In Vivo Regulation of the dsRNA-Dependent Protein Kinase PKR by the Cellular Glycoprotein P67†

Jesús Gil,‡ Mariano Esteban,‡ and Don Roth*,‡,§

Department of Molecular and Cellular Biology, Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas, Campus Universidad Autónoma, 28049 Madrid, Spain, and Department of Molecular Biology, University of Wyoming, Laramie, Wyoming 82071

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ABSTRACT: Regulation of eIF2α phosphorylation is critical to the maintenance of cellular homeostasis, and eIF2α kinases are subject to complex and multidimensional controls. A cellular 67 kDa glycoprotein (p67) has been proposed to have an important role in regulating the activity of eIF2α kinases including the interferon-induced, dsRNA-stimulated protein kinase PKR. To dissect p67−PKR interactions and evaluate their significance in vivo, we have used a vaccinia virus (VV) expression system that successfully mimics PKR control pathways. Recombinant VV were constructed that constitutively express p67 and inducibly express PKR in BSC-40 cells. Stable expression of p67 reduced the PKR-mediated antiviral response and apoptosis. These effects correlated with decreased eIF2α phosphorylation, with rescue of PKR-mediated inhibition of protein synthesis, and with partial inhibition of PKR-triggered activation of NF-κB. The direct interaction between PKR and p67 was suggested by in vivo and in vitro analyses. These data demonstrate that in vivo p67 is an important modulator of PKR-mediated signal transduction pathways and may provide a useful tool to dissect the relative contributions of PKR to cell growth and stress response.

Phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF2α)[1] is an important mechanism to regulate protein synthesis and maintain cellular homeostasis (reviewed in *1*−*3*). Formation of the preinitiation ternary complex of eIF2−GTP−$tRNA^{i}_{met}$ is inhibited as a direct result of specific eIF2α phosphorylation on serine 51 (*3*−*5*). In yeast, decreased ternary complex activity results in the specific translational upregulation of GCN4, a transcriptional activator of amino acid biosynthesis genes, although overexpression of eIF2α kinases can lead to global decreases in protein synthesis (*6*−*8*). In mammalian cells, eIF2α phosphorylation generally causes a global reduction in mRNA translation; however, there is evidence suggesting specific effects on mRNA (*9*−*12*). This contributes, in part, to the cellular antiviral and abiotic stress responses and the control of apoptosis and proliferation (*1, 13*−*21*). The kinases that specifically phosphorylate eIF2α on serine 51 include the double-stranded RNA-stimulated kinase (PKR), the heme-dependent inhibitor (HRI), the plant-encoded homologue of PKR (pPKR), the endoplasmic reticulum resident kinase (PERK, also called PEK), and the GCN2 gene product (*2, 7, 22*−*26*).

PKR is a major mediator of dsRNA and interferon-associated effects in cells (*2, 26, 27*). Binding to dsRNA promotes PKR dimerization, induces autophosphorylation, and activates exogenous substrate phosphotransferase activity. Additionally, PKR dimerization occurs following binding to the protein activator PACT/RAX (*28, 29*). The best characterized PKR substrate is eIF2α (*2, 3, 26*); however, it is now recognized that PKR regulates the action of several transcription factors (*30*−*34*). Thus, the relative contribution of translational and transcriptional activity to PKR-mediated control of biotic and abiotic stress, cell cycling, differentiation, proliferation, and apoptosis remains a major issue (*16*−*19, 35*−*40*). Clearly the inappropriate regulation of PKR activity has dramatic and severe consequences on cell homeostasis. Overexpression of wild-type PKR in mammalian, insect, and yeast cells drastically inhibits cell growth and induces apoptosis (*8, 18, 26, 38*−*40*), whereas expression of a dominant negative mutant causes malignant transformation in NIH 3T3 cells (*17*).

The importance of PKR is also supported by the presence of numerous cellular and viral modulators of its activity (*2, 41*). Among these is a M_r 67 000 glycoprotein (p67) first identified as a copurifying component in eIF2 fractions (*42*). P67 contains multiple O-linked *N*-acetyl-β-D-glucosamine

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* Address correspondence to this author at the Department of Molecular Biology, University of Wyoming, Laramie, WY 82071. Phone: 307-766-5719; Fax: 307-766-5098; E-mail: rothdon@uwyo.edu.

‡ Consejo Superior de Investigaciones Científicas, Campus Universidad Autónoma.

§ University of Wyoming.

[1] Abbreviations: eIF2α, α subunit of eukaryotic initiation factor 2; DMEM, Dulbecco's modified Eagle's medium; WR, western reserve; CPRG, chlorophenol red-β-D-galactopyranoside; NCS, newborn calf serum; PKR, interferon-induced dsRNA dependent protein kinase; TCA, trichloroacetic acid; BSA, bovine serum albumin; VV, vaccinia virus; dsRNA, double-stranded RNA.; BLOTTO, PBS containing 5% nonfat dry milk; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Tk, thymidine kinase.

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(GlcNAc) residues that are critical for inhibition of eIF2α phosphorylation (*43*). In vitro decreased protein synthesis levels and increased eIF2α phosphorylation are correlated with decreased p67 levels or deglycosylation (*43, 44*). Similarly in vivo, correlative evidence has been presented that p67 has a role in control of protein synthesis, although interaction with PKR has not been clearly established (*45*−*48*). Wu et al. (*48*) found that transient expression of p67 in COS cells reduced eIF2α phosphorylation and increased expression of plasmid-derived reporter mRNA relative to control cells transfected with empty vector.

To understand the contribution of p67 to the regulation of cellular homeostasis, it is critical to directly evaluate the functional consequences of p67−PKR interactions in vivo. To address this issue, we have taken advantage of a unique vaccinia virus (VV)−cell system that allows the constitutive expression of p67 and the inducible expression of PKR. Expression of p67 rescues BSC-40 cells from the antiviral effects of PKR induction following VV infection, and PKR-mediated decreases in protein synthesis are specifically abrogated by p67. These effects are directly correlated with p67 protection of eIF2α phosphorylation and, in part, by inhibition of PKR-mediated activation of NF-κB. The association between p67 and PKR is supported by in vitro and in vivo binding assays. Taken together these results support the concept that p67 is an in vivo modifier of PKR activity.

EXPERIMENTAL PROCEDURES

Materials. Chemicals were from Sigma (St. Louis, MO) unless otherwise specified. Monoclonal antiserum to glycosylated p67 was a gift from N. Gupta (*49*, University of Nebraska, Lincoln). A peptide spanning amino acids 464−478 of the human p67 was synthesized at the CNB to produce antibodies against p67. Polyclonal rabbit antibody specific for p67 was obtained after repeated immunizations with the purified peptide coupled to keyhole limpet hemocyanin. Polyclonal antisera specific for PKR and VV proteins have been previously described (*18, 50*). Monoclonal antiserum recognizing eIF2α was a gift from Cesar de Haro. Antiserum specific for the phosphorylated form of eIF2α was purchased from Research Genetics (Huntsville, AL). Secondary antibodies were from Cappel (Durham, NC). Plasmid pGEMp67 containing the 1.9 kb cDNA encoding p67 was obtained from N. Gupta (*51*). The p67 cDNA was excised with *Pst*I/*Eco*RV, purified, and subcloned into the *Pst*I−*Sma*I site of the hemagglutinin insertional VV vector pHLZ (*50*).

