Phosphorylation of Serine 51 in Initiation Factor 2α (eIF2α) Promotes Complex Formation between eIF2α(P) and eIF2B and Causes Inhibition in the Guanine Nucleotide Exchange Activity of eIF2B†

Akulapalli Sudhakar,‡ Aruna Ramachandran,§,|| Sudip Ghosh,§,|| Seyed E. Hasnain,§,|| Randal J. Kaufman,⊥ and Kolluru V. A. Ramaiah*,‡

Department of Biochemistry, University of Hyderabad, Hyderabad 500 046, Andhra Pradesh, India, National Institute of Immunology, Aruna Asaf Ali Marg, New-Delhi 100 067, India, Laboratory of Molecular and Cellular Biology, CDFD, Nacharam, Hyderabad 500 076, India, Department of Biological Chemistry, Howard Hughes Medical Institute, University of Michigan Medical Centre, Ann Arbor, Michigan 48109

Received April 17, 2000; Revised Manuscript Received August 15, 2000

ABSTRACT: Phosphorylation of serine 51 residue on the α-subunit of eukaryotic initiation factor 2 (eIF2α) inhibits the guanine nucleotide exchange (GNE) activity of eIF2B, presumably, by forming a tight complex with eIF2B. Inhibition of the GNE activity of eIF2B leads to impairment in eIF2 recycling and protein synthesis. We have partially purified the wild-type (wt) and mutants of eIF2α in which the serine 51 residue was replaced with alanine (51A mutant) or aspartic acid (51D mutant) in the baculovirus system. Analysis of these mutants has provided novel insight into the role of 51 serine in the interaction between eIF2 and eIF2B. Neither mutant was phosphorylated in vitro. Both mutants decreased eIF2α phosphorylation occurring in hemin and poly(IC)-treated reticulocyte lysates due to the activation of double-stranded RNA-dependent protein kinase (PKR). However, addition of 51D, but not 51A mutant eIF2α protein promoted inhibition of the GNE activity of eIF2B in hemin-supplemented rabbit reticulocyte lysates in which relatively little or no endogenous eIF2α phosphorylation occurred. The 51D mutant enhanced the inhibition in GNE activity of eIF2B that occurred in hemin and poly(IC)-treated reticulocyte lysates where PKR is active. Our results show that the increased interaction between eIF2 and eIF2B protein, occurring in reticulocyte lysates due to increased eIF2α phosphorylation, is decreased significantly by the addition of mutant 51A protein but not 51D. Consistent with the idea that mutant 51D protein behaves like a phosphorylated eIF2α, addition of this partially purified recombinant subunit, but not 51A or wt eIF2α, increases the interaction between eIF2 and 2B proteins in actively translating hemin-supplemented lysates. These findings support the idea that phosphorylation of the serine 51 residue in eIF2α promotes complex formation between eIF2α(P) and eIF2B and thereby inhibits the GNE activity of eIF2B.

Eukaryotic initiation factor 2 (eIF2)[1] is a three-subunit protein composed of α-, *β*-, and *γ*-subunits with different molecular masses and is required in the first step of initiation of protein synthesis (reviewed in refs *1*−*4*). eIF2 promotes the joining of initiator tRNA, Met-tRNAi, to 40S ribosomal subunit to form the 43S preinitiation complex. Upon joining to the 60S ribosomal subunit, GTP is hydrolyzed and eIF2 is released as eIF2·GDP. Prior to joining Met-tRNAi for another round of initiation, the GDP in eIF2 must be exchanged for GTP. This guanine nuucleotide exchange (GNE) reaction is catalyzed by eIF2B, an important rate-limiting heteropentameric protein involved in polypeptide chain initiation (reviewed in refs *5* and *6*).

Phosphorylation of the α-subunit in eIF2 occurs in cells or in cell-free translational systems in response to various stimuli. These are heme-deficiency (*7*−*10*), viral infection, or low levels of double-stranded RNA (*9*, *11*), amino acid and nutrient starvation (*12*−*15*), purine limitation (*16*), serum and growth factor deprivation (*17*−*19*), transient transfection of certain plasmids (*20*), cerebral ischemia (*21*), exercise (*22*), heat shock (*23*, *24*), heavy metals (*25*−*27*), and release of calcium from the endoplasmic reticulum (ER) or ER-stress (*28*−*32*). In addition, oxidizing agents such as oxidized glutathione (*33*, *34*), pyrroloquinoline quinone (*35*), sodium arsenite (*32*), denatured proteins (*36*), nitric oxide (*37*) etc., also are found to stimulate eIF2α phosphorylation. Phosphorylation is correlated with a global inhibition of protein

† This work was supported by a grant (Bt/R&D/15/17/95) from the Department of Biotechnology, New-Delhi, India, to K.V.A.R. Mr. A.S. was supported by a fellowship from C.S.I.R, New-Delhi. S.E.H. lab at CDFD and NII was supported by core grant from the DBT.

* To whom correspondence should be addressed. Fax: 011-91-40-3010145/3010120. E-mail: kvarsl@uohyd.ernet.in.

‡ University of Hyderabad.

§ National Institute of Immunology.

|| Laboratory of Molecular and Cellular Biology.

⊥ University of Michigan Medical Centre.

[1] Abbreviations: eIF2α, the α-subunit (38 kDa) of eukaryotic translational initiation factor 2; eIF2α(P), phosphorylated eIF2α; eIF2B*ϵ*, *ϵ* subunit of eIF2B; dsRNA, double-stranded RNA; HRI, heme-regulated inhibitor; PKR, double-stranded RNA dependent protein kinase; Met-tRNAi, initiator methionyl tRNA; AcNPV, *Autographa californica nuclear polyhedrosis virus*; SDS−PAGE, sodium dodecyl sulfate-polyacralamide gel electrophoresis; wt, wild-type; Sf9, *Spodoptera frugiperda;* PI, postinfection.

10.1021/bi0008682 CCC: $19.00
Published on Web 09/27/2000

synthesis in cell-free translational systems obtained from reticulocyte lysates, or, selective stimulation of certain mRNAs over the others as in the case of yeast subjected to amino acid starvation (reviewed in refs *38*−*40*). Recent studies suggest that eIF2α phosphorylation plays an important role in growth and development and in apoptosis (*41*−*45*). Phosphorylation of eIF2α is now clearly recognized as a major mechanism in the regulation of initiation step of eukaryotic protein synthesis.

Several specific eIF2α kinases have been identified and cloned to date (reviewed in refs *46*−*48*). These include heme-regulated kinase (HRI) (reviewed in ref *49*), double-stranded RNA-induced protein kinase (PKR) (reviewed in ref *50*), GCN2 kinase from yeast (reviewed in ref *51*), *Drosophila* and mouse (*52*, *53*), PfPK4 from malarial parasite (*54)*, pancreatic eIF2α kinase, and PEK (*55*, *56*) also called PERK, an ER-resident kinase (*57*). All eIF2α kinases share sequence and structural similarities distinguishable from those of other serine-threonine kinases, phosphorylate serine 51 residue in eIF2α and are regulated by different stress signals.

Phosphorylation of eIF2α inhibits the GNE activity eIF2B in vitro (*58*) and in translating extracts (*59*−*61*). Phosphorylation of a small portion (20−30%) of total eIF2α can efficiently sequester the less abundant eIF2B and form a 15S complex, eIF2α(P)·eIF2B in which eIF2B becomes nonfunctional (*62*). This event leads to an impairment in the recycling of eIF2 (*62*, *63*) and is the cause for inhibition of protein synthesis. Expression of mutants of eIF2α, in which the putative phosphorylation sites, serine residues at 48 and 51, were changed to alanine, mitigates the inhibition of protein synthesis (*64*, *65*) and the reduction in GNE activity of eIF2B (*66*) caused by eIF2α phosphorylation in cultured mammalian cells. While 51A mutant was not phosphorylated, 48A mutant was used as a substrate. In contrast, expression of a serine 51 to aspartic acid mutant (51D) inhibited protein synthesis. The mutants of eIF2α were useful in resolving the phosphorylation sites in mammalian eIF2α (*67*, *64*), in elucidating the protein synthesis defects caused due to eIF2α phosphorylation in such cases as heat shock and calcium sequestration (*65*, *68*), in determining the importance of eIF2α phosphorylation in growth and development and in apoptosis (*41*, *44*) and also in expressing eIF2α kinases that are inhibitory for protein synthesis (*69*).

