Serine 48 in Initiation Factor 2α (eIF2α) Is Required for High-Affinity Interaction between eIF2α(P) and eIF2B†

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ABSTRACT: Phosphorylation of the serine 51 residue in the α-subunit of translational initiation factor 2 in eukaryotes (eIF2α) impairs protein synthesis presumably by sequestering eIF2B, a rate-limiting pentameric guanine nucleotide exchange protein which catalyzes the exchange of GTP for GDP in the eIF2−GDP binary complex. To further understand the importance of eIF2α phosphorylation in the interaction between eIF2α(P) and eIF2B proteins and thereby the regulation of eIF2B activity, we expressed the wild type (wt) and a mutant eIF2α in which the serine 48 residue was replaced with alanine (48A mutant) in the baculovirus system. The findings reveal that the expression of both of these recombinant subunits was very efficient (15−20% of the total protein) and both proteins were recognized by an eIF2α monoclonal antibody and were phosphorylated to the same extent by reticulocyte eIF2α kinases. However, partially purified recombinant subunits (wt or 48A mutant) were not phosphorylated as efficiently as the eIF2α subunit present in the purified reticulocyte trimeric eIF2 complex and were also found to inhibit the phosphorylation of eIF2α of the trimeric complex. Furthermore, the extents of inhibition of eIF2B activity and formation of the eIF2α(P)−eIF2B complex that occurs due to eIF2α phosphorylation in poly(IC)-treated rabbit reticulocyte lysates were decreased significantly in the presence of insect cell extracts expressing the 48A mutant eIF2α compared to those for wt. These findings support the hypothesis that the serine 48 residue is required for high-affinity interaction between eIF2α(P) and eIF2B.

Translational initiation factor 2 in eukaryotes (eIF2)[1] is a heterotrimer composed of α-, *β*-, and *γ*-subunits. It plays a central role in the translational initiation, forming a ternary complex with GTP and Met-tRNAi which then joins 40S ribosomes to form the 43S initiation complex. Following the joining of messenger RNA, the GTP in the ternary complex is hydrolyzed and the inactive eIF2−GDP binary complex is released at the end of the initiation step in protein synthesis (reviewed in refs *1*−*3*). For eIF2 to enter another round of initiation, the GDP in the eIF2 binary complex must be exchanged for GTP, a reaction that is catalyzed by a heteropentameric protein called eIF2B (previously called the reversing factor or guanine nucleotide exchange factor) (*4*−*8*). One of the most important ways through which the recycling of eIF2 and thereby protein synthesis is regulated occurs through phosphorylation of the small or α-subunit in eIF2 (eIF2α) (reviewed in refs *9*−*12*).

Several physiological stimuli such as heme deficiency, viral infection, amino acid starvation, heavy metal stress, heat shock, serum and calcium deprivation, or mobilization of intracellular calcium inhibit protein synthesis through the activation of eIF2α kinases which stimulate eIF2α phosphorylation (reviewed in refs *13*−*15*). Some of the eIF2α kinases such as heme-regulated kinase (HRI) (reviewed in refs *13* and *14*), the double-stranded RNA-induced inhibitor (PKR) (*16*), and the GCN2 kinase in yeast and higher eukaryotes which become activated in response to amino acid starvation (reviewed in ref *15*) have been well characterized. In addition, two more eIF2α kinases have been recently characterized. These are PEK, a pancreatic kinase, and PERK, an endoplasmic reticulum resident kinase (*43*, *44*).

Phosphorylation of a small portion of the total eIF2α inhibits the catalytic ability of purified and lysate eIF2B to exchange guanine nucleotides on eIF2 in vitro (*6*, *17*−*23*). Phosphorylated eIF2α is a strong competitive inhibitor of eIF2B (*24*, *25*). The affinity of eIF2B for phosphorylated eIF2 (in which the α-subunit is phosphorylated) is found to be much higher than that for unphosphorylated eIF2 (*24*),

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

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and phosphorylated eIF2 forms a tight complex with eIF2B in which eIF2B becomes nonfunctional (*26*). Since eIF2B does not exceed 20−30% of the total eIF2, a small proportion of eIF2α phosphorylation sequesters all of the available eIF2B into an inactive complex and prevents the recycling of eIF2 (*26*, *27*).

The availability of site-specific mutants of eIF2α like the 48A or 51A mutant in which the serine residues in the respective positions of eIF2α have been replaced with alanine has advanced our understanding in identifying that (a) the serine 51 residue in eIF2α is the only site for phosphorylation in mammalian eIF2α (*28*), (b) the translational block caused by adenoviral mRNAs, plasmid-derived mRNAs, heat shock, or calcium release from the endoplasmic reticulum is due to an increased level of eIF2α phosphorylation (*29*−*32*) or to localizing the translational inhibition caused by eIF2α phosphorylation, and (c) phosphorylation of eIF2α plays a critical role in cell proliferation and development (*33*, *34*). In addition, the coexpression of a mutant eIF2α which cannot be phosphorylated has facilitated the expression of mammalian eIF2α kinases such as the heme-regulated kinase in insect cells (*35*).

To further understand the mechanism of regulation of eIF2B activity by phosphorylated eIF2α and the protein−protein interactions, we have used the baculovirus system to produce the eIF2α wild type (wt) and the 48A mutant. The latter can be phosphorylated on its serine 51 residue and has been shown to rescue protein synthesis inhibition caused by PKR and heat shock (*29*, *30*). Consistent with these earlier observations, our findings here indicate that baculovirus-expressd 48A mutant eIF2α mitigates the inhibition of eIF2B activity in reticulocyte lysates caused by eIF2α phosphorylation and reduces the extent of formation of the 15S complex that occurs between eIF2 and eIF2B when the α-subunit in eIF2 is phosphorylated, thereby suggesting that the mutant eIF2α(P) cannot interact with eIF2B as efficiently as wt eIF2α(P). Our studies additionally demonstrate here that AcNPV-infected insect cell extracts inhibit eIF2α phosphorylation in vitro.