Cells and Viruses. African green monkey kidney cells BSC-40 (ATCC CCL-26) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated newborn calf serum (NCS). After mock-inoculation or viral adsorption, cells were maintained with DMEM supplemented with 2% NCS. VV recombinant expressing PKR (K296R) mutant has been previously described (*52*). The recombinant VV expressing IPTG inducible PKR from the HA site (termed VV PKR) was generated as described below by recombination of empty plasmid pHLZ with the western reserve (WR) strain of VV expressing PKR (WR 68K) (*13*). VV−p67 was generated by homologous recombination of the pHLZ−p67 with wild-type VV in BSC-40 cells, as previously described (*53*), and selected by blue plaque formation in response to addition of X-gal. VV PKR−p67 was generated by recombination of pHLZ−p67 with the WR 68K virus, following standard procedures (*54*). Viruses were subjected to 5 rounds of plaque purification to generate homogeneous population of recombinants. A scheme representing vectors introduced into the different viruses is presented in Figure 1A.

Measurement of β-Galactosidase Activity. Confluent BSC-40 cells seeded in 24 well plates were infected with 5 pfu per cell of the indicated viruses. After 1 h of viral adsorption, 5 mM IPTG was added to induce PKR expression. Cells were collected at the indicated times, resuspended in 100 μL of 0.25 M Tris, pH 7.8, and lysed by three freeze−thaw cycles. After lysis, extracts were diluted to 1 mL with water and centrifuged, and 10 μL of supernatant was used for β-galactosidase determination, performed in duplicate. Supernatants from cell lysates (10 μL) were mixed with 150 μL of chlorophenol red−β-D-galactopyranoside (CPRG) solution [1 mM $MgCl_2$, 45 mM β-mercaptoethanol, 0.1 M sodium phosphate (pH 7.5), 5 mM CPRG] in a 96 well plate and incubated at 37 °C for 1 h, and the absorbance at 540 nm was determined. Experiments were repeated at least twice.

Measurement of Apoptosis. The Cell Death Detection Enzyme-linked immunosorbent assay (ELISA) kit (Roche) was used according to the manufacturer's instructions. This assay is based on the quantitative sandwich-enzyme-immunoassay-principle and uses mouse monoclonal antibodies directed against DNA and histones to estimate the amount of cytoplasmic histone-associated DNA.

For measurement of caspase-3 activity, 3×10^6 BSC-40 cells were collected, lysed in lysis buffer (150 mM KCl, 10% glycerol, 1 mM dithiothreitol, 5 mM magnesium acetate, 0.5% Nonidet P-40), and clarified by centrifugation. Equal amounts of supernatant and 2× reaction buffer (100 mM HEPES, pH 75, 20% glycerol, 5 mM dithiothreitol, 0.5 mM EDTA) were mixed and assayed for caspase-3 activity using 200 μM DEVD-pNA (Calbiochem) as substrate. Free pNA produced by caspase activity was determined by measuring the absorbance at 405 nm. All apoptosis analyses were repeated at least twice.

Metabolic Labeling of Proteins. BSC-40 cells cultured in 12 well plates were infected with the viruses indicated and rinsed 3 times with Met-Cys-free DMEM 30 min prior to labeling. Following incubation for an additional 30 min at 37 °C with Met-Cys-free DMEM, medium was removed and 50 μCi/mL [^{35}S]Met-Cys promix (Amersham) in Met-Cys-free DMEM was added for an additional hour. After 3 washes with PBS, cells were harvested in lysis buffer followed by SDS−PAGE and autoradiography. Protein concentrations were determined by the bicinchoninic acid assay (Pierce) with bovine serum albumin (BSA) as a standard. An aliquot of the cell lysate was diluted in 0.1 mg/mL BSA solution, and proteins were precipitated with 5% trichloroacetic acid (TCA) and collected on glass fiber filters with a vacuum manifold instrument (Millipore). Filters were dried, and radioactivity was counted in a scintillation counter with liquid scintillation cocktail. Experiments were repeated at least twice.

Immunoblotting. For immunoblot analysis, total cell extracts were boiled in Laemmli sample buffer, and proteins were fractionated by SDS−PAGE. After electrophoresis,

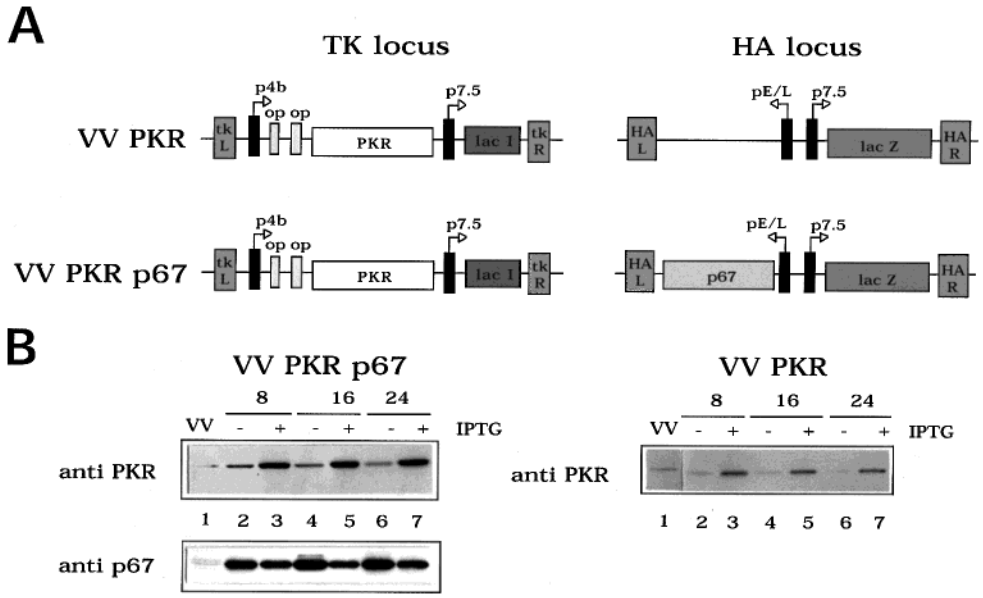

FIGURE 1: Generation of VV recombinants that express the human 67 kDa inhibitor of eIF2α phosphorylation together with PKR. (A) Scheme showing insertional vectors used for generating the different VV double-recombinants. All viruses contain an IPTG-inducible copy of PKR under the control of a hybrid operator−vaccinia late promoter inserted in the thymidine kinase (tk) locus. In the hemagglutinin (HA) locus, different pHLZ-derived insertional plasmids are inserted in each virus. The control VV PKR strain harbors an empty pHLZ vector. VV PKR−p67 harbors a copy of the rat 67 kDa inhibitor of eIF2α phosphorylation, under the control of a constitutive strong early/late VV promoter. (B) Immunoblot analysis showing PKR and p67 produced following infection by recombinant VV PKR−p67 or VV PKR. BSC-40 cells were infected with the indicated viruses (5 pfu/cell), and following viral adsorption for 1 h, inoculum was removed and PKR expression induced by adding (+) or not adding (−) 5 mM IPTG. At selected times, cells were harvested and subjected to western blot analyses using p67 or PKR antisera.

proteins were transferred to nitrocellulose paper with a semidry blotting apparatus (Gelman Sciences). Filters were mixed with antiserum in PBS containing 5% nonfat dry milk (BLOTTO), incubated overnight at 4 °C, washed 3 times with PBS, and further incubated with secondary antibody coupled to horseradish peroxidase in Blotto. After being washed with PBS, the immunocomplexes were detected with ECL Western blotting reagents (Amersham). Exposure of filters to Kodak X-OMAT films was performed for times varying from 3 s to 5 min, as needed. Experiments were repeated at least 3 times.