In a recent study, we have shown that the serine 48 residue, which is not phosphorylated, is however required for the formation of a complex between eIF2α(P) and eIF2B when the serine residue in 51 is phosphorylated (*70*). To further understand the importance of serine 51 phosphorylation in the formation of eIF2α(P)·eIF2B complex and intersubunit protein interactions, we studied the phosphorylation of eIF2α, GNE activity of eIF2B, and formation of eIF2α(P)·eIF2B complex in hemin or hemin and poly(IC)-treated reticulocyte lysates in the presence of partially purified recombinant human eIF2α wt, mutants of 48A, 51A, and 51D proteins. Our findings support the hypothesis that phosphorylation of serine 51 in wt or native eIF2α promotes complex formation between eIF2 and eIF2B, and thereby impairs the GNE activity of eIF2B.

MATERIALS AND METHODS

Materials. The pETFVA$^-$ vector harboring wild-type or mutants of human eIF2α was described previously (*71*). Baculovirus transfer vector pBakPAK8 and pBakPAK6 (*Bsu* 36I digest), the transfecting agent lipofectin were obtained from Clonetech. Random primer labeling kit, hybond N$^+$ membrane, [α-^{32}P]dCTP, were purchased from Amersham. Poly(IC), a synthetic double-stranded RNA, was obtained from Calbiochem. Restriction enzymes, anti-mouse IgG raised as a rabbit-alkaline phosphatase conjugate, and Western developing agents NBT and BCIP were obtained from Promega Corp. A polyclonal phosphospecific anti-eIF2α antibody was purchased from Research Genetics. Monoclonal antibodies for eIF2α, eIF2Bϵ, and HRI proteins produced respectively in Drs. E. C. Henshaw, C. Proud, and I. M. London's laboratories were kindly provided by Dr. J.-J. Chen (**M**assachusetts Institute of Technology, Cambridge, MA). GDP, CPK, DTT, and protease solutions were purchased from Boehringer and Mannheim, GmbH. Fetal calf serum and antibiotics were from GIBCO BRL. Insect cell culture medium (TNM-FH) and various other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). [8-^{3}H]-GDP (2 mM, 9 Ci/mmol) was obtained from Dupont, NEN, while [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from BRIT (Mumbai, India).

Preparation and Identification of the Recombinant Baculovirus Transfer Vector. Amplification of parent vector harboring wt or 51A or 51D in DH5α cells, isolation of 1.6 kb eIF2α insert from the vector DNA, cloning of the same into pBacPAK8 transfer vector, and amplification of transfer vector carrying the insert in the right orientation were carried out as described earlier (*70*). The *Spodoptera frugiperda* (Sf9) cell line was maintained in complete TNM-FH medium containing 10% fetal calf serum and anti-mycotic and antibiotic solutions as described previously (*69*, *72*). Recombinant baculoviruses were generated in vitro by transfecting *Bsu*36I-digested AcNPV virus DNA (Clonetech) into Sf9 cells as described previously (*73*).

Plaque assays were carried out to obtain recombinant viruses from a single clone, and the positive plaques were identified by dot blot hybridization using [α-^{32}P]dCTP-labeled cDNA. Amplification of recombinant viruses were carried out to increase the titer of the recombinant virus in a stepwise manner.

Determination of eIF2α Expression. Expression of recombinant eIF2α in the Sf9 cell extracts transfected with the recombinant viruses was checked using a monoclonal anti-eIF2α antibody as described earlier (*70*). The secondary antibody used here was alkaline phosphatase conjugated anti-mouse IgG.

Preparation of Reticulocyte Lysates, eIF2 Purification, and GNE Activity of eIF2B. Heme-deficient reticulocyte lysates which respond to added hemin were prepared from anemic male New Zealand rabbits, and the lysates were used as a source for measuring the GNE activity of eIF2B and also for the purification of rabbit reticulocyte eIF2 as described (*61*, *66*).The GNE activity of eIF2B in hemin or heme and poly(IC)-treated reticulocyte lysates in the presence and absence of Sf9 cell extracts expressing the wt or mutants of eIF2α was measured by monitoring the dissociation of the preformed labeled reticulocyte eIF2-[^{3}H]GDP binary complex, as described earlier (*61*, *66*, *70*). Further the recombinant eIF2α expressed in Sf9 cell extracts was partially purified using S-300 (Pharmacia) gel filtration column and DEAE-ion exchange chromatography. In the DEAE chro-

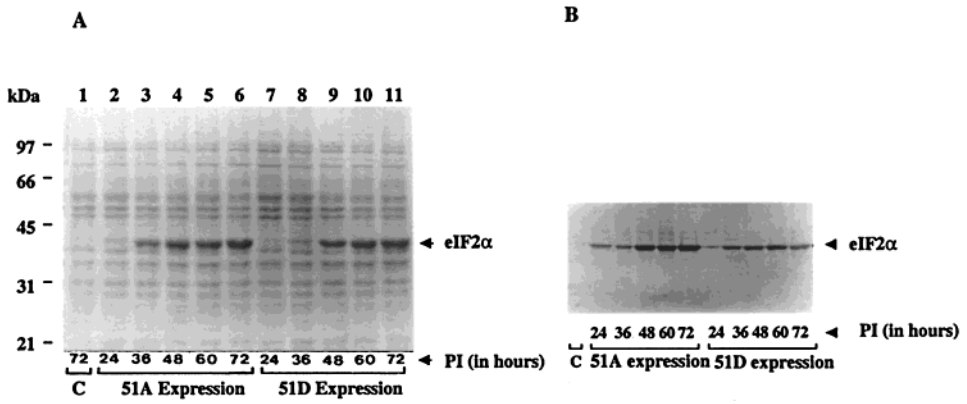

FIGURE 1: Expression and immuno reactivity of recombinant human eIF2α 51A and 51D in Sf9 insect cells using baculovirus. Kinetics of eIF2α 51A and 51D protein expression at different time points, 24−72 h post infection (PI), can be seen in lanes 2−6, and 7−11 in panel A, respectively. Uninfected control Sf9 cell extracts were prepared and loaded in lane 1 (C). Each lane contains approximately 30 μg of extract protein and the figure is a coomassie stained 10% SDS−Polyacrylamide gel. (B) Western immunoblot analysis of recombinant eIF2α 51A and 51D. The recombinant proteins expressed in Sf9 insect cells were separated by 10% SDS−PAGE as shown in panel A and transferred to a nitrocellulose membrane. Membrane was then probed with an eIF2α monoclonal antibody. The immunoreactivity of the recombinant eIF2α was detected with the help of rabbit anti-mouse alkaline phosphatase conjugated secondary antibody (Promega). Lanes 1−11 represent as in panel A.

matography, the recombinant protein is eluted out using 0.25 M KCl.

Phosphorylation of Recombinant eIF2α in Vitro, in Poly-(IC)-Treated Reticulocyte Lysates and Insect Cell Extracts. Phosphorylation of partially purified recombinant eIF2α wt, 48A, 51A, 51D mutants was carried out by purified heme-regulated kinase (HRI) and [γ-^{32}P]ATP in the presence and absence of purified rabbit trimeric eIF2 as described earlier (*35*, *70*). Phosphorylated extracts were then resolved by 10% SDS−PAGE and analyzed by autoradiography. Phosphorylation of baculovirus-expressed recombinant eIF2α in heme and poly(IC)-treated reticulocyte lysates and also uninfected and virus-infected Sf9 cellular eIF2α in the presence and absence of heme-regulated eIF2α kinase was carried out as described earlier (*61*, *70*) but with out the addition of labeled ATP. Phosphorylation of eIF2α in the reaction mixtures were analyzed by Western immunoblot analysis using a polyclonal eIF2α antibody that recognizes the phosphorylated form of the protein. The levels of eIF2α and HRI kinase proteins present in the reaction mixtures were analyzed by immunoblot analysis with the help of their respective monoclonal antibodies using alkaline phosphatase conjugated secondary anti-mouse IgG as decribed previously (*69*, *74*).

Analysis of eIF2α(P)·eIF2B Complex Formation in Translating Reticulocyte Lysates. Protein synthesis reactions (100 μL) were carried out at 30 °C for 15 min in 20 μM heme or heme and poly(IC)-treated (20 μM and 300 ng/mL) reticulocyte lysates in the presence of partially purified recombinant baculovirus-expressed eIF2α wt, 48A, 51A, and 51D proteins (∼6 μg). At the end of the protein synthesis reaction, the lysates were diluted with equal volume of chilled TKM buffer consisting of 20 mM Tris-HCl, pH 7.6, 100 mM KCl, and 2 mM $Mg(OAc)_2$ to terminate the reaction. Samples were layered on 4.5 mL exponential sucrose gradients (10 to 30%) which were prepared with the above dilution buffer. Samples were run at 40 000 rpm for 6 h at 4 °C in a SW 50.1 rotor to separate free eIF2 from [eIF2α(P)·eIF2B] complex as described previously (*22*). Fractions (400 μL) were collected by upward displacement of the gradients with the help of an ISCO gradient fractionator. Fractions were concentrated by pH 5.0 precipitation in the presence of 50 mM NaF and 5 mM EDTA to prevent the dephosphorylation of eIF2α. Samples were suspended in sample buffer, briefly boiled, and separated by 10% SDS−PAGE. Proteins were transferred to nitrocellulose membranes. and eIF2α and eIF2B of the various gradient fractions were detected by using an anti-eIF2α and eIF2Bε monoclonal antibodies as described earlier (*70*, *75*).

