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In Vivo Regulation of Protein Synthesis by Phosphorylation of the α Subunit of Wheat Eukaryotic Initiation Factor 2†

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ABSTRACT: The regulation of protein synthesis is a critical component in the maintenance of cellular homeostasis. A major mechanism of translational control in response to diverse abiotic and biotic stress signals involves the phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF2α). The pathway has been demonstrated in all eukaryotes except plants, although components of a putative plant pathway have been characterized. To evaluate the in vivo capability of plant eIF2α to participate in the translation pathway, we have used vaccinia virus recombinants that constitutively express wheat eIF2α and inducibly express the eIF2α dsRNA-stimulated protein kinase, PKR, in BSC-40 cells. Activation of PKR in cells expressing wild-type wheat eIF2α resulted in an inhibition of cellular and viral protein synthesis and an induction of cellular apoptosis correlating with phosphorylation of eIF2α on serine 51. Expression of a nonphosphorylatable mutant (51A) of plant eIF2α reversed the PKR-mediated translational block as well as the PKR-induced apoptosis. A direct interaction of the plant proteins with the mammalian translational initiation apparatus is supported by coimmunoprecipitation of wild-type plant eIF2α and the 51A mutant with mammalian eIF2γ and the localization of the plant proteins in ribosome fractions. These findings suggest that plant eIF2α is capable of interacting with the guanine nucleotide exchange factor eIF2B within the context of the eIF2 holoenzyme and provide direct evidence for its ability to participate in phosphorylation-mediated translational control in vivo.

The regulation of protein synthesis is a fundamental mechanism by which cells modulate gene expression. One of the most important translational control mechanisms is mediated by the phosphorylation of eIF2α[1] (*1*, *2*). This event is of critical importance in the regulation of global as well as specific protein levels and is essential for the maintenance of cellular homeostasis in response to diverse abiotic and biotic stresses. It is involved in the regulation of viral pathogenesis (*3*), cell growth (4), differentiation (5), the nutritional deprivation response (*6*), and apoptosis (*7*, *8*). The pathway has been well characterized in all eukaryotes with the major exception of plants. Indeed, the necessity for a plant eIF2α phosphorylation pathway has been questioned (*9*, *10*). Krishna et al. (*10*) suggest that even though wheat germ eIF2α can be phosphorylated in vitro it is unable to interact with eIF2B in vivo. Although the protein synthesis framework is functionally similar between plants and other eukaryotes, there are distinct differences that likely reflect the unique challenges facing plants in a sessile environment (*11*).

Members of the eIF2α kinase family are the key guardians of the eIF2α phosphorylation pathway and include the dsRNA-dependent protein kinase PKR (*12*, reviewed in ref *13*), the heme-dependent protein kinase HRI (*14*), and the GCN2 gene product (*15*, *16*). The 68 kDa human PKR is a major regulator of dsRNA responses in cells (*17*). It is activated by autophosphorylation following dsRNA binding and then is capable of dsRNA-independent exogenous substrate phosphorylation (reviewed in ref *18*). The primary target for PKR phosphotransferase activity is serine 51 of eIF2α, although other PKR substrates have been suggested (*19*−*22*). Plants encode a biochemical and immunological homologue of PKR termed pPKR (*23*). In vitro phosphorylation of the M_r 68 000 pPKR is stimulated by dsRNA, select polyanions, or intramolecularly base-paired, single-stranded RNAs of sufficient length but not by DNA or RNA−DNA hybrids (*24*). Similarly to mammalian PKR (*18*), autophosphorylation is inhibited by high levels of dsRNA (*23*, *25*). Both pPKR and mammalian PKR are localized in the

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[1] Abbrevations: eIF2α, α subunit of eukaryotic initiation factor 2; DMEM, Dulbecco's modified Eagle's medium; IPTG, isopropyl β-D-thiogalactoside; WR, western reserve; CPRG, chlorophenol red β-D-galactopyranoside; NCS, newborn calf serum; pPKR, plant-encoded double-stranded RNA-dependent protein kinase; TCA, trichloroacetic acid; BSA, bovine serum albumin; VV, vaccinia virus; dsRNA, double-stranded RNA.; Blotto, phosphate-buffered saline containing 5% nonfat dry milk; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; tk, thymidine kinase; moi, multiplicity of infection; pfu, plaque-forming units; pi, postinfection.

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cytoplasm and are ribosome-associated; however, they have also been found in the nucleus (*26*, *27*; Yang et al., unpublished results). Plant PKR activity is stimulated by virus and viroid infection and is differentially regulated during plant development (*28*−*30*; Yang et al., unpublished results). Still the in vivo significance of pPKR activation remains unclear at this time.

Recently, the cDNA for wheat eIF2α has been cloned (Metz and Browning, unpublished results). It encodes a M_r 42 000 protein and ca. 50% of the amino acid residues are identical with residues in the human M_r 36 000 homologue. Despite these differences, the plant protein contains domains associated with eIF2α phosphorylation and in vitro it is phosphorylated specifically on serine 51 by pPKR, GCN2, and human PKR (*31*). However, the physiological significance of eIF2α phosphorylation is unclear. Whereas in mammals eIF2α phosphorylation in response to virus infection results in the severe downregulation of protein synthesis, a similar shutdown of translation has not been observed following synchronized infection of plant protoplasts with tobacco mosaic virus (Hu and Roth, unpublished results). Further, activation of pPKR occurs in compatible host-virus interactions, suggesting it may not be involved in a plant antiviral response mechanism (*29*).

In view of the physiological importance of eIF2α phosphorylation it is critical to establish whether a similar pathway exists in plants. Further, understanding processes involved in plant translational control and identifying unique features may contribute to the overall knowledge of eukaryotic protein synthesis. Unfortunately, at this time there is not a plant system comparable to the mammalian cell culture or the yeast model to dissect the functional significance of eIF2α phosphorylation in vivo. Thus, we have taken advantage of a model vaccinia virus (VV) cell system designed to allow the timely and localized expression of eIF2α in the presence or absence of PKR. Previous results have demonstrated that this system mimics the eIF2α phosphorylation control pathway and is useful to probe specific functional relationships operative in the pathway (*32*, *33*). Herein, we provide evidence that wheat eIF2α functionally interacts in the eIF2α phosphorylation pathway in mammalian cells and demonstrate that the nonphosphorylatable mutant 51A behaves as a dominant negative regulator of the pathway, similarly to its mammalian homologue (*33*). These findings support the concept that initiation of plant protein translation may be regulated by eIF2α phosphorylation and establish the conservation between mechanisms of initiation among all eukaryotes.

