Regulation of eIF-2 α-Subunit Phosphorylation in Reticulocyte Lysate†

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ABSTRACT: An eIF-2 associated 67-kDa protein (p^{67}) protects eIF-2 α-subunit from eIF-2 kinase(s) catalyzed phosphorylation and promotes protein synthesis in the presence of active eIF-2 kinase(s). p^{67} is a glycoprotein and contains multiple O-linked GlcNAc moieties. We have now studied the roles of hemin, p^{67}, and the glycosyl residues on p^{67} in the regulation of eIF-2 α-subunit phosphorylation in reticulocyte lysates. The results are as follows: (i) Both hemin and p^{67} inhibited HRI (heme-regulated protein synthesis inhibitor) and dsI (double-stranded RNA activated protein synthesis inhibitor) catalyzed phosphorylation of eIF-2 α-subunit *in vitro*. However, only hemin, and not p^{67}, inhibited casein kinase catalyzed phosphorylation of eIF-2 β-subunit. (ii) Only p^{67}, and not hemin, inhibited eIF-2 α-subunit phosphorylation by eIF-2 kinase(s) in reticulocyte lysate. Significant eIF-2 α-subunit phosphorylation was observed even in the presence of hemin when p^{67} in the reticulocyte lysate was removed by treatment with p^{67} antibodies. (iii) Reticulocyte lysate contains a p^{67}-deglycosylase in latent form, and hemin prevents activation of this deglycosylase. In the absence of hemin, this p^{67}-deglycosylase is activated. Once activated in the absence of hemin, the activated deglycosylase deglycosylates p^{67}, even in the presence of hemin. This inactivates p^{67} and allows eIF-2 kinase to phosphorylate eIF-2 α-subunit and inhibit protein synthesis. Protein synthesis in reticulocyte lysate is thus regulated by two novel cascades of covalent modifications: protein deglycosylation leading to protein phosphorylation.

Heme-supplemented reticulocyte lysate is widely used in protein synthesis experiments. In the absence of hemin, protein synthesis in reticulocyte lysate continues for a short period and then abruptly ceases. This cessation of protein synthesis is accompanied by phosphorylation of a key peptide chain initiation factor eIF-2[1] (eukaryotic initiation factor 2) and consequent inactivation of eIF-2 activity. It is widely believed that an eIF-2 kinase (HRI, heme-regulated protein synthesis inhibitor) is present in reticulocyte lysate in latent form and hemin prevents activation of this inhibitor [for recent reviews, see London et al. (1987), Hershey (1991), and Gupta et al. (1993)]. The precise mechanism of HRI activation is not fully understood, but possibly involves HRI autophosphorylation. Several laboratories reported that hemin inhibits *in vitro* autophosphorylation of HRI (Yang et al., 1992) and also HRI-catalyzed phosphorylation of eIF-2 α-subunit (Hronis & Traugh, 1981, 1986; Yang et al., 1992; Mendez et al., 1992).

Our laboratory has reported that the reticulocyte lysate contains an eIF-2 kinase in active form (Ray et al., 1992). However, this eIF-2 kinase cannot phosphorylate eIF-2 α-subunit since this lysate also contains a 67-kDa glycoprotein, p^{67} (Datta et al., 1988, 1989). p^{67} protects 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) in the reticulocyte lysate. Removal of endogenous p^{67} in reticulocyte lysate by treatment with p^{67} antibodies led to extensive eIF-2 α-subunit phosphorylation, even in the presence of hemin. We also reported that p^{67} is possibly deglycosylated and subsequently degraded in heme-deficient lysate (Ray et al., 1992). Here we present the results of our studies on the roles of hemin, heme-regulated eIF-2 kinase, p^{67}, and a p^{67}-deglycosylase in the regulation of eIF-2 α-subunit phosphorylation in reticulocyte lysate.