RESULTS

Expression, Immunoreactivity, and Partial Purification of Recombinant 51A and 51D Mutants of eIF2α. Extracts of Sf9 cells transfected with recombinant AcNPV harboring 51A and 51D mutants of human eIF2α were prepared at different time points postinfection (24, 36, 48, 60, and 72 h) and were analyzed by 10% SDS−polyacrylamide gel (Figure 1A) to monitor the expression of eIF2α. A single protein with a molecular mass of 38 kDa was detected that increased with time up to 72 h. Uninfected or AcNPV-infected cells do not produce a protein of similar molecular mass in large amounts where expression is increased with time postinfection. Expressed recombinant protein crossreacts with a monoclonal anti-eIF2α antibody in the Western immunoblot analysis, and the intensity of the eIF2α signal is related to the level of protein expression which increases with time postinfection (Figure 1B). Although moderate levels of 51D protein is produced in Sf9 cells, it is noted, however, that 51D expression is slightly lower than 51A protein (Figure 1). The expression level is dramatically increased over previous methods (*20*, *44*). The recombinant eIF2α wt, 48A, 51A, and 51D proteins expressed by Sf9 cells were partially purified using a gel filtration and DEAE 52 ion-exchange columns (Figure 2). The protein was eluted in the 0.2−0.25 M KCl fraction of DEAE cellulose column.

Phosphorylation of Baculovirus-Expressed Recombinant eIF2α Protein. Earlier studies demonstrated that recombinant wt and 48A mutant eIF2α proteins expressed in Sf9 cells using baculovirus (*70*) and in mammalian cells (*64*, *66*) can serve as substrates for eIF2α kinases such as HRI and PKR, whereas, the mutants of 51A and 51D expressed in mammalian cells are not phosphorylated. We have expressed, for

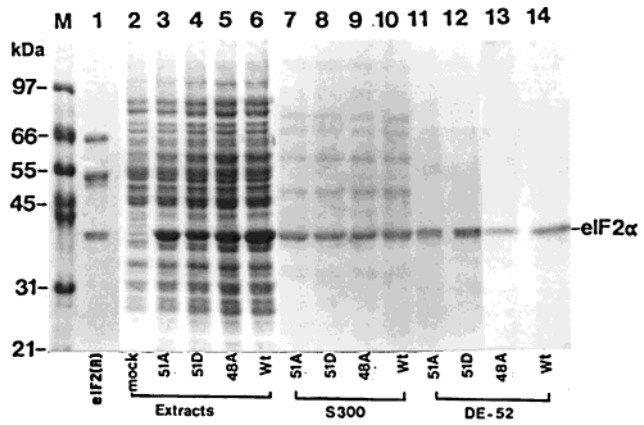

FIGURE 2: Partial purification of recombinant eIF2α. Extracts of Sf9 cells expressing wt, 48A, 51A, and 51D eIF2α mutant proteins were prepared and passed through Sephacryl S-300 (Pharmacia) and DEAE Cellulose-52 columns as described in Materials and Methods. The figure is a coomassie stained 10% SDS−Polyacrylamide gel. Lane 1 contains purified reticulocyte eIF2[eIF2(R)]. Lanes 2−6 contain extracts of Sf9 cells as indicated. Lanes 7−10 contain proteins of S-300 fractions of the cell extracts expressing 51A, 51D, 48A, and wt eIF2α, respectively. Lanes 11−14 contain proteins of S-300 fraction that was eluted from DEAE-52 with 0.25 M KCl.

the first time, 51A and 51D mutants of eIF2α in Sf9 cells using baculovirus. The phosphorylation of partially purified 51A and 51D mutants of recombinant eIF2α was studied by addition of purified reticulocyte heme-regulated eIF2α kinase (HRI) and labeled ATP (Figure. 3A). The 51D and 51A mutants (Figure 3A, lanes 1 and 2) of eIF2α were not phosphorylated, whereas the wt and 48A mutants (lanes 3 and 4) were. The phosphorylation of trimeric purified eIF2 by HRI occurred more efficiently (Figure 3, lane 9) than the free eIF2α mutant 51A and 51D subunits (lanes 1 and 2). The phosphorylation of trimeric eIF2 was reduced in the presence of the recombinant 51D, 51A, wt, or 48A mutant eIF2α (lanes 5, 6, 7, and 8, respectively) as previously described for the 48A mutant and wild-type subunits (*70*). The differences observed in the phosphorylation could not be explained by the levels of HRI and eIF2 present in the reactions as analyzed by their respective monoclonal antibodies (Figure 3B). In addition, the phosphorylation of HRI kinase that occurred in the presence of trimeric eIF2 (lane 9, Figure 3A) was decreased in the presence of partially purified recombinant eIF2α protein (lanes 1−8) and was not proportional to the actual levels of HRI present as analyzed by western blot analysis in the reactions (lanes 1−8, Figure 3B).

The phosphorylation of reticulocyte eIF2α in heme-deficient, hemin, or hemin and poly(IC)-treated reticulocyte lysates was also evaluated in the presence and absence of partially purified recombinant eIF2α by immunoblot analysis using a phosphopeptide-specific anti-eIF2α antibody [Figure 4, eIF2α(P)] and the levels of eIF2α and HRI in the reactions were analyzed by their respective monoclonal antibodies (data not shown). Results demonstrate that eIF2α phosphorylation [eIF2α(P)] decreased substantially in the presence of hemin as compared with the absence of hemin or heme-deficiency (Figure 4, compare lane 2 vs lane 1). In the absence of hemin, HRI kinase is activated and phosphorylates eIF2α. Similarly, addition of poly(IC) to hemin-supplemented lysates activates yet another eIF2α kinase called PKR and increased eIF2α phosphorylation (lane 3). Addition of recombinant eIF2α wt and 48A to hemin and poly(IC)-treated lysates decreased the phosphorylation of lysate eIF2α to some extent (lanes 4 and 5 vs lane 3), but addition of recombinant mutant proteins of eIF2α 51A and 51D decreased the phosphorylation of reticulocyte lysate eIF2α much more significantly (lanes 6 and 7 vs lane, 3). The results on the kinetics of lysate eIF2α phosphorylation in heme and poly(IC)-treated lysates in the presence and absence of partially purified wt, and mutants of eIF2α proteins are shown in Figure 4B. In this batch of lysates, a higher basal level of eIF2α phosphorylation is observed in the presence of hemin (lane 1, Figure 4B) compared to earlier Figure 4A. Addition of poly(IC) increases the phosphorylation of lysate eIF2α with time (lanes 2, 3, and 4) as expected. Addition of the recombinant subunit of eIF2α wt or mutants to poly(IC)-treated lysates, however, decreases significantly the phosphorylation of lysate eIF2α (lanes 5−16 vs lanes 2−4). In reactions where the nonphosphorylatable eIF2α 51A or 51D is supplemented, a time-dependent increase in phosphorylation of lysate eIF2α is also evident. This is possible if the exchange of recombinant human eIF2α 51A or 51D into the trimeric endogenous lysate eIF2 is not complete as has been shown earlier (*64*). Further, the phosphorylation of lysate eIF2α is not different in the presence of partially purified recombinant wt and 48A mutant eIF2α (Figure 4B, lanes 5−7, and 8−10, respectively). Similarly, the phosphorylation of reticulocyte lysate eIF2α in the presence of 51A and 51D mutants appears to be similar (lanes 11−13 and 14−16, respectively). These differences in phosphorylation cannot be explained by the levels of HRI and eIF2α present in the reaction mixtures by two methods of analysis (see Discussion).