MATERIALS AND METHODS

Materials. The pETFVA$^-$ vector harboring wild type or human mutant eIF2α has been described previously (*45*). pBakPAK8, pBakPAK6 (*Bsu*36I digest), lipofectin, and Sf9 cells were obtained from Clonetech. The random primer labeling kit, the hybond N$^+$ membrane, and [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 AP conjugate, NBT, and BCIP were obtained from Promega Corp. A monoclonal anti-eIF2α antibody produced in E. C. Henshaw's laboratory and purified heme-regulated eIF2α kinase (HRI) were kindly provided by J.-J. Chen (Massachusetts Institute of Technology, Cambridge, MA). GDP, CPK, DTT, and protease solutions were purchased from Boehringer 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 of the Recombinant Baculovirus Transfer Vector. DH5α cells were transformed with the parent vector harboring wt or 48A mutant eIF2α cDNA to amplify the parent vector. Vector DNA was isolated using the Qiagen column and digested with *Eco*RI to separate the 1.6 kb eIF2α insert. The insert was electroeluted from a 1% agarose gel and was purified by Qiaquick Spin columns. PBacPAK8, a baculovirus transfer vector, was linearized with *Eco*RI and ligated to the eIF2α cDNA. DH5α cells were transformed with the recombinant pBacPAK8 vector, and the positive colonies containing the vector with the eIF2α insert were identified by using the colony hybridization technique. The insert orientation in the vector was checked by double-restriction digestion using *Mlu*I and *Sca*I, or *Sph*I and *Bam*HI, enzymes for pBacPAK8 vector carrying wt or 48A mutant eIF2α cDNA, respectively, and the recombinant constructs with the insert in the right orientation were used for further work.

Maintenance of Insect Cells, Cotransfection, and Identification of Recombinant Baculoviruses. The *Spodoptera frugiperda* (Sf9) cell line was maintained in complete TNM-FH medium containing 10% fetal calf serum and antimycotic and antibiotic solutions as described previously (*35*). Recombinant baculoviruses were generated in vitro by transfecting *Bsu*36I-digested AcNPV virus DNA (Clonetech) into Sf9 cells as described previously (*37*).

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 was carried out to increase the titer of the recombinant virus in a stepwise manner.

Determination of the Level of eIF2α Expression. Uninfected insect cells as well as infected cells (infected with AcNPV or eIF2α wt or eIF2α 48A mutant recombinant viruses) were washed with ice-cold PBS (pH 6.2). The cells were lysed in ice-cold lysis buffer [20 mM Tris-HCl (pH 7.8), 1 mM Mg^{2+}, 1 mM DTT, and protease inhibitors such as pepstatin, leupeptin, and aproteinin] and centrifuged at 10 000 rpm for 10 min. To the lysate supernatant were added PMSF (1 mM) and KCl (80 mM), and the mixture was immediately aliquoted and stored in liquid N_2. Samples of the concentrated extracts were separated on 10% SDS−PAGE and were also transferred to a nitrocellulose membrane to determine the level of expression of eIF2α based on its migration on SDS−PAGE and also on its ability to interact with a monoclonal eIF2α antibody (*22*, *35*).

Preparation of Reticulocyte Lysates and eIF2. Heme-deficient reticulocyte lysates which respond to added hemin were prepared and were used as a source for measuring eIF2B activity and also for the purification of eIF2 as described previously (*20*, *23*).

Measurement of eIF2B Activity. The eIF2B activity of hemin or heme and poly(IC)-treated reticulocyte lysates in the presence and absence of insect cell extracts expressing the wt or mutant eIF2α was measured by monitoring the level of dissociation of the preformed labeled reticulocyte eIF2−[^{3}H]GDP binary complex, as described previously (*20*, *23*, *35*, *38*).

Phosphorylation of Recombinant eIF2α in Vitro, in Poly-(IC)-Treated Reticulocyte Lysates, and in Insect Cell Extracts. Purified heme-regulated kinase (HRI) and [γ-^{32}P]ATP

