A Eukaryotic Translation Initiation Factor 2-Associated 67 kDa Glycoprotein Partially Reverses Protein Synthesis Inhibition by Activated Double-Stranded RNA-Dependent Protein Kinase in Intact Cells

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ABSTRACT: A eukaryotic translation initiation factor 2 (eIF-2)-associated 67 kDa glycoprotein (p67) protects the eIF-2 α-subunit from inhibitory phosphorylation by eIF-2 kinases, and this promotes protein synthesis in the presence of active eIF-2α kinases *in vitro* [Ray, M. K., et al. (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89, 539−543]. We have now examined the effect of overexpression of this cellular eIF-2 kinase inhibitor in an *in vivo* system using transiently transfected COS-1 cells. In this system, coexpression of genes that inhibit PKR activity restores translation of plasmid-derived mRNA. We now report the following. (1) Transient transfection of COS-1 cells with a p67 expression vector increased p67 synthesis by 20-fold over endogenous levels in the isolated subpopulation of transfected cells. (2) Cotransfection of p67 cDNA increased translation of plasmid-derived mRNAs. (3) Overexpression of p67 reduced phosphorylation of coexpressed eIF-2α. (4) p67 synthesis was inhibited by cotransfection with an eIF-2α mutant S51D, a mutant that mimics phosphorylated eIF-2α, indicating that p67 cannot bypass translational inhibition mediated by phosphorylation of the eIF-2 α-subunit. These results show that the cellular protein p67 can reverse PKR-mediated translational inhibition in intact cells.

Phosphorylation of the α-subunit of heterotrimeric eIF-2 (eIF-2α) is an important regulatory mechanism in the initiation of protein synthesis (Gupta et al., 1987; Hershey, 1991). Under certain physiological conditions, kinases such as the double-stranded RNA (dsRNA)-activated inhibitor (PKR) and heme-regulated inhibitor (HRI) phosphorylate eIF-2α and inhibit protein synthesis. The synthesis of PKR is induced by interferon, and its kinase activity is dependent upon dsRNA. Various inhibitors of PKR-mediated eIF-2α phosphorylation have been identified within interferon-resistant viral genomes, including the small RNA polymerase III transcripts encoded by adenovirus (VA RNAs I and II) and Epstein Barr virus (EBER RNAs I and II) (Schneider et al., 1985; Siekierka et al., 1985) as well as protein products such as E3L and K3L that are encoded by vaccinia virus (Chang et al., 1992; Davies et al., 1992). Whereas E3L is a double-stranded RNA binding protein that competes with PKR for binding to dsRNA, K3L bears amino acid sequence homology to the phosphorylation site on the eIF-2 α-subunit and competitively inhibits the interaction of PKR with the eIF-2 α-subunit (Davies et al., 1992). In addition to its role in the antiviral activity of interferon, PKR is likely also important as a major regulator of cell growth. This is most strongly supported by the observation that mutations in the serine/threonine catalytic domain of PKR can act in a dominant manner to transform NIH 3T3 cells (Koromilas et al., 1992; Meurs et al., 1993). These results implicate PKR as a tumor supressor gene. Therefore, there is much interest in identifying and characterizing the activity of proteins that may regulate PKR activity. To date, several cellular gene products that inhibit PKR activity have been characterized. (1) A cellular 58 kDa protein is activated upon influenza virus infection (Lee et al., 1992; Barber et al., 1994). (2) A protein factor is synthesized in polio virus-infected cells (Ransoni & Dasgupta, 1988). (3) The cellular HIV TAR RNA binding protein (TRBP) binds RNAs containing specific secondary structure(s) and inhibits PKR activation (Park et al., 1994). (4) A 100 kDa PKR inhibitor is associated with Ha-*ras*-mediated transformation of NIH3T3 cells (Mundschau & Faller, 1992). (5) A 15 kDa protein (dRF) is expressed in 3T3 F442A upon growth arrest with subsequent induction of adipocyte differentiation (Judware & Petryshyn, 1992).