EXPERIMENTAL PROCEDURES

Materials. Chemicals were from Sigma (St. Louis, MO) unless otherwise specified. Polyclonal rabbit antibodies against plant eIF2 were a gift from Karen Browning (University of Texas, Austin). Rabbit polyclonal antiserum recognizing specifically the phosphorylated form of both mammalian and plant eIF2α were purchased from Research Genetics (Huntsville, AL). Rabbit polyclonal antibodies directed against VV proteins have been used previously (*34*). Polyclonal rabbit antibody specific for PKR has been previously described (*33*). Antiserum to mammalian eIF2γ was provided by J. Hershey (University of California, Davis).Secondary antibodies were purchased from Cappel (Durham, NC).

Plasmids. Wild-type wheat eIF2α is encoded within a 1.02 kb *Nco*I−*Bam*HI fragment. This cDNA was subcloned into the *Nco*I−*Bam*HI site of pAlter-EX2 (Promega, Madison, WI). Single-stranded DNA was prepared and mutagenesis was performed with the Altered Sites II in vitro system (Promega) with the following oligonucleotide (boldface nucleotides indicate change): 51A- 5′ TCT CCG AGC TC**G CC**C GCC GCC GCA TCC 3′. The resultant 51 alanine mutant (51A) as well as the wild-type 51 serine (51S) cDNAs were subcloned into the *Hin*dIII site of pBSII-SK(+) (Stratagene). Sequencing confirmed the fidelity of each eIF2α cDNA subclone. Wheat eIF2α 51S and 51A cDNAs were excised from pBS-51S and pBS-51A, respectively, by digestion with *Hin*dIII. A 1.1 kb fragment was purified, repaired with Klenow, and cloned into the hemagglutinin insertional VV vector pHLZ (*35*), previously digested with *Sma*I and dephosphorylated with alkaline phosphatase, to generate pHLZ-51A and pHLZ-51S, respectively. Sense orientation of the inserts was checked by restriction analysis.

Cells and Viruses. African green monkey kidney cells BSC-40 (ATCC CCL-26) were grown in DMEM supplemented with 10% heat-inactivated newborn calf serum (NCS). HeLa cells (ECACC 85060701) were grown in DMEM supplemented with 10% NCS. After mock inoculation or viral adsorption, cells were maintained with DMEM supplemented with 2% NCS. The recombinant VV PKR HA^- virus expressing IPTG-inducible PKR (called VV PKR for clarity in this study) was generated as described below by recombination of empty plasmid pHLZ (*35*) with WR 68K virus, expressing IPTG-inducible PKR (*34*). VV-α51S and VV-α51A were generated by homologous recombination of their respective pHLZ-derived plasmids with the WR strain of VV in BSC-40 cells, as previously described (*36*) and selected by blue plaque formation upon X-gal addition. VV PKR-α51S and VV PKR-α51A were generated by recombination of their respective pHLZ-based vectors with WR 68K virus, following standard procedures (*36*). Viruses were subjected to 5 rounds of plaque purification to generate homogeneous recombinants. A scheme representing vectors introduced into the different viruses is presented in Figure 1B.

Measurement of β-Galactosidase Activity. Confluent BSC-40 cells seeded in 24-well plates were infected with 5 pfu/cell of the indicated viruses. After 1 h of viral adsorption, 5 mM IPTG was added to induce PKR expression. Cells were collected at 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. Cell lysate supernatants (10 μL) were mixed with 150 μL of CPRG solution [1 mM Mg Cl_2, 45 mM β-mercaptoethanol, 0.1 M sodium phosphate (pH 7.5), and 5 mM CPRG] in a 96-well plate and incubated at 37 °C for 1 h, and absorbance at 540 nm was determined. Experiments were repeated at least twice.

Measurement of Apoptosis. The cell death detection 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