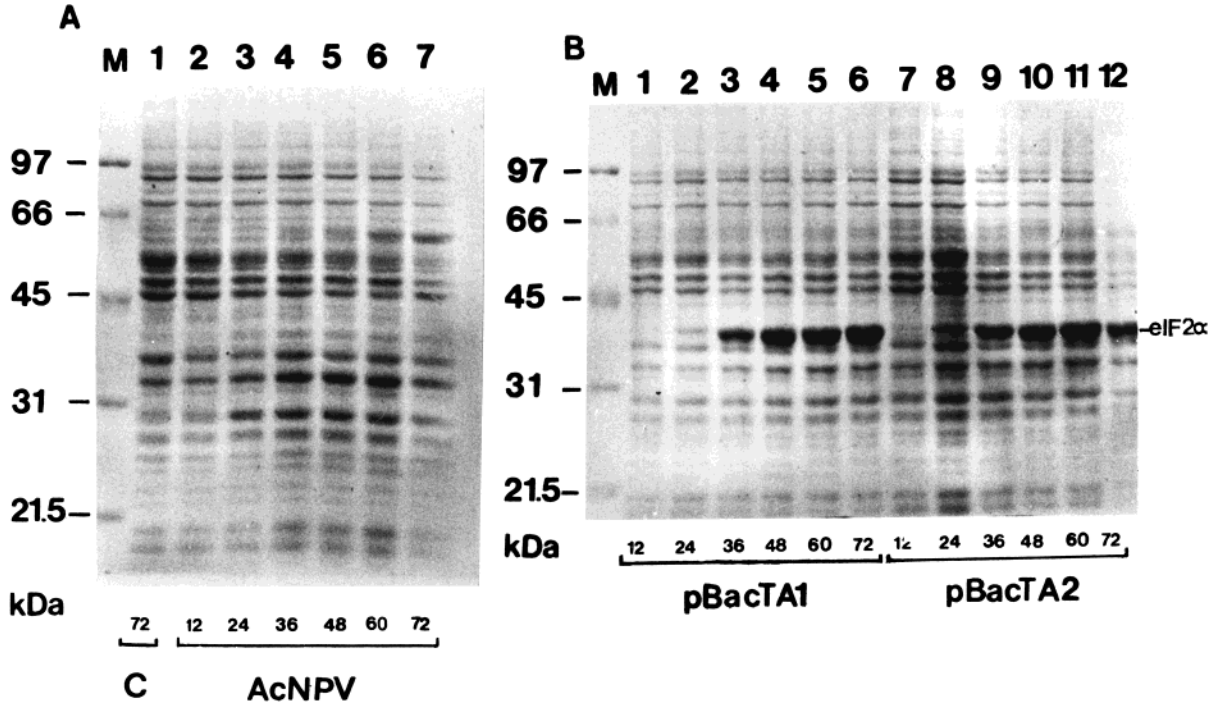

FIGURE 1: Expression of wild type and 48A mutant human eIF2α in insect cells using recombinant baculovirus. Time course of protein expression. Insect cell extracts were prepared as described in Materials and Methods from Sf9 cells infected at a multiplicity of infection (MOI) of 10, with recombinant viruses harboring wt or 48A mutant eIF2α (B) or with wild type AcNPV (A) at different time points as shown in the figure. Extract from uninfected cells (control, C, in panel A) was prepared for only one time point (72 h postinfection). Each extract was prepared from 2×10^6 Sf9 cells. The viruses had an MOI of 10 in each case. An equal amount of protein extract (25 μg) was loaded in each well of a 10% SDS−PAGE and stained with Coomassie blue.

were used to phosphorylate the expressed recombinant eIF2α in insect cell extracts. The phosphorylated extracts were then resolved by 10% SDS−PAGE and analyzed by autoradiography (*20*−*23*). Also, the insect cell extracts or the partially purified eIF2α wt or 48A mutant (purified using Sephacryl 300 and DEAE cellulose 52) was phosphorylated with or without HRI, respectively, in the presence of [γ-^{32}P]ATP. In addition, phosphorylation of poly(IC)-treated hemin-supplemented reticulocyte lysates was carried out in the presence and absence of insect cell extracts expressing wt or 48A mutant eIF2α.

Analysis of the eIF2α(P)−*eIF2B Complex in Reticulocyte Lysates*. Reticulocyte lysate 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 nonrecombinant or recombinant virus-infected cell extracts (25 μg of protein) as described in the legend of Figure 5. At the end of the protein synthesis reaction, the lysates were diluted with an 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 dilution buffer described above. Samples were run at 40 000 rpm for 6 h at 4 °C in a SW 50.1 rotor to separate free eIF2 from the 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 fractinator. 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 on 10% SDS−PAGE. Proteins were transferred to nitrocellulose membranes, and eIF2α of the various gradient fractions was detected by using an anti-human eIF2α monoclonal antibody as described previously (*22*).

RESULTS

Expression of Wild Type and 48A Mutant Human eIF2α. Extracts of uninfected and infected Sf9 cells (infected with wild type AcNPV or with recombinant, pBacTA1, wild type eIF2α or pBacTA2, 48A mutant eIF2α) were prepared at different time points post-infection (12, 24, 36, 48, 60, and 72 h) and were analyzed by 10% SDS−PAGE (Figure 1). A protein with a molecular mass of 38 kDa is expressed in the cells infected with the recombinant virus from 24 h postinfection onward up to 72 h (Figure 1B). This protein is not found in uninfected cells or in cells infected with the wild type AcNPV virus (Figure 1A).

Immunoreactivity of the Recombinant eIF2α. Immunoreactivity of the expressed protein was tested by Western blot analysis using anti-eIF2α monoclonal antibody (Figure 2). Both the wild type (Figure 2, lanes 3−7) and the 48A mutant (lanes 8−12) react equally well with the antibody, and the magnitude of the signal is proportional to the level of expression of the eIF2α protein. At 24 h postinfection, the extracts contain low levels of eIF2α, and accordingly, the reactivity of the antibody is poor in these lanes (lanes 3 and 8). In contrast, a strong signal appeared between 36 and 72 h (lanes 4−7 and 9−12) postinfection and is consistent with the previous results which showed that expression starts around 24 h and increases with time up to 72 h postinfection. Neither control Sf9 cell extracts (lane 1) nor wild-type AcNPV-infected cell extracts (lane 2) contain any polypeptide that is immunoreactive with respect to the eIF2α monoclonal antibody described above.

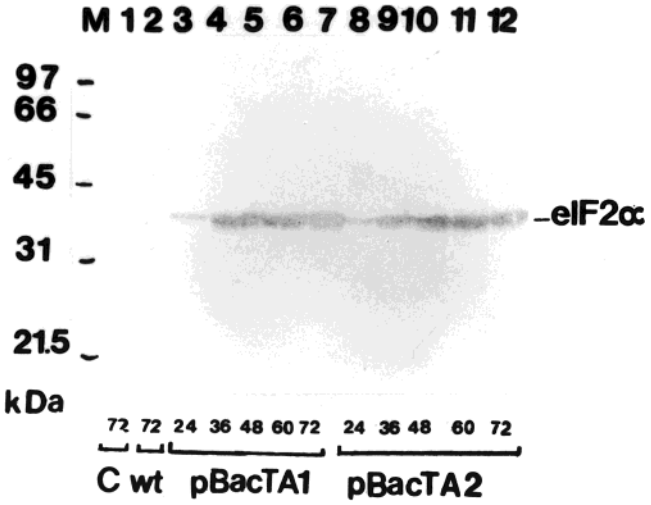

FIGURE 2: Immunoreactivity of the recombinant eIF2α. Cell extracts were prepared as described in the legend of Figure 1. The protein extracts (25 μg) were separated on a 10% SDS−PAGE, transferred to a nitrocellulose membrane, and probed with an eIF2α monoclonal antibody. The signal was detected with the help of rabbit anti-mouse alkaline phosphatase-conjugated secondary antibody (Promega). In the case of uninfected cells (C) and of cells infected with wild type AcNPV, extracts from only one time point (72 h postinfection) were used.