Gupta and co-workers have previously described the isolation and cloning of a gene encoding a 67 kDa glycoprotein (p67) that copurifies with eIF-2 (Wu et al., 1993). They provided evidence that p67 protects the eIF-2 α-subunit from inhibitory phosphorylation by eIF-2 kinases (Datta et al., 1988, 1989). This protein is, presumably, necessary for protein synthesis in the presence of active eIF-2 kinases (Ray et al., 1992). In the present study, we describe expression of the cloned p67 (Wu et al., 1993) in COS-1 cells. We provide evidence that the expressed protein inhibits PKR-catalyzed eIF-2 α-subunit phosphorylation and also reverses PKR-mediated inhibition of protein synthesis in intact cells.

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[1] Abbreviations: PKR, double-stranded RNA-activated protein kinase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DHFR, dihydrofolate reductase; ADA, adenosine deaminase; eIF-2α, eukaryotic translation initiation factor 2 α; TF, tissue factor.

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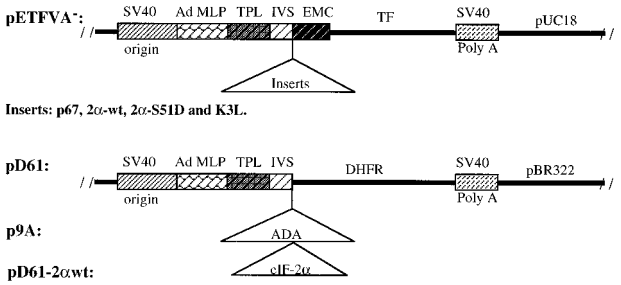

FIGURE 1: Expression vectors used in this study. The construction of the vectors was described in Materials and Methods.

MATERIALS AND METHODS

Vector Constructions. The expression vectors used in this study are shown in Figure 1. All of the vectors contain the same transcription unit utilizing the adenovirus major late promoter and simian virus 40 (SV40) enhancer element for transcription initiation. In addition, the vectors contain the SV40 origin for replication in COS-1 cells. The dihydrofolate reductase (DHFR) expression plasmid pD61 (Davies et al., 1993) and the tissue factor (TF) (Rehemtulla et al., 1991) expression plasmid pETFVA$^-$ that utilizes the encephalomyelocarditis internal ribosomal entry site to initiate translation of TF (Davies et al., 1993) were previously described. The eIF-2α expression vectors encoding the wild type pETFVA$^-$-2αwt and the Ser-51 to Asp mutant pETFVA$^-$-51D were described elsewhere (Kaufman et al., 1989). The adenosine deaminase (ADA) expression plasmid p9A (Kaufman & Murtha, 1987) and the eIF-2α expression plasmid pD61-2αwt (Davies et al., 1993) were previously described.

For expression of p67, the coding region of p67 was excised from the p67 bacculovirus expression vector pBB-p67 (S. Wu et al., unpublished data) by first digesting with *Hin*dIII. The 5′ overhangs were made flush-ended using T4 DNA polymerase. The p67 fragment was then excised by *Pst*I digestion. pETFVA$^-$ was digested with *Pst*I and *Sal*I. The *Sal*I site was blunt-ended using T4 DNA polymerase. The *Pst*I blunt fragment of p67 was ligated to the vector to form the p67 expression plasmid pETFVA$^-$-p67.

DNA Transfection and Analysis of Expression. COS-1 monkey kidney cells were transfected using the DEAE-dextran procedure as described previously (Kaufman, 1990). After 48 h, cells were labeled with Expre [^{35}S][^{35}S] protein-labeling mixture (100 μCi/mL; 1000 Ci/mmol, NEN, Boston, MA) for 20 min in methionine/cysteine-free minimal essential medium (GIBCO BRL, Gaithersburg, MD). Cell extracts were prepared by lysis in Nonidet P-40 lysis buffer as described elsewhere (Kaufman et al., 1989) and analyzed by SDS−PAGE (Laemmli, 1970). Gels were fixed in 40% methanol−10% acetic acid, prepared for fluorography by treatment with EnHance (New England Nuclear Corp., Boston, MA), and dried. Dried gels were autoradiographed with Kodak XAR-5 film with a Dupont Cronex Lightning-plus screen. Protein levels of DHFR were quantitated by PhosphorImage scanning (Molecular Dynamics, Sunnyvale, CA), and band intensities were measured using the ImageQuant program (Molecular Dynamics, Sunnyvale, CA).

