Characteristics of the Eukaryotic Initiation Factor 2 Associated 67-kDa Polypeptide†

Manas K. Ray,‡,§ Arup Chakraborty,§ Bansidhar Datta,§,∥ Ansuman Chattopadhyay,§ Debabrata Saha,§ Avirup Bose,§ Terry G. Kinzy,⊥ Shiyong Wu,§ Ronald E. Hileman,§ William C. Merrick,⊥ and Naba K. Gupta*,§

Department of Chemistry, University of Nebraska, Lincoln, Nebraska 68588, and Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106

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ABSTRACT: A eukaryotic initiation factor 2 (eIF-2) associated 67-kDa polypeptide (p^{67}) protects the eIF-2 α-subunit from eIF-2 kinase(s) catalyzed phosphorylation, and this promotes protein synthesis in the presence of active eIF-2 kinase(s), [Datta, B., *et al.* (1988) *Proc. Natl. Acad. Sci. U.S.A. 85,* 3324–3328]. This report presents the results of studies related to characteristics of p^{67} action and the mechanism of p^{67}:eIF-2 interaction: (1) p^{67} antibodies inhibited protein synthesis in hemin-supplemented rabbit reticulocyte lysates, and such inhibition was reversed by preincubation of the antibodies, specifically with p^{67}. (2) p^{67} inhibited HRI- and dsI-catalyzed phosphorylations of the eIF-2 α-subunit and histones, but it did not inhibit casein kinase catalyzed phosphorylation of the eIF-2 β-subunit. (3) p^{67} bound specifically to the eIF-2 γ-subunit. p^{67} co-immunoprecipitated with the eIF-2 subunits when a p^{67}/eIF-2 mixture was treated with p^{67} or eIF-2 subunit antibodies and protein A agarose. However, when eIF-2 was preincubated specifically with the eIF-2 γ-subunit antibodies, subsequent co-immunoprecipitation of p^{67} with the eIF-2 subunits was completely inhibited. Similarly, preincubation of p^{67} and p^{67} antibodies prevented subsequent p^{67} binding to eIF-2. Preincubation of eIF-2, with either eIF-2 α- or β-subunit antibodies, had no effect on p^{67} co-immunoprecipitation with the eIF-2 subunits. (4) p^{67}:eIF-2 interaction is necessary for p^{67} activity to protect the eIF-2 α-subunit from eIF-2 kinase(s) catalyzed phosphorylation. p^{67} bound to eIF-2 only at low Mg^{2+} concentrations (0.5–2 mM) and protected the eIF-2 α-subunit at these Mg^{2+} concentrations. High Mg^{2+} (3–5 mM) completely inhibited p^{67} binding to eIF-2 and also inhibited p^{67} activity to protect the eIF-2 α-subunit. These results suggest that p^{67} binding to eIF-2 is necessary for p^{67} activity to protect the eIF-2 α-subunit from inhibitory phosphorylation. Several factors, such as the p^{67} level in the cell, may affect the equilibrium between p^{67}-bound eIF-2 and free eIF-2 and thus regulate protein synthesis.

Animal cells contain one or more protein synthesis inhibitors, such as HRI (heme-regulated inhibitor) and dsI[1] (double-stranded RNA activated inhibitor). These inhibitors are also protein kinases, and they specifically phosphorylate the α-subunit of eukaryotic initiation factor 2 (eIF-2). This phosphorylation inactivates eIF-2 activity and inhibits protein synthesis. It is generally believed that these inhibitors remain in latent forms and are activated under certain physiological conditions to inhibit protein synthesis [for recent reviews, see Gupta *et al.* (1987) and Hershey (1991)]. In reticulocyte lysates, one of these inhibitors, HRI, is activated in the absence of hemin, and the other inhibitor, dsI, is activated in the presence of double-stranded RNA and ATP. Numerous reports have indicated that animal cells widely use this eIF-2 α-subunit phosphorylation mechanism to regulate protein synthesis under different physiological conditions, which include heat shock (Duncan & Hershey, 1985; Scorsone *et al.*, 1987), nutritional deprivation (Scorsone *et al.*, 1987; Duncan & Hershey, 1984, and virus infection (Whitaker-Dowling & Younger, 1984; Rice & Kerr, 1984; Schneider *et al.*, 1985; Sierkiera *et al.*, 1985).

Recently several laboratories also reported the isolation of several eIF-2 kinase inhibitors from animal cells and provided evidence that these eIF-2 kinase inhibitors promote protein synthesis in the presence of active eIF-2 kinases. These inhibitors are either small RNA molecules (Schneider *et al.*, 1985; Siekierka *et al.*, 1985) or proteins (Chang *et al.*, 1992; Davis *et al.*, 1992; Lee *et al.*, 1992) which inhibit activation of dsI. A protein factor which inhibits the activity of an activated eIF-2 kinase has also been reported to be present in polio virus infected cells (Ransoni & Dasgupta, 1988).

In 1988, Datta *et al.* reported (1988a,b) that a 67-kDa protein (p^{67}) which copurifies with reticulocyte eIF-2 protects the eIF-2 α-subunit from eIF-2 kinase catalyzed phosphorylation, and this promotes protein synthesis in the presence of activated eIF-2 kinase(s). The authors also reported (Datta *et al.*, 1989) that p^{67} is a glycoprotein and contains multiple O-linked GlcNAc residues and that these GlcNAc residues on p^{67} may be necessary for p^{67} activity to protect the eIF-2 α-subunit from eIF-2 kinase(s) catalyzed phosphorylation. An important characteristic of p^{67} is that this protein is easily degradable and also inducible under different physiological conditions, and the level of this protein in the cell correlates directly with the protein synthesis activity of the cells (Ray *et al.*, 1992). For example, it has been observed that p^{67} is deglycosylated and subsequently degraded in heme-deficient reticulocyte lysates and also in serum-starved animal cells in

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‡ Present address: Dept. of Cell Biology, Baylor College of Medicine, Houston, TX 77030.

§ University of Nebraska.

∥ Present address: Dept. of Molecular Biophysics & Biochemistry, Yale University, New Haven, CT.

⊥ Case Western Reserve University.

[1] Abbreviations: eIF-2, eukaryotic peptide chain initiation factor 2 which forms Met-tRNA$_f$·eIF-2·GTP (ternary complex); p^{67}, eIF-2 associated 67-kDa polypeptide; HRI, heme-regulated protein synthesis inhibitor (eIF-2 kinase); dsI, double-stranded RNA activated protein synthesis inhibitor (eIF-2 kinase); WGA, wheat germ agglutinin.

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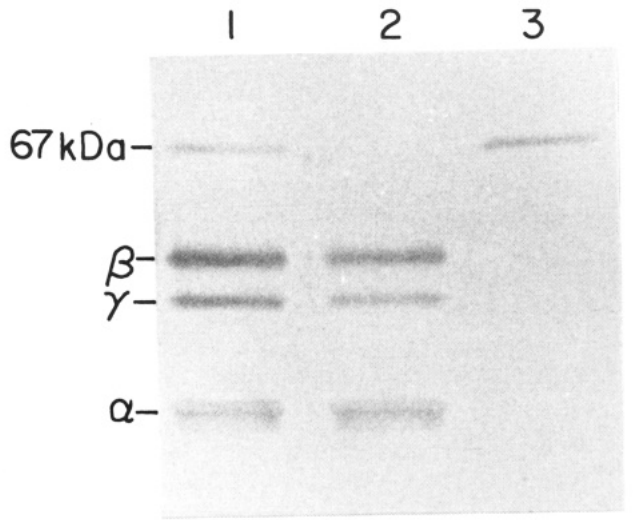

FIGURE 1: SDS–polyacrylamide gel electrophoresis of eIF-2 and p^{67}. The purified proteins were used for polyacrylamide (15%) gel electrophoresis in the presence of sodium dodecyl sulfate at pH 8.3. The electrophoresis was performed at 150 V for 6 h followed by staining using the Bio-Rad silver stain method originally described by Merril *et al.* (1981). Concentrations of the proteins used were as follows: lane 1, fraction IV eIF-2 (1.3 μg); lane 2, fraction V eIF-2 (1 μg); lane 3, p^{67} (0.3 μg).

culture with accompanying inhibition of protein synthesis in both systems. Again, it has been observed that the p^{67} level increases significantly after a mitogen (PMA, phorbol 12-myristate 13-acetate) addition to serum-starved animal cells in culture with an accompanying increase in the protein synthesis activity of the cells. These results indicate that p^{67} plays a critical role in the regulation of protein synthesis in animal cells.