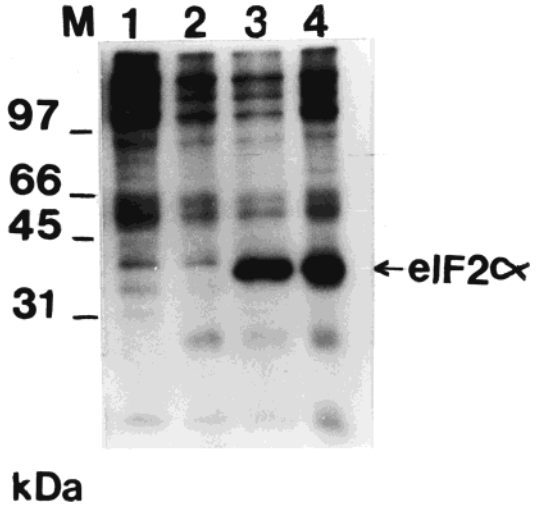

FIGURE 3: Phosphorylation of the recombinant human eIF2α wt and 48A mutant in insect cell extracts by HRI. Cell extracts (25 μg) from uninfected and infected cells (infected with wt or recombinant viruses) prepared 48 h postinfection were incubated at 30 °C for 5 min prior to the addition of HRI. HRI was preincubated in a reaction mixture containing 20 mM Tris-HCl (pH 7.6), 2 mM Mg^{2+}, 80 mM KCl, and 30 μM ATP at 30 °C for 5 min. The extracts were incubated for an additional 5 min before they were pulsed with [γ-^{32}P]ATP (10 μCi). Reactions were terminated 5 min after the addition of the labeled ATP by adding 2× SDS sample buffer and the mixtures heated for 2 min in boiling water. The samples were separated on 10% SDS−PAGE, and the gel was analyzed by autoradiography: lane 1, uninfected cell extract; lane 2, wild type AcNPV-infected cell extract; lane 3, recombinant virus-infected cell extract expressing the eIF2α wt; and lane 4, recombinant virus-infected cell extract expressing the eIF2α 48A mutant.

Phosphorylation of the Recombinant eIF2α wt and 48A Mutant. Since wild type eIF2α and 48A mutant eIF2α expressed in mammalian systems are shown to be substrates for eIF2α kinases (*30*, *39*), the ability of baculovirus-expressed eIF2α subunits to serve as substrates for phosphorylation in the presence of purified HRI is tested (Figure 3). Both the recombinant wild type and 48A mutant eIF2α are found to be accessible for phosphorylation (lanes 3 and 4). A similar phosphorylated protein corresponding to human eIF2α is lacking in the control and AcNPV-infected cell extracts (lanes 1 and 2). These findings suggest that baculovirus-expressed eIF2α truly represents human eIF2α. In the absence of added HRI, phosphorylation of recombinant eIF2α could not be detected in insect cell extracts (data not shown).

The 48A Mutant Decreases the Level of Inhibition of eIF2B Activity in Poly(IC)-Treated Reticulocyte Lysates. Earlier studies with Chinese hamster ovary cells (*38*) have shown that the level of inhibition of eIF2B activity via phosphorylation of eIF2α, either by purified reticulocyte HRI or by endogenous eIF2α kinase activated by heat shock, was reduced by mutations replacing serine residues 48 and 51 with alanine compared to that with wild type eIF2α. The functional characteristics of baculovirus-expressed human wild type and 48A mutant eIF2α, and their effects on the inhibition of eIF2B activity that occurs in poly(IC)-treated reticulocyte lysates, were also studied (Figure 4). The kinetics of eIF2B GDP−GTP exchange activity in poly(IC) and hemin-supplemented lysates is shown in Figure 4. The guanine nucleotide exchange activity of eIF2B in lysates is measured from the extent of dissociation of labeled GDP in the preformed eIF2−[^{3}H]GDP binary complex. The extent of dissociation of the labeled GDP is higher in hemin-supplemented lysates than in poly(IC)-treated lysates. The inhibition of eIF2B activity in hemin and poly(IC)-treated reticulocyte lysates is consistent with the earlier findings that poly(IC) induces eIF2α phosphorylation via double-stranded RNA-dependent kinase (PKR) and causes inhibition of eIF2B activity in reticulocyte lysates (*18*, *20*−*23*). Poly(IC)-treated reticulocyte lysates were supplemented with insect cell extracts (50 μg) prepared from control Sf9 cells, wild type AcNPV-infected cells, or recombinant virus-infected cells expressing wild type or mutant eIF2α. It is observed that the virus-infected cell extracts instead of the uninfected extracts can relieve to some extent the inhibition in eIF2B activity of reticulocyte lysates caused by the addition of poly-(IC). However, the decrease in the level of inhibition of eIF2B activity is relatively higher in the presence of cell extracts expressing the 48A mutant than in the presence of wt eIF2α (Figure 4). These experiments were performed with equal amounts of extract protein in each of the reaction mixtures that contained fairly equal amounts of expressed eIF2α protein (wild type or 48A mutant eIF2α) (data not shown).