Analysis of the mRNA. Total RNAs were prepared using Trizol (GIBCO, Bethesda, MD) and analyzed by Northern blot hybridization (Thomas, 1980) following electrophoresis on formaldehyde−formamide denaturing agarose gels as described elsewhere (Derman et al., 1981). Hybridizations were performed using two probes (DHFR and β-actin) prepared by random priming with [^{32}P]dCTP and oligonucleotides as described by the supplier (Pharmacia Biotech. Inc., Piscataway, NJ).

In vivo Phosphorylation of eIF-2α. To monitor *in vivo* phosphorylation of the eIF-2 α-subunit, COS-1 cells were cotransfected with pETFVA$^-$-2αwt in the presence of an equal amount of pETFVA$^-$-p67, pETFVA$^-$-K3L, or the pETFVA$^-$ vector DNA. The transfected cells were labeled at 48 h post-transfection with 2 mL of $^{32}PO_4$ (200 μCi/mL, NEN, Boston, MA) for 4 h in phosphate-free medium (GIBCO BRL, Gaithersburg, MD). Cell extracts were prepared by lysis in Nonidet P-40 lysis buffer. The eIF-2 α-subunit was immunoprecipitated with anti-eIF-2α monoclonal antibody (kindly provided by Dr. Henshaw), resolved by SDS−PAGE, and electroblotted to nitrocellulose. The membrane was immunoblotted with anti-eIF-2α monoclonal antibody, and the eIF-2α protein level was visualized by the alkaline phosphatase immunoblotting detection system as described by the supplier (Sigma Corp., St. Louis, MO). $^{32}PO_4$ incorporation was quantitated by autoradiography of the nitrocellulose membrane using Kodak XAR-5 film and a Dupont Cronex Lightning-plus screen. Protein levels and phosphorylation of eIF-2α were quantitated using NIH-Image (Version 1.55b, NIH, Bethesda, MD).

Analysis of p67 Expression in the Subpopulation of Transfected Cells. COS-1 cells were transfected and labeled as described above. The cells were then detached from the plates using 10 mM EDTA and incubated for 0.5 h in polyethylene dishes previously coated with anti-TF monoclonal antibody (Davies et al., 1993). Unbound cells were collected and saved. Bound cells were washed two times to remove nonspecifically bound cells. Both the unbound and bound cells were collected and lysed using NP-40 lysis buffer. The total cell extracts were analyzed on SDS−PAGE.

Western Blot Analysis of p67. The steady state expression level of p67 was measured in cell extracts obtained as described above. Equal amounts of cell extract protein were resolved by SDS−PAGE and electroblotted to nitrocellulose membranes. The membranes were probed with anti-p67 mono- or polyclonal antibodies and analyzed with the alkaline phosphatase detection system as recommended by the supplier (Sigma Corp., St. Louis, MO).

RESULTS

Overexpression of p67 in COS-1 Cells. To elucidate the role of p67 in regulating initiation of protein synthesis, we analyzed the effect of p67 overexpression on translation in intact cells. The p67 cDNA was subcloned into an expression vector pETFVA$^-$ that allows for a high level expression in COS-1 cells (Davies et al., 1993). Cell extracts were prepared 48 h post-transfection and analyzed by immunoblotting using either a polyclonal antibody or a monoclonal antibody that specifically reacts with the O-linked glycosylated forms of p67 (Datta et al., 1989). A 67 kDa band was detected by Western immunoblotting using a polyclonal antibody in cell extracts from the transfected cells. As shown, this protein level was significantly increased in cells transfected with the expression construct pETFVA$^-$-p67 (Figure 2B, lane 3 vs lanes 1 and 2). A similar increase in p67 level was observed in pETFVA$^-$-p67-transfected cells when the p67 level was analyzed using p67 monoclonal