Plaque Assays. Confluent monolayers of BSC-40 cells grown in a 6 well plate were infected with 200 pfu/well of the indicated viruses. After 1 h of viral adsorption, the inoculum was removed, cells were washed, and medium was replaced by a mixture consisting of DMEM, 2% NCS, and 0.9% agar, in the presence or absence of 5 mM IPTG as indicated. At 72 hpi, medium was removed, and the monolayers were stained with 1% crystal violet in 2% ethanol. Experiments were repeated at least 3 times.

One-Step Virus Growth Curves. Confluent monolayers of BSC-40 cells were infected with 5 pfu/cell of the indicated viruses. After 1 h, the inoculum was removed, cells were washed twice with DMEM and DMEM + 2% NCS, and where indicated 5 mM IPTG was added to cells. At selected times, cells were harvested and subjected to 3 freeze−thaw cycles, and supernatants were titrated by plaque assays using BSC-40 cells. Experiments were repeated at least 3 times.

Gel Retardation Assays. HeLa cells were infected with the indicated viruses (at a total MOI of 10 pfu/cell) or mock-inoculated, and nuclear extracts were prepared as described previously, (*55*). These were then incubated with a [α-^{32}P]-dCTP-labeled double-stranded synthetic wild-type HIV enhancer oligonucleotide (*13*) that contains two κB consensus motifs. The binding buffer consisted of 25 mM HEPES, 1 mM EDTA, 3.5 mM spermidine, 6 mM $MgCl_2$, 100 mM NaCl, 0.15% Nonidet P-40, 10% glycerol, 5 mM DTT, 0.5 mg of BSA/mL, and 25 μg/mL poly(dI-dC). Complexes were then resolved by electrophoresis on a 6% native gel.

PKR−P67 Interaction Analyses. For in vivo co-immunoprecipitations, confluent PKR$^{0/0}$ cells were grown in 60 mm plates and infected for 20 h with the VV PKR or VV PKR−p67. Cells were scraped, and the clarified supernatant was mixed with 150 μL of protein A Sepharose, previously incubated with specific antibodies directed against p67, and further incubated overnight. Immunocomplexes were resolved by SDS−PAGE followed by immunoblot analysis with anti-PKR and anti-p67 specific antibodies.

For in vitro association studies, confluent PKR$^{0/0}$ cells infected for 20 h with VV p67 or with VV were subject to immunoprecipitation as outlined above with anti-p67 serum. Washed immunocomplexes were then incubated for 1 h at 25 °C with equal amounts of ^{35}S-labeled protein obtained from in vitro transcription−translations of plasmids carrying either PKR or MC159L from *Molluscum contagiosum* virus (MCV) (negative control) under the transcriptional control of a T7 promoter using the TNT T7 Quickcoupled system (Promega). After incubation, resins were extensively washed and bound proteins analyzed by SDS−PAGE followed by autoradiography.

RESULTS

Expression of P67 from a VV Recombinant. We have previously described an inducible system based on VV recombinants capable of expressing PKR and other proteins under regulation of the LacI operator/repressor system and

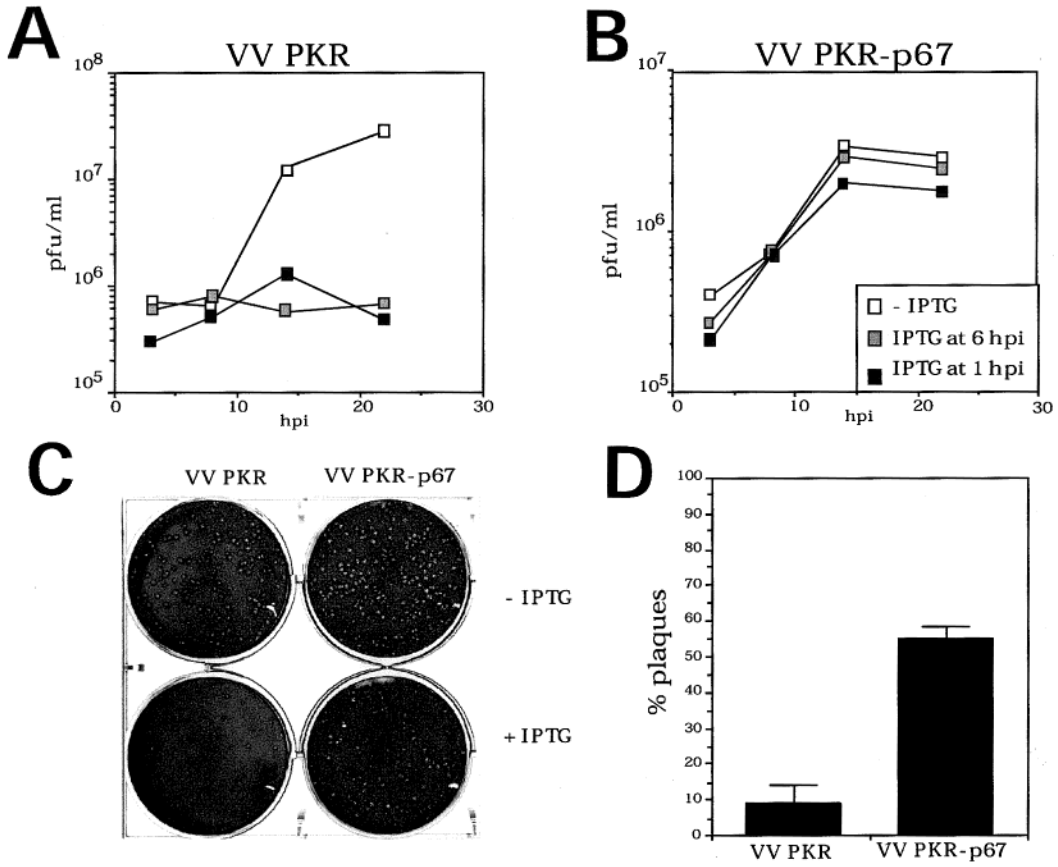

FIGURE 2: Expression of p67 overrides the antiviral effects exerted by PKR expression from VV recombinants. BSC-40 cells were infected (5 pfu/cell) with (A) VV PKR or (B) VV PKR−p67, and one-step virus growth curves were determined in the presence or absence of 5 mM IPTG. (C) Plaque formation by VV PKR or VV PKR−p67 in the presence of absence of IPTG. Confluent BSC-40 cells were infected with ca. 200 pfu/well of the indicated viruses. After virus adsorption, cells were overlaid with media containing 0.9% agar, in the presence or absence of 5 mM IPTG. Three days later the overlay was removed, and cells were stained with 1% crystal violet in 2% ethanol. (D) Quantitation of p67-mediated inhibition of the PKR-induced antiviral effect from (C).