Analysis of eIF2α(P)−eIF2B Complex Formation in Reticulocyte Lysates. Previously, it was hypothesised that the 48A mutation in eIF2α decreases the extent of interaction of eIF2α(P) with eIF2B (*38*). To assess such a possibility, the eIF2α(P)−eIF2B complex that forms in poly(IC)-inhibited reticulocyte lysates was analyzed by sucrose gradient centrifugation, as described previously (*22*) in the presence of insect cell extracts containing the eIF2α wild type or 48A mutant (Figure 5). Since free eIF2 has a significantly lower molecular mass than the eIF2α(P)−eIF2B complex, the top fractions of the 10 to 30% sucrose gradient contain free eIF2, whereas the bottom fractions contain the complex. The free eIF2 and eIF2α(P)−eIF2B complex in

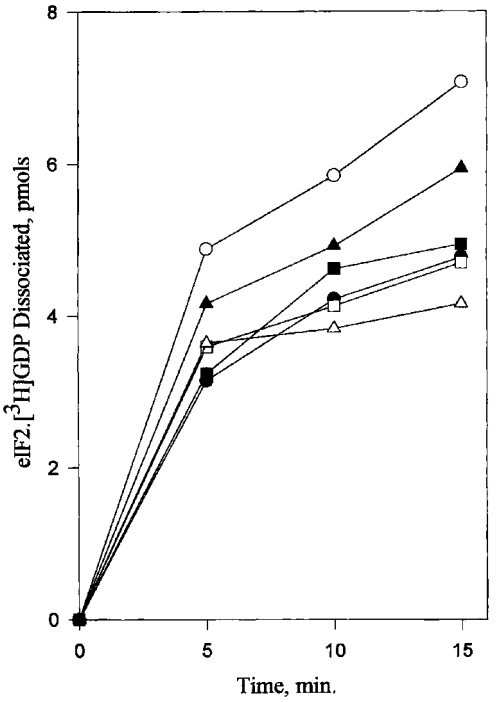

FIGURE 4: Kinetics of eIF2−[^{3}H]GDP dissociation in hemin-treated or hemin- and poly(IC)-treated reticulocyte lysates in the presence of insect cell extracts expressing the eIF2α wt or 48A mutant. Protein synthesis was carried out in 70 μL of rabbit reticulocyte lysates at 30 °C for 10 min as described previously (*20*, *23*) under the following conditions: (○) with heme (20 μM), (●) with heme and poly(IC) (300 ng/mL), (△) with heme, poly(IC), and uninfected cell extract, (□) with heme, poly(IC), and virus-infected cell extract, (■) with heme, poly(IC), and virus-infected cell extract expressing eIF2α wt, and (▲) with heme, poly(IC), and virus-infected cell extract expressing the eIF2α 48A mutant. The insect cell extracts (175 μg in 35.25 μL) were prepared 48 h postinfection. Seventy microliters (35.0 pmol) of the preformed binary complex was added to the above reticulocyte lysate reaction mixtures (70 μL) and incubated at 30 °C. At various time intervals, 50 μL aliquots were taken to determine the amount of eIF2−[^{3}H]GDP dissociated as described in Materials and Methods.

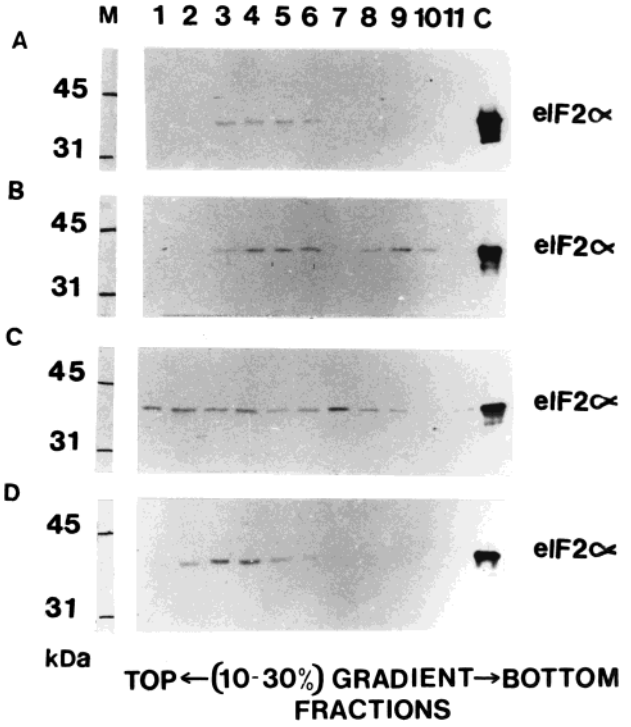

FIGURE 5: Detection of the eIF2α(P)−eIF2B complex in reticulocyte lysates by immunoblot analysis of eIF2 in the 10 to 30% gradient fractions. Protein synthesis reactions of reticulocyte lysates were carried out in 100 μL for 15 min at 30 °C in the presence of (A) 20 μM heme (h) and wild type AcNPV-infected Sf9 cell extracts (25 μg), (B) h, poly(IC) (300 ng/mL), and wild type AcNPV-infected cell extract, (C) h, poly(IC), and recombinant virus-infected cell extract overexpressing eIF2α wt (25 μg), and (D) h, poly(IC), and recombinant virus-infected cell extract overexpressing the 48A mutant. Reaction mixtures were diluted with an equal volume of TKM buffer [20 mM Tris-HCl (pH 7.8), 80 mM KCl, and 2.5 mM $Mg(OAc)_2$] and layered on a 10 to 30% sucrose gradient. The gradients were prepared in TKM buffer and centrifuged with the samples for 6 h at 40 000 rpm in a SW 50.1 rotor. The gradient fractions (400 μL) were collected, concentrated by pH 5.0 precipitation, separated on 10% SDS−PAGE, and transferred to a nitrocellulose membrane. eIF2α in the transferred proteins of the different gradient fractions was identified with the help of anti-mouse human eIF2α monoclonal antibodies as described previously (*22*). Purified eIF2 (250 ng) was loaded at the end of each gel to serve as a control.