Phosphorylation of Sf9 Cell eIF2α and Purified Trimeric Reticulocyte eIF2α by Mock and Wild-Type AcNPV-Infected Extracts. Earlier observations suggested that AcNPV-infection leads to the synthesis of PK2 protein, a truncated protein which is homologous to eIF2α kinases and blocks PKR kinase autophosphorylation and thereby eIF2α phosphorylation (*76*). It was suggested that the presence of PK2-like protein enables the virus to withstand activated eIF2α kinase-(s) like GCN2 probably present in insect cells (*52*). Since it is observed here that purified reticulocyte eIF2α phosphorylation decreased in the presence of partially purified recombinant eIF2α preparations, we have analyzed eIF2α phosphorylation in the mock and AcNPV-infected Sf9 cell extracts and the ability of these extracts to phosphorylate purified reticulocyte eIF2α (Figure 5). The phosphorylation status of both the cellular and added eIF2α were assessed by a phosphopeptide-specific anti-eIF2α antibody as above. Results indicate that the phosphospecific polyclonal anti-eIF2α antibody recognized the phosphorylated eIF2α of the reticulocytes and Sf9 cells (Figure 5A) whereas the eIF2α monoclonal antibody used here recognizes only mammalian eIF2α (Figure 5B). The phosphorylation of insect cell eIF2α was significantly reduced upon AcNPV infection (Figure 5A, lane 1 vs lane 2). Further, added purified reticulocyte eIF2α was efficiently phosphorylated (lanes 3 and 4) in insect cell extracts which were not infected with the virus and in the absence of any added kinase. In contrast, AcNPV-infected cell extracts were unable to efficiently phosphorylate added purified reticulocyte eIF2α (lanes 5 and 6). Purified eIF2α

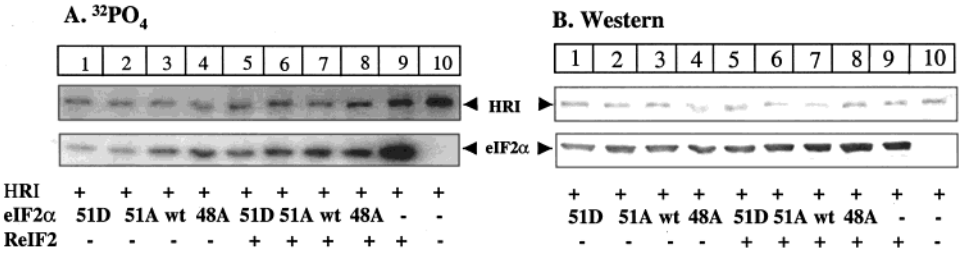

FIGURE 3: Phosphorylation of recombinant and reticulocyte eIF2α in vitro. Phosphorylation of (a) purified reticulocyte eIF2 (lane 9, panel A, ReIF2), (b) recombinant eIF2α 51D, 51A, wt, and 48A (lanes 1−4, panel A), and (c) purified reticulocyte eIF2 in the presence of recombinant eIF2α 51D, 51A, wt, and 48A (lanes 5−8, panel A) was performed in the presence of HRI and [γ-^{32}P]ATP at 30 °C for 10 min as described in Materials and Methods. The reaction mixtures were separated by 10% SDS−PAGE. Lane 10 contains HRI alone. Panel A is an autoradiogram showing the phosphorylation of eIF2α and HRI. Panel B is an immunoblot of panel A indicating eIF2α and HRI protein levels used in the reaction mixtures as judged by their respective monoclonal antibodies.

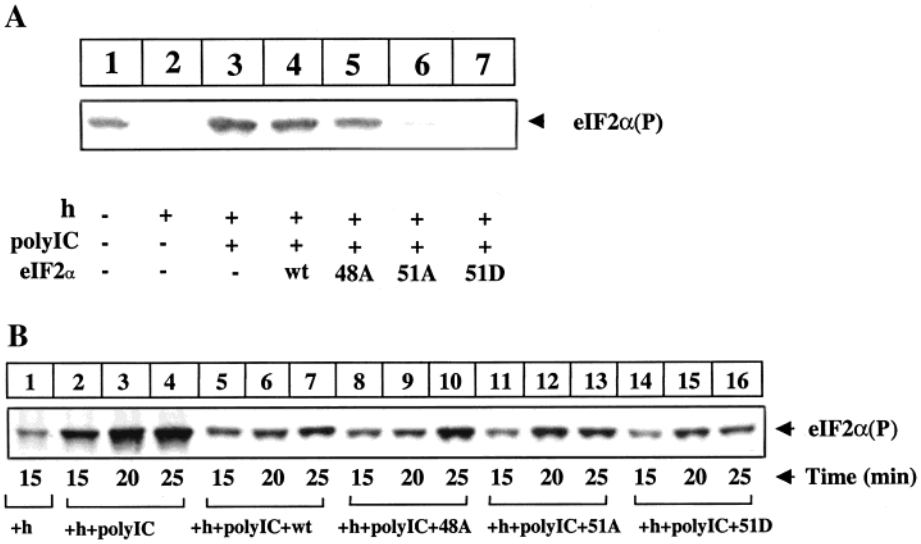

FIGURE 4: Phosphorylation of recombinant eIF2α in hemin and poly(IC)-treated reticulocyte lysates. Partially purified recombinant eIF2α wt, 48A, 51A, and 51D proteins (∼6 μg) were added to hemin and poly(IC)-treated reticulocyte lysates and incubated at 30 °C for 10 min. A total of 10 μL of the respective lysate reaction mixtures was precipitated at pH 5.0 and separated by 10% SDS−PAGE. Phosphorylated eIF2α, eIF2α(P), was analyzed by western immunoblotting using a phophospecific eIF2α antibody. The various lanes in panel are as follows: lane 1, with out hemin; lane 2, with hemin; lane 3, heme and poly(IC); lane 4, heme, poly(IC) and wt eIF2α; lane 5, heme, poly(IC) and 48A eIF2α; lane 6, heme, poly(IC) and 51A eIF2α; lane 7, heme, poly(IC) and 51D eIF2α. Panel B represents kinetics of reticulocyte eIF2α phosphorylation in hemin and poly(IC)-treated lysates in the presence and absence of partially purified recombinant wt, 48A, 51A, and 51D eIF2α. Reaction mixtures were processed and eIF2α phosphorylation of the reactions were analyzed as stated in panel A. Various lanes are as follows: lane 1, heme; lanes 2, 3, and 4, heme and poly(IC)-treated lysates for 15, 20, and 25 min, respectively; lanes 5−16 represent heme and poly(IC)-treated lysates for 15, 20, and 25 min with wt or mutants of 48A, 51A, and 51D eIF2α, respectively.

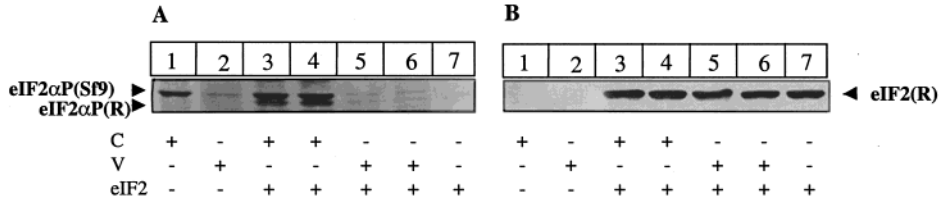

FIGURE 5: Phosphorylation of Sf9 and reticulocyte eIF2 in uninfected and virus-infected extracts without added kinase. (1) Uninfected (C) and AcNPV-virus infected (V) Sf9 cell extracts were prepared as described in Materials and Methods and the proteins were separated by 10% SDS−PAGE to determine the eIF2α phosphorylation of the cell extracts by western analysis (lanes 1 and 2; panel A). The above extracts were also used to phosphorylate purified reticulocyte eIF2 in reaction mixtures containing 20 mM Tris-HCl, 1 mM Mg^{2+}, 1 mM DTT, 80 mM KCl and 100 μM unlabeled ATP at 30 °C for 10 min. The reaction mixtures were then separated by 10% SDS−PAGE and transferred to nitrocellulose membrane to determine phosphorylation of reticulocyte eIF2 [eIF2αP (R)] by western analysis. Lanes 3 and 4 represent duplicate set of reticulocyte eIF2α phosphorylation in uninfected cell extracts and lanes 5 and 6 represent the same in the presence of virus-infected extracts. Lane 7 contains purified reticulocyte eIF2 alone. Panel A represents western analysis of phosphorylated Sf9 [eIF2αP(Sf9)] and added reticulocyte eIF2α [eIF2α P(R)] using a phosphospecific antibody. Panel B represents western analysis of reticulocyte eIF2α [eIF2(R)] levels used in the reaction mixtures as assessed by an eIF2α monoclonal antibody.

is not recognized by the phoshospecific anti-eIF2α antibody (lane 7), suggesting that this purified preparation of trimeric reticulocyte eIF2 is not phosphorylated without the addition of eIF2α kinase and ATP. These results are thus consistent with the idea that the uninfected insect cells contain an active eIF2α kinase that can phosphorylate added eIF2 and produce an eIF2α kinase inhibitor (*76*) upon baculovirus infection.