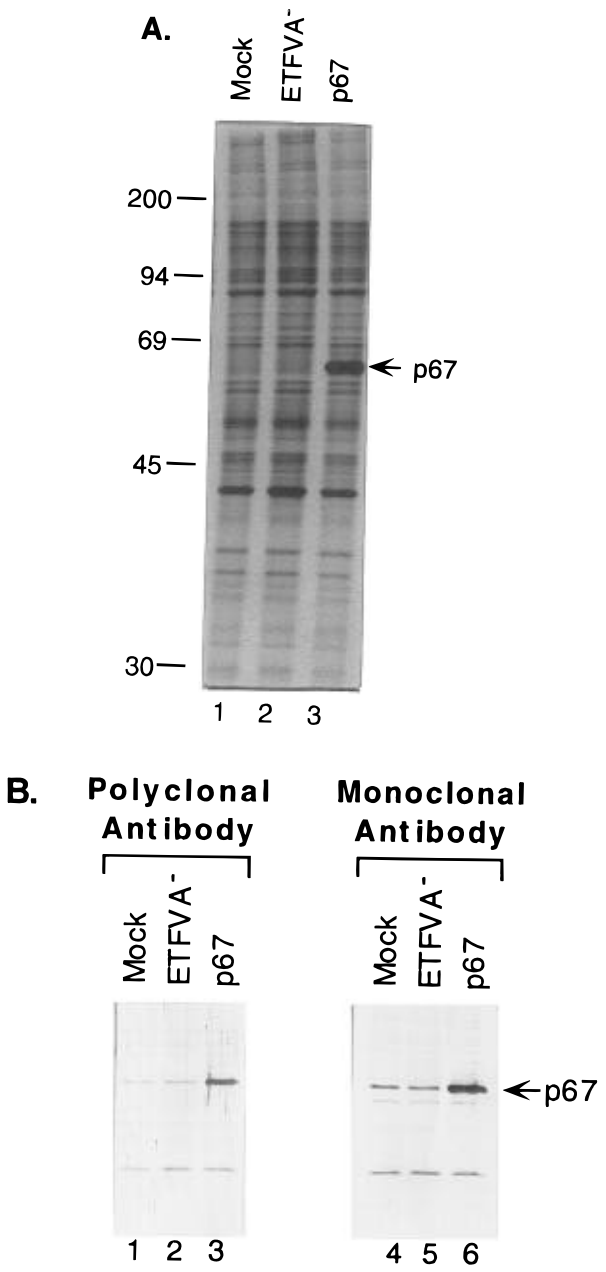

FIGURE 2: Overexpression of p67 in COS-1 cells. (A) Cells were transfected with pETFVA$^-$ (lane 2) or pETFVA$^-$-p67 (lane 3) using the DEAE-dextran method. After 42 h, cells were pulse-labeled with [^{35}S]methionine/cysteine for 20 min. The labeled cells were lysed with NP-40 lysis buffer. The expression of p67 in cell extracts was analyzed by SDS−PAGE. (B) The steady state level of p67 was measured by Western blot analysis using polyclonal (lanes 1−3) and monoclonal (lanes 4−6) antibodies against p67. Densitometric scanning showed transfection of pETFVA$^-$-p67 yielded a 5-fold increase in p67 level using the polyclonal antibody (lane 3 vs 2) and a 4-fold increase in p67 level using the monoclonal antibody (lane 6 vs 5).

antibodies (Figure 2B, lane 6 vs lanes 4 and 5). These results demonstrate that the overexpressed protein in COS-1 cells was glycosylated in a manner similar to that of native p67, indicating that p67 overexpression did not significantly saturate the glycosylation machinery.

To determine if the increased steady state level of p67 was due to an increase in the p67 protein synthetic rate or a decrease in the p67 degradation rate, pulse labeling with [^{35}S]-methionine/cysteine was performed. A 67 kDa polypeptide was observed only in the cells that were transfected with pETFVA$^-$-p67 (Figure 2A, lane 3 vs lanes 1 and 2). Since