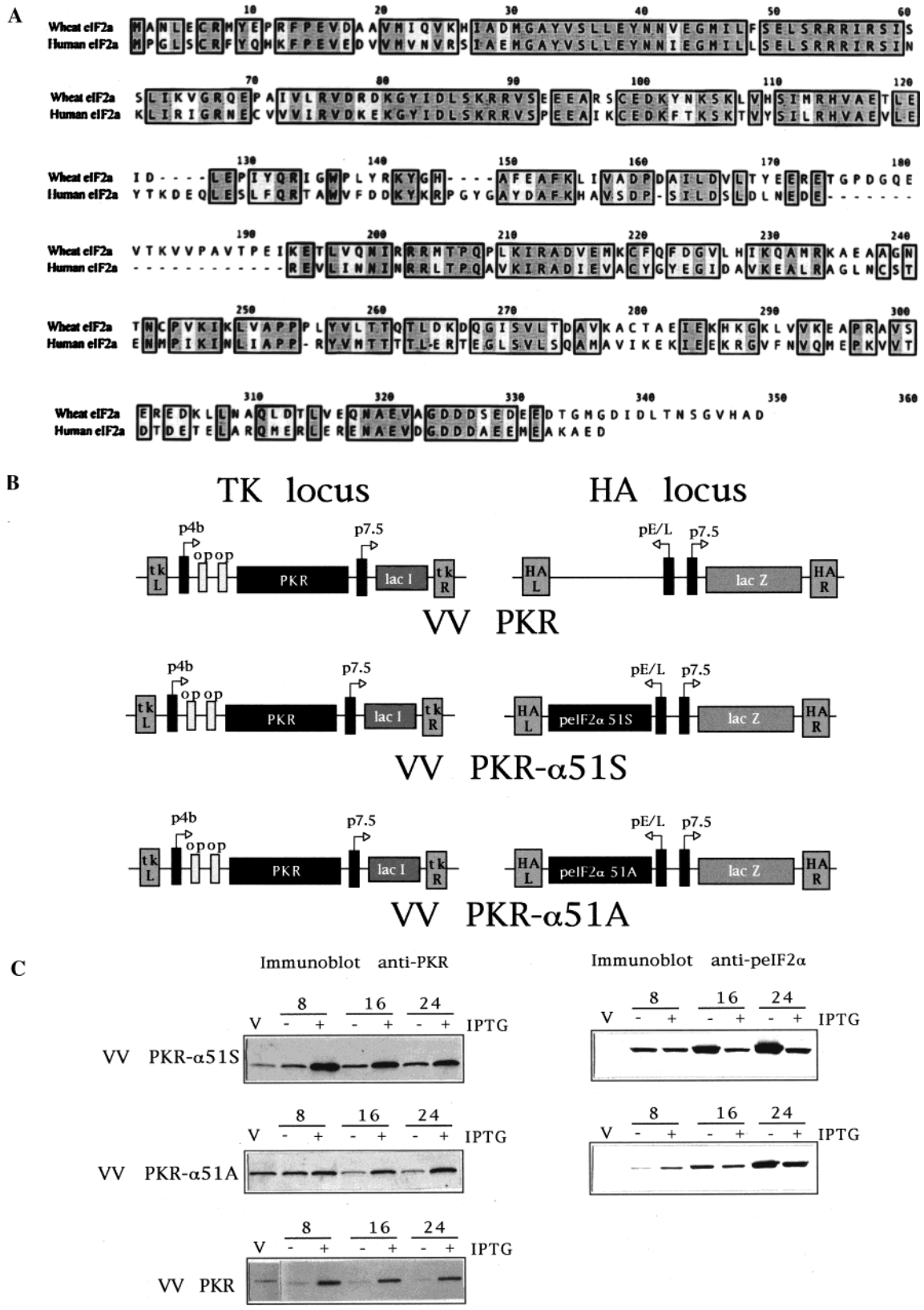

FIGURE 1: Generation of VV recombinants that express 51A and 51S forms of wheat eIF2α together with PKR. (A) Homology between human and wheat eIF2α subunits. An optimized alignment of the protein sequences of human and wheat eIF2α is shown. Identical amino acids are boxed. (B) Scheme showing insertional 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-α51S and VV PKR-α51A harbor copies of wild-type or 51 serine to alanine (51A) mutant of eIF2α, respectively, under the control of a constitutive strong early/late VV promoter. (C) Immunoblot analysis showing PKR (left panel) and plant eIF2α (right panel) proteins produced following infection by recombinant VV. BSC-40 cells were infected with indicated viruses (5 pfu/cell), and following viral adsorption for 1 h, inoculum was removed and PKR expression and induced by adding 5 mM IPTG (+) or not (−). At selected times cells were harvested and subjected to western blot analyses with PKR or plant eIF2 antisera.

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, and 0.5% Nonidet P-40) and clarified by centrifugation. Equal amounts of supernatant and 2× reaction buffer (100 mM HEPES, pH 7.5, 20% glycerol, 5 mM dithiothreitol, and 0.5 mM EDTA) were mixed and assayed for caspase 3 activity with as substrate 200 μM DEVD-pNA from Calbiochem. Free pNA produced by caspase activity was determined by measuring 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 three 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 of [^{35}S]Met−Cys promix (Amersham) in Met−Cys-free DMEM was added for an additional hour. After three washes with PBS, cells were harvested in lysis buffer. 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 precipitated with 5% TCA and collected on glass fiber filters by use of 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−10% or 12% PAGE. After electrophoresis, proteins were transferred to nitrocellulose paper with a semidry blotting apparatus (Gelman Sciences). Filters were mixed with antiserum in Blotto, incubated overnight at 4 °C, washed three 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 enhanced chemiluminescence (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 three times.

Plaque Assays. Confluent monolayers of BSC-40 cells grown in a 6-well plate were infected with 200 pfu/well of indicated viruses. After 1 h of viral adsorption, the inoculum was removed, cells were washed and the 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 h pi, medium was removed and the monolayers were stained with 1% crystal violet in 2% ethanol. Experiments were repeated at least three 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 three freeze−thawing cycles, and supernatants were titrated by plaque assays with BSC-40 cells. Experiments were repeated at least three times.

RESULTS

Wheat Wild-Type eIF2α and Mutant 51A Proteins Are Efficiently Expressed from Recombinant Vaccinia Viruses in Mammalian Cells. Although only ca. 50% of the amino acid residues in the M_r 42 000 wild-type (51S) wheat eIF2α are identical with residues in the M_r 36 000 human eIF2α, it contains motifs associated with eIF2α phosphorylation (Figure 1A). Most importantly, the domain surrounding serine 51 and the KGYID putative kinase docking domain (*37*) are entirely conserved. To determine whether wheat eIF2α can function in the eIF2α phosphorylation pathway, we used a system based on the coexpression of the wheat protein together with PKR driven from VV recombinants. We have previously used PKR expressed from VV recombinants to check its function in the regulation of protein synthesis, antiviral activity induced following VV and vesicular stomatis virus infection and apoptosis induction (*7*, *34*, *38*−*40*). To analyze the function of plant eIF2α, wild-type and nonphosphorylatable mutant (51A) cDNAs were subcloned into the VV vector pHLZ (*35*). The construction of the VV vectors is shown in Figure 1B. Recombinant VVs were generated to allow the expression of 51A or 51S from the HA locus under the control of a VV early−late synthetic promoter while PKR was inserted into the tk locus of the virus genome under the control of an *Escherichia coli lacI* operator−repressor system−VV hybrid promoter allowing IPTG-inducible expression of PKR (*39*). Figure 1C shows that both wheat eIF2α 51S and 51A proteins are constitutively expressed following infection of BSC-40 cells by recombinant vaccinia viruses VV PKR-α51S and VV PKR-α51A, respectively. Immunoblotting with antiserum that specifically recognized the M_r 42 000 plant eIF2α shows expression of the eIF2α proteins at 8 h and maximum accumulation at 24 h postinfection (Figure 1C). The M_r 36 000 mammalian eIF2α did not cross-react with this antiserum. Although a background of PKR expression occurs in VV-infected cells as a result of induction of endogenous PKR and leakiness of the system (Figure 1C, lane 1), PKR accumulation from VV PKR-α51S, VV PKR-α51A, and VV PKR was significantly induced following IPTG treatment (Figure 1C, lanes 3, 5, and 7). IPTG-inducible PKR expression was observed at 8 h pi and levels continued to accumulate during infection. The decrease in 51S expression in cells treated with IPTG compared with uninduced cells is consistent with global decreases in protein synthesis following PKR induction and activation of the eIF2α phosphorylation pathway (*34*) and has been observed for other proteins coexpressed together with PKR by this system (*33*; unpublished observations). However, the expression pattern for 51A was only slightly altered by addition of IPTG, suggesting that PKR-mediated inhibition of protein synthesis is abrogated by 51A.

Plant eIF2α Is Phosphorylated on Serine 51 by PKR in Mammalian Cells. Given the fact that the plant eIF2α wild-type and 51A mutant cDNAs are correctly expressed in mammalian cells alone or together with PKR, it was important to determine if wheat eIF2α is phosphorylated in vivo on the conserved serine 51 by PKR, a key requirement of the eIF2α translational regulation pathway. To address this and to evaluate the effect of overexpression of the plant proteins on endogenous eIF2α, HeLa cells were infected with

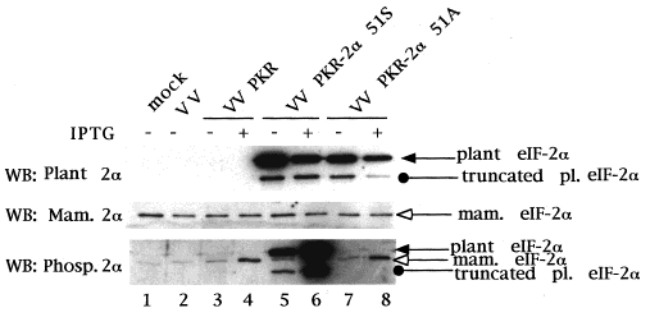

FIGURE 2: Plant eIF2α is phosphorylated on serine 51 by PKR in mammalian cells. HeLa cells were infected with the indicated viruses (5 pfu/cell) or mock-inoculated, and PKR expression was induced or not with 5 mM IPTG after viral adsorption for 1 h. Cells were collected at 16 h pi and subjected to immunoblot analysis with polyclonal antisera against plant eIF2 (upper panel), mammalian eIF2α (middle panel), or a polyclonal antibody that specifically recognizes the phosphorylated form of eIF2α (lower panel). WB, western blot.

