after treatment with PBS, Ad-Luc, or Ad-mda7. The expression level of actin was used as a control.

duction resulted in apoptosis in the lung cancer cells (Fig. 1A). We also noted the inhibition of cellular viability by XTT assay after infection of Ad-mda-7, Ad-Luc, or PBS control. Consistent with the FACS results, Ad-mda7-infected cells showed significant inhibition of cell growth 48 h after transduction (data not shown). Western blot

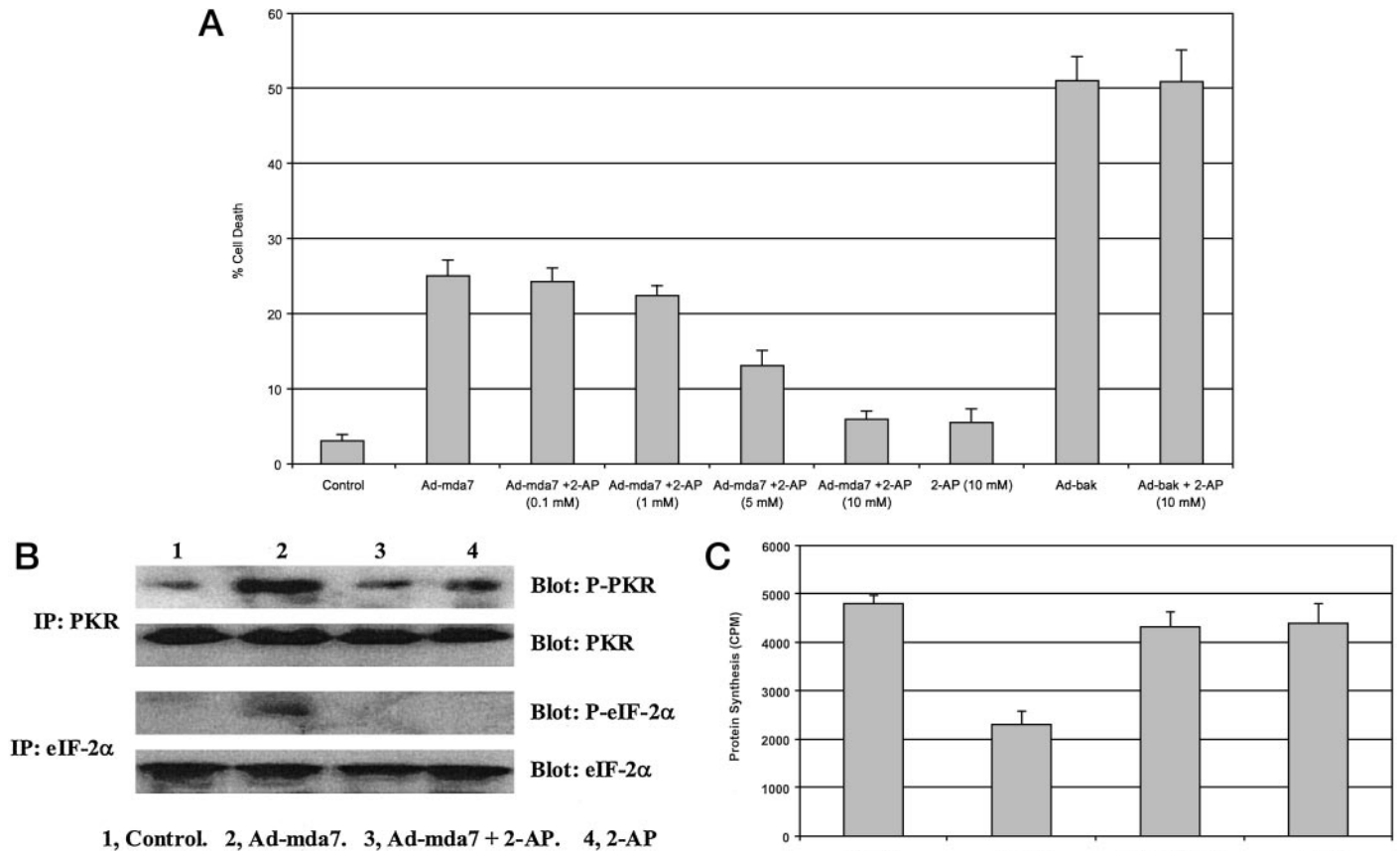


Fig. 3. Treatment of lung cancer cells with 2-AP (serine/threonine kinase inhibitor) after Ad-mda7 transduction blocks apoptosis induction, eIF-2 α phosphorylation, and eIF-2 α inhibition of protein synthesis. **A**, percentage of cell death in A549 cells after treatment with PBS, Ad-Luc, Ad-Bak, or Ad-mda7 in combination with increasing doses of the PKR inhibitor, 2-AP, administered 30 min before Ad-mda7 treatment. **B**, protein fractions, obtained from A549 cells, treated with PBS, Ad-Luc, or Ad-mda7 in combination with 10 mM 2-AP for 48 h were immunoprecipitated with antihuman PKR and eIF-2 α and assayed for phospho-PKR or phospho-eIF-2 α . Results of one representative experiment of three are shown. **C**, cells were treated with Ad-mda7 or Ad-Luc in combination with 10 mM 2-AP for 48 h. Measurement of protein synthesis was performed as described in "Materials and Methods."

expression demonstrated dose-dependent increases in PKR (Fig. 1B) after Ad-mda7 transduction but not after transduction with control vectors (Ad-Luc), PBS, or other pro-apoptotic vectors such as Ad-Bak (Fig. 1C).

Ad-mda7 Activates PKR, eIF2 α , and Other PKR Substrate Targets. To determine whether Ad-mda7 could activate PKR *in vitro*, Ad-mda7-treated cells (A549) were assessed by immunoblot assay for the presence of phosphorylated PKR. Only Ad-mda7-treated cells demonstrated increased expression of PKR and its active phosphorylated form (Fig. 2A). Activation of the serine/threonine kinase was also demonstrated by phosphorylation of PKR's downstream targets eIF-2 α , Tyk2, Stat1, Stat3, and p38 (Fig. 2, A and B). Treatment with Ad-mda7 led to subsequent apoptosis induction with caspase 3, 8, and 9 activation, and Bid and PARP cleavage (Fig. 2C). The activation of PKR appeared to be specific for Ad-mda7 and upstream of caspase activation because pretreatment with caspase inhibitors failed to block PKR phosphorylation (data not shown).