have shown that expression of PKR in an activated form triggers apoptosis, induces antiviral activity against VV and VSV, blocks translation, and activates NF-κB through the IκB kinase complex (*13, 18, 32, 52, 56*−*58*). Results obtained using this inducible virus−cell system have been confirmed by others with transfected cells or with cells derived from PKR gene knock-out mice (*19, 40*). The VV inducible system was also used to identify apoptotic viral genes (*59*) and cellular and viral inhibitors of apoptosis (*58, 60*). Because of the biological relevance of the VV system, in this investigation we have analyzed the in vivo role of the cellular protein p67 on PKR action. To this end, p67 was subcloned into the vaccinia virus vector pHLZ and shuttled into vaccinia virus according to the procedures of (*54*). Recombinant VV's were generated to allow the constitutive expression of p67 from the HA locus under the control of a VV early−late synthetic promoter. In these recombinants, PKR was inserted into the tk (thymidine kinase) locus of the virus genome under the control of an *Escherichia coli lacI* operator−repressor system−VV hybrid promoter, allowing inducible expression (Figure 1A). This arrangement permits differential PKR expression based upon concentration and timing of IPTG treatment in order to manipulate relative molar ratios of PKR and p67. We reasoned that this strategy might be particularly useful because the inhibitory activity of p67 in vitro appears to be dependent upon the relative kinase:p67 ratios (*44*). As shown in Figure 1B, p67 is constitutively expressed from the VV early−late promoter in cells infected with VV PKR−p67 in the presence or absence of IPTG at approximately 8 h, and expression levels increased with time of infection. A low level of endogenous p67 also was detectable in immunoblots of extracts from VV infected cells. Monoclonal antiserum (*49*) that detects the glycosylated, active p67 form yielded the same results (data not shown). A slight decrease in steady-state p67 levels following PKR induction with IPTG was observed particularly at 16−24 hpi and is likely due to PKR-mediated inhibition of protein synthesis and induction of apoptosis (*18*). In addition, immunofluorescence staining of cells (*61*) overexpressing p67 distinctly showed that p67 was expressed solely in the cytoplasm but not in the nucleus (data not shown).

At 8 hpi, PKR was detected in cells infected with VV PKR−p67 or VV PKR and treated with IPTG (Figure 1B). PKR levels remained constant through 24 hpi. A low level of PKR also was detectable in cells in the absence of IPTG treatment probably as a result of some leakiness of the system as noted previously (*18*).

Expression of P67 Reverses PKR-Mediated Antiviral Effects. Lee and Esteban (*52*) found that the inducible expression of PKR from VV recombinants inhibits viral pathogenesis. This and other studies (*13, 50, 53*) demonstrate that the VV recombinant system is an effective mimic of the PKR regulatory pathway. To determine if expression of p67 can rescue VV pathogenesis from the effects of PKR

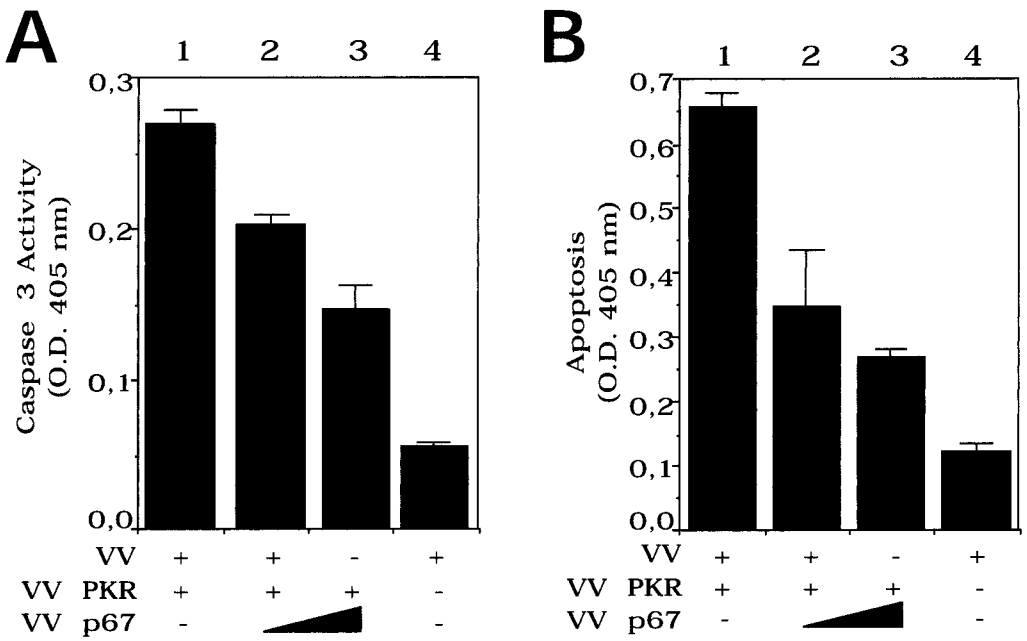

FIGURE 3: Inhibition of PKR-induced apoptosis by coexpression of p67. (A) Caspase-3 activity. BSC-40 cells were infected with the indicated viruses, and at 24 hpi, cells were collected in lysis buffer. Cellular extracts were used to determine caspase-3 activity as described under Experimental Procedures. Each point represents duplicate samples. Cells in all treatments were infected at a total moi of 9 pfu/cell, completed when needed with VV. Cells in lanes 1−3 were infected with 3 pfu/cell of VV PKR. Cells in lanes 2 and 3 were co-infected with 3 or 6 pfu/cell, respectively, of VV p67. Cells in lane 4 were infected with 9 pfu/cell of VV. (B) Cell-Death ELISA. BSC-40 cells were infected with the same viruses as in (A), and at 24 hpi, cells were collected in lysis buffer. Cytoplasmic extracts were prepared and used to determine the extent of apoptosis as described under Experimental Procedures. Each point represents triplicate samples.

expression, BSC-40 cells were infected with VV PKR or VV PKR−p67 in the presence or absence of IPTG, and viral growth rates were determined at various times post-infection. As expected, VV pathogenesis was severely inhibited by expression of PKR from VV PKR recombinants (Figure 2A,C). At 24 hpi, viral growth was decreased approximately 90% by PKR expression (Figure 2D). However, expression of p67 significantly abrogated the antiviral effect of PKR (Figure 2B,C). In a virus plaque formation assay, coexpression of p67 with PKR resulted in an approximate 4−5-fold increase in virus plaques relative to cells infected with VV PKR in the presence of IPTG (Figure 2D). Varying the time of PKR expression between 1 and 6 hpi via the addition of IPTG had not discernible effect. This p67-associated barrier to the PKR-mediated antivirus reaction was consistent in three independent experiments although variation was noted in absolute plaque numbers.