VV, VV PKR-α51A, or VV PKR-α51S or mock-inoculated and the phosphorylation state of the eIF2α species was analyzed. As shown in Figure 2 (upper panel), both plant proteins are expressed efficiently, and as noted previously, IPTG induction of PKR provokes a decrease in steady-state levels (33% less than in the absence of IPTG), probably as a result of the block in protein synthesis caused by PKR expression. Further, a lower band that is likely a truncated form of plant eIF2α is detectable. However, when extracts were immunoblotted with antiserum that specifically recognizes the phosphorylated form of eIF2α, an immunoreactive band from cells infected with VV PKR-α51A at the position expected for plant eIF2α was not detectable regardless of IPTG or exposure time (lower panel, lanes 7 and 8). Wild-type plant eIF2α (51S) was heavily phosphorylated in cells infected with VV PKR-α51S and treated with IPTG (lanes 5 and 6). The detection of phosphorylated 51S in the absence of IPTG (lane 5) was likely due to leakage of the inducible system and by the presence of endogenous eIF2α kinases. However, the steady-state level of wild-type peIF2α(PO_4) significantly increased when PKR expression was induced, even though 51S protein levels are lower than in the absence of PKR induction. Steady-state levels of endogenous eIF2α were not affected by expression of plant proteins (middle panel, compare lanes 1−4 with lanes 5−8). Further, endogenous eIF2α phosphorylation levels were similar in VV-infected and VV PKR-α51S- or VV PKR-α51A-infected cells upon PKR induction, suggesting that expression of plant proteins did directly interfere with expression or activity of the endogenous eIF2α.

Expression of eIF2α 51A Rescues PKR-Mediated Antiviral Effects. On the bais of demonstration of in vivo PKR-mediated peIF2α phosphorylation and the fact that this is the hallmark of the PKR-based pathway for translational regulation, we next evaluated the ability of plant eIF2α to influence PKR antiviral activity in VV-infected cells. Viral growth curves were determined in cells infected with the various VV recombinants in the presence or absence of IPTG. Induction of PKR resulted in a ca. 35-fold inhibition of VV replication at 24 h (Figure 3A). These results are in agreement with those of Lee and Esteban (*38*). When a similar analysis was performed with VV PKR-α51S-infected cells, the difference in viral yields between PKR-induced and uninduced treatments was reduced (3-fold inhibition of VV replication at 24 h, Figure 3B) compared to VV PKR-infected cells. Significantly, no difference in viral yields was observed between PKR-induced or uninduced treatments in cells infected with VV PKR-α51A (Figure 3C), suggesting that expression of the nonphosphorylatable form of peIF2α suppressed the PKR-mediated eIF2α phosphorylation pathway. To confirm these observations, we compared plaque numbers from cells infected with the various viruses and cultured for 3 days in the continuous presence or absence of IPTG. As previously noted (*38*), a dramatic reduction in plaque number and size was observed in VV PKR-infected cells growing in the presence of IPTG (Figure 3D, left). A similar result was observed in cells infected with VV PKR-α51S (Figure 3D, middle). However, when plaque formation was analyzed in cells infected with VV PKR-α51A in the presence of IPTG, a rescue of approximately 50% of the plaques was observed (Figure 3D, right). These data suggest not only that plant eIF2α 51A expression abrogates the PKR-mediated antiviral response but also that wild-type peIF2α 51S functions within the translation initiation mammalian pathway.

Expression of eIF2α 51A Abrogates the Translational Block Caused by PKR Expression. The mechanism responsible for the PKR-mediated effects on viral growth in cells expressing wheat eIF2α 51S or wheat eIF2α 51A was evaluated by assaying protein synthesis patterns in the presence and absence of PKR induction. In mammalian cells, decreased VV pathogenesis as a result of PKR activation is correlated with a global decrease in protein synthesis (*34*, *38*). An initial and very sensitive determination of protein synthesis levels was made on the basis of expression of *β*-galactosidase driven from a VV 7.5 promoter incorporated in the VV recombinants. Figure 4A shows that *β*-galactosidase production was severely inhibited (ca. 80%) in cells infected with VV PKR 20 h after IPTG treatment. Similarly, induction of PKR in cells infected with VV PKR-α51S inhibited *β*-galactosidase expression, consistent with results from growth curve studies. However, *β*-galactosidase activity in cells expressing plant 51A was rescued from induction of PKR (Figure 4C). This rescue could account for the absence of reduction in viral yields upon PKR expression and supports the concept that plant proteins are functionally interacting in the mammalian pathway.

In addition, specific VV protein production in cells infected with VV recombinants in the presence or absence of IPTG was determined to check if there was a correlation between levels of VV proteins expressed and the effect on viral yields. In VV PKR-infected cells, addition of IPTG resulted in a severe decrease in steady state levels of VV proteins, specifically late viral proteins, since PKR expression was driven from a VV late promoter (Figure 5A). Also, a significant decrease in VV protein production at late times of infection was observed in cells infected with VV PKR-α51S when PKR was induced by IPTG treatment, although it was not as dramatic as in VV PKR-infected cells (Figure 5B). However, this reduction was not observed in cells infected with VV PKR-α51A (Figure 5C), supporting previous results.