Ad-mda7 Apoptosis Induction Is Dependent on PKR Activation. To assess whether Ad-mda7 apoptosis induction was dependent on PKR activation, we evaluated the effect of the specific serine/threonine kinase inhibitor 2-AP. A high concentration of 2-AP (10 mM) alone was found to have no effect on cell viability. However, in cells treated with Ad-mda7, 2-AP blocked apoptosis induction and cell killing in a dose-dependent manner (Fig. 3A). The inhibition of apoptosis by 2-AP appeared specific to Ad-mda7 and was not seen after treatment with control vectors (Ad-Luc) or Ad-Bak (Fig. 3A). Immunoprecipitation studies demonstrated that 2-AP treatments at-

tenuated PKR activation with the inhibition of both PKR and eIF-2 α phosphorylation (Fig. 3B). The ability of 2-AP to block eIF-2 α phosphorylation was manifested by normal protein synthesis and apoptotic induction (Fig. 3, A and C). To confirm the contribution of PKR-activation to Ad-mda7 apoptotic activity, MEFs from PKR knockout mice were evaluated. Despite adequate transduction and expression of mda-7 protein in both PKR null ($-/-$) and wild-type MEFs (Fig. 4A), only PKR wild-type MEFs underwent apoptosis induction after Ad-mda7 treatment (Fig. 4, B and C), which suggested that Ad-mda7-induced cell killing was dependent on PKR. Unlike Ad-mda7, Ad-Bak apoptosis induction did not appear to be dependent on PKR genomic status, with apoptosis occurring in both PKR null and wild-type MEFs (Fig. 4B).

Discussion

Adenoviral-mediated gene transfer of the tumor suppressor p53 gene has demonstrated tumor regression in selected patients with lung cancer (16). Because not all patients respond to Ad-p53, there is a need for other tumor suppressor genes to be developed for adenoviral gene transfer in lung cancer. mda-7 is a novel tumor suppressor gene that was first identified by subtraction hybridization of a melanoma cell line induced to terminally differentiate IFN- β and mezerin (1). Adenoviral overexpression of mda-7 leads to tumor-selective growth suppression and apoptosis induction in various tumor types, including colorectal, breast, and lung carcinoma (3, 5, 6). The mechanism by which Ad-mda7 induces apoptosis, however, is not well defined.