the gradient fractions were detected by Western analysis using an eIF2α monoclonal antibody as previously described (*22*). The eIF2α signal could be seen only in the top fractions of the gradient that contains hemin-supplemented lysates treated with wild type AcNPV-infected insect cell extracts (Figure 5A). This is because in the presence of hemin and without poly(IC) or dsRNA being included in the reaction, reticulocyte lysates contain very little or no eIF2α kinase activity which can phosphorylate eIF2α and facilitate the formation of a complex between eIF2α(P) and eIF2B. Hence, very little reticulocyte eIF2 is bound to eIF2B which can be detected in the bottom fractions of these gradients. In contrast, eIF2α is detected in both the top and bottom fractions of the gradients containing lysates treated with hemin, poly(IC), and the wild type AcNPV-infected cell extracts (Figure 5B). A similar result was obtained, indicating the presence of the eIF2α signal in the top and bottom fractions of the gradients for the fractions of hemin and poly-(IC)-treated lysates that were supplemented with insect cell extracts containing wild type eIF2α (Figure 5C). This indicates the presence of free eIF2α and eIF2α in complex with eIF2B. However, the intensity of the eIF2α signal was greater in the top fractions because the reaction mixtures contain the overexpressed wild type eIF2α subunit, and the signal can be seen even in the first fraction of the gradient (Figure 5C). This suggests that the overexpressed subunit form of eIF2α can also be separated from the eIF2α present in the trimeric eIF2 complex in the lysates. In contrast, it was not possible to detect eIF2α in the bottom fractions of the gradient in the hemin and poly(IC)-treated reticulocyte lysates which were supplemented with insect cell extracts expressing 48A mutant eIF2α (Figure 5D). These findings suggest that the 48A mutation in mammalian eIF2α reduces the extent of formation of the complex that exists between eIF2α(P) and eIF2B in hemin and poly(IC)-treated reticulocyte lysates. These findings are consistent with the eIF2B activity measurements (Figure 4).

Phosphorylation of Recombinant eIF2α, eIF2α in a Trimeric Complex in Vitro, and in the Presence of Uninfected and AcNPV-Infected Insect Cell Extracts. To understand if the phosphorylation of recombinant eIF2α, wt, or 48A mutant was in any way different from that of the α-subunit in the trimeric complex of eIF2, the phosphorylation of partially purified recombinant eIF2α (purified using Sephacryl-300 and the DEAE cellulose 52 column) was studied in vitro

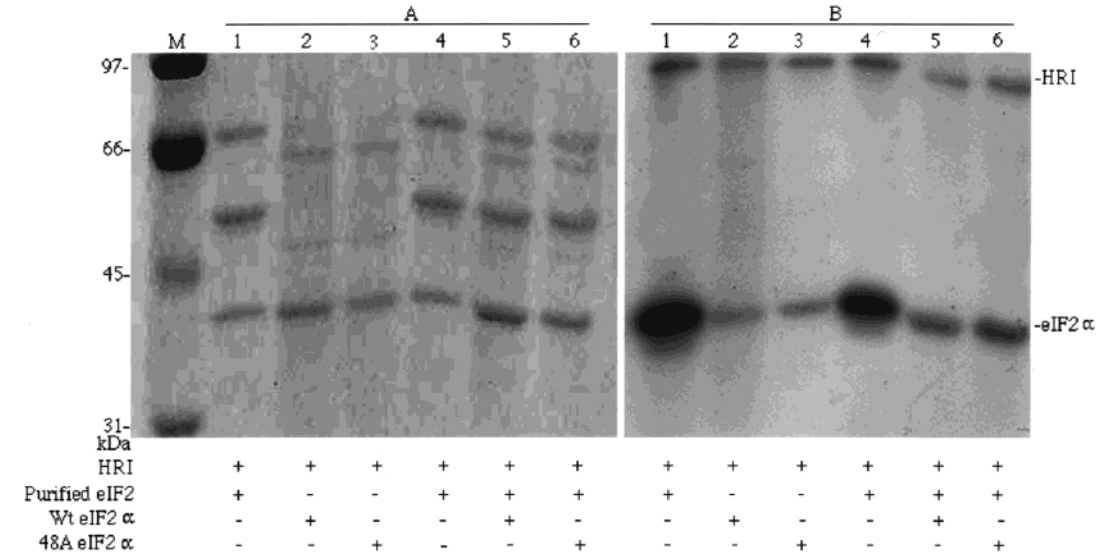

FIGURE 6: Phosphorylation of the α-subunit in purified rabbit reticulocyte trimeric eIF2 in the presence and absence of recombinant subunit eIF2α (wt or 48A mutant) and by purified HRI and [γ-^{32}P]ATP in vitro. Purified eIF2 and partially purified recombinant subunits of eIF2α wt and 48A mutants were phosphorylated as described previously (*23*), and the reaction mixtures were loaded on a 10% SDS−PAGE gel. Panel A is a stained gel, and panel B is the corresponding autoradiogram indicating HRI and eIF2α phosphorylation: lane 1, purified rabbit reticulocyte eIF2 complex; lane 2, partially purified wt recombinant eIF2α; lane 3, partially purified 48A mutant eIF2α; lane 4, purified rabbit reticulocyte eIF2; lane 5, purified rabbit eIF2 and wt eIF2α; and lane 6, purified rabbit eIF2 and 48A mutant eIF2α. HRI is present in all reaction mixtures.