To gain further insights into the mechanism of p^{67} action, several important characteristics of this protein factor have been studied. Presented in this article are the results of these studies related to the mechanism of p^{67} inhibition of eIF-2 kinases and also the characteristics and requirements of p^{67}: eIF-2 interaction.

MATERIALS AND METHODS

Most of the materials and methods used in this work were the same as described previously (Datta *et al.*, 1988a,b; Ray *et al.*, 1992).

The different eIF-2 preparations (with or without associated p^{67}, isolated p^{67}, and the eIF-2 γ-subunit) used in this work were purified following the procedures described previously (Datta *et al.*, 1988a,b, 1989; Ray *et al.*, 1992; Merrick, 1979). Figure 1 shows a silver-stained SDS–PAGE picture of the purified eIF-2 and p^{67} preparations. No other polypeptide band was visible in the gel picture. HRI was purified as described previously (Trachsel *et al.*, 1978). A sample of dsI, prepared according to Kuklicki *et al.* (1987), was generously donated by Dr. Gisela Kramer (Clayton Foundation, Biochemistry Inst., University of Texas, Austin, TX). Dr. David W. Litchfield (University of Washington, Seattle, WA) kindly supplied us with a sample of casein kinase II prepared according to a previously described procedure (Litchfield *et al.*, 1990).

Preparation of Polyclonal Antibodies against Isolated eIF-2 Subunits and p^{67}. Polyclonal antibodies against eIF-2 α-, β-, and γ-subunits and p^{67} were prepared using a modification of the procedure described previously (Datta *et al.*, 1988a). As before, the fraction IV eIF-2 preparation was subjected to $NaDodSO_4$/15% PAGE, and the individual polypeptide bands were isolated from the gel by electroelution. Each electroeluted protein (25 μg) was then emulsified with an equal volume of Freund's complete adjuvant and injected into the peritoneal cavity of a mouse. After 15 days, three booster injections of 25 μg of isolated polypeptide in Freund's incomplete adjuvant were given at 1-week intervals. Blood was collected by tail bleeding, and the antibody titer in the serum was tested by ELISA using a β-galactosidase conjugate. When antibody titer in the serum was over 1:1000, the mouse was injected with TG-180 sarcoma cells (obtained from American Type Cell Culture) for the generation of ascitic fluid. Antibodies from the ascitic fluid were purified by the use of DEAE Affi-Gel Blue, according to the procedure supplied by the manufacturer (Bio-Rad), and were characterized by $NaDodSO_4$/PAGE followed by immunoblotting. The results of this immunoblot experiment are shown in Figure 2. Fraction IV eIF-2 containing p^{67} was used as the antigen in this experiment. As shown in Figure 2, all of the polyclonal antibodies were monospecific and reacted only with the corresponding antigen. In control experiments, we also used preimmune mouse serum, which did not react with either eIF-2 subunits or p^{67}.

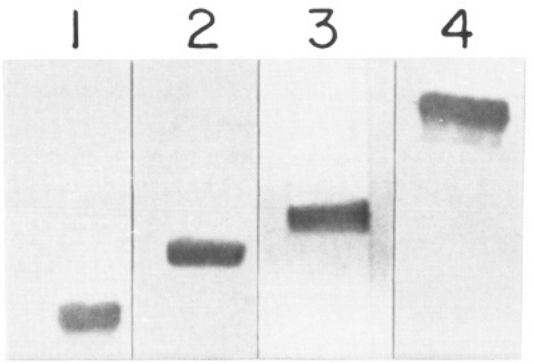

FIGURE 2: Characterization of the antibodies. Approximately 2 μg of fraction IV eIF-2 (eIF-2 containing p^{67}) was subjected to SDS–PAGE and electrotransferred to nitrocellulose sheets following the procedure provided by Bio-Rad (Trans-Blot Cell Operating Instruction, 1985). The nitrocellulose sheets were then incubated with the antibodies following the procedure described by the Bio-Rad Immunoblot Assay Kit. The antigen–antibody complexes were then detected using goat anti-mouse IgG conjugated horseradish peroxidase (Bio-Rad). Lane 1, eIF-2 α antibody; lane 2, eIF-2 γ antibody; lane 3, eIF-2 β antibody; lane 4, p^{67} antibody.

Phosphorylation of eIF-2 α-Subunits Using eIF-2 Kinases. Phosphorylation of the eIF-2 α-subunit using purified eIF-2 kinases (HRI and dsI) was carried out using the procedure described (Datta *et al.*, 1988a).

Interaction of p^{67} with eIF-2 Subunits. Interaction of p^{67} with eIF-2 subunits was studied using a co-immunoprecipitation method previously described by Bauer *et al.*, (1980). Standard reaction mixtures contained, in a total volume of 25 μL, 20 mM Tris-HCl, pH 7.8, 100 mM potassium chloride, 1 mM $MgCl_2$, 10 μg of bovine serum albumin, 2 mM dithiothreitol, 3 μg of eIF-2 (fraction V), and 1 μg of p^{67}. The reaction mixtures were incubated in ice for 1 h. Polyclonal antibodies (10 μg) against each of the eIF-2 subunits were then added separately to each tube, and the reactions were further incubated overnight at 4 °C. The reaction mixtures were then mixed with 25 μL of protein A agarose (BRL) and further incubated at room temperature for 2 h. Next the antigen–antibody complexes were precipitated by centrifugation, and the precipitates were washed extensively with phosphate-buffered saline (PBS, GIBCO) containing 0.05% NP-40 and subjected to SDS–PAGE. Proteins were then electrolytically transferred onto nitrocellulose papers and

Table I: Effects of Control and Immune Serum against p^{67} on Protein Synthesis in Reticulocyte Lysates[a]

samples	additions	[^{14}C]leucine incorporation/ 20-μL incubation mixtures (pmol) –hemin	+hemin
1	none	31	97
2	preimmune serum	–	93
3	p^{67}	–	99
4	anti-p^{67}	–	36
5	anti-p^{67} + p^{67} (1 μg)	–	78
6	anti-p^{67} + p^{67} (1.6 μg)	–	97
7	anti-p^{67} + three-subunit eIF-2 (2.4 μg)	–	42
8	anti-p^{67} + four-subunit eIF-2 (2.4 μg)	–	96

[a] Reaction mixtures (25 μL) contained 50% by volume reticulocyte lysate, 10 mM Tris-HCl, pH 7.8, 1 mM magnesium acetate, 100 mM potassium chloride, 0.2 mM GTP, 30 μM concentration each of 19 amino acids (leucine), 56 μM [^{14}C]leucine (250 cpm/pmol), 5 mM creatine phosphate, 10 μg/mL creatine phosphokinase, 20 μM hemin, and where indicated, preimmune serum (20 μg) or Affi-Gel Blue purified anti-p^{67}IgG (10 μg), 6 μg of p^{67}, three-subunit eIF-2, and four-subunit eIF-2. For samples 5–8, the peptide chain initiation factors (p^{67} and eIF-2) were preincubated with Affi-Gel Blue purified anti-p^{67}IgG at 30 °C for 30 min and then further incubated in ice for 2 h. The reaction mixtures were then mixed with other components of protein synthesis and were further incubated at 30 °C for 40 min. [^{14}C]Leucine incorporation into protein was determined in 20 μL of the reaction mixture.

analyzed by immunoblotting using either p^{67} antibodies or eIF-2 α-subunit antibodies.