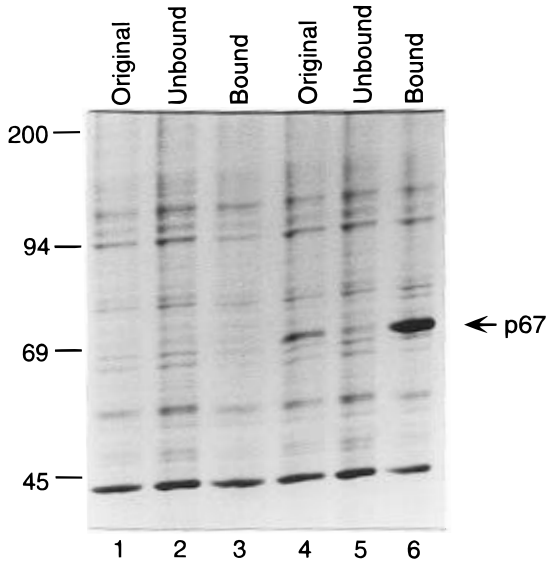

FIGURE 3: Analysis of protein synthesis in the subpopulation of p67-transfected cells. COS-1 cells were transfected with pETFVA$^-$ (lanes 1−3) or pETFVA$^-$-p67 (lanes 4−6) as described. After 42 h, cells were pulse-labeled with [^{35}S]methionine/cysteine for 20 min. The labeled cells were then collected and panned on plates coated with monoclonal antibody anti-TF. Both the bound and unbound cells were collected and lysed using NP-40 buffer and analyzed by SDS−PAGE. Equal amounts of cell lysate were loaded for SDS−PAGE: lanes 1−3, cells transfected with pETFVA$^-$; lanes 4−6, cells transfected with pETFVA$^-$-p67; lanes 1 and 4, original cells without panning; lanes 2 and 5, unbound cells; and lanes 3 and 6, bound cells. Densitometric scanning showed a 36-fold increase in p67 synthesis in bound p67-transfected cells over that of bound TF-transfected cells that did not receive p67.

the expression vector contains a dicistronic mRNA transcriptional cassette, cells that take up the DNA would express the inserted cDNA as well as tissue factor (TF), a cell surface molecule. Hence, the subpopulation of transfected cells can be isolated by panning with an antibody against TF. At 48 h post-transfection, cells were pulse-labeled with [^{35}S]-methionine/cysteine and cell extracts were prepared before and after panning with anti-TF antibody. A protein migrating at 67 kDa was detected in cells transfected with pETFVA$^-$-p67 (Figure 3, lane 4) that was not in cells that received the vector pETFVA$^-$ alone (Figure 3, lane 1) compared to the original cell population. Analysis of the subpopulation of cells after panning with anti-TF demonstrated a 4-fold increase in p67 synthesis compared to that of the original culture (Figure 3, lane 6) which was reduced to 0.2-fold in the unbound cells (Figure 3, lane 5), consistent with enrichment of the subpopulation of transfected cells. Quantitation of p67 synthesis in the transfected cells demonstrated a 20-fold increase over endogenous p67 (Figure 3, lane 3 vs 6). Analysis of the total cellular polypeptides synthesized in the subpopulation of cells overexpressing p67 indicated no detectable change in the spectrum of polypeptides synthesized compared to that of cells not overexpressing p67 (Figure 3, lane 3 vs 6).

p67 Overexpression Derepresses PKR-Dependent Inhibition of Protein Synthesis. To evaluate the effect of p67 overexpression on protein synthesis, we have utilized the transfection system previously described (Kaufman et al., 1989). This system exploits the property that some expression plasmids, such as pD61 which encodes DHFR, produce mRNAs that are selectively inefficiently translated due to PKR activation and eIF-2α phosphorylation (Kaufman et al., 1989). The unique feature of this system is that inefficient