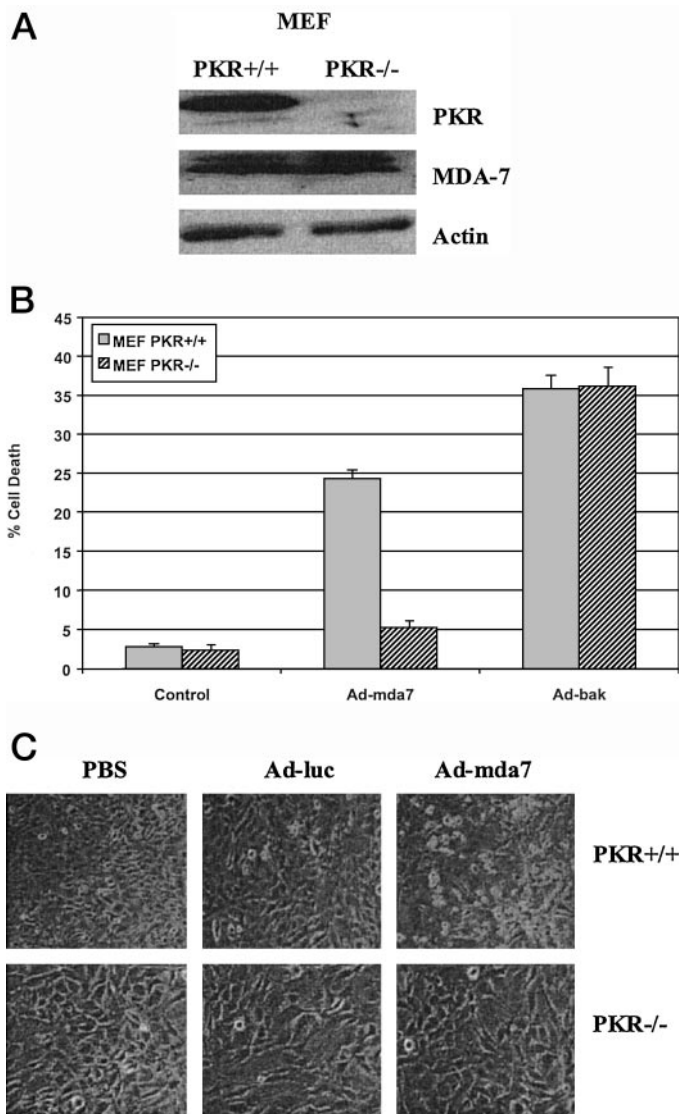


Fig. 4. PKR-dependent induction of Ad-mda7-induced apoptosis in MEFs from PKR^{+/+} and PKR^{-/-} cells. **A**, Western blot analysis of the expression of PKR, MDA-7, and actin in MEF PKR^{+/+} and PKR^{-/-} cell lysates 48 h after treatment with Ad-mda7, demonstrating similar levels of transduced MDA-7. **B**, percentage of cell death in MEF PKR^{+/+} and PKR^{-/-} cells after treatment with PBS, Ad-Bak, and Ad-mda7. The cells were analyzed by flow cytometry 48 h after transduction. **C**, morphological signs of apoptosis were seen only in PKR^{+/+} cells treated with Ad-mda7 and not in PKR^{-/-} cells or after treatment with Ad-Luc or PBS.

Some authors have noted increases in pro-apoptotic proteins such as p53, Bax, and Bak after Ad-mda7 transduction (6), whereas others have observed sharp rises in the pro-inflammatory cytokines IL-6, IL-12, and IFN- γ (4). These studies, however, have been largely correlative and have not addressed which, if any, upstream genes Ad-mda7 engages before caspase activation and apoptosis.

In this paper, we demonstrate for the first time clear up-regulation of the IFN-induced, serine/threonine protein kinase PKR. Ad-mda7 transduction of lung cancer cells leads to PKR induction in a p53-independent manner (Fig. 1, **A** and **B**). We have also seen PKR up-regulation in other types of cancer cells, including colorectal and breast, which suggests that this relationship is not specific to lung cancer cells (data not shown). Up-regulation of PKR also appears specific for Ad-mda7 because little PKR change is seen after transduction of cancer cells with adenoviral vectors containing the luciferase reporter gene or other pro-apoptotic genes such as *Ad-Bak* (Fig. 1, **B** and **C**). In addition, PKR induction does not appear to be

attributable to nonspecific caspase cleavage because blockage with caspase inhibitors did not abrogate PKR up-regulation, and Ad-Bak apoptosis induction failed to induce PKR (Fig. 1C).