Modulation of PKR-Mediated Apoptosis by P67. The induction of apoptosis is a major consequence of PKR activity and is involved in the antiviral response (*13*, *18*, *32*, *50*, *62*). To assess the effect of different relative p67: PKR ratios on apoptosis, single VV recombinants expressing either p67 or PKR were generated according to (*18*). BSC-40 cells were infected with VV, VV PKR, or VV p67 singly or in co-infections at different virus multiplicities. The level of apoptosis was determined by the caspase-3 activity assay and an ELISA designed to measure the amount of cytoplasmic histone-associated DNA. Results from both assays were consistent and, in support of (*13*), demonstrate that infection of cells with VV PKR induces cellular apoptosis (Figure 3A,B). In this system, wild-type VV infection of BSC-40 cells does not induce apoptosis (*13*). Further, coexpression of p67 from VV−p67 significantly decreased the level of apoptosis by both assays, in agreement with the abrogation of the PKR-mediated antiviral effect. The inhibition of apoptosis was dependent upon relative VV p67/VV PKR multiplicities of infection, suggesting that p67 levels, at least in part, are important in the interaction with PKR. When cells were co-infected with VV PKR (3 pfu/cell) and VV p67 (6 pfu/cell), respectively, there was an approximate 2-fold decrease in measurable apoptosis compared to VV PKR infected cells.

P67 Expression Abrogates PKR-Mediated Inhibition of Protein Synthesis. The p67-associated abrogation of the PKR-mediated antiviral response and apoptosis could be due to translational and/or transcriptional mechanisms (*32*). Translational activity was measured by three assays: reporter gene expression, western blotting of VV proteins, and metabolic labeling to measure total protein synthesis levels in infected cells. The design of the VV recombinant system permits analysis of β-galactosidase expression driven from a VV 7.5 promoter as a quantitative and very sensitive measurement of levels of protein synthesis. Figure 4A shows that PKR expression from VV PKR caused a significant decrease (ca. 80%) in β-galactosidase activity relative to uninduced controls. These data are consistent with results from one-step viral growth assays and previous reports (*13*, *18*) and indicate a severe block of protein synthesis by PKR. However, in cells infected with VV PKR−p67 where PKR expression was induced by IPTG, β-galactosidase activity increased to approximately 80% of uninduced controls, supporting the concept that p67 can prevent the PKR-mediated inhibition of protein synthesis (Figure 4B).

Steady-state VV protein levels were determined by immunoblotting using antiserum specific for VV proteins. In accordance with previous reports (*18*, *52*), induction of PKR in cells infected with VV PKR leads to a marked decrease in synthesis of vaccinia virus-encoded proteins (Figure 4C,

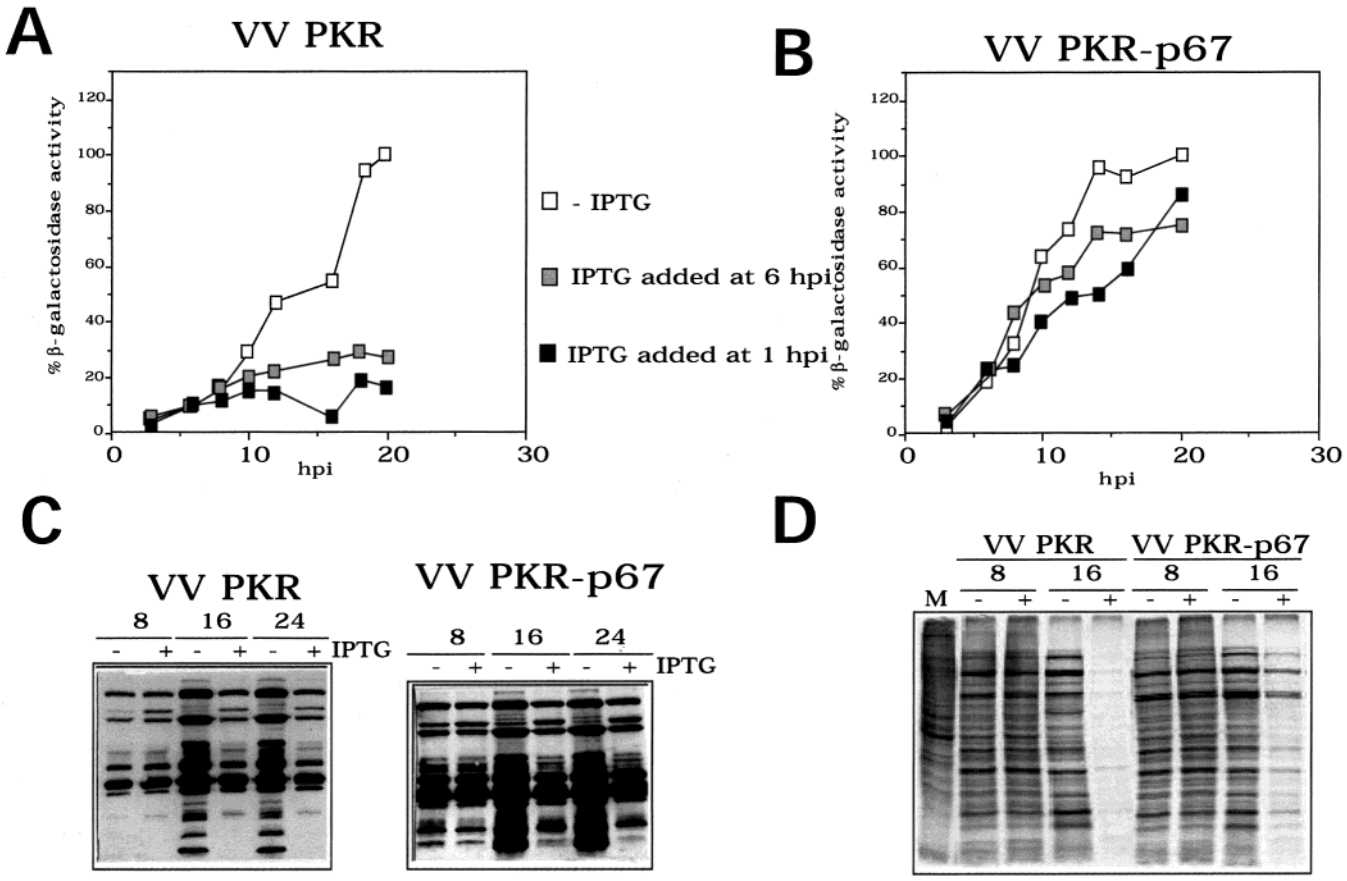

FIGURE 4: P67-mediated regulation of β-galactosidase activity, vaccinia virus specific and total protein synthesis. Monolayers of BSC-40 cells were infected with VV PKR or VV PKR−p67 viruses (5 pfu/cell), and after 1 h of virus adsorption, inoculum was removed and new medium ± 5 mM IPTG was added. At selected times, cells were harvested and extracts (A, B) were prepared for β-galactosidase activity measurement or (C) were subjected to immunoblot analyses using polyclonal antibodies against VV (1:1000 dilution). β-Glactosidase data are presented as a percentage of the maximum measured activity. (D) Cells were infected with VV PKR or VV PKR−p67, labeled at the indicated times with [^{35}S]methionine-cysteine for 1 h, and collected in lysis buffer, and protein synthesis was analyzed by SDS−PAGE, followed by autoradiography.