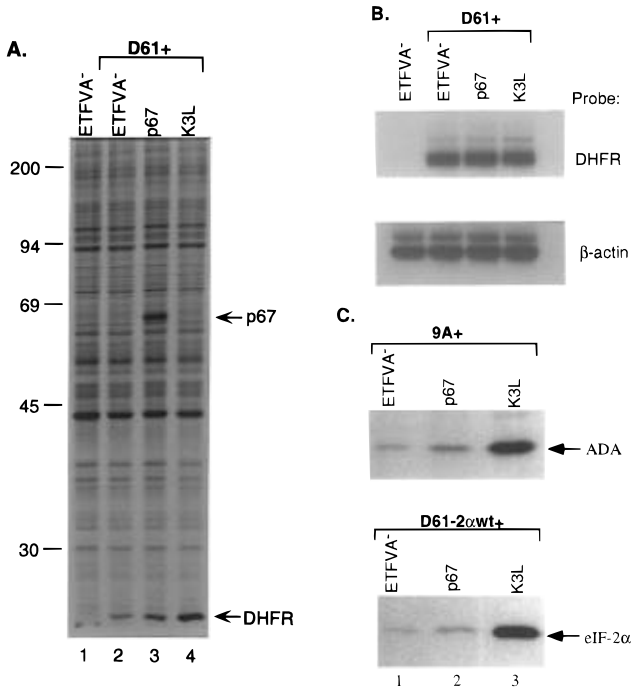

FIGURE 4: p67 overexpression increases translation of a reporter mRNA. (A) COS-1 cells were cotransfected with the DHFR expression vector pD61 in the presence of pETFVA$^-$ (lane 2), pETFVA$^-$-p67 (lane 3), or pETFVA$^-$-K3L (lane 4). Equal amounts (4 μg) of the two plasmid DNAs were used. The transfected cells were labeled and lysed using NP-40 buffer as described in Materials and Methods. DHFR was analyzed by SDS−PAGE and autoradiography (panel A). PhosphorImage scanning showed DHFR synthesis increased 1.5-fold in the presence of pETFVA$^-$-p67 (lane 3) and 3.1-fold in the presence of pETFVA$^-$-K3L (lane 4). K3L migrates at 11 kDa and is not detectable on this gel. (B) Total RNA from cells transfected in parallel was isolated, and the mRNA levels for DHFR and β-actin were analyzed by Northern blot hybridization. (C) COS-1 cells were cotransfected with pETFVA-p67 or pETFVA$^-$-K3L in the presence of p9A (ADA) or pD61-2αwt (eIF-2α). Equal amounts of cell extracts (counts per minute) were quantitatively immunoprecipitated using excess anti-ADA polyclonal antibody or anti-eIF-2α monoclonal antibody to quantitate ADA or eIF-2α synthesis in the presence of pETFVA$^-$-p67 (lane 2) and pETFVA$^-$-K3L (lane 3).

translation occurs only in *cis* with respect to the plasmid-derived mRNA. COS-1 cells were cotransfected with pD61 and pETFVA$^-$ without or with the p67- or K3L-coding sequences. The ability of the cells to express DHFR was then measured. DHFR synthesis was quantitated by [^{35}S]-methionine/cysteine pulse labeling of the cells followed by analysis of the radioactivity in the cell extracts by SDS−PAGE and autoradiography (Figure 4A). DHFR translation in control cells that did not receive pD61 was not detectable (lane 1), whereas in cells cotransfected with the control vector pETFVA$^-$ in the presence of pD61, DHFR synthesis was detectable at a low level (Figure 4A, lane 2). DHFR translation was increased 1.5-fold upon cotransfection with pETFVA$^-$-p67 (lane 3) or 3.1-fold upon cotransfection with pETFVA$^-$-K3L (lane 4).

To demonstrate that the increased DHFR synthesis in cotransfected cells was indeed regulated at the translational level and was not due to variation in the DHFR mRNA level, the DHFR mRNA level in the transfected cells was quantitated by Northern blot analysis (Figure 4B). The DHFR mRNA level was essentially the same when pD61 was cotransfected with the control vector pETFVA$^-$, pETFVA$^-$-p67, or pETFVA$^-$-K3L. To control for RNA loading and transfer, we performed hybridization of the same filters with a β-actin cDNA probe. These results indicate that increased DHFR synthesis in COS-1 cells cotransfected with either pETFVA$^-$-p67 or pETFVA$^-$-K3L was, indeed, due to increased translation of DHFR mRNA.

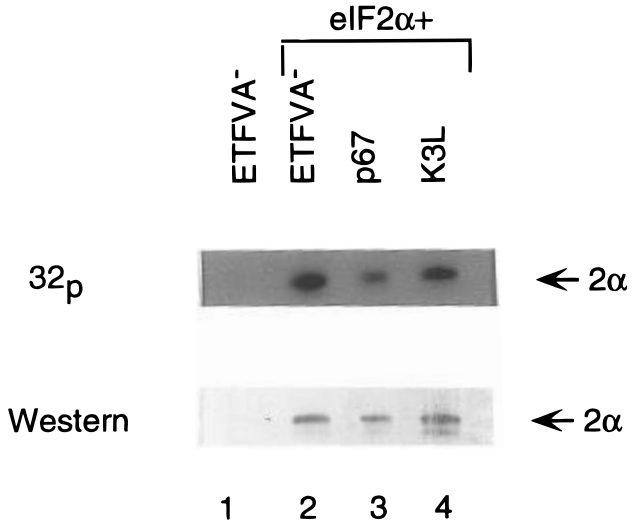

FIGURE 5: p67 overexpression reduces eIF-2α phosphorylation. COS-1 cells were cotransfected with the eIF-2 α-subunit expression vector pD61-2αwt in the presence of pETFVA$^-$ (lane 2), pETFVA$^-$-p67 (lane 3), or pETFVA$^-$-K3L (lane 4). After 42 h, cells were labeled with [^{32}P]phosphoric acid for 4 h and lysed using NP-40 buffer. The labeled eIF-2α was immunoprecipitated using anti-eIF-2α monoclonal antibody. The immunoprecipitates were analyzed by SDS−PAGE and Western blot analysis using anti-eIF-2α monoclonal antibody as indicated. The same membrane was autoradiographed to measure the incorporation of $^{32}PO_4$ into the eIF-2 α-subunit.