RESULTS

Inhibition of Protein Synthesis by p^{67} Antibodies. We previously reported that the addition of p^{67} antibodies to hemin-supplemented reticulocyte lysate promoted eIF-2 α-subunit phosphorylation, presumably by depletion of endogenous p^{67}. Consistent with that report, we have now observed that the addition of p^{67} antibodies strongly inhibited protein synthesis in hemin-supplemented reticulocyte lysate (Table I). As can be seen, addition of preimmune serum (sample 2) or p^{67} (sample 3) had no effect on protein synthesis. However, addition of Affi-Gel Blue purified anti-p^{67}IgG against p^{67} strongly inhibited protein synthesis (sample 4), and such inhibition was overcome by preincubation of the purified IgG, specifically with isolated p^{67} (samples 5 and 6) or eIF-2 containing p^{67} (sample 8). A homogeneous eIF-2 preparation devoid of p^{67} was ineffective in this reversal reaction (sample 7).

Specificity of p^{67} Action. We studied the specificity of p^{67} action using three different eIF-2 kinases (Figure 3). In Figure 3A we present the results of our studies using HRI and casein kinase. HRI efficiently phosphorylated the eIF-2 α-subunit (lane 1), and the addition of p^{67} at or near concentrations equimolar to eIF-2 almost completely inhibited this phosphorylation reaction (lanes 2 and 3). However, as shown in lanes 4–6, casein kinase phosphorylated the eIF-2 β-subunit (lane 4), and p^{67} at concentrations similar to those used in the experiments described in lanes 2 and 3 had no effect on this phosphorylation reaction. Figure 3B shows that dsI, like HRI, also phosphorylated the eIF-2 α-subunit (lane 3), and again p^{67} at near equimolar concentrations to eIF-2 inhibited this phosphorylation reaction (lanes 4 and 5). The results presented in Figure 3C show the effects of addition of p^{67} on dsI-catalyzed phosphorylation of histones at varying histone concentrations. Two or more species of histones were phosphorylated with varying efficiencies. The lower molecular weight histone migrating closely with the dye front was phosphorylated more efficiently than higher molecular weight histones. Again, addition of p^{67} at or near equimolar concentrations efficiently

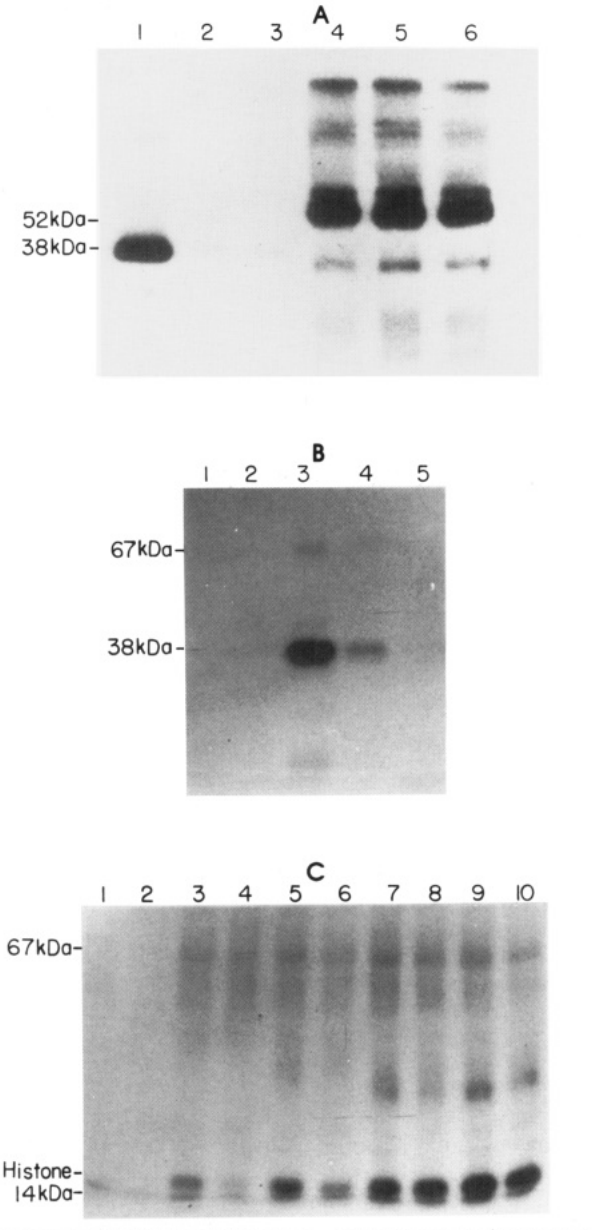

FIGURE 3: Specificities of p^{67} action. (A) Effects of p^{67} on HRI-catalyzed phosphorylation of the eIF-2 α-subunit and casein kinase catalyzed phosphorylation of the eIF-2 β-subunit. The reaction conditions for eIF-2 kinase(s) catalyzed phosphorylation were similar to those described previously (Datta *et al.*, 1988; Ray *et al.*, 1992). The reaction mixtures contained (total volume, 25 μL) 20 μM Tris-HCl, pH 7.8, 100 mM potassium chloride, 10 μg of bovine serum albumin, 2 mM dithiothreitol and, where indicated, 3 μg of eIF-2 (fraction V), and p^{67}. The reactions were started by addition of either 0.02 μg of HRI or 6 μg of casein kinase and 10 μCi of [γ-^{32}P]-ATP (sp act 4500 Ci/mmol; ICN), and the mixtures were further incubated at 37 °C for 10 min. Lane 1, eIF-2 + HRI; lane 2, eIF-2 + HRI + 1.2 μg of p^{67}; lane 3, eIF-2 + HRI + 1.6 μg of p^{67}; lane 4, eIF-2 + casein kinase; lane 5, eIF-2 + casein kinase + 1.2 μg of p^{67}; lane 6, eIF-2 + casein kinase + 1.6 μg of p^{67}. (B) Effects of p^{67} on dsI-catalyzed phosphorylation of the eIF-2 α-subunit. The reaction conditions were the same as those described in A. Approximately 3 μg of eIF-2 and 0.05 μg of dsI were used. Lane 1, dsI alone; lane 2, dsI + 1.2 μg of p^{67}; lane 3, eIF-2 + dsI; lane 4, eIF-2 + dsI + 1.2 μg of p^{67}; lane 5, eIF-2 + dsI + dsI + 2.4 μg of p^{67}. (C) Effects of p^{67} on dsI-catalyzed phosphorylation of histones. The reaction conditions were the same as those described in A. Approximately 0.05 μg of dsI and 1 μg of p^{67} were used in the presence of varying concentrations of histones. Lane 1, dsI alone; lane 2, dsI + p^{67}; lane 3, dsI + 0.1 μg of histones; lane 4, dsI + 0.1 μg of histones + p^{67}; lane 5, dsI + 0.2 μg of histones; lane 6, dsI + 0.2 μg of histones + p^{67}; lane 7, dsI + 0.3 μg of histones; lane 8, dsI + 0.3 μg of histones + p^{67}; lane 9, dsI + 0.4 μg of histones; lane 10, dsI + 0.4 μg of histones + p^{67}.