Global protein synthesis levels were determined by metabolically labeling cells infected with VV recombinants followed by SDS−PAGE analysis. Figure 6A,B demonstrates

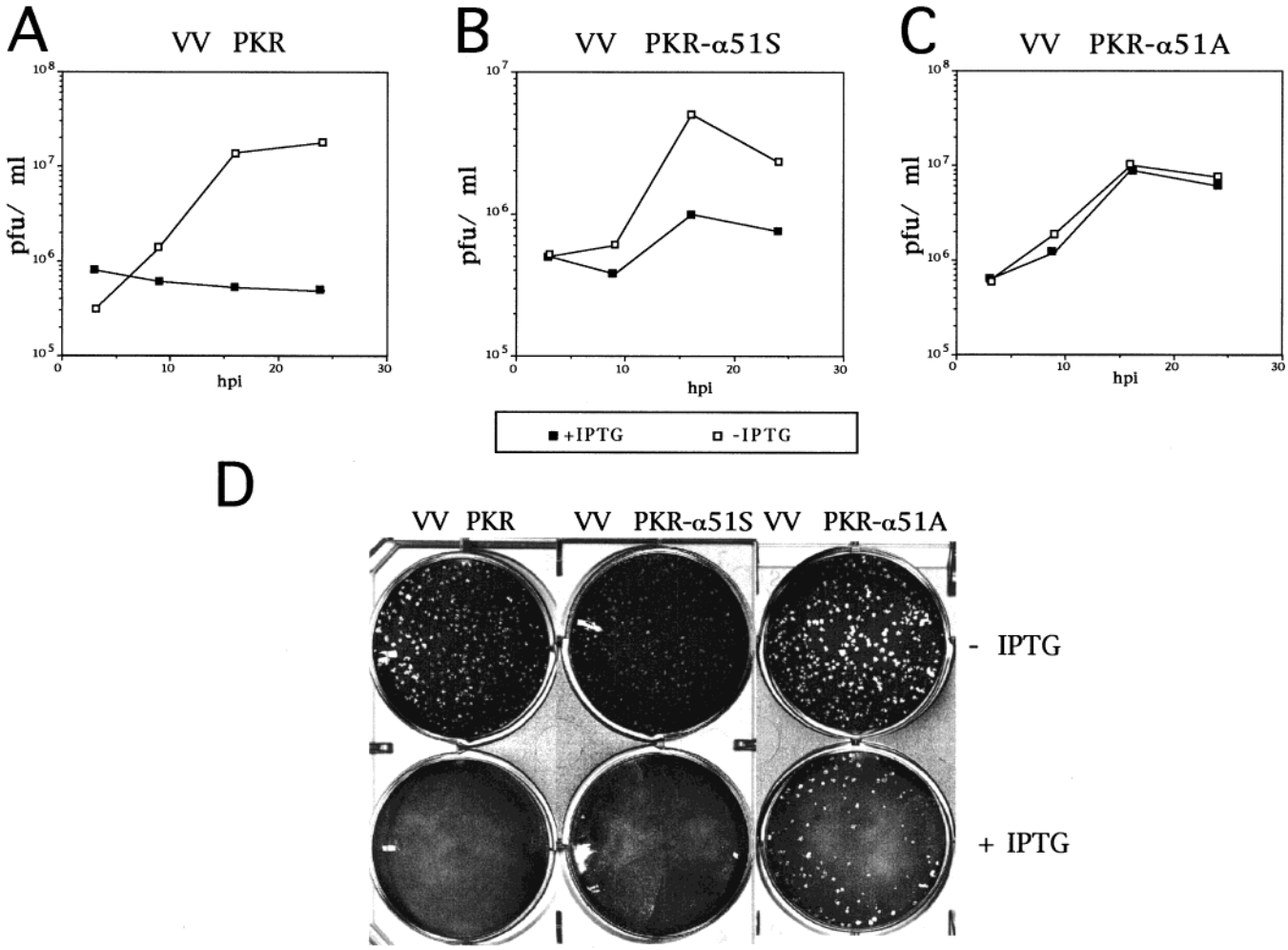

FIGURE 3: Expression of 51A overrides the antiviral effects exerted by PKR expression from VV recombinants. BSC-40 cells were infected (5 pfu/cell) with (A) VV PKR, (B) VV PKR-α51S, or (C) VV PKR-α51A as indicated under Experimental Procedures and one-step virus growth curves were determined in the presence (■) or absence (□) of 5 mM IPTG. (D) Plaque formation by VV PKR, VV PKR-α51S, and VV PKR-α51A in the presence or absence of IPTG. Confluent BSC-40 cells were infected with ca. 200 pfu/cell of the indicated viruses. After virus adsorption, cells were overlaid with medium 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.

that there was a significant decrease in protein synthesis in cells infected with VV PKR and VV PKR-α51S at late times (16 or 24 h pi) following PKR induction with IPTG. At earlier times (4 or 8 h pi) no differences were observed because active PKR only begins to accumulate at 10−12 h pi (*38*). This is consistent with the kinetics observed in experiments analyzing viral protein production and *β*-galactosidase activity. Similar results were observed in cells infected with VV PKR-α51S (Figure 6B). However, in cells infected with the VV PKR-α51A recombinant, inhibition of protein synthesis was alleviated to a significant extent (Figure 6C). To quantitate these results, labeled proteins were precipitated and counted in a scintillation counter. In cells infected with VV PKR or VV PKR-α51S, protein synthesis at 16 hpi decreased ca. 95% upon PKR induction relative to uninduced levels (Figure 6D). However, expression of plant eIF2α-51A partially rescued the protein synthesis inhibition caused by PKR expression. In cells infected with VV−PKR α51A at 16 h pi there was a ca. 4-fold increase in protein synthesis compared with VV PKR-infected cells. At 24 h pi in cells infected with VV PKR-α51A, there still was a 20% rescue of protein synthesis upon PKR induction compared with uninduced cells. These results are consistent with those of Gil et al. (*33*) for mammalian eIF2α 51A and PKR coexpressed in infected cells from separate VV recombinants. Together these results strongly support the concept that a functional interference by the plant eIF2α 51A mutant helps to partially suppress the PKR-imposed translational block, causing also a reversion in antiviral effects induced by PKR expression.

PKR-Induced Apoptosis Is Decreased by Expression of Plant eIF2α 51A. Lee et al. (*7*) showed that induction of PKR results in the development of apoptosis in cells infected with VV PKR. Recently, we and others (*33*, *40*) also showed that inhibiting PKR-mediated phosphorylation of eIF2α by expression of mammalian eIF2α 51A resulted in a significant decrease in PKR-induced apoptosis. Thus, it was predicted that expression of plant eIF2α 51A would similarly inhibit PKR-induced apoptosis. To determine the effect of different doses of plant eIF2α while maintaining a constant PKR amount, we generated with the same VV insertional vectors described in Figure 1B recombinant VVs expressing only the wheat eIF2α proteins (designated VV α51S and VV α51A). Thus, coinfection experiments to evaluate PKR-induced apoptosis could be performed with VV PKR and varying inoculum levels of VV α51S or VV α51A. Induction