PKR is well characterized in the literature as an antiviral immune mediator responding to viral dsRNA by activation of eIF-2 α , protein synthesis inhibition, and blockage of viral protein production. Many viruses contain inhibitory proteins to block PKR activity (7). Our studies suggest that Ad-mda7-induced PKR up-regulation is not solely a response to the adenoviral construct, because Ad-Luc and Ad-Bak fail to induce PKR activation and Ad-Bak is not dependent on PKR genomic status to induce apoptosis in MEFs. Indeed, our studies give further support to the premise that one of PKR's main cellular functions is as a regulator of tumorigenesis (7). Activation of PKR in HeLa, Cos1, U937, and NIH3T3 tumor cells leads to apoptosis induction and cellular death (7). Additionally, MEFs from PKR knockout mice are resistant to apoptotic cell death in response to a variety of stimuli including dsRNA, tumor necrosis factor α , and lipopolysaccharide (12). Further evidence of PKR's antitumorigenic function includes critical tumorigenic deletions in myelodysplasias of the *IRF-1* gene that appear to be associated with decreased PKR levels (7, 17) and in associations with PKR expression and prolonged lung and colorectal cancer survival (18).

PKR appears to mediate antitumorigenic activity through the activation of multiple transduction pathways culminating in growth inhibition and apoptosis induction. Activation of these pathways occurs after the latent, inactive homodimeric form, located predominantly in the cytoplasm, is induced by activating signals to undergo conformational changes leading to auto-phosphorylation and activation (9). Once activated, PKR is able to phosphorylate various substrate targets that are important in growth control and apoptosis induction (10, 11). Our immunoprecipitation studies are consistent with this model showing PKR activation after Ad-mda7 transduction leading to increases in phosphorylated (active) PKR and phosphorylated eIF-2 α (Fig. 2A). We, also, demonstrate after Ad-mda7 transduction, the phosphorylation of several other PKR substrate targets that may be associated with apoptosis induction and growth control, including Stat1, Stat3, and p38 (19, 20). The activation of PKR appears to be a critical event in Ad-mda7 apoptosis (as opposed to Ad-Bak) because the inhibition of PKR with the specific threonine/kinase inhibitor, 2-AP leads to the inhibition of Ad-mda7 apoptosis, eIF-2 α phosphorylation, and protein synthesis inhibition. The inhibition of protein synthesis may be critical to the induction of apoptosis possibly because of the regulation of one or more short-lived proteins involved in apoptosis inhibition. Alternatively, other pathways controlled by PKR may be important, such as those involved in the regulation of NF- κ B, p53, MEK, IRF-1, or FADD (7, 21–23).

Although multiple pathways may be involved, PKR activation appears to be critical for Ad-mda7 apoptosis because MEFs lacking PKR were unable to undergo apoptosis as opposed to MEFs with wild-type PKR. This inhibition of apoptosis appeared specific to MDA-7 because transduction of MEFs lacking PKR with the pro-apoptotic Ad-Bak vector led to unimpaired apoptosis. These observations can be explained by a model in which MDA-7 and PKR are upstream of the caspases and the pro-apoptotic *Bak* gene. In this model, mda-7 induces PKR up-regulation with subsequent activation of various cellular pathways that lead to caspase activation and apoptosis induction. Bak being downstream of PKR and MDA-7 is not dependent on PKR activation to induce apoptosis or caspase cleavage. It is, also, interesting to note that we saw clear evidence of Bid and caspase 8 cleavage, which is consistent with other laboratories that have suggested PKR apoptosis is often mediated through activation of the Fas/FADD death receptor pathway with caspase-8

and Bid cleavage leading to subsequent caspase 3 and 9 activation (23).

In summary, our paper demonstrates for the first time that Ad-mda7 leads to the rapid induction of PKR with subsequent phosphorylation of eIF-2 α , other PKR target substrates, and apoptosis induction. Specific inhibition of PKR by 2-AP in lung cancer cells inhibits Ad-mda7-induced PKR activation, PKR substrate target phosphorylation, and apoptosis induction. As evidenced by PKR null fibroblasts, Ad-mda7 apoptosis appears dependent on a functional PKR pathway. These results suggest a novel role for the multifunctional PKR gene as a critical mediator of Ad-mda7 apoptosis and give insight into the mechanism of action of Ad-mda7. In the future, novel therapeutic strategies aimed at activating PKR or blocking PKR inhibitors may be able to further enhance the antitumoral efficacy of Ad-mda7.

Acknowledgments

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