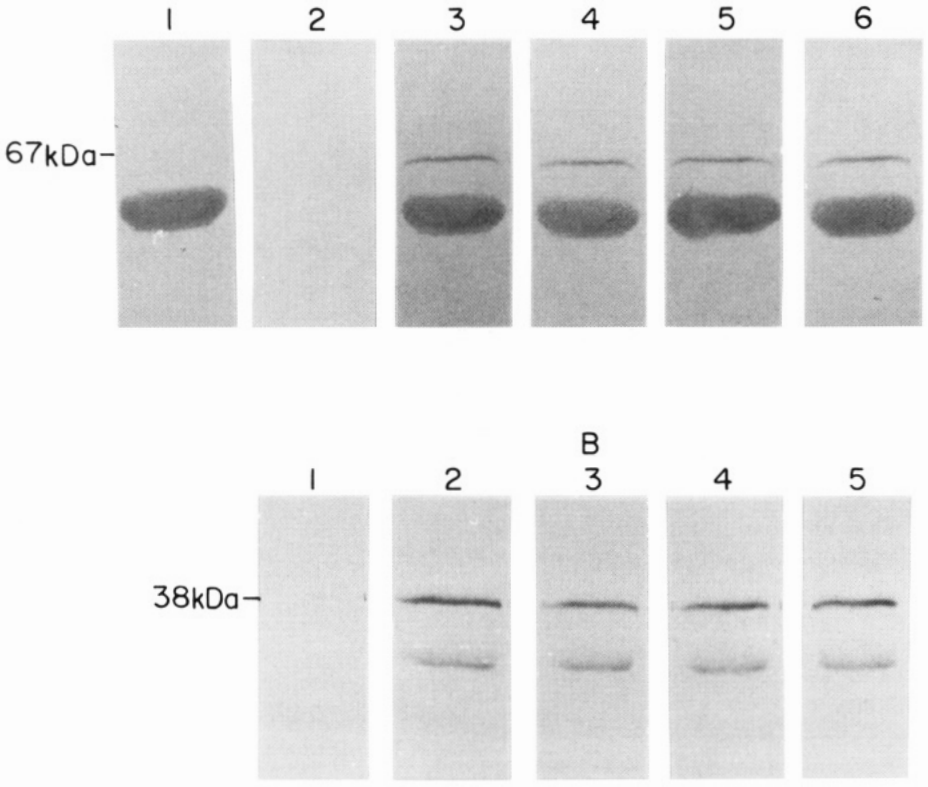

FIGURE 4: Co-immunoprecipitation of p^{67} with the eIF-2 subunits present in reticulocyte lysates. The reactions were carried out in two steps. In step I, reticulocyte lysates (40 μL) were mixed with antibodies (10 μg) against either eIF-2 α, β, γ, or p^{67}, and the reaction mixtures were incubated at 4 °C overnight. In step II, 25 μL of protein A agarose (BRL) was added to each reaction mixture, and the reaction mixtures were further incubated at room temperature for 1 h. The precipitates containing antigen–antibody complexes were then washed extensively with 1× phosphate-buffered saline (PBS, GIBCO) containing 0.05% NP-40 and were then analyzed by SDS–PAGE followed by immunoblotting. For immunoblotting, the nitrocellulose papers were cut into two halves. The upper half, containing p^{67}, was developed using p^{67} antibodies, and the lower half, containing the eIF-2 α-subunit, was developed using eIF-2 α-subunit antibodies. (A) Lane 1, –lysate + p^{67} antibodies; lane 2, +lysate + preimmune serum; lane 3, +lysate + p^{67} antibodies; lane 4, +lysate + eIF-2 γ antibodies; lane 5, +lysate + eIF-2 β antibodies; lane 6, lysate + eIF-2 α antibodies. (B) Lane 1, lysate + preimmune serum; lane 2, lysate + p^{67} antibodies; lane 3, lysate + eIF-2 γ antibodies; lane 4, lysate + eIF-2 β antibodies; lane 5, lysate + eIF-2 α antibodies.

inhibited the phosphorylation of this lower molecular weight histone (lanes 4 and 6). However, at higher histone concentrations p^{67} failed to inhibit this phosphorylation reaction.

We previously reported that WGA inhibited p^{67} activity to protect the eIF-2 α-subunit from HRI-catalyzed phosphorylation (Datta *et al.*, 1989). This result indicated that the glycosyl residues on p^{67} may be necessary for p^{67} activity. We have now observed that addition of WGA similarly inhibited p^{67} activity to protect both the eIF-2 α-subunit and histones from dsI-catalyzed phosphorylation (data not shown). A similar mechanism, possibly involving the glycosyl residues, may be responsible for p^{67} activity in all of these cases.

As shown in Figure 3, one or more polypeptides were also phosphorylated under the experimental conditions, and p^{67} protected these phosphorylation reactions. For example, significant phosphorylation of a 67-kDa protein, presumably dsI in this case, was observed (panel B, lane 3), and this phosphorylation reaction was also inhibited by p^{67} (panel B, lanes 4 and 5). Interestingly, this phosphorylation of dsI appeared to be linked to eIF-2 α-subunit phosphorylation. No phosphorylation of dsI was observed when dsI alone was incubated with [γ-^{32}P]ATP in the absence (panel B, lane 1) or presence of p^{67} (panel B, lane 2). The significance of this dsI phosphorylation dependence on eIF-2 α-subunit phosphorylation will be discussed later.

Mechanism of p^{67}:eIF-2 Interaction. The p^{67} binds to eIF-2. The results presented in Figure 4 provide evidence that the p^{67} in reticulocyte lysate remains associated with three-subunit eIF-2 and can be co-immunoprecipitated with the eIF-2 subunits. In these experiments, reticulocyte lysate was mixed with either preimmune serum or with polyclonal antibodies against p^{67} and eIF-2 γ-, β-, and α-subunit antibodies. The antigen–antibody complexes were then precipitated using protein A agarose, and the precipitates were analyzed by immunoblotting using either p^{67} antibodies (panel A) or eIF-2 α-antibodies (panel B).

As shown in Figure 4A, p^{67} in the lysate was precipitated not only with p^{67} antibodies (lane 3) but also with eIF-2 γ (lane 4), β (lane 5), and α (lane 6) antibodies. No precipitation was observed when preimmune serum was used (lane 2) or when an antibody alone (lane 1) was used. The lower molecular weight band in panel A represents the heavy chain of immunoglobulin. Similarly, the results in Figure 4B show that the eIF-2 α-subunit is precipitated, not only with the eIF-2 γ (lane 3), β (lane 4), and α (lane 5) antibodies but also with p^{67} antibodies (lane 2). Again, no precipitate was observed when preimmune serum was used (lane 1). In this figure, the lower molecular weight band represents the light chain of immunoglobulin.

These results suggest that p^{67} in the reticulocyte lysates remains associated with three-subunit eIF-2. We performed similar experiments using isolated three-subunit eIF-2 and p^{67} and the results were similar. p^{67} bound to the three-subunit eIF-2, and both p^{67} and the eIF-2 α-subunit in a mixture could be immunoprecipitated with either eIF-2 subunit antibodies of p^{67} antibodies.

In a similar series of experiments using purified eIF-2 and p^{67}, we included WGA during preincubation of p^{67} with eIF-2