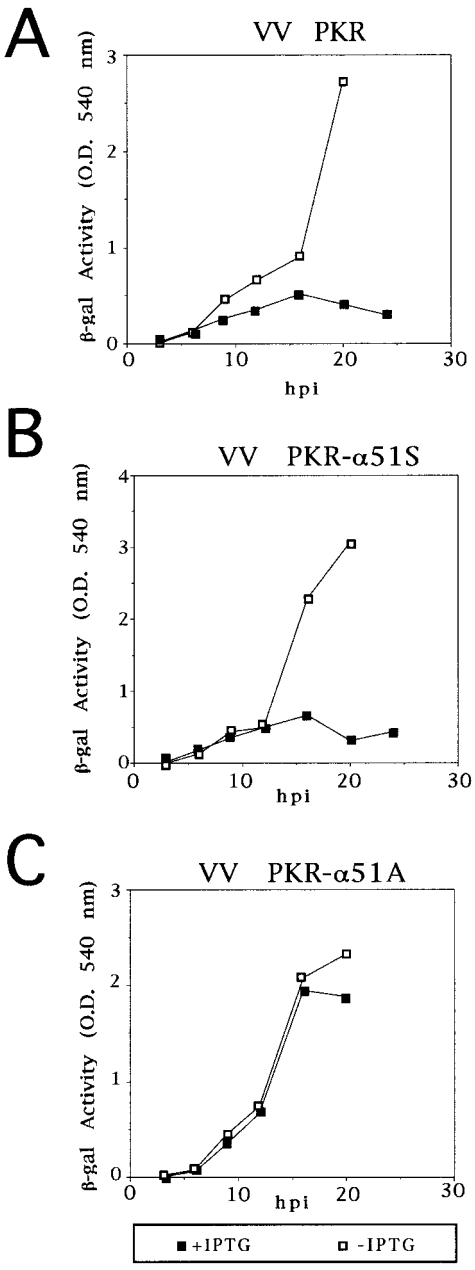

FIGURE 4: Time course of β-galactosidase activity driven from a VV promoter. Monolayers of BSC-40 cells were infected with (A) VV PKR, (B) VV PKR-α51S, or (C) VV PKR-α51A viruses (5 pfu/cell), and after 1 h of virus adsorption, inoculum was removed and new medium ± 5 mM IPTG was added. At selected times cells were harvested and extracts were prepared for β-galactosidase activity measurement as described under Experimental Procedures.

of PKR in cells infected with VV PKR resulted in apoptosis, based upon analysis of caspase 3 activity (Figure 7A) and a photometric immunoassay for cytoplasmic histone-associated DNA fragments (Figure 7B). A slight decrease in apoptosis induction was observed in cells expressing plant eIF2α 51S relative to controls consisting of cells infected with VV PKR and may suggest an incomplete complementation between plant eIF2α and mammalian eIF2 β and γ subunits. However, when plant eIF2α 51A was expressed, caspase 3 activity and DNA fragmentation were significantly reduced (Figure 7). This effect was dose-dependent, and at higher levels of 51A expression, the degree of apoptosis inhibition as measured by the caspase 3 assay was essentially identical to that of control cells infected solely with VV. Together these results suggest that, similarly to the mammalian homologue, expression of a nonphosphorylatable plant eIF2α mutant can block apoptosis induction triggered by PKR.

Interaction of Plant eIF2α 51S and 51A Proteins in the Mammalian Translational Initiation Apparatus. Localization and coimmunoprecipitation experiments were preformed in order to determine if the observed effects of plant eIF2α 51S and 51A expression in the VV system were due to their direct interaction in mammalian translational initiation complexes. For localization studies, cytosolic and ribosomal fractions were prepared from cells that were infected with VV PKR-α51S, VV PKR-α51A, or VV or were mock-inoculated. Western blots presented in Figure 8A show that plant eIF2α wild-type and the 51A mutant proteins are not only present in the soluble fraction but also localized to ribosomal fractions. As these fractions contain translationally active complexes, identification of the plant proteins is consistent with their interaction in protein synthesis. Further support is provided by coimmunoprecipitation experiments where VV PKR-α51S-, VV PKR-α51A-, or VV-infected cells or mock-inoculated cells were metabolically labeled with [^{35}S]Met−Cys prior to immunoprecipitation with antiserum to mammalian eIF2γ. Following thorough washing, immunocomplexes were resolved by SDS−PAGE and subjected to immunoblotting with antiserum to plant eIF2. Immunoreactive bands were detected in extracts from cells infected with VV PKR-α51S and VV PKR-α51A but not in controls consisting of cells infected with VV or mock-inoculated (Figure 8B).

DISCUSSION

Although the plant translational machinery is functionally similar to that of other eukaryotes, significant differences have been documented that suggest qualitative and quantitative differences in the mechanisms of regulation (*11*). Gallie et al. (*41*) found that heat shock in plants does not induce eIF2α phosphorylation in a manner similar to mammalian cells (*42*, *43*). Further, on the basis of measurements of relative affinities of eIF2 for GDP and GTP, Shaikhin et al. (*9*) proposed that plants have no need for eIF2B or eIF2α-mediated regulation. Krishna et al. (*10*) suggested that although wheat germ eIF2α can be phosphorylated in vitro it is nonfunctional in terms of translational control in reticulocytes. A mitigating factor in the studies of Shaikhin and Krishna may be that they used eIF2α purified from wheat germ that is a dormant, nonmetabolizing tissue. However, the presence of a plant eIF2α phosphorylation pathway has been suggested by characterization studies demonstrating the presence of a plant homologue to the mammalian dsRNA-dependent protein kinase PKR (*23*) and the specific in vitro phosphorylation of plant eIF2α (*31*). Further, putative subunits of the guanine nucleotide exchange factor eIF2B have been identified from the plant-expressed sequence tag database. Yet, it is not clear if plant eIF2α and plant eIF2α-(PO_4) are capable of interacting with eIF2B, and the regulation of protein synthesis by plant eIF2α has not been demonstrated in vivo. The presence of PKR substrates in addition to eIF2α suggests roles for pPKR other than translational control. The vaccinia virus−cell system described herein provides an excellent model to address issues related to the role of plant eIF2α in translational control. In

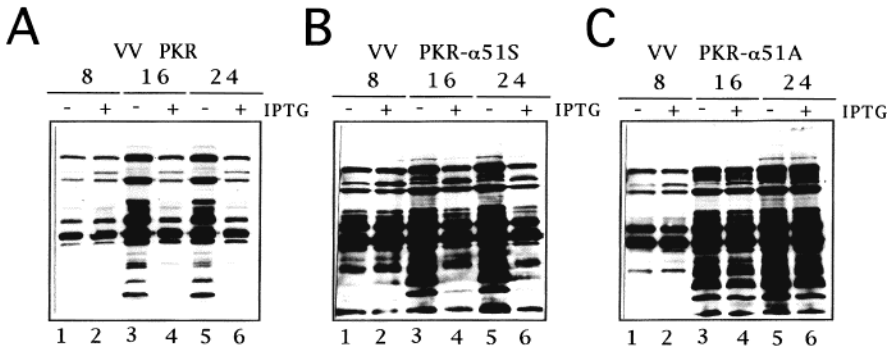

FIGURE 5: Regulation of VV protein synthesis by wheat eIF2α proteins. Extracts from BSC-40 cells infected with (A) VV PKR, (B) VV PKR-α51S, or (C) VV PKR-α51A viruses (5 pfu/cell) were subjected to immunoblot analyses with polyclonal antibodies against VV (1:1000 dilution).