left panel); however, in cells infected with VV PKR−p67 and treated with IPTG, viral protein synthesis was partially rescued (right panel). In vivo metabolic labeling assays showed that at 16 hpi, total protein synthesis in cells infected with VV PKR in the presence of IPTG was inhibited by 93% (Figure 4D). In comparison, the PKR-mediated block of protein synthesis due to coexpression of p67 from VV PKR−p67-infected cells resulted in a 44% increase in protein synthesis (as quantitated by acid precipitation and scintillation counting). The 1 h pulse-labeling used in these experiments yields a view of translation within a small window of time whereas the β-galactosidase measurements reflect the total impact or cumulative effect. Taken together, these data indicate that p67 extends the cell's ability to engage in protein synthesis in the presence of PKR expression.

P67 Inhibition of PKR-Mediated Translational Regulation Is Due to Inhibition of eIF2α Phosphorylation. To evaluate if the inhibition of PKR translational control by p67 is directly linked to eIF2α phosphorylation levels, cells infected with 5 pfu/cell of VV PKR or VV PKR−p67 were exposed to varying IPTG concentrations to differentially induce PKR. Cells were collected at 10 hpi, and extracts were subjected to immunoblotting using antisera against PKR, p67, eIF2α-(PO_4), or eIF2α. Steady-state eIF2α(PO_4):eIF2α(total) ratios were normalized to levels from untreated cells. PKR was efficiently expressed in VV PKR and VV PKR p67-infected cells in an IPTG-dependent manner (Figure 5A). Levels of eIF2α protein were uniform over the time course in cells infected by the recombinant viruses, whereas eIF2α phosphorylation increased approximately 3-fold in cells infected with VV PKR and treated with 1 mM IPTG as compared to untreated cells (Figure 5A,B). In addition to the endogenous p67 detectable in all treatments, infection of cells with VV PKR−p67 resulted in a significant increase in steady-state p67 levels. Expression of p67 resulted in an approximate 50% decrease in eIF2α phosphorylation in the presence of PKR regardless of PKR expression level (Figure 5B). The background level of eIF2α phosphorylation observed in VV PKR infected cells without IPTG treatment corresponds to both endogenous eIF2α kinase activity and leaky expression of PKR, as noted previously. Interestingly, p67 overexpression was also able to inhibit the background levels of eIF2α phosphorylation. These data indicate that the relative ability of p67 to interfere with PKR-mediated eIF2α phosphorylation is dependent upon relative PKR concentrations.

P67 Affects PKR-Mediated NF-κB Binding Activity. PKR is a multifunctional regulator of cellular activity with effects on transcriptional activation via NF-κB activity and on translational control. Gil et al. (*13*) showed that induction of PKR from VV PKR infected cells markedly increased NF-κB activity, as measured by gel shift assays. Activation of NF-κB binding activity correlated with increased NF-κB-

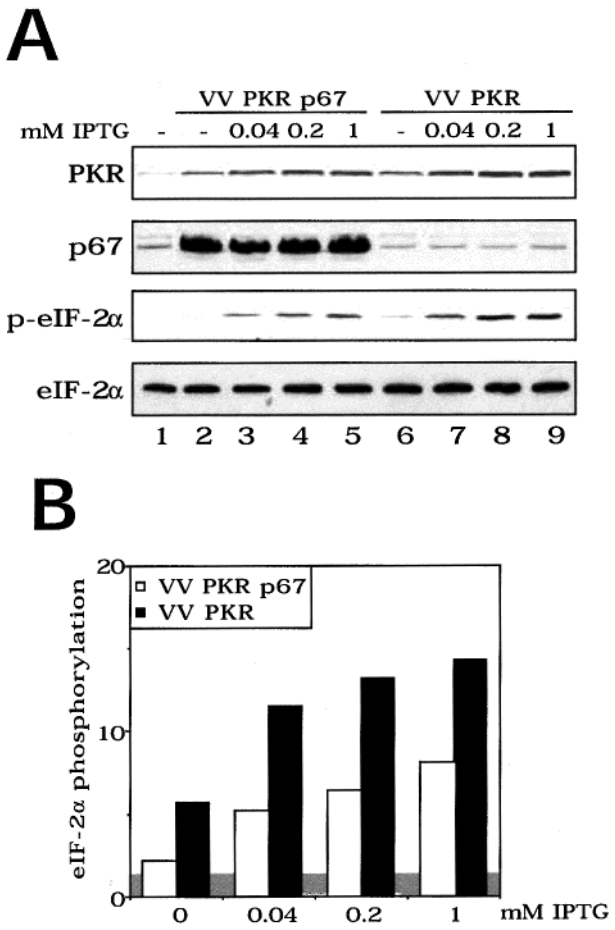

FIGURE 5: P67 protects eIF2α from PKR-mediated phosphorylation in HeLa cells. (A) HeLa cells were infected with the indicated viruses (5 pfu/cell), and PKR expression was induced or not induced by adding the indicated concentrations of IPTG after viral adsorption for 1 h. Cells were collected at 10 hpi and subjected to immunoblot analysis using anti-eIF2α-specific monoclonal antibodies, a polyclonal antiserum that specifically recognizes the phosphorylated form of eIF2α, or polyclonal antisera to PKR or p67. (B) Phosphorylation of eIF2α was measured by densitometric analysis and is presented as a ratio of phosphorylated eIF2α to total eIF2α, normalized to the value obtained from mock-infected cells.

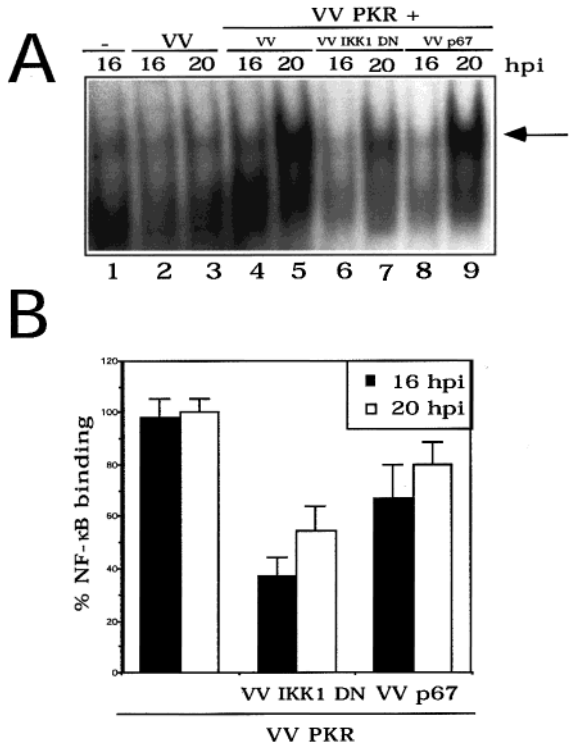

FIGURE 6: P67 interference of PKR activation of NF-κB. HeLa cells were infected with the combinations of VV recombinants indicated (at a total moi of 10 pfu/cell), and nuclear extracts were prepared at 16 or 20 hpi and (A) analyzed by a gel shift assay with a probe specific for NF-κB. VV IKKI DN, VV expressing a dominant negative mutant of IKK1. The effect of p67 over PKR-induced activation of NF-κB was quantified by densitometric analyses of autoradiograms from four independent experiments and expressed as a percentage of maximum NF-κB binding. The quantitation is shown in panel B.