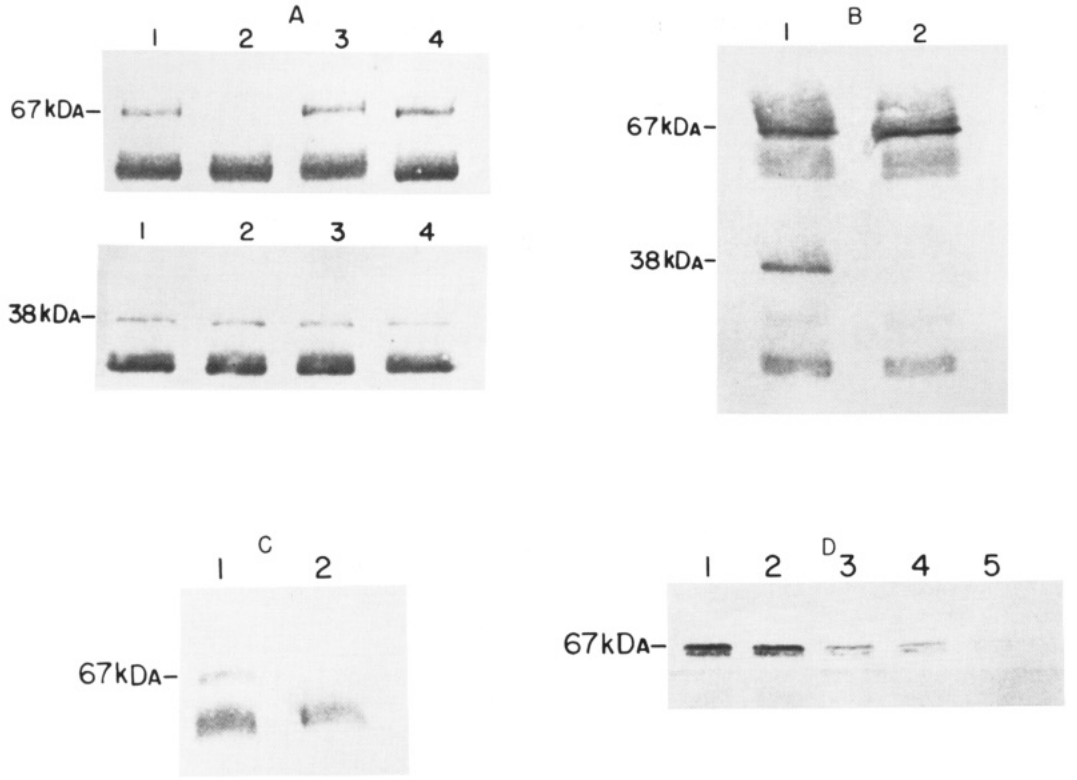

FIGURE 5: Specific interaction between p^{67} and the eIF-2 γ-subunit. (A) Effects of preincubation of eIF-2 with different antibodies on subsequent p^{67} binding to eIF-2. We carried out the reaction in three steps. In step I, the reaction mixtures contained (total volume, 25 μL) 20 mM Tris-HCl, pH 7.8, 100 mM potassium chloride, 10 μg of bovine serum albumin, and 2 nM dithiothreitol. In addition, in the experiments described in lane 1, the reaction mixtures contained 3 μg of eIF-2 (fraction V) and 1 μg of p^{67}, and in the experiments described in lanes 2–4, the reaction mixtures contained 3 μg of eIF-2 and different antibodies (10 μg). The reaction mixtures were incubated in ice for 1 h. In step II, the reaction mixture described in lane 1 was mixed with eIF-2 γ-subunit antibodies, and the reaction mixtures described in lanes 2–4 were mixed with 1 μg of p^{67}. The reaction mixtures were then incubated at 4 °C overnight. In step III, the antigen-antibody complex were precipitated using protein A agarose (BRL) and were analyzed by immunoblotting using either p^{67} (panel A) or eIF-2 α (panel B) antibodies, as described in Figure 4. The antibodies used during preincubation with eIF-2 in step I were as follows: lane 2, eIF-2 γ antibodies; lane 3, eIF-2 α antibodies; lane 4, eIF-2 β antibodies. (B) Effects of preincubation of p^{67} with p^{67} antibodies on subsequent p^{67} binding to eIF-2. The reaction conditions were the same as those described in A. In the experiment described in lane 1, eIF-2 + p^{67} were premixed and were then coimmunoprecipitated with p^{67} antibodies. In the experiment described in lane 2, p^{67} was preincubated with p^{67} antibodies and was then mixed with eIF-2. For immunoblotting, the nitrocellulose paper, in each case, was developed using a mixture of p^{67}/eIF-2 α-subunit antibodies. (C) Effects of preincubation of the eIF-2 γ-subunit with eIF-2 γ-subunit antibodies on subsequent eIF-2 γ-subunit:p^{67} interaction. The reaction conditions were the same as those described in A. In the experiment described in lane 1, 1 μg of isolated eIF-2 γ-subunit and p^{67} were premixed and were then co-immunoprecipitated with eIF-2 γ-subunit antibodies. In the experiment described in lane 2, isolated eIF-2 γ-subunit was preincubated with eIF-2 γ-subunit antibodies and was then mixed with isolated p^{67}. The reaction mixtures were then used for immunoblotting using p^{67} antibodies. (D) Effects of preincubation of p^{67} with increasing concentrations of isolated eIF-2 γ-subunit on subsequent binding of p^{67} to eIF-2. The reaction conditions were the same as those described in A. p^{67} (1 μg) was preincubated with increasing concentrations of isolated eIF-2 γ-subunit at room temperature for 1 h. This reaction mixture was then mixed with eIF-2 (fraction V, 3 μg). The reaction mixtures were then treated with eIF-2 α-subunit antibodies, and p^{67} binding to eIF-2 was assayed by immunoblotting using p^{67} antibodies. Lane 1, p^{67} alone; lane 2, p^{67} + 0.2 μg of eIF-2 γ-subunit; lane 3, p^{67} + 0.4 μg of eIF-2 γ-subunit; lane 4, p^{67} + 0.8 μg of eIF-2 γ-subunit; lane 5, p^{67} + 1 μg of eIF-2 γ-subunit.

and studied the effects of WGA addition on p^{67} binding to eIF-2. The results were precisely the same as those described in Figure 4A. WGA had no effect on p^{67} binding to eIF-2.

The p^{67} Binds Specifically to the eIF-2 γ-Subunit. A modification of the co-immunoprecipitation method described above was used to demonstrate that p^{67} binds specifically to the eIF-2 γ-subunit (Figure 5). In the experiments described in Figure 5A–C, one of the interacting proteins, either three-subunit eIF-2, isolated eIF-2 γ-subunit, or p^{67}, was preincubated with a specific antibody (eIF-2 α, β, or γ or p^{67}) to block the protein binding site. The effect of such preincubation on subsequent p^{67}:eIF-2 interaction was studied using the co-immunoprecipitation and immunoblotting procedure described in Figure 4.

In the experiments described in Figure 5A, eIF-2 containing three subunits was preincubated with either eIF-2 γ (lane 2), α (lane 3), or β (lane 4) antibodies. The reaction mixtures were then mixed with p^{67}, and subsequent p^{67} binding to eIF-2 subunits was studied. In the experiment described in panel A, lane 1 served as a control. In this experiment, eIF-2 and p^{67} were premixed and the mixture was then treated with eIF-2 γ-subunit antibodies. p^{67} co-immunoprecipitated with the eIF-2 subunit, when it was premixed with eIF-2 and subsequently treated with eIF-2 γ-subunit antibodies (lane 1). However, when the three-subunit eIF-2 was preincubated specifically with the eIF-2 γ-subunit antibodies, co-immunoprecipitation of p^{67} with the eIF-2 subunits was completely inhibited (Figure 5A, top, lane 2). Preincubation of eIF-2 with eIF-2 α- and β-subunit antibodies had no effect on co-immunprecipitation of p^{67} with the eIF-2 subunits (Figure 5A, top, lanes 3 and 4). The experiments described in the bottom panel showed that preincubation of eIF-2 with these antibodies had no effect on co-immunoprecipitation of the eIF-2 α-subunit with other eIF-2 subunits.

In the experiments described in Figure 5B, the effects of preincubation of p^{67} with p^{67} antibodies on subsequent binding of p^{67} to eIF-2 were studied. As described in panel B, lane 1, in which p^{67} was initially mixed with eIF-2 and then treated

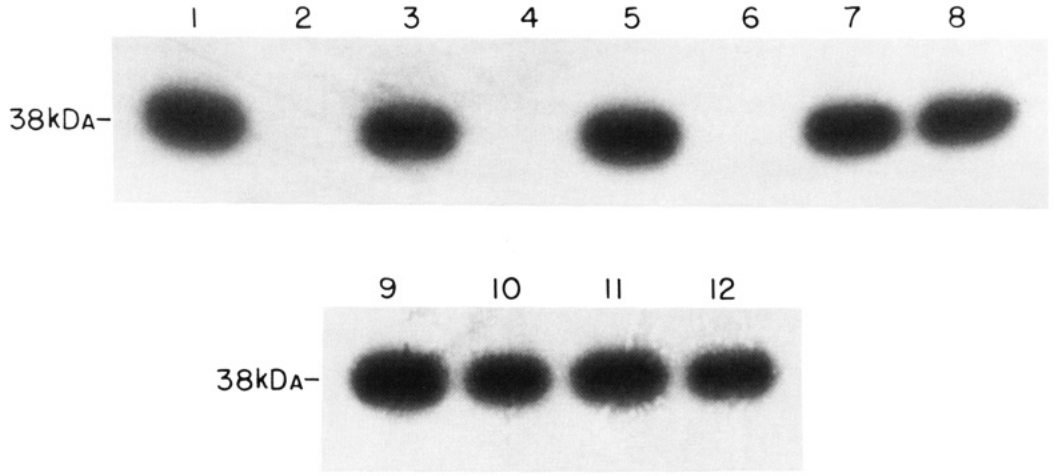

FIGURE 6: Effects of the addition of different antibodies and WGA on p^{67} activity to protect the eIF-2 α-subunit from HRI-catalyzed phosphorylation. The reaction conditions were the same as those described in Figure 3A. Where indicated, different antibodies (10 μg) or WGA was added. All of the reaction mixtures contained eIF-2 (fraction V, 3 μg) and HRI (0.02 μg). In addition, the reaction mixtures also contained the following: lane 1, nothing else; lane 2, p^{67}; lane 3, eIF-2 α-antibodies; lane 4, p^{67} + eIF-2 α antibody; lane 5, eIF-2 β antibodies; lane 6, p^{67} + eIF-2 β antibodies; lane 7, eIF-2 γ antibodies; lane 8, p^{67} + eIF-2 γ antibodies; lane 9, p^{67} antibodies; lane 10, p^{67} + p^{67} antibodies; lane 11, WGA; lane 12, p^{67} + WGA.

with p^{67} antibodies, p^{67} and the eIF-2 α-subunit were both co-immunoprecipitated. However, prior incubation of p^{67} with p^{67} antibodies almost completely inhibited subsequent p^{67} binding to eIF-2, as evidenced by the complete absence of the eIF-2 α-subunit in the immunoprecipitate (panel B, lane 2).