Since the eIF2α kinase activity of uninfected insect cells was decreased upon viral infection, we also studied the phosphorylation of purified reticulocyte eIF2α in vitro by

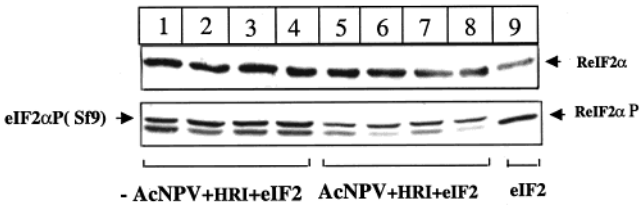

FIGURE 6: Phosphorylation of reticulocyte eIF2α by HRI kinase in the presence of increasing concentrations of uninfected and virus-infected Sf9 extracts. Increasing concentration of uninfected (−AcNPV) and virus-infected (+AcNPV) Sf9 extracts (15−30 μg) were added to the phosphorylation reaction mixtures containing purified reticulocyte eIF2 and HRI kinase. Phosphorylation was carried out with the addition of 20 mM Tris-HCl, pH 7.8, 1 mM Mg^{2+}, DTT at 1 mM, 80 mM KCl, and 100 μM unlabeled ATP at 30 °C for 10 min and the mixtures were separated by 10% SDS−PAGE. Gels were analyzed by western immunoblot analysis. Reticulocyte eIF2α (ReIF2α) levels present in the reaction mixtures were analyzed by western immunoblotting using an eIF2α monoclonal antibody. Phosphorylation of eIF2α of unifected, AcNPV-infected Sf9 cell extracts [eIF2αP(Sf9)] and of the trimeric reticulocyte eIF2 (ReIF2αP) were assessed by using a phosphospecific eIF2α antibody. The various lanes are as follows: lanes 1−4 of the bottom panel represent phosphorylation of reticulocyte and insect cell eIF2α by purified HRI in the presence of increasing concentration (15, 20, 25, and 30 μg protein respectively) of uninfected Sf9 extract and lanes 5−8 represent the same in the presence of AcNPV-infected extracts. Lane 9 represents the phosphorylation of reticulocyte eIF2α alone by purified HRI kinase.

added HRI kinase or in the presence of increasing concentrations of mock and AcNPV-infected cell extracts (Figure 6). Phosphorylation of reticulocyte and insect cell eIF2α was assessed by using a phosphopeptide-specific anti-eIF2α antibody (Figure 6, lower panel). Our results show that the phosphorylation of reticulocyte and insect cell eIF2α was decreased in the presence of increasing concentrations of AcNPV-infected extracts significantly greater than in the presence of uninfected extracts (Figure 6, compare lanes 5−8 vs 1−4). These observations are consistent with our earlier findings (*70*) that virus-infected extracts decrease eIF2α phosphorylation by blocking the autophosphorylation of HRI as measured by ^{32}P labeling.

eIF2·[^{3}H]GDP Dissociation in Hemin or Hemin and Poly(IC)-Treated Reticulocyte Lysates in the Presence of Recombinant eIF2α. Previous studies established that the GNE activity of eIF2B in translating lysates can be measured by the dissociation or exchange of labeled GDP for unlabeled GDP in the preformed eIF2·[^{3}H]GDP binary complex and is decreased under conditions that promote eIF2α phosphorylation (*59*, *61*, *78*). Using the lysate eIF2B activity, it was shown that the inhibition of eIF2B activity that occurred due to eIF2α phosphorylation could be partially reduced by addition of the 48A and 51A mutant eIF2α compared to that with wild-type eIF2α (*66*, *70*). Previously, it has not been possible to study eIF2B activity in the presence of 51D mutant protein because the expression of this protein leads to cell death in mammalian cells (*44*). Although the expression of 51D in insect cells is lower than the 51A protein (Figure 1), sufficient accumulation of 51D could be detected (see also Discussion). Hence, we studied the effects of these partially purified baculovirus-expressed recombinant human eIF2α wt and its mutants on eIF2B activity in reticulocyte lysates treated with hemin alone or hemin and poly(IC) (Figure 7). The dissociation of preformed reticulocyte labeled eIF2·GDP binary complex is high in the presence of hemin-

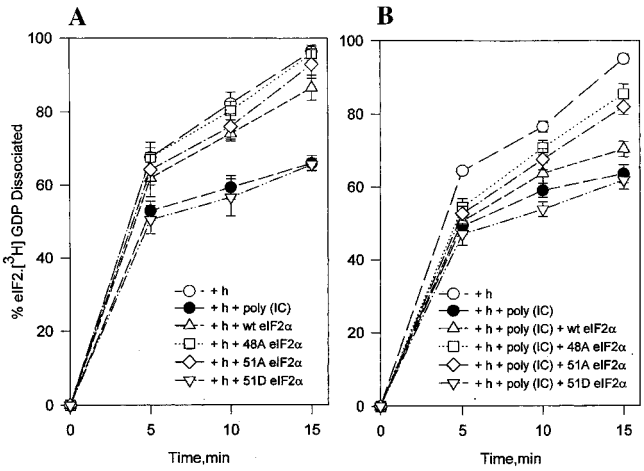

FIGURE 7: Kinetics of eIF2·[^{3}H]GDP dissociation. Dissociation of preformed labeled eIF2·GDP binary complex was studied in hemin (+h) or hemin and poly(IC)-treated reticulocyte lysates in the presence of partially purified recombinant wt and or mutants of eIF2α as indicated in panels A and B, respectively. The experiment was carried out as described earlier (*70*) but in the presence of 6 μg of partially purified recombinant eIF2α protein. A total of 70 μL of preformed eIF2·[^{3}H]GDP binary complex was added to 70 μL translating lysates. The amount of labeled binary complex present in the three independent experiments was 30.8, 29.75, and 28.87 pmols (panel A) and was 28.35, 30.1, and 27.3 pmol (panel B), respectively. At every time point, 40 μL aliquots were taken out and the percent eIF2·[^{3}H]GDP dissociated was measured as described in Materials and Methods. Data are presented as a percentage mean of three independent experiments and standard errors are shown against each value.

supplemented lysates and is low in hemin and poly(IC)-treated lysates (Figure 7) and is therefore related to the extent of lysate eIF2α phosphorylation (Figure 4). Addition of partially purified 51D protein which reduces lysate eIF2α phosphorylation (Figure 4), however, inhibited eIF2B activity of control hemin-supplemented lysates (Figure 7A) severely like the addition of poly(IC) and is consistent with the idea that it behaves like phosphorylated eIF2α (*64*). In contrast, addition of nonphosphorylatable 51A or phosphorylatable wt eIF2α to hemin-supplemented control lysates (Figure 7A) did not inhibit eIF2B activity as significantly observed by the addition of poly(IC) (Figure 7B) or partially purified 51D mutant protein (Figure 7).

The decrease in the GNE activity of eIF2B caused by addition of poly(IC) to hemin-supplemented lysates was mitigated partially in the presence of 48A and 51A proteins compared to wt eIF2α (Figure 7B). Although 48A is phosphorylated on its 51 serine residue like the wt eIF2α, it reduced partially the inhibition in the GNE activity of eIF2B that occurred due to eIF2α phosphorylation, consistent with our earlier studies (*66*, *70*). These studies support that the 48 serine residue is required for high affinity interaction between eIF2α(P) and 2B. Addition of 51D protein, however, enhanced further the inhibition of eIF2B activity in poly(IC)-treated lysates (Figure 7B).

eIF2α(P)·eIF2B Complex Formation. eIF2α(P) can form a complex with eIF2B that can be separated on a sucrose gradient (*62*, *75*, *70*) or using the purified poly-histidine tagged phosphorylated and unphosphorylated eIF2 that binds to Ni-NTA-agarose affinity resin (*78*). The latter study demonstrated that eIF2 can form a stable complex with the overexpressed yeast eIF2B subunits that can be captured