In another series of experiments, the effect of p67 overexpression on the translation of two different products, ADA and eIF-2α, was studied. The ADA expression plasmid p9A and the eIF-2α expression plasmid pD61-2αwt were cotransfected with pETFVA$^-$-p67 or pETFVA-K3L. ADA and eIF-2α synthesis in [^{35}S]methionine/cysteine pulse-labeled cotransfected cells were analyzed by immunoprecipitation of cell extracts using ADA antibody or eIF-2α antibody (Figure 4C). ADA synthesis was increased 2.5-fold in cells cotransfected with pETFVA$^-$-p67 (lane 2) and 16-fold in cells cotransfected with pETFVA$^-$-K3L (lane 3). eIF-2α synthesis was increased 2-fold in cells cotransfected with pETFVA$^-$-p67 (lane 2) and 7.6-fold in cells cotransfected with pETFVA$^-$-K3L (lane 3).

Overexpression of p67 Inhibits eIF-2α Phosphorylation. To further delineate the mechanism of enhanced DHFR translation, we studied the phosphorylation status of coexpressed eIF-2 α-subunit. COS-1 cells were cotransfected with the eIF-2 α-subunit expression vector pD61-2αwt with either pETFVA$^-$, pETFVA$^-$-p67, or pETFVA$^-$-K3L. Cells were labeled with $^{32}PO_4$, and the cell extracts were prepared. The phosphorylation state of the overexpressed eIF-2α in the cotransfected cells was measured by immunoprecipitation using eIF-2α antibodies followed by analysis of the immunoprecipitates by SDS−PAGE and autoradiography (Figure 5). The total amounts of eIF-2α polypeptide in different immunoprecipitates were measured by subsequent immunoblotting using eIF-2α monoclonal antibody (Figure 5). The intensities of the phosphorylated eIF-2α bands and the eIF-2α immunoblots were scanned to determine the ratios of ^{32}P incorporation to the amount of eIF-2α protein. Averaging results from two independent transfection experiments

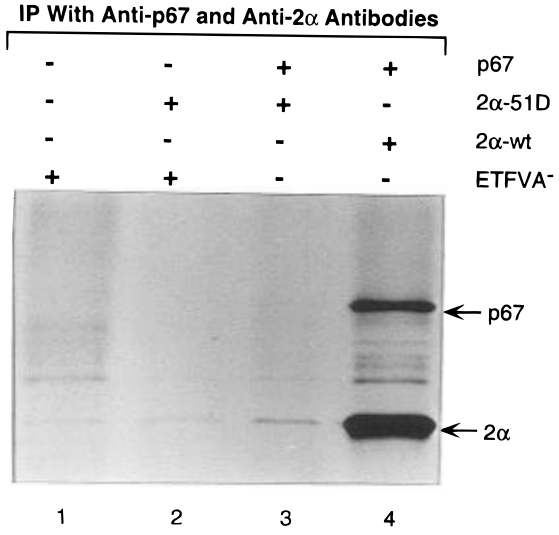

FIGURE 6: p67 synthesis cannot bypass translational inhibitory effects of eIF-2α51D mutant expression. COS-1 cells were transfected with the indicated plasmid DNAs. The expressed eIF-2α and p67 were immunoprecipitated using anti-eIF-2α and anti-p67 monoclonal antibodies. The immunoprecipitates were analyzed by SDS−PAGE and autoradiography.

demonstrated that eIF-2α phosphorylation was reduced to 66.5 ± 11.5 and to 48.5 ± 12.5% in cells cotransfected with pETFVA⁻-p67, and pETFVA⁻-K3L, respectively. These results show that p67 overexpression can inhibit eIF-2 α-subunit phosphorylation in intact cells.