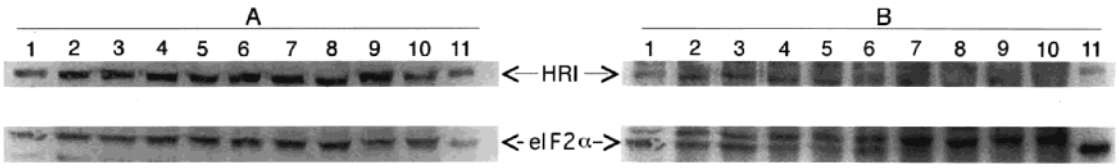

FIGURE 7: Reticulocyte eIF2α phosphorylation by HRI in the presence of uninfected and AcNPV-infected cell extracts. Phosphorylation of reticulocyte eIF2 was carried out in vitro by HRI and [γ-^{32}P]ATP (10 μCi) in a total volume of 20 μL as described previously (*23*) in the presence of different concentrations of uninfected and virus-infected cell extracts. The samples were separated on 10% SDS−PAGE and transferred to a nitrocellulose membrane. The eIF2 and HRI levels were analyzed by using respective monoclonal antibodies as described previously (*38*, *45*), and the immunoblot was analyzed by autoradiography to determine the levels of eIF2 and HRI phosphorylation. Panel A is an immunoblot indicating eIF2α and HRI levels present in the reaction mixtures. Panel B is an autoradiogram of the same gel indicating the phosphorylation of eIF2α and HRI in the presence of insect cell extracts in vitro: lanes 1−5, uninfected cell extracts containing 10, 15, 20, 25, and 30 μg of protein, respectively; lanes 6−10, AcNPV-infected cell extracts containing 10, 15, 20, 25, and 30 μg of protein, respectively; and lane 11, control lane containing HRI and reticulocyte eIF2.

with HRI kinase (Figure 6A,B). Interestingly, it is observed here that the recombinant eIF2α (both wt and 48A mutant) is less efficiently phosphorylated than the eIF2α present in the purified reticulocyte eIF2 complex (Figure 6B, lanes 2 and 3 vs lane 1). In addition, the level of phosphorylation of HRI kinase is also decreased in the presence of recombinant wt or 48A mutant protein (Figure 6B). These experiments were carried out in such a way that protein levels of recombinant eIF2α and the eIF2α in the purified complex were fairly similar as judged by the stain intensity in the gels (Figure 6A). These findings raise the possibility that virus infection may inhibit HRI kinase and thereby eIF2α phosphorylation. This possibility is supported by the observation that phosphorylation of eIF2α in the purified trimeric reticulocyte eIF2 complex is not enhanced but inhibited in the presence of the recombinant eIF2α wt or 48A mutant in vitro (Figure 6B, lane 4 vs lanes 5 and 6). However, the decrease in the level of phosphorylation of eIF2α in the purified complex is similar in the presence of partially purified recombinant wt and 48A mutant eIF2α (lanes 5 and 6). Also, the phosphorylation of the partially purified recombinant wt eIF2α subunit is not different from that of the 48A mutant eIF2α in vitro (Figure 6B, lanes 2 and 3).

To further understand if virus-infected cell extracts reduce the level of eIF2α phosphorylation, different amounts (10−30 μg of protein) of uninfected and AcNPV-infected insect cell extracts were added to the phosphorylation reaction mixtures containing purified reticulocyte eIF2 and HRI (Figure 7). It is observed that the level of reticulocyte eIF2α phosphorylation is reduced significantly (the lower band in the gel in Figure 7B that aligns with the eIF2α of the immunoblot in Figure 7A) in the presence of AcNPV-infected extracts containing 15−30 μg of protein (Figure 7B, lanes 7−10), whereas a similar decrease in the level of eIF2α phosphorylation is not seen in the presence of uninfected cell extracts (Figure 7B, lanes, 1−5) or in the presence of a low concentration (10 μg) of virus-infected extracts (Figure 7B, lane 6). The decrease in the level of eIF2α phosphorylation in the presence of virus-infected extracts appears to be due to a decrease in the level of HRI kinase phosphorylation. Corresponding immunoblots indicating HRI and eIF2 levels are presented in Figure 7A.

Phosphorylation of Recombinant eIF2α in Inhibited Reticulocyte Lysates. In addition, the phosphorylation of the purified recombinant eIF2α wt or 48A mutant is found to be similar in translating hemin-supplemented lysates which

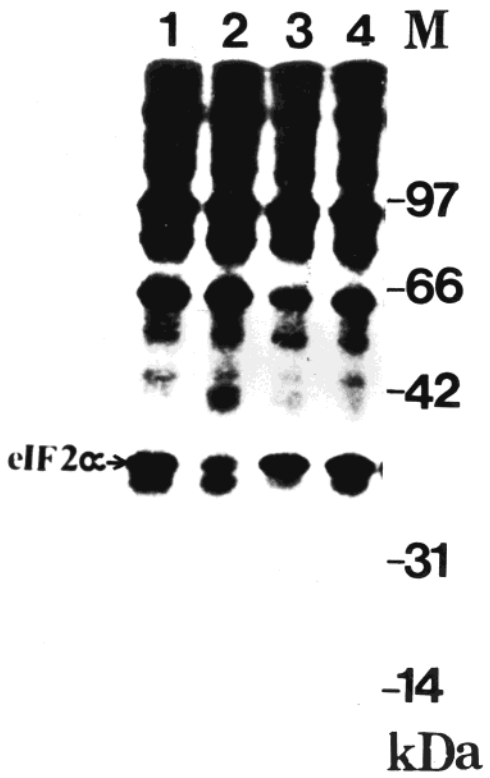

FIGURE 8: Phosphorylation of wt and 48A mutant eIF2α in heme- and poly(IC)-treated reticulocyte lysates. Heme-deficient reticulocyte lysates (20 μL) were prepared, supplemented with hemin (20 μM) or hemin and poly(IC) (300 ng/mL), and incubated with the protein synthesis cocktail at 30 °C for 10 min. The lysates were supplemented, whereever indicated, with partially purified recombinant wt or 48A mutant eIF2α. Phosphorylation of lysates was carried out in the presence of [γ-^{32}P]ATP (20 μCi) for 5 min between 10 and 15 min of protein synthesis. Afterward, 10 μL of the reaction mixtures was taken and precipitated at pH 5.0 as described previously (*23*). Samples were then resuspended in SDS sample buffer and separated on 10% SDS−PAGE. The gel was dried and analyzed by autoradiography: lane 1, heme and poly-(IC); lane 2, heme; lane 3, heme, poly(IC), and wt eIF2α; and lane 4, heme, poly(IC), and the 48A mutant eIF2α.