In the experiments described in Figure 5C, a similar experimental procedure was used to study interaction between isolated eIF-2 γ-subunit and isolated p^{67}. Again, when the eIF-2 γ-subunit was preincubated with p^{67} and the mixture was subsequently treated with eIF-2 γ-subunit antibodies, p^{67} co-immunoprecipitated with the eIF-2 γ-subunit (panel C, lane 1). However, when the eIF-2 γ-subunit was preincubated with eIF-2 γ-subunit antibodies, subsequent binding of p^{67} to the eIF-2 γ-subunit was completely inhibited (panel C, lane 2). It is expected that eIF-2 γ-subunit antibodies would react with the eIF-2 γ-subunit and would be precipitated with protein A agarose under the experimental conditions. However, this experiment was not done as the eIF-2 γ-subunit would overlap with the heavy chain of IgG.

The effects of the addition of increasing concentrations of isolated eIF-2 γ-subunit on subsequent p^{67} binding to three-subunit eIF-2 were studied, and the results are presented in Figure 5D. In these experiments isolated eIF-2 γ-subunit in increasing concentrations and isolated p^{67} were preincubated, and the reaction mixtures were mixed with three-subunit eIF-2. The binding of p^{67} to three-subunit eIF-2 was then studied after immunoprecipitation with eIF-2 α-subunit antibodies and subsequent immunoblotting using the p^{67} antibodies. As shown in panel D, addition of increasing concentrations of isolated eIF-2 γ-subunit progressively inhibited p^{67} binding to three-subunit eIF-2.

Requirement of eIF-2:p^{67} Interaction for p^{67} Action To Protect the eIF-2 α-Subunit from eIF-2 Kinase Catalyzed Phosphorylation. Two different lines of investigations were carried out to demonstrate that eIF-2:p^{67} interaction is necessary for p^{67} action to protect the eIF-2 α-subunit from eIF-2 kinase catalyzed phosphorylation.

(*1*) *Effects of Antibodies.* The results presented in Figure 6 show the effects of the addition of different antibodies on p^{67} activity to protect the eIF-2 α-subunit from HRI-catalyzed phosphorylation. As before, HRI efficiently phosphorylated the eIF-2 α-subunit (lane 1), and p^{67} inhibited this phosphorylation reaction (lane 2). Addition of eIF-2 α-subunit antibodies (lane 4) and β-subunit antibodies (lane 6) had no effect on p^{67} protection of the eIF-2 α-subunit from phosphorylation. However, addition of either eIF-2 γ-subunit antibodies (lane 8), p^{67} antibodies (lane 10), or WGA (lane 12) almost completely inhibited p^{67} activity to protect the eIF-2 α-subunit from eIF-2 kinase catalyzed phosphorylation. None of these antibodies (α, β, γ, or p^{67}) or WGA, when added in the absence of p^{67}, had any effect on HRI-catalyzed phosphorylation of the eIF-2 α-subunit (lanes 3, 5, 7, 9, and 11).

It is expected that p^{67} antibodies bound to p^{67} and thus inactivated p^{67} activity to protect the eIF-2 α-subunit (lane 10). We previously reported (Datta *et al.*, 1989) that WGA bound to the glycosyl residues on p^{67} and also inhibited p^{67} activity (lane 12). The mechanism of inhibition of p^{67} activity by eIF-2 γ-subunit antibodies is not clear. In these experiments, eIF-2 and p^{67} were premixed, and as described in Figure 5 (panel A, lane 1), subsequent addition of eIF-2 γ-subunit antibodies did not inhibit p^{67} binding to the eIF-2 γ-subunit. The inhibition of p^{67} activity observed with eIF-2 γ-subunit antibodies may, therefore, indicate that p^{67} needs to interact at more than one site on eIF-2. eIF-2 γ-subunit antibodies interfere with one ore more of these interactions necessary for p^{67} activity to protect eIF-2 α-subunits from phosphorylation. Another possibility would be that the binding of the eIF-2 γ-subunit antibodies causes the subunit to undergo a conformational change. This change could alter the way that p^{67} binds to the eIF-2 γ-subunit, or it could alter the conformation of the eIF-2 α-subunit and expose the phosphorylation site.

(*2*) *Mg^{2+} Effect.* We previously reported that a high Mg^{2+} concentration (5 mM) inhibits interactions between eIF-2 and eIF-2 ancillary protein factors (Bagchi *et al.*, 1985). The results presented in Figure 7 show that high Mg^{2+} concentrations (3–5 mM) also inhibit p^{67} binding to eIF-2. In these experiments, eIF-2 was preincubated with p^{67} in the presence of varying Mg^{2+} concentrations in ice for 2 h. p^{67} binding to eIF-2 was subsequently assayed by the standard co-immunoprecipitation method using eIF-2 γ-subunit antibodies, as described in Figure 4. As shown in Figure 7, p^{67} bound efficiently to eIF-2 at 1 and 2 mM Mg^{2+} (panel A, lanes 1 and 2), and this binding was completely inhibited at 3 and 5 mM Mg^{2+} (panel A, lanes 3 and 4). High Mg^{2+} concen-

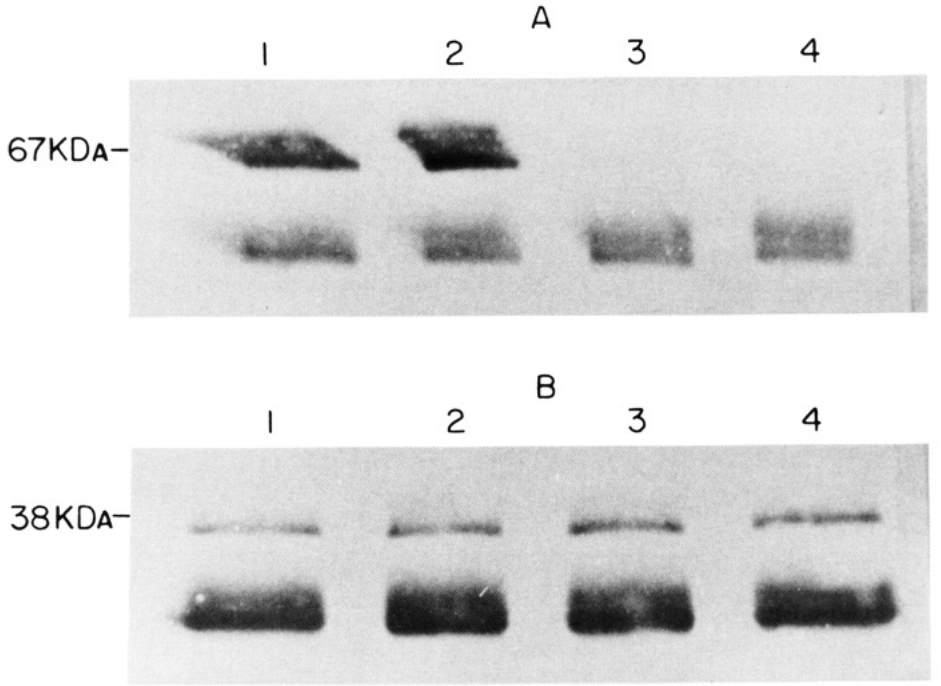

FIGURE 7: Effects of Mg^{2+} on p^{67} binding to eIF-2. The reaction conditions were the same as those described in Figure 5A, except that the Mg^{2+} concentration was varied as indicated. eIF-2/p^{67} mixtures were precipitated using eIF-2 γ antibodies and protein A agarose and were immunoblotted using either p^{67} antibodies (A) or eIF-2 α antibodies (B). Mg^{2+} concentrations used in different experiments were as follows: lane 1, 1 mM Mg^{2+}; lane 2, 2 mM Mg^{2+}; lane 3, 3 mM Mg^{2+}; lane 4, 5 mM Mg^{2+}.