dependent transcription of reporter genes (*13*, *63*). Whereas previous in vitro studies show that p67 protects eIF2α from PKR-mediated phosphorylation, it is unclear if p67 influences other PKR-mediated activities. To address this issue, cells were infected with VV PKR alone or together with VV p67 and NF-κB induction as determined by gel shift assays. As previously shown (*13*), NF-κB binding activity was not detectable in cells infected with VV whereas induction of PKR in cells infected with VV PKR increased NF-κB activity (Figure 6A, lanes 4, 5). This effect was particularly evident at 20 hpi. As a control, coexpression of a dominant negative mutant of IKK1, a subunit of the kinase complex involved in transmitting the NF-κB activation signal triggered by PKR (*63*), significantly inhibited NF-κB activation induced by PKR as would be expected (lanes 6, 7). Coexpression of p67 resulted in an intermediate effect (lanes 8, 9) whereby there was a detectable but not dramatic decrease in PKR-mediated NF-κB activation. Quantitation of NF-κB binding from four independent experiments indicates that at 16 hpi, there was an approximate 20−25% decrease in activity associated with p67 coexpression compared with cells expressing PKR only (Figure 6B).

Association of P67 with PKR. Purified p67 is able to inhibit PKR autophosphorylation in the absence of eIF2 (*64*), it co-purifies with PKR on dsRNA agarose despite the failure of purified p67 to bind dsRNA agarose directly (data not shown), and it does not contain the conserved dsRNA binding domain (*65*). These results suggest a direct interaction between PKR and p67. To further characterize the association between p67 and PKR, in vivo co-immunoprecipitation assays and in vitro binding assays were performed. The catalytically inactive K296R mutant was used in the co-immunoprecipitation assays in order to reduce self-regulation and maximize expression of PKR. Extracts from cells infected with VV p67 and/or VV PKR (K296R) were subject to immunoprecipitation using anti-p67 serum/protein A Sepharose, and immunocomplexes were resolved by SDS−PAGE and immunoblotted with anti-PKR or anti-p67 sera (Figure 7). Detectable concentrations of p67 were found in cells infected or co-infected with VV−p67 (Figure 7A, right panel, lanes 3−5). Western blotting of total extracts also showed that PKR was present in cells infected with VV PKR (K296R) or coinfected with VV PKR (K296R) and VV p67 in the presence of IPTG (Figure 7A, left panel, lanes 2, 5) but not from cells infected with VV or VV p67 (lanes 1, 3). The western blot analysis of anti-p67 immunocomplexes showed that PKR co-immunoprecipitates with p67 in a specific manner (Figure 7B, lane 5). The PKR band observed in immunoprecipitates from lane 2 is likely due to its

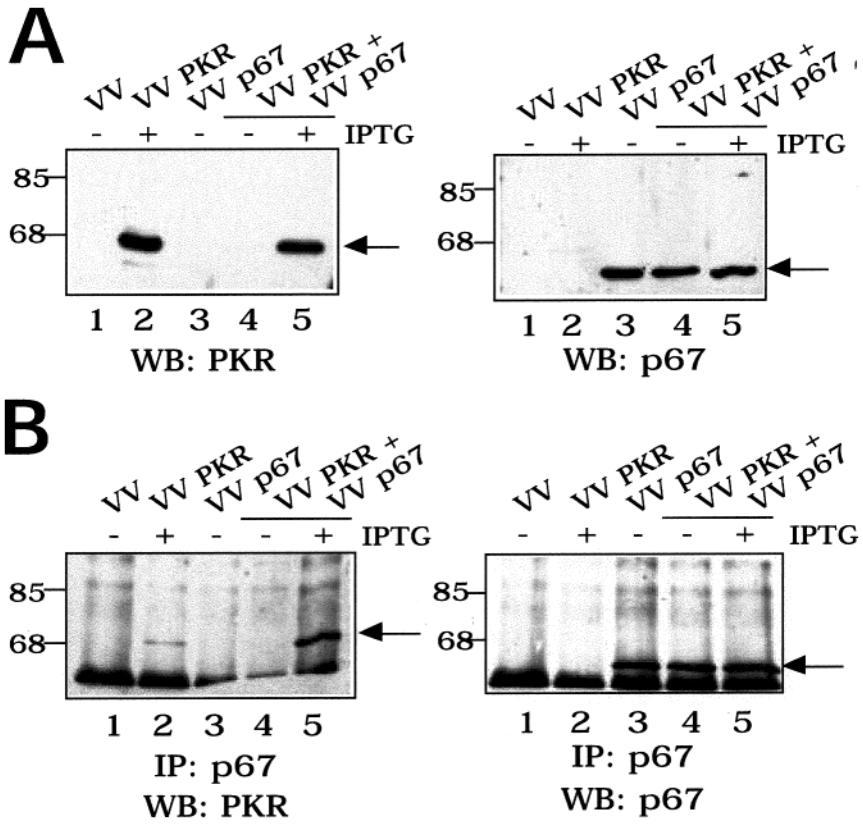

FIGURE 7: Co-immunoprecipitation of PKR and p67. (A) PKR$^{0/0}$ cells were infected with VV (lane 1), VV−PKR (K296R) (lane 2), VV−p67 (lane 3), or VV−PKR (K296R) plus VV−p67 (lanes 4, 5) in the presence (lanes 2, 5) or absence (lanes 1, 3, 4) of IPTG, followed by immunoblot analysis with anti-PKR or anti-p67 antiserum. (B) Extracts prepared as in (A) were immunoprecipitated using anti-p67 serum and thoroughly washed and immunocomplexes analyzed by SDS−PAGE and subjected to immunoblotting with antiserum to PKR or p67. IP, immunoprecipitation; WB, western blot.