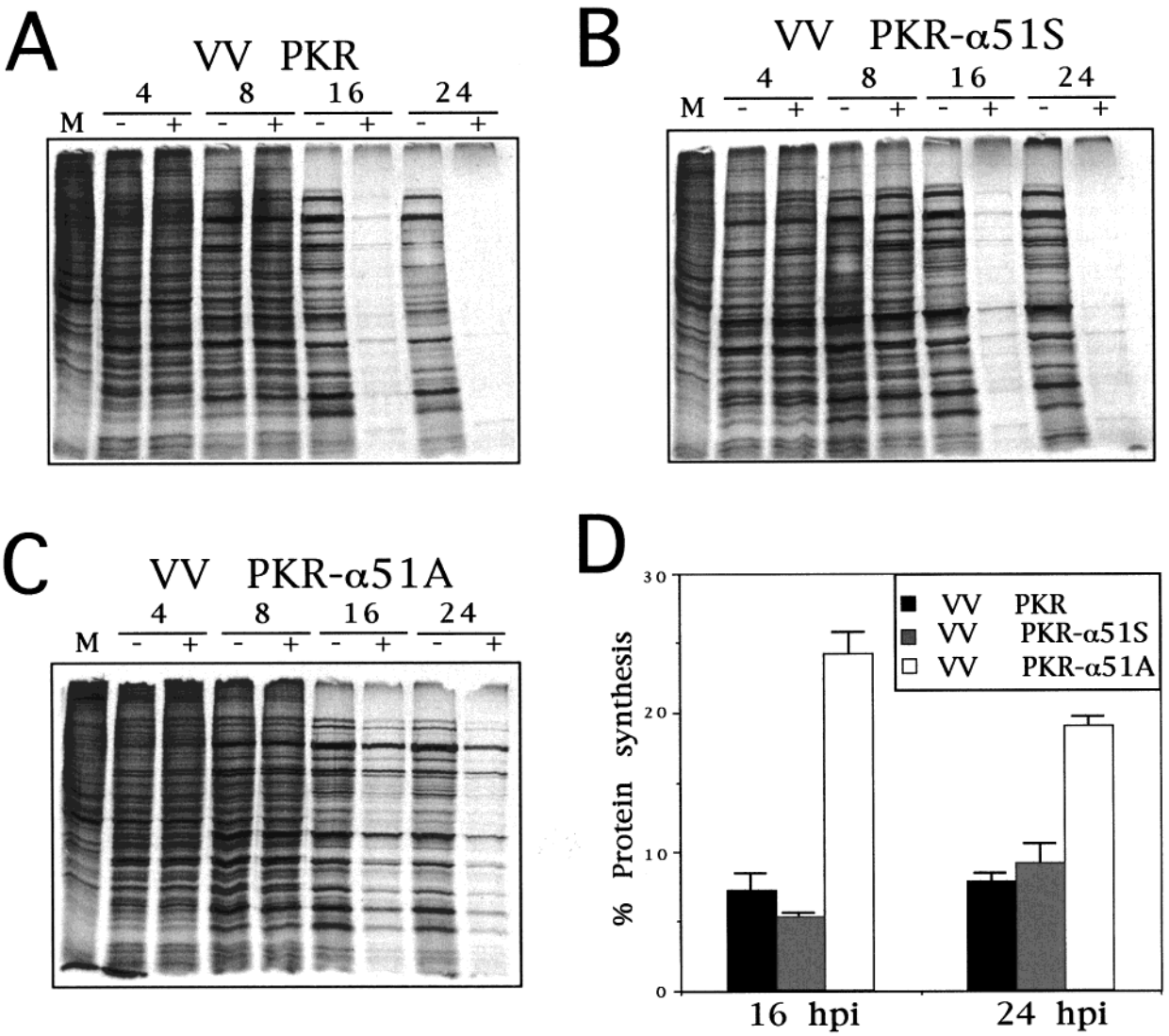

FIGURE 6: Effect of wheat eIF2α proteins on PKR-mediated inhibition of total protein synthesis. Monolayers of BSC-40 cells were infected (5 pfu/cell) with (A) VV PKR, (B) VV PKR-α51S, or (C) VV PKR-α51A viruses, labeled at indicated times with [^{35}S]methionine for 1 h and cells were collected in lysis buffer. Protein synthesis was analyzed by SDS−PAGE, followed by autoradiography. (D) Proteins from cell lysates were precipitated with 5% TCA and counted in a scintillation counter. Protein synthesis levels in cells infected with the various VV recombinants in the presence of IPTG (+PKR) relative to those in the absence of IPTG (−PKR) are given. M, mock-inoculated treatment.

this system, eIF2α is constitutively expressed from the virus vector whereas PKR expression is inducible with IPTG and the consequences of PKR-mediated eIF2α phosphorylation have been well documented (*7*, *34*, *38*). As a result, the effect of plant eIF2α expression can be evaluated in the presence or absence of PKR. In this study, the physiological effects of wheat eIF2α expression were directly related to its ability to act as a substrate for PKR. Phosphorylation of wheat eIF2α in VV PKR-α51S infected cells was dependent upon PKR induction and resulted in the decreased synthesis of