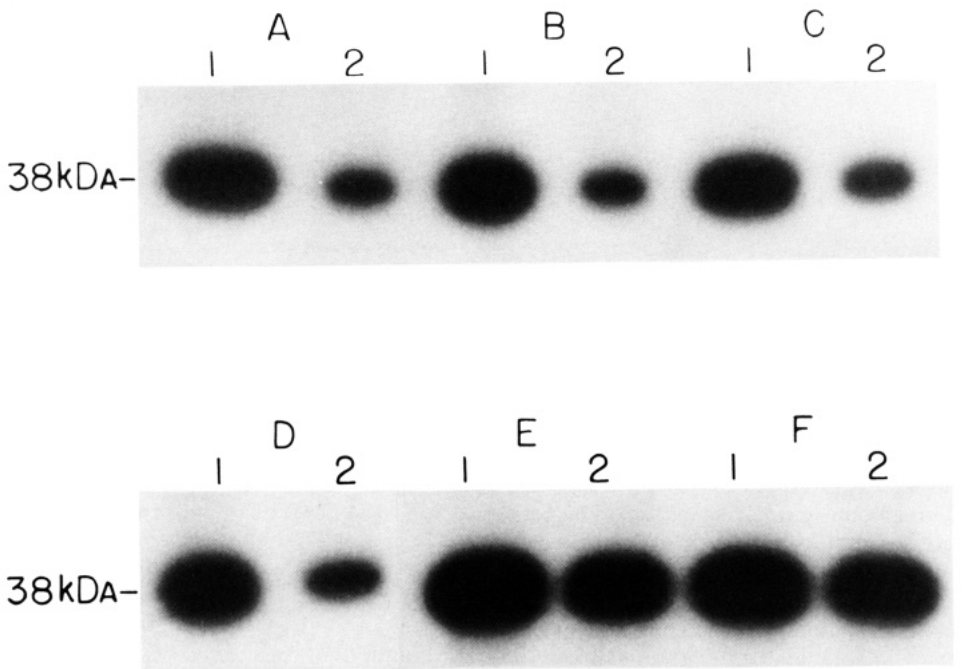

FIGURE 8: Effects of Mg^{2+} concentrations of p^{67} activity to protect the eIF-2 α-subunit from HRI-catalyzed phosphorylation. The reaction conditions were the same as those described in Figure 3, except that different Mg^{2+} concentrations were used as indicated. All of the reactions described in lane 1 in each panel contained eIF-2 alone and those described in lanes 2 contained eIF-2 + p^{67}. (A) 0.5 mM Mg^{2+}; (B) 1 mM Mg^{2+}; (C) 1.5 mM Mg^{2+}; (D) 2 mM Mg^{2+}; (E) 3 mM Mg^{2+}; (F) 5 mM Mg^{2+}.

trations, however, did not dissociate the eIF-2 subunits. The eIF-2 α-subunit was co-immunoprecipitated with the γ-subunit antibodies at all of the Mg^{2+} concentrations tested (panel B, lanes 1–4).

The results presented in Figure 8 show the effects of the addition of different Mg^{2+} concentrations on p^{67} activity to protect the eIF-2 α-subunit from eIF-2 kinase catalyzed phosphorylation. HRI phosphorylated eIF-2 at all Mg^{2+} concentrations used, and the extent of this phosphorylation was somewhat greater at higher Mg^{2+} concentrations (lane 1, panels A–F). However, p^{67} activity to protect the eIF-2 α-subunit was observed only at low Mg^{2+} (lanes 2: panel A, 0.5 mM Mg^{2+}; panel B, 1 mM Mg^{2+}; panel C, 1.5 mM Mg^{2+}; and panel D, 2 mM) and not at high Mg^{2+} (lanes 2: panel E, 3 mM Mg^{2+}; panel F, 5 mM Mg^{2+}). These results, in agreement with the co-immunoprecipitation data shown in Figure 7, suggest that p^{67}:eIF-2 interaction is necessary for p^{67} activity to protect the eIF-2 α-subunit from eIF-2 kinase catalyzed phosphorylation and that this interaction is unstable at high Mg^{2+}.

We have performed experiments similar to those described in Figure 8 using dsI and the results were similar. p^{67} protected

the eIF-2 α-subunit from dsI-catalyzed phosphorylation only at low Mg^{2+} (1 mM) and not at high Mg^{2+} (5 mM) (data not shown).

DISCUSSION

Data presented in this article provide the following information regarding p^{67} action and the mechanism of p^{67}:eIF-2 interactions.

(*1*) *Requirement of p^{67} for Protein Synthesis.* Addition of p^{67} antibodies to hemin-supplemented reticulocyte lysates enhanced eIF-2 α-subunit phosphorylation, presumably as these antibodies inactivated endogenous p^{67} (Ray *et al.*, 1992). It was expected that such phosphorylation would lead to protein synthesis inhibition in heme-supplemented reticulocyte lysate. Consistent with that report, we have now observed that p^{67} antibodies also inhibit protein synthesis in heme-supplemented reticulocyte lysate. These two related observations suggest that p^{67} is an essential factor for protein synthesis. This factor, p^{67}, must be present to protect the eIF-2 α-subunit and to promote protein synthesis in the presence of inhibitory eIF-2 kinases. It should be emphasized, however, that p^{67} antibody inhibition of protein synthesis alone does not provide conclusive evidence for the p^{67} requirement, as alternative explanations for such inhibition may be offered. For example, we have provided evidence in this article that p^{67} in reticulocyte lysates remains bound to three-subunit eIF-2. p^{67} antibodies may bind to p^{67} in association with eIF-2 and indirectly inhibit the activity of the bound eIF-2.

(*2*) *Characteristics of p^{67} and eIF-2 Kinase Activities.* The following have been previously reported: (i) p^{67} protects the eIF-2 α-subunit from HRI-catalyzed phosphorylation, but it does not directly inhibit HRI activity (Datta *et al.*, 1988). HRI did not phosphorylate the eIF-2 α-subunit in the presence of near-equimolar concentrations of p^{67}, but instead efficiently phosphorylated the eIF-2 α-subunit when excess three-subunit eIF-2 devoid of p^{67} was subsequently added. (ii) p^{67} also inhibited phosphorylation of one or more cellular proteins besides eIF-2 (Ray *et al.*, 1992). It was suggested that p^{67} may use its glycosyl residues to mask the phosphorylation sites of one or more closely related proteins.

The results presented in Figure 3 are consistent with the above reports. We have now studied the characteristics and specificities of p^{67} action using three different eIF-2 kinases. At near-equimolar concentrations to eIF-2, p^{67} efficiently protected the eIF-2 α-subunit from phosphorylation by both HRI and dsI, but had no significant effect on eIF-2 β-subunit phosphorylation by casein kinase. Also, at near-equimolar concentrations to histones, p^{67} protected one or more species of histones from phosphorylation by dsI (Figure 3C, lanes 4 and 6). However, p^{67} did not inhibit dsI-catalyzed phosphorylation of histones when excess histones were added, indicating that as in the case of eIF-2 reported before (Datta *et al.*, 1988) p^{67} protects equimolar concentrations of histones from dsI-catalyzed phosphorylation, but does not directly inhibit dsI activity.

p^{67} protection of histones suggests that these histones may possess phosphorylation sites similar to those of the eIF-2 α-subunit. p^{67} activities to protect the eIF-2 α-subunit from HRI- and dsI-catalyzed phosphorylations and the histones from dsI-catalyzed phosphorylation were similarly inhibited by WGA, indicating that a similar mechanism involving the glycosyl residues on p^{67} may be operative in all three cases. However, p^{67} protection of histones, and also possibly other proteins reported previously (Ray *et al.*, 1992), raises a serious question regarding the precise role(s) of p^{67}. On the basis of several important characteristics of p^{67} reported in this article and reported previously (Datta *et al.*, 1988; Ray *et al.*, 1992), we believe that an important role of p^{67} is to protect the eIF-2 α-subunit from inhibitory phosphorylation and thus to promote protein synthesis in the presence of eIF-2 kinases. However, the possibility that p^{67} may inhibit phosphorylation of other key proteins and thus serve other regulatory roles may remain open.