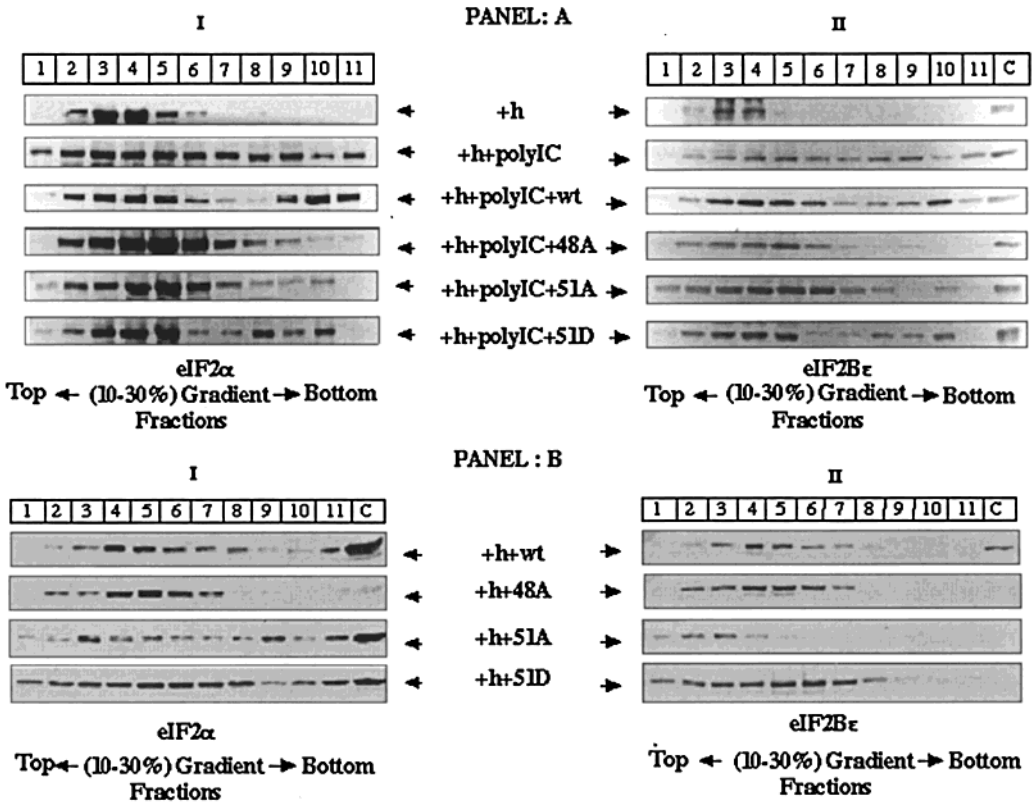

FIGURE 8: eIF2α(P)−eIF2B complex formation in reticulocyte lysates. Complex formation between eIF2 and eIF2B was studied by western analysis of eIF2α and eIF2Bε subunits in the 10−30% sucrose gradient fractions of translating reticulocyte lysates. Protein synthesis reactions of reticulocyte lysates were performed in 100 μL for 15 min at 30 °C in the presence of hemin (20 μM) or hemin and poly(IC) (300 ng/mL) with and with out the addition of recombinant eIF2α wt, 48A, 51A, and 51D proteins (~6 μg) as indicated. The complex, eIF2−eIF2B, was fractionated and fractions were collected using a ISCO-gradient fractionator as described in Materials and Methods. Proteins were separated by 10% SDS−PAGE and analyzed by Western analysis. Panel A represents western analysis of eIF2α (I) and eIF2Bε (II) subunits in the fractions of 10−30% gradients containing reaction mixtures of hemin and poly(IC)-treated reticulocyte lysates in the presence of partially purified recombinant eIF2α wt, 48A, 51A, or 51D proteins as shown in the figure. Panel B represents western analysis of eIF2α (I) and eIF2Bε (II) subunits in the fractions of 10 to 30% gradients containing reaction mixtures of hemin-supplemented reticulocyte lysates treated with the recombinant eIF2α wt, 48A, 51A, or 51D proteins. Purified eIF2 or eIF2B was loaded in lane C, wherever indicated.

using the affinity matrix. It was shown that the binding of all five subunits of eIF2B to eIF2α(P) was about 2-fold higher than to unphosphorylated eIF2. Here, we have analyzed the eIF2α(P)·eIF2B complex formation in hemin or hemin and poly(IC)-treated reticulocyte lysates in the presence and absence of recombinant eIF2α wt, 48A, 51A, and 51D mutants on 10 to 30% gradients (Figure 8) as previously described (*70*). The eIF2 signal in the gradient fractions is detected by using a monoclonal anti-eIF2α antibody that cross-reacts with the α-subunit of eIF2 (Figure 8, part I of panels A and B). Lysate eIF2 migrated mostly in the top of the gradient fractions (Figure 8A, part I, +h), if it was not phosphorylated, as in hemin-supplemented lysates (Figure 4). This is because free eIF2 has a lower molecular mass than eIF2α(P)·eIF2B complex. In addition to analyzing the presence of eIF2, we have also analyzed the gradient fractions for the presence of eIF2B using a monoclonal antibody against eIF2Bε subunit (Figure 8, part II of panels A and B). Our observations suggest that eIF2 (I) and eIF2B (II) are present mainly as a single peak spreading about the top two to six fractions in control hemin-supplemented lysates (panel A, +h). These lysates are translationally active (data not shown), show high eIF2B activity (Figure 7), and display little eIF2α phosphorylation (Figure 4A, lane, 2). In contrast, eIF2 and 2B presence was detected at either the top and or the bottom of the gradient fractions of hemin-supplemented lysates which were treated with poly(IC) [Figure 8A, +h + poly(IC)]. Typically, two peaks of eIF2α are observed in the gradient fractions of lysates that are treated with hemin and poly(IC). The first peak comes between fractions 2 and 6 and the second peak is seen at the bottom between fractions 8 and 11. These lysates showed enhanced eIF2α phosphorylation (Figure 4) and low levels of eIF2B activity (Figure 7).

Addition of 48A or 51A recombinant proteins to hemin and poly(IC)-treated lysates decreased the amount of eIF2α (I) and 2Bε (II) protein present at the bottom of the gradient fractions, the second peak of fractions in the gradient (Figure 8A). Addition of recombinant wt eIF2α, however, to hemin and poly(IC)-treated lysates resolved the bottom and top peaks of eIF2α and 2Bε, thereby, suggesting that complex formation between eIF2α(P) and 2B occurred in the presence of wt eIF2α. The signal intensity of eIF2 and 2B in these fractions was reduced significantly in the presence of 48A and 51A mutants of eIF2α (Figure 8A). The findings suggest that the complex formation between eIF2 and 2B requires the phosphorylation of serine 51 residue and also the presence of adjacent unphosphorylated serine 48 residue. This was further substantiated by using 51D mutant protein, which does not get phosphorylated but mimcs the phosphorylated form with the 48 serine residue being conserved. Addition of this mutant protein to hemin and poly(IC)-treated lysates,

enhanced the eIF2α and eIF2Bϵ signal in the bottom fractions of the gradients such as wt eIF2α [Figure 8A, +h + poly-(IC) + 51D/or wt].

Complex formation was also analyzed in control hemin-supplemented lysates treated with the recombinants of eIF2α. Addition of wt eIF2α, 48A, and 51A produced a different effect compared to 51D mutant protein in the control hemin-supplemented lysates which were not treated with poly(IC) (Figure 8B). In control hemin-supplemented lysates, eIF2α and 2Bϵ sedimented in the gradient as a single peak in the presence of either recombinant wt 48A or 51A eIF2α . While eIF2α was detected in both the top and the bottom of the gradient fractions without significant separation, eIF2Bϵ was found mostly in the top fractions. This may have resulted from the absence of eIF2α phosphorylation in hemin-supplemented lysates that reduces the interaction between eIF2 and 2B (*78*) and/or from the presence of high concentrations of added overexpressed variant subunits of eIF2α in the reactions. In contrast, addition of 51D mutant protein to control hemin-supplemented lysates produced two separate peaks detected by increased intensity of the eIF2α and 2Bϵ signal in the bottom gradient fractions (Figure 8B).

DISCUSSION

Regulation of translation mediated by eIF2α phosphorylation is one of the best characterized control mechanisms operating at the initiation step of protein synthesis (reviewed in refs *2*, *6*, *38*, *39*, and *79*). Several studies suggested that phosphorylation of a small portion of eIF2α can inhibit protein synthesis by inhibiting the GNE activity of limiting amounts of eIF2B (reviewed in refs *5* and *39*). Active eIF2B is required to convert inactive eIF2·GDP binary complex to active eIF2·GTP complex that can interact with Met-tRNAi and 40S ribosomal subunits and to facilitate the recycling of eIF2 (*62*, *63*, *81*). Further, earlier studies suggested that phosphorylated eIF2α sequesters eIF2B into a complex in which eIF2B is nonfunctional (*62*). By use of enzyme kinetic methods, it was demonstrated that eIF2α(P) is not a substrate for eIF2B and that eIF2B has a higher affinity for the inhibitor eIF2α(P) than for the substrate, eIF2 (*80*, *82*). Using an affinity matrix assay, it has been shown recently that binding of all five subunits of yeast eIF2B to yeast poly-histidine-tagged eIF2α(P) is about 2-fold higher than to unphosphorylated eIF2 (*78*). Using mutants of human eIF2α, earlier studies (*66*) hypothesized that phosphorylation of serine 51 promotes the complex formation between eIF2 and 2B.