p67 Does Not Bypass Translational Inhibition Mediated by eIF-2 α*-Subunit Phosphorylation.* We have asked whether p67 can be synthesized in the presence of a phosphorylated eIF-2α by measuring synthesis of p67 in the presence of an eIF-2α Ser51Asp mutant. Mutation of serine 51 to aspartic acid in eIF-2 results in an eIF-2 molecule that constitutively inhibits translation since the aspartic acid mimics a phosphoserine (Kaufman et al., 1989; Choi et al., 1992). COS-1 cells were cotransfected with the p67 expression vector in the presence of either a wild type or Ser51Asp mutant eIF-2α expression vector. Whereas translation of p67 and eIF-2 were efficient in the presence of wild type eIF-2α cotransfection (Figure 6, lane 4), cotransfection of pETFVA⁻-p67 with pETFVA⁻-2α51D inhibited translation of p67 (Figure 6, lane 2) as well as eIF-2α51D (Figure 6, lane 3). These results indicate that the synthesis of p67 cannot bypass the translational inhibition imposed by eIF-2α51D.

DISCUSSION

The COS-1 cell system has been conveniently used to analyze eIF-2 kinase inhibitors, such as K3L and E3L (Kaufman et al., 1989; Davies et al., 1993), by two different assay methods: (a) restoration of translation of plasmid-derived mRNAs and (b) inhibition of PKR-catalyzed phosphorylation of a coexpressed eIF-2 α-subunit. In the present study, we have used the same experimental procedures to analyze a cellular eIF-2 kinase inhibitor, p67. p67 has been previously reported to protect the eIF-2 α-subunit from phosphorylation by eIF-2 kinases *in vitro* (Datta et al., 1988, 1989; Wu et al., 1993; Ray et al., 1992). This protein can also reverse protein synthesis inhibition in heme-deficient reticulocyte lysate, i.e., in the presence of active eIF-2 kinases. We now provide evidence that overexpression of p67 in COS-1 cells partially reverses translational inhibition of reporter mRNAs and also inhibits eIF-2 α-subunit phosphorylation. In the present study, we have used as a positive control a vaccinia virus gene-coded PKR inhibitor, K3L protein. Our results show that both p67 and K3L inhibited eIF-2 α-subunit phosphorylation in intact cells to similar degrees (Figure 6). Although p67 was significantly less effective than K3L in reversal of PKR inhibition of protein synthesis, p67 stimulation of protein synthesis was consistently reproducible in several experiments. In addition, p67 increased translation of several reporter mRNAs, including DHFR, ADA, and eIF-2α. The disproportionate decrease in eIF-2 phosphorylation compared to reversal of protein synthesis might reflect that we have measured the phosphorylation status of the free eIF-2α subunit and not that of eIF-2α associated with trimeric eIF-2. However, previous studies have indicated that this measure correlates well with the phosphorylation status of eIF-2α in heterotrimeric eIF-2 (Choi et al., 1993).

Our results indicate that, although p67 can inhibit PKR-catalyzed eIF-2 α-subunit phosphorylation in a manner similar to that of K3L *in vivo*, it is less effective than K3L in reversal of PKR inhibition of protein synthesis. The weaker ability of p67 to stimulate translation compared to K3L may be explained by recent observations (Gupta et al., 1995). These authors reported that p67 is necessary in trace amounts for protein synthesis. It was proposed that high levels of p67 saturated the p67 requirement and that other factors became limiting for protein synthesis. Expression of an antisense-p67 DNA almost completely eliminated endogenous p67 mRNA and inhibited low levels of p67 synthesis. This led to an inhibition of protein synthesis. These results indicate that, although p67 is necessary for protein synthesis, this protein may be only necessary in trace amounts and that overexpression of this protein does not similarly enhance protein synthesis in the cells.

We have shown that p67 is present in significant amounts in wild type COS-1 cells and transfection of p67 increased p67 synthesis by 4−5-fold (Figure 2). Increased p67 synthesis in the transfected cells led to significant inhibition of PKR-catalyzed eIF-2 α-subunit phosphorylation. However, this increase in p67 level stimulated the translation of reporter mRNAs by 1.5−2.0-fold. As discussed above, this marginal stimulation of protein synthesis was reproducible. Although the reduced eIF-2α phosphorylation was observed upon supraphysiological levels of p67 expression, these results do provide evidence that the cellular protein p67 can be an inhibitor of eIF-2α phosphorylation under these conditions *in vivo*.

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