An interesting observation described in Figure 3 is that dsI, and also possibly HRI, is phosphorylated during eIF-2 α-subunit phosphorylation, and in both cases this phosphorylation is inhibited by p^{67} in a manner similar to that of the eIF-2 α-subunit phosphorylation. No phosphorylation of dsI was observed when dsI was incubated alone with radioactively labeled ATP (Figure 3B, lane 1). The significance of this result is not apparent at the present time. The results of our preliminary experiments are consistent with the following mechanism: Activated dsI used in these experiments is already phosphorylated. During eIF-2 α-subunit phosphorylation this phosphorylated dsI transfers its phosphoryl residues to the eIF-2 α-subunit. Dephosphorylated dsI is then autophosphorylated using radioactively labeled ATP and the cycle continues. Inhibition of eIF-2 α-subunit phosphorylation by p^{67} also inhibits this phosphorylation cycle and consequent dsI phosphorylation with radioactive phosphate.

(*3*) *p^{67} Binds Specifically to the eIF-2 γ-Subunit.* We previously reported (Datta *et al.*, 1988a) that eIF-2 remained associated with p^{67} through several purification steps. Also, a nearly homogeneous eIF-2 preparation contains, besides the three standard eIF-2 subunits (α, β, and γ), a nearly equimolar amount of p^{67}. These results suggested a strong association of eIF-2 to p^{67}. Using a co-immunoprecipitation procedure, we now provide evidence that p^{67} does indeed bind to eIF-2 in reticulocyte lysate. The p^{67}:eIF-2 complex in reticulocyte lysate was co-immunoprecipitated with antibodies against any of the eIF-2 subunits (eIF-2 α, β, and γ) or p^{67} and protein A agarose (Figure 4). Using a modification of the above co-immunoprecipitation procedure, we also provide evidence that p^{67} binds specifically to the eIF-2 γ-subunit. In these experiments, one of the interacting components (three-subunit eIF-2, isolated eIF-2 γ-subunit, or p^{67}) was preincubated with a specific antibody (eIF-2 α, β, or γ or p^{67}) to block the binding sites, and then the effects of such preincubation on p^{67}:eIF-2 complex formation were studied. Only when eIF-2 or the eIF-2 γ-subunit was preincubated with eIF-2 γ-subunit antibodies was subsequent binding of p^{67} to three-subunit eIF-2 (Figure 5A) or the eIF-2 γ-subunit (Figure 5C) inhibited. Preincubation of eIF-2 with eIF-2 α- or β-subunit antibodies had no effect on p^{67} binding to eIF-2 (Figure 5B). Similarly, preincubation of free p^{67} with p^{67} antibodies inhibited p^{67} binding to eIF-2 (Figure 5A). These results suggest that p^{67} and eIF-2 γ-subunit antibodies blocked the binding sites on the respective polypeptides and this inhibited the p^{67}:eIF-2 interaction.

The inhibition of the p^{67}:eIF-2 interaction was observed only when the interacting proteins were preincubated specifically with the eIF-2 γ-subunit antibodies or p^{67} antibodies. This inhibition was not observed when eIF-2 was preincubated with either eIF-2 α- or β-subunit antibodies. This inhibition, therefore, indicates preblocking of the interaction site(s) on the specific interacting protein(s) and is not merely due to steric hindrance by unspecific antibody molecules. This antibody preblocking technique is new and could possibly be used similarly for studies of other interactions between protein complexes. However, it should be noted that preincubation

of eIF-2 with the eIF-2 α-subunit antibodies did not inhibit either eIF-2 kinase(s) catalyzed phosphorylation of the eIF-2 α-subunit or p^{67} activity to protect the eIF-2 α-subunit from eIF-2 kinase(s) catalyzed phosphorylation. It is possible that the antibodies against this phosphorylation site are not available or that the phosphorylation site is not accessible to the antibodies under the *in vitro* assay condition. It may, therefore, be assumed that the antibody preblocking technique may be useful only when the antibodies inhibit the protein: protein interaction and that the negative results may not necessarily indicate a lack of interaction.

(*4*) *Glycosyl Residues on p^{67} Are Not Involved in p^{67}:eIF-2 γ-Subunit Interactions.* The p^{67} contains multiple O-linked GlcNAc residues. It was previously postulated that p^{67} uses these glycosyl residues to mask the phosphorylation site on eIF-2; WGA binds to these glycosyl residues and inhibits p^{67} activity to protect the eIF-2 α-subunit from eIF-2 kinase(s) catalyzed phosphorylation (Datta *et al.*, 1989). However, in this work we have observed that WGA does not inhibit p^{67} binding to eIF-2. This observation suggests multiple p^{67} binding sites on eIF-2. It may be that p^{67} initially binds to the eIF-2 γ-subunit and subsequently uses the glycosyl residues to bind to the eIF-2 α-subunit phosphorylation site to protect this site from eIF-2 kinase(s) catalyzed phosphorylation.

(*5*) *p^{67} Binding to the eIF-2 γ-Subunit Is Necessary for p^{67} Activity To Protect the eIF-2 α-Subunit from eIF-2 Kinase(s) Catalyzed Phosphorylation.* Factors which inhibit the p^{67}:eIF-2 γ-subunit interaction, such as preincubation with the eIF-2 γ-subunit antibodies (Figure 5) and high Mg^{2+} concentration (Figure 7), also inhibited p^{67} activity to protect the eIF-2 α-subunit. These results suggest that p^{67} binding to the eIF-2 γ-subunit is essential for subsequent p^{67} activity to protect the eIF-2 α-subunit from eIF-2 kinase(s) catalyzed phosphorylation.

p^{67} also protects histones, and possibly several other proteins, from the phosphorylation reaction. It is not known whether a similar mechanism requiring p^{67} interaction with a distal site is also necessary for p^{67} activity to protect these proteins from the phosphorylation reaction. It is conceivable, however, that p^{67} interaction with the eIF-2 γ-subunit provides an additional regulatory mechanism and is operative only in protection of the eIF-2 α-subunit from inhibitory phosphorylation.

Concluding Remarks. The results presented in this article suggest that p^{67} binds in an equimolar concentration to eIF-2 and thus protects the eIF-2 α-subunit from inhibitory phosphorylation by eIF-2 kinases. This binding of p^{67} to eIF-2 is reversible, and several factors, such as p^{67} level in the cell, possibly influence the position of this equilibrium. Our previous reports indicated that the p^{67} level in the cell may vary widely under different physiological conditions, and this level correlates directly with the protein synthesis activity of the cell (Ray *et al.*, 1992). There has been no conclusive evidence for significant variations in the eIF-2 kinase levels in the cells. Our previous reports indicated that dsI remains essentially unchanged during several phases of cell growth in culture (Ray *et al.*, 1992). However, the results of numerous *in vitro* studies have indicated that the addition of excess exogenous eIF-2 kinases inhibits protein synthesis (Maxwell & Rabinovitz, 1969; Gross & Rabinovitz, 1972; Trachsel *et al.*, 1978; Fagard & London, 1981). Consistent with these reports, we have now observed that an eIF-2 kinase, such as dsI when added in excess *in vitro*, can effectively phosphorylate the eIF-2 α-subunit even in the presence of an equimolar concentration of p^{67} (data not shown). Apparently, eIF-2 kinase(s) at higher concentrations can phosphorylate three-subunit eIF-2 in equilibrium with eIF-2 containing p^{67} and shift the equilibrium in favor of the phosphorylated form, leading to protein synthesis inhibition. The possibility, therefore, remains that protein synthesis in the cell may also be regulated by relative concentrations of both eIF-2 kinases and p^{67}.

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