This hypothesis was tested here by studying complex formation between eIF2α(P) and eIF2B that occurs in inhibited hemin and poly(IC)-treated reticulocyte lysates in the presence of recombinant human eIF2α wt and 48A, 51A, and 51D mutants of eIF2α. The results presented here suggest that eIF2α subunit in the purified trimeric reticulocyte eIF2 complex is phosphorylated more efficiently than the baculovirus-expressed single subunit wt or variant eIF2α forms (Figure 3A). This is probably because the single subunit of eIF2α is a poor substrate for eIF2α kinases as has been suggested previously (*64*). However, addition of partially purified recombinant subunits of wt and or mutants of eIF2α is found to inhibit HRI autokinase activity in vitro and thereby inhibits reticulocyte eIF2α phosphorylation (Figure 3A). The diminution in the autokinase activity of HRI in the presence of baculovirus-expressed wt and mutant forms of eIF2α suggests that these partially purified recombinant preparations carry a contaminant inhibitor of eIF2α kinases. This idea is consistent with the results presented here in Figures 3−6. It is observed here that eIF2α phosphorylation is significantly higher in uninfected Sf9 cells than in virus-infected cells (Figure 5). This is due to a potent eIF2α kinase activity in uninfected Sf9 cells which can also phosphorylate reticulocyte eIF2α in vitro (Figure 5). Virus infection reduces cellular eIF2α phosphorylation (Figure 5), and the virus-infected cell extracts also reduce the phosphorylation of reticulocyte eIF2α that is mediated by HRI kinase in vitro (Figure 6). All these results are consistent with the idea that baculovirus infection produces a truncated eIF2α kinase homologue that inhibits eIF2α kinase activity of Sf9 cells (*76*). Apparently the inhibitor is comigrating with the partially purified baculovirus expressed wt and mutants of eIF2α. This is evident because the eIF2α phosphorylation that occurs in hemin and poly(IC)-treated reticulocyte lysates due to the activation of PKR is reduced in the presence of baculovirus expressed eIF2α wt and or mutants (Figure 4B).

However, reticulocyte eIF2α phosphorylation in these above lysates is found similar in the presence of wt and 48A mutant eIF2α and is significantly higher than in the presence of nonphosphorylatable 51A and 51D mutants of eIF2α. The decrease in reticulocyte eIF2 phosphorylation in the presence of 51A and 51D eIF2α subunit is due to formation of a mutant reticulocyte eIF2 complex, which is generated by an exchange mechanism that replaces the reticulocyte eIF2α subunit with the recombinant variant form as has been suggested previously (*64*). With increasing time, however, a significant increase in lysate eIF2 phosphorylation is evident even in the presence of 51A and 51D mutants of eIF2α (Figure 4B). This is very likely due to the incomplete exchange of recombinant variant form of eIF2 into the reticulocyte endogenous eIF2 complex.

Further, the results presented here suggest that phosphorylation of the 48A mutant eIF2α is comparable to the wt form but the former reduces significantly the inhibition in the GNE activity of eIF2B (Figure 7B) and the complex formation between eIF2α(P) and eIF2B that occurs in hemin and poly(IC)-treated reticulocyte lysates (Figure 8A). These findings suggest that the serine 48 residue is required for high affinity interaction between eIF2α(P)·eIF2B. In contrast, the nonphosphorylatable 51D mutant but not 51A and wt, inhibited the GNE activity of eIF2B (Figure 7A) and enhanced significantly the complex formation between eIF2 and eIF2B in uninhibited or hemin-supplemented control reticulocyte lysates (Figure 8B). While 51A mutant reduced significantly the complex formation between eIF2α(P) and 2B in hemin and poly(IC)-treated reticulocyte lysates, 51D mutant and wt eIF2α proteins were unable to cause a similar effect (Figure 8A), thereby suggesting that the aspartic acid in the place of 51 serine residue mimcs the charge of a phosphorylated serine. Previously (*66*), and also here, it is observed that 48A mutant is somewhat better and offers more protection to the GNE activity of eIF2B in poly(IC)-treated lysates than the nonphosphorylatable 51A mutant of eIF2α. It is not known if this is due to the presence of trimeric mutant 48A in reticulocyte lysates that results from the exchange of free 48A subunits into endogenous trimeric eIF2

(*64*). This chimeric eIF2 may be able to perform the GDP/GTP exchange independent of eIF2B.

Surprisingly, despite significant levels of eIF2α phosphorylation, the uninfected cells were able to survive. As has been suggested by Dever et al. (*76*), this may be due to species differences. eIF2B activity of Sf9 cells is less sensitive to regulation by phosphorylated eIF2 than the eIF2B from mammalian systems where small changes (10−20%) in eIF2α phosphorylation can drastically inhibit the GNE activity of eIF2B. This is also consistent with species specific reactivity of the eIF2 monoclonal antibody. This species specificity may also explain the better ability of Sf9 cells to tolerate moderate expression of 51D mutant of eIF2α than mammalian systems.

ACKNOWLEDGMENT

We are thankful to Dr. Jane-Jane Chen and Professor Irving M. London at the Massachusetts Institute of Technology for providing us with purified HRI and monoclonal antibodies for eIF2α, eIF2Bϵ, and HRI.