are treated with poly(IC) (Figure 8, compare lanes 3 and 4). In the absence of poly(IC), eIF2α is less efficiently phosphorylated in hemin-supplemented lysates (Figure 8, lane 2) as expected, and this is consistent with the presence of higher eIF2B activity (Figure 4) and a reduced level of formation of the 15S eIF2α(P)−eIF2B complex (Figure 5A). Although the recombinant eIF2α wt and 48A mutant are phosphorylated to the same extent in poly(IC)-treated reticulocyte lysates (Figure 8, compare lanes 3 and 4), the extents of inhibition of eIF2B activity and formation of 15S complex that occurs in poly(IC)-treated reticulocyte lysates are reduced significantly in the presence of extracts expressing 48A mutant eIF2α compared to those of wt (Figures 4 and 5). These findings thus suggest that phophorylated 48A mutant eIF2α does not inhibit the GDP−GTP exchange activity of eIF2B and is unable to form a 15S complex with eIF2B as efficiently as the phosphorylated wt eIF2α (Figures 4 and 5).

DISCUSSION

Recombinant wild type human eIF2α and a mutant form having a serine replaced with an alanine at position 48 were expressed in insect cells using the baculovirus system to determine the importance of the serine 48 residue in eIF2α in the interaction between eIF2α(P) and eIF2B. Recombinant eIF2α was expressed as approximately 15−20% of the total protein (Figure 1B). The baculovirus system which allows simultaneous expression of multiple genes (*37*) to produce multimeric protein complexes has been used recently to produce wt and Ser51Ala mutant forms of eIF2α for evaluating the phosphorylation site of the variant eIF2α (*39*) and for determining the requirements for the subunit assembly into a functional pentameric eIF2B protein (*40*). This system has previously been used by us to express wild type HRI kinase by cotransfecting the cells with the 51A mutant of eIF2α which bypasses the protein synthesis inhibition caused by the overexpression of wild type HRI (*35*).

The importance of eIF2α phosphorylation in translational control was highlighted through the expression of wt human eIF2α and serine to alanine mutants at residues 48 and 51 (48A and 51A mutants) in mammalian and insect systems (*28*−*33*). Although the 51A mutant cannot be phosphorylated, the 48A mutant can be phosphorylated at its serine 51 residue. Interestingly, expression of either mutant protects protein synthesis in mammalian cells caused by PKR and heat shock (*29*−*31*). These findings suggest that in addition to serine 51, serine 48 is required in inhibiting translation when residue 51 is phosphorylated. Previous biochemical studies using cell free systems derived from rabbit reticulocytes (*18*, *20*) and through genetic and biochemical experiments conducted in yeast (*24*) have shown that phosphorylation of eIF2α results in the inhibition of guanine nucleotide exchange activity of eIF2B and thereby protein synthesis. Results presented here indicate that the baculovirus-expressed 48A mutant of eIF2α decreases the level of inhibition of eIF2B activity caused by poly(IC) treatment in reticulocyte lysates that occurs via the activation of PKR (Figure 4). Phosphorylation of recombinanant eIF2α, wt or 48A mutant, was found not to be different in vitro or in insect cell extracts in the presence of purified HRI kinase or in hemin- and poly(IC)-treated reticulocyte lysates (Figure 3, lanes 3 and 4; Figure 6B, lanes 2 and 3; Figure 8, lanes 3 and 4).

These results are consistent with our previous observations (*38*) showing that the level of inhibition of eIF2B activity that occurs upon heat shock in CHO cells or by addition of purified HRI to cell extracts can be decreased in the presence of the 48A mutant eIF2α. In this latter study (*38*), Ramaiah et al. speculated that the serine 48 residue is required for maintaining a high-affinity interaction between phosphorylated eIF2α and eIF2B. Therefore, we analyzed the formation of eIF2α(P)−eIF2B complexes in hemin- and poly(IC)-treated reticulocyte lysates. Consistent with our earlier prediction (*38*), the extent of formation of the 15S complex between eIF2α(P) and eIF2B was decreased significantly in the presence of insect cell extracts expressing 48A mutant eIF2α, but not in the presence of insect cell extracts from cells expressing wt eIF2α (Figure 5, compare panels D and C).

We additionally observed that the partially purified recombinant subunit of eIF2α was not phosphorylated by HRI as efficiently as the α-subunit present in the trimeric purified reticulocyte eIF2 complex (Figure 6B). The level of phosphorylation of HRI kinase was also decreased under these conditions. It is not clear if this is due to the lack of a

proper conformation in the subunit form due to the absence of the other two subunits as has been suggested previously (*30*) or due to the association of some kind of an inhibitory material with the partially purified recombinant form. However, the intensity of phosphorylation of eIF2α in the three-subunit eIF2 complex was reduced in the presence of either recombinant subunit eIF2α wt or the 48A mutant, and the phosphorylation was not cumulative (Figure 6B). It is likely, therefore, that the recombinant form of eIF2α may be contaminated with an eIF2α kinase inhibitor expressed in insect cells in response to viral infection as suggested recently (*42*). This latter interpretation is consistent with the reduction in the level of eIF2α phosphorylation of purified eIF2 in vitro by HRI kinase in the presence of virus-infected cell extracts (Figure 7B). This appears to be due to a reduction in the level of HRI phosphorylation in the presence of virus-infected extracts. However, the results do not rule out the possibility that the reduction in the level of phosphorylation of the free eIF2α subunit in vitro by HRI may also be due to lack of proper conformation comparable to that in the trimeric eIF2 complex.

The phosphorylation of the 48A mutant was not however significantly different from that of the wt eIF2α in the presence of reticulocyte lysates. Previously, the expressed free subunits were shown to be incorporated into trimeric eIF2 (*30*). Therefore, our results support the possibility that the reduced ability of the 48A mutant to inhibit eIF2B activity is due to the inability of the eIF2 mutant complex to interact with the reticulocyte eIF2B as efficiently as the trimeric complex containing phosphorylated eIF2α wt.

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