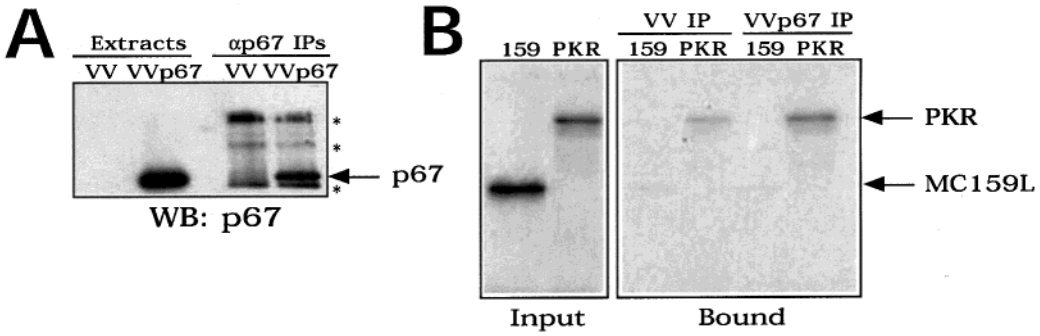

FIGURE 8: In vitro interaction between PKR and p67. (A) PKR$^{0/0}$ cells were infected with VV or VV p67, and extracts were subjected to immunoblotting with anti-p67 serum or to immunoprecipitation with p67 antiserum followed by immunoblotting. Asterisks (*) mark nonspecific proteins appearing in immunoblots of immunoprecipitations. (B) PKR and a control protein (MC159L) were in vitro transcribed/translated, labeled with [^{35}S]Met-Cys (inputs), and incubated with p67 immunoprecipitates from cells infected with the indicated viruses. Aliquots of the inputs and proteins bound to p67-containing immunocomplexes following incubation and washing were analyzed by SDS−PAGE followed by autoradiography. WB, western blotting; 159, MCV protein MC159L; IP, immunoprecipitation.

association with endogenous p67, since PKR does not bind to protein A Sepharose alone or protein A Sepharose bound to a control serum (data not shown).

In vitro binding assays further support the interaction between PKR and p67. In these studies, PKR$^{0/0}$ cells were infected with VV p67 or VV for 20 h followed by incubation of cell extracts with anti-p67 serum−protein A Sepharose. The immunocomplexes were then incubated with [^{35}S]-methionine-cysteine-labeled, in vitro transcribed and translated PKR or, as a negative control, MC159L protein (from *Molluscum contagiosum* virus). Following thorough washing, SDS−PAGE, and autoradiography, PKR was detectable in p67 immunocomplexes from VV p67 and VV infected cells (Figure 8), in a manner dependent on p67 levels. Specificity of association is demonstrated by the inability of MC159L to bind to p67 immunocomplexes and by previously mentioned controls that exclude the nonspecific association of PKR to protein A Sepharose resin. The above findings suggest that a physical interaction between p67 and PKR can explain inhibitory effects exerted by this glycoprotein.

DISCUSSION

PKR is a multidimensional regulator of cell activity, and the diversity of cellular and viral modulators of PKR testifies

to its physiological importance. Using a VV recombinant system that effectively mimics PKR-mediated pathways in vivo, we demonstrate that in the presence of PKR, p67 specifically reduced eIF2α phosphorylation and rescued protein synthesis. As a result, both PKR-mediated apoptosis and antiviral responses were down-regulated. In vitro studies previously suggested that the host-encoded glycoprotein p67 inhibited PKR-mediated eIF2α phosphorylation (*42*−*44*). However, in these studies the activation state of PKR was unclear, and exogenous dsRNA was not added, complicating interpretation. Other studies provided evidence that p67 levels and glycosylation state were correlated with in vivo protein synthesis levels (*46*, *47*). Wu et al. (*48*) found that transient overexpression of p67 in COS cells increased p67 expression 4−5-fold and resulted in an approximate 40% inhibition of eIF2α phosphorylation. However, only a 1.5−2-fold increase was observed in expression of a reporter mRNA. In our system, p67 overexpression had a substantially greater impact on the rescue of protein synthesis. This may be due to the concomitant induction of PKR expression from VV recombinants. Although p67 expression prolongs the cell's translational ability in the presence of PKR, it does not impose an absolute constraint on PKR translational inhibition. More likely, expression and activation of p67 guides rather than dominates PKR-mediated translational control, and effects may be dependent upon timing and/or localization of the interaction. However, this may be significant because in reticulocyte lysates an increase of 5−10% in eIF2α phosphorylation is sufficient to dramatically increase the level of translational inhibition (*66*).

In addition, stably expressed p67 also had a slight but detectable effect on activation of the NF-κB pathway as observed by gel shift analyses. This extends the range previously reported for p67-mediated effects. The relative level of p67-mediated influence on PKR activities may indicate that eIF2α phosphorylation is more sensitive than NF-κB activation to regulation of PKR activity. $p58^{IPK}$, another PKR inhibitor that is able to inhibit PKR activation by blocking its dimerization, has been shown to also inhibit NF-κB activation triggered by PKR (*67*).

The mechanism of p67-mediated inhibition of PKR action is not understood. A significant degree of amino acid identity (66%) exists between p67 and methionine aminopeptidases, suggesting multifunctional activity (*68*, *69*). In fact, Bazan et al. (*70*) proposed that p67 attacks eIF2α kinases as a metalloproteinase. An alternative model (*44*, *71*) proposes that p67 binds to the γ subunit of eIF2 and shelters the eIF2α phosphorylation site. They suggest that PKR does not directly interact with p67 and that p67 has no effect on PKR autophosphorylation but functions against activated PKR by inhibiting phosphoryl transfer between phosphorylated PKR and eIF2α. In contrast, data presented herein together with the finding that purified p67 inhibits dsRNA-dependent PKR autophosphorylation in the absence of eIF2 (*64*, Chang et al., unpublished results) support a more direct interaction between p67 and PKR. This is also consistent with the p67 impact on PKR-mediated NF-κB activity. However, at this time we cannot exclude the possibility that p67 also may interact directly with eIF2.

Multiple eIF2α kinases are present in mammalian cells, including HRI, PKR, GCN2, and PERK/PEK. The redundancy of their activities is suggested by studies with $PKR^{0/0}$ mice and $PERK^{0/0}$ cells (*72*, *73*). Datta et al. (*43*) have previously shown that p67 inhibits not only PKR-mediated but also HRI-mediated eIF2α phosphorylation. Although multiple cellular and viral inhibitors of eIF2α kinase activity have been identified, only p67 and the VV-encoded K3L protein (*74*) are known to have the ability to regulate eIF2α phosphorylation by multiple kinases. Unlike p67, the VV-encoded K3L protein contains a domain homologous to eIF2α that acts as a pseudosubstrate to limit phosphorylation (*75*). Interestingly, K3L has been shown to interact with PKR through the PKR catalytic domain (*76*, *77*), and this seems to confer the ability to interact and inhibit not only PKR but also other eIF2α kinases. It will be of interest to know if, similar to K3L, p67 interacts with eIF2α kinases via the kinase domain. This interaction would likely contribute to inhibition of PKR-mediated NF-κB activation. The broad-spectrum anti-eIF2α kinase activity of p67 also may be exploited in dissecting the regulatory role of eIF2α phosphorylation in response to diverse abiotic and biotic stresses.

In conclusion, in this investigation we have demonstrated with a virus−cell system that the cellular glycoprotein p67 reverses the antiviral and proapoptotic effects of PKR. This reversal correlates with a p67-mediated decrease of PKR-induced eIF2α phosphorylation and, in part, NF-κB binding activity. By in vitro and in vivo approaches, we documented interaction between p67 and PKR. Whether there is direct or indirect p67 interaction and what domains are involved remain to be determined. Our findings highlight the importance of p67 as a regulator of PKR functions.

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