REFERENCES

1. Ochoa, S. (1983) *Arch. Biochem. Biophys. 223*, 325−349.
2. Hershey, J. W. B. (1991) *Annu. Rev. Biochem. 60*, 717−755.
3. Merrick, W. E. (1992) *Microbiol. Rev. 56*, 291−315.
4. Rhoads, B. E. (1993) *J. Biol. Chem. 268*, 3017−3020.
5. Webb, B. L., and Proud, C. G. (1997) *Int. J. Biochem. Cell. Biol. 10*, 1127−1130.
6. Clemens, M. J. (1996) in *Translational Control* (Hershey, J. W. B., Mathews, M. B., and Sonenberg, N., Eds.) pp 139−172, Cold Spring Harbor Laboratory Press, Plainview, NY.
7. Levin, D. H., Ranu, R. S., Ernst, V., and London, I. M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3112−3116.
8. Kramer, G., Cimadevilla, M., and Hardesty, B. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3078−3082.
9. Farrell, P. J., Balkow, K., Hunt, T., Jackson, R. J., and Trachsel, H. (1977) *Cell 11*, 187−200.
10. Surolia, N., and Padmanaban, G. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4786−4790.
11. Levin, D. H., and London, I. M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1121−1125.
12. Dever, T. E., Feng, L., Wek, R. C., Cigan, A. M., Donahue, T. D., and Hinnebusch, A. G. (1992) *Cell 68*, 585−596.
13. Scorsone, K. A., Panniers, R., Rowlands, A. G., and Henshaw, E. C. (1987) *J. Biol. Chem. 262*, 14538−14543.
14. Clemens, M. J., Galpine, A., Austin, S. A., Panniers, R., and Henshaw, E. C. (1987) *J. Biol. Chem. 262*, 767−771.
15. Alcazar, A., Rivera, J., Gomez-Calcerrada, M., Munoz, F., and Salinas, M., and Fando, J. L. (1996) *Brain Res. Mol. Brain. Res. 38*, 101−108.
16. Rolfes, R. J., and Hinnebusch, A. G. (1993) *Mol. Cell. Biol. 13*, 5099−6111.
17. Duncan, R., and Hershey, J. W. B. (1985) *J. Biol. Chem. 260*, 5493−5497.
18. Montine, K. S., and Henshaw, E. C. (1987) *Biochim. Biophys. Acta. 1014*, 282−288.
19. Ito, T., Jagus, R., and May, W. S. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 7455−7459.
20. Kaufman, R. J., Davies, M. V., Pathak, P. K., and Hershey, J. W. B. (1989) *Mol. Cell. Biol. 9*, 946−958.
21. Burda, J., Martin, M. E., Gottlieb. M., Marsala, J., Alcazar, A., Pavon, M., Fando, J. L., and Salinas, M. (1998) *J. Cereb. Blood. Flow. Metab. 18*, 59−66.
22. Menon, V., and Thomason, D. B. (1995) *Am. J. Physiol. 269*, C802−804.
23. Duncan, R., and Hershey, J. W. B. (1984) *J. Biol. Chem. 259*, 11882−11889.
24. De Benedetti, A., and Baglioni, C. (1986). *J. Biol. Chem. 261*, 338−342.
25. Hurst, R., Schatz, J. R., and Matts, R. L. (1987) *J. Biol. Chem. 262*, 15939−15945.
26. Matts, R. L., Schatz, J. R., Hurst, R., and Kagen, R. (1991) *J. Biol. Chem. 266*, 12695−12702.
27. Alirezaei, M., Nairn, A., Glowinski, J., Premont, J., and Marin, P. (1999) *J. Biol. Chem. 274*, 32433−32438.
28. Prostko, C. R., Brostrom, M. A., Malara, E. M., and Brostrom, C. O. (1992) *J. Biol. Chem. 267*, 16751−16754.
29. Prostko, C. R., Brostrom, M. A., and Brostrom, C. O. (1993) *Mol. Cell. Biochem. 127*, 255−265.
30. Prostko, C. R., Dholakia, J. N., Brostrom, M. A., and Brostrom, C. O. (1995) *J. Biol. Chem. 270*, 6211−6215.
31. Aktas, H., Fluckiger, R., Acosta, J. A., Savage, J. M., Palakurthi, S. S., and Halperin, J. A. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 8280−8285.
32. Laitusis, A. l., Brostrom, M. A., and Brostrom, C. O. (1999) *J. Biol. Chem. 274*, 486−493.
33. Ernst, V., Levin, D. H., and London, I. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2118−2122.
34. Kan, B., London, I. M., and Levin, D. H. (1988) *J. Biol. Chem. 263*, 15652−15656.
35. Ramaiah, K. V. A., Chen, J.-J., Gallop, P. M., and London, I. M. (1997) *Blood Cells, Mol. Dis. 23*, 177−187.
36. Matts, R. L., Hurst, R., and Xu, Z. (1993) *Biochemistry 32*, 7323−7328.
37. Kim, Y. M., Son, K., Hong, S. J., Green, A., Chen, J.-J., Tzeng, E., Hierholzer, C., and Billiar, T. R. (1998) *Mol. Med. 4*, 179−190.
38. London, I. M., Levin, D. H., Matts, R. L., Thomas, N. S. B., Petryshyn, R., and Chen, J.-J. (1987) in *The Enzymes* (Boyer, P. D., and Krebs, E. G., Eds.) Vol. 17, pp 359−380, Academic Press, New York.
39. Jackson, R. J. (1991) in *Translation in Eukaryotes* (Trachsel, H., Ed.) pp 193−229, CRC Press, Boca Raton, FL.
40. Hinnebusch, A. G. (1993) *Mol. Microbiol. 10*, 215−223.
41. Donze, O., Jagus, R., Koromilas, A. E., Hershey, J. W. B., and Sonenberg, N. (1995) *EMBO J. 14*, 3828−3834.
42. Qu, S., Perlaky, S. E., Organ, E. L., Crawford, D., and Cavener, D. R. (1997) *Gene Expression 6*, 349−360.
43. Der, S. D., Yang, Y.-L., Weissmann, C., and Williams, B. R. G. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 3279−3283.
44. Srivastava, S. P., Kumar, K. U., and Kaufman, R. J. (1998) *J. Biol. Chem. 273*, 2416−2423.
45. Alcazar, A., Bazan, E., Rivera, J., and Salinas, M. (1995) *Neurosci. Lett. 201*, 215−218.
46. Samuel, C. E. (1993) *J. Biol. Chem. 268*, 7603−7606.
47. Hinnebusch, A. G. (1994) *Trends. Biochem. Sci. 19*, 409−414.
48. de Haro, C., Mendez, R., and Santoyo. J. (1996) *FASEB. J. 10*, 1378−1388.
49. Chen, J.-J., and London, I. M. (1995) *Trends. Biochem. Sci. 20*, 105−108.
50. Clemens, M. J., and Elia, A. (1997) *J. Interferon Cytokine Res. 17*, 503−524.
51. Wek, R. C. (1994) *Trends. Biochem. Sci. 19*, 491−496.
52. Santoyo, J., Alcalde, J., Mendez, R., Pulido, D., and de Haro, C. (1997) *J. Biol. Chem. 272*, 12544−12550.
53. Berlanga, J. J., Santoyo, J., and De Haro, C. (1999) *Eur. J. Biochem. 265*, 754−762.
54. Mohrle, J. J., Zhao, Y., Wernli, B., Franklin, R. M., and Kappes, B. (1997) *Biochem. J. 328*, 677−687.
55. Shi, Y., An, J., Liang, J., Hayes, S. E., Sandusky, G. E., Stramm L. E., and Yang, N. N. (1999) *J. Biol. Chem. 274*, 5723−5730.
56. Shi, Y., Vattem, K. M., Sood, R., An, J., Liang, J., Stramm, L., and Wek, R. C. (1998) *Mol. Cell. Biol. 18*, 7499−7509.
57. Harding, H. P., Zhang, Y., and Ron, D. (1999) *Nature 397*, 271−274.
58. Clemens, M. J., Pain, V. M., Wong, S., and Henshaw, E. C. (1982) *Nature 296*, 93−95.
59. Matts, R. L., and London, I. M. (1984) *J. Biol. Chem. 259*, 6708−6711.
60. Rowlands, A. G., Montine, K. S., Henshaw, E. C., and Panniers, R. (1988b) *Eur. J. Biochem. 175*, 93−99.

61. Babu, S. V. N., and Ramaiah, K. V. A. (1996) *Arch. Biochem. Biophys. 327*, 201−208.
62. Thomas, N. S. B., Matts, R. L., Levin, D. H., and London, I. M.(1985) *J. Biol. Chem. 260*, 9860−9866.
63. Ramaiah, K. V. A., Dhindsa, R. S., Chen, J.-J., London, I. M., and Levin, D. H. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 12063−12067.
64. Choi, S. Y., Scherer, B. J., Schnier, J., Davies, M. V., Kaufman, R. J., and Hershey, J. W. B. (1992) *J. Biol. Chem. 267*, 286−293.
65. Murtha-Riel, P., Davies, M. V., Scherer, B. J., Choi, S. Y., Hershey, J. W. B. and Kaufman, R. J. (1993) *J. Biol. Chem. 268*, 12946−12951.
66. Ramaiah, K. V. A., Davies, M. V., Chen, J.-J., and Kaufman, R. J. (1994) *Mol. Cell. Biol. 14*, 4546−4553.
67. Pathak, V. K., Schindler, D., and Hershey, J. W. B. (1988) *Mol. Cell. Biol. 8*, 993−995.
68. Srivastava, S. P., Davies, M. V., and Kaufman, R. J. (1995) *J. Biol. Chem. 28*, 16619−16624.
69. Chefalo, P. J., Yang, J. M., Ramaiah, K. V. A., Gehrke, L., and Chen, J.-J. (1994) *J. Biol. Chem. 269*, 25788−25794.
70. Sudhakar, A., Krishnamoorthy, T., Jain, A., Chatterjee, U., Hasnain, S. E., Kaufman, R. J., and Ramaiah, K. V. A. (1999) *Biochemistry 38*, 15398−15405.
71. Davies, M. V., Chang, H. W., Jacobs, B. L., and Kaufman, R. J. (1993) *J. Virol. 67*, 1688−1692.
72. Summers, M. D., and Smith, G. E. (1987) *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*, Texas Agric. Station. Bulletin No. 1555, College Station, TX.
73. Chatterjee, U., Ahmed, R., Venkaiah, B., and Hasnain, S. E. (1996) *Gene 171*, 209−213.
74. Pal, J. K., Chen, J. J., and London, I. M. (1991) *Biochemistry 30*, 2555−2562.
75. Krishna, V. M., Janaki, N., and Ramaiah, K. V. A. (1997) *Arch. Biochem. Biophys. 346*, 28−36.
76. Dever, T. E., Sripriya, R., McLahlin, J. R., Fabian, J. R., Kimball, S. R., and Miller, L. K. (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95, 4164−4169.
77. Matts, R. L., Levin, D. H., and London, I. M. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2559−2563.
78. Pavitt, G. D., Ramaiah, K. V. A., Kimball, S. R., and Hinnebusch, A. G. (1998) *Genes Dev. 12*, 514−526.
79. Hershey, J. W. B. (1989) *J. Biol. Chem. 264*, 20824−20826.
80. Rowlands, A. G., Panniers, R., and Henshaw, E. C. (1988) *J. Biol. Chem. 263*, 5526−5533.
81. Gross., M, Wing, M., Rundquist, C., and Rubino, M. S. (1987) *J. Biol. Chem.* 262, 6899−6907.
82. Goss, D. J., Parkhurst, L. J., Mehta, H. D., Woodley, C. L., and Wahba, A. J. (1984) *J. Biol. Chem. 259*, 7374−7377.

BI0008682