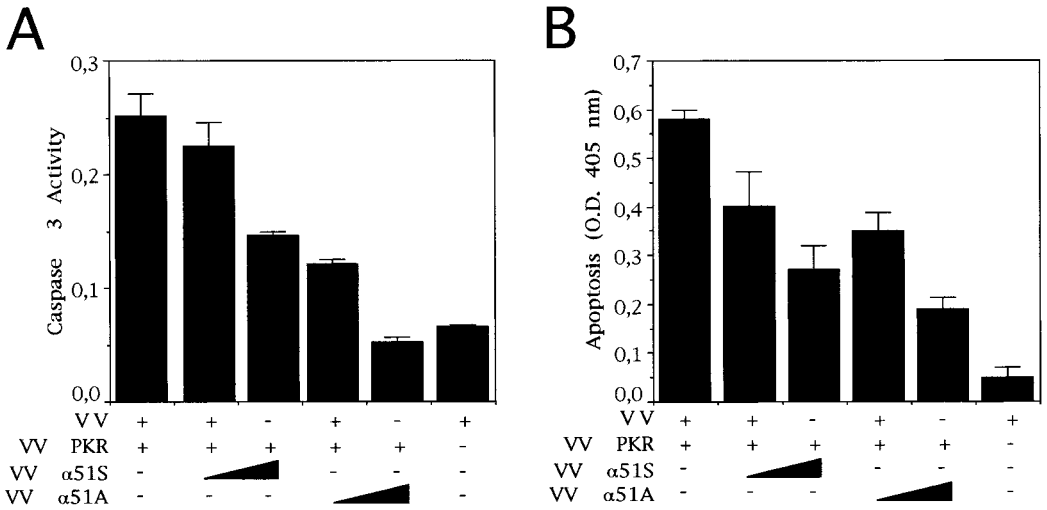

FIGURE 7: Inhibition of PKR-induced apoptosis by coexpression of plant eIF2α 51A. (A) Caspase 3 activity. BSC-40 cells were infected with the indicated viruses and at 24 h pi cells were collected in lysis buffer. Cellular extracts were used to determine caspase 3 activity as described under Experimental 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 from columns 1 to 5 were infected with 3 pfu/cell of VV PKR. Cells from column 2 and 3 were coinfected with 3 or 6 pfu/cell, respectively, of VV α51S. Cells from column 4 and 5 were coinfected with 3 or 6 pfu/cell, respectively, of VV α51A. Cells from column 6 were infected with 9 pfu/cell of VV. (B) Cell-death ELISA. BSC-40 cells were infected with the same viruses as in panel A and at 24 h pi cells were collected in lysis buffer. Cytoplasmic extracts were prepared and used to determine the extent of apoptosis. Each point represents triplicate samples.

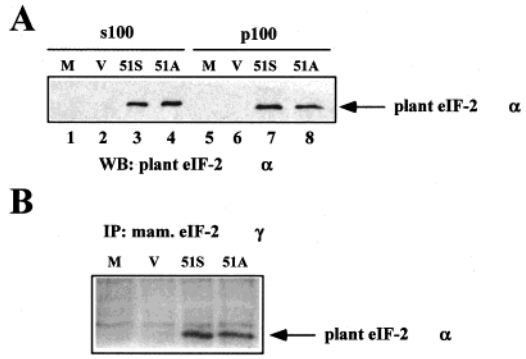

FIGURE 8: Interaction of plant eIF2α 51S and 51A in mammalian translational complexes. (A) Extracts from cells infected with VV (V), VV PKR-α51S (51S), or VV PKR-α51A (51A) or mock-inoculated (M) were fractionated into cytosolic (s100) and ribosomal (p100) fractions and subject to immunoblot analysis with plant eIF2 antiserum. (B) Extracts from cells infected with viruses described in panel A or mock-inoculated were metabolically labeled with [^{35}S]-Met-Cys and immunoprecipitated with antiserum to mammalian eIF2γ. Following thorough washing, immunocomplexes were resolved by SDS−PAGE, immunoblotted with antiserum to plant eIF2, and subjected to autoradiography.

viral and cellular proteins as well as β-galactosidase. The consequences of wheat 51S phosphorylation culminated in an increased level of apoptosis and decreased viral growth relative to 51A expression. Wheat 51A expression abrogated PKR-mediated inhibition of protein synthesis and apoptosis. The effects of 51A expression were specifically related to translational control as no differences in NF-κB-mediated transcriptional control were observed by gel shift analysis (data not shown).

The interaction of the plant eIF2 proteins in ternary complex formation and activity is supported by the presence of plant 51S and 51A proteins in fractions containing polysomes with initiating 40S subunits and, most strongly, by the coimmunoprecipitation of the plant proteins with mammalian eIF2γ. However, the partial inhibitory effect on protein synthesis and on apoptosis related to expression of wild-type plant eIF2α suggests incomplete complementation. These results may be due to the fact that plant and mammalian eIF2α are only approximately 51% identical. Similarly in yeast, recessive mutations in the eIF2α gene cause a phosphorylation-independent decrease in ternary complex activity that inhibits cell growth (*44*). The fact that expression of the plant proteins does not interfere with endogenous mammalian eIF2α expression and PKR-mediated phosphorylation suggests that the effects are not due to direct inhibition of the endogenous protein.

Although it is likely that endogenous eIF2α contributed to initiation events, taken together, these data support the interaction of plant eIF2α in the phosphorylation pathway and the ability of plant 51A to serve as a dominant negative inhibitor of PKR. These results are consistent with those of Gil et al. (*33*) using PKR and mammalian eIF2α-51A coexpressed in cells doubly infected with separate VV recombinant viruses. However, in the experiments described herein, cells were infected with pHLZ-based double VV recombinants allowing for the simultaneous expression of PKR (under an IPTG-inducible promoter) and putative modulators of its activity (expressed constitutively) from the same virus. By using this approach it is possible to measure the impact exerted by modulator proteins on PKR-mediated antiviral effects. Thus, the use of a VV recombinant expressing within the same genome a modulator like eIF2α constitutively under an early/late promoter and PKR under regulation of an inducible late promoter is more efficient and superior for the analysis of protein modulators of PKR activity.

These data also support the homology between the essential translational processes of plants, yeast, and mammals. This apparent conservation of function between components of the eukaryotic translational apparatus permits the use of heterologous systems to probe structure−function characteristics. However, distinct differences are also ap-

parent. Phosphorylation of eIF2α in plants does not apparently shut off protein synthesis similarly to that described in mammalian cell culture (Hu and Roth, unpublished results). Further, increased pPKR phosphorylation following plant virus infection does not appear to have antiviral consequences (*25*, *29*). The physiological significance of plant eIF2α phosphorylation *in planta* has not been demonstrated, although differential eIF2α phosphorylation has been shown during plant development (*30*, *41*). The critical importance of eIF2α phosphorylation in control of cell stress responses has important implications in the potential role of translational control in plant growth and development. Ongoing studies with transgenic plants expressing nonphosphorylatable 51A mutants may help to determine the physiological significance of plant eIF2α phosphorylation.

In addition, the link between plant eIF2α phosphorylation and induction of apoptosis suggests a physiological role for eIF2α phosphorylation in plant development. Apoptosis is a widely conserved mechanism of cell regulation in all eukaryotes and similar pathways appear to be operative in plants and mammals (*45*, *46*). Since PKR has been shown to play a role in apoptosis induction in mammalian cells (*7*, *8*), conservation of PKR and eIF2α proteins suggests the existence of a conserved apoptosis pathway in plants involving these molecules. This is further supported by the findings that the mammalian 2−5A system that exerts antiviral effects and induces apoptosis (*47*, *48*) is also functional when expressed in plants, triggering antiviral effects (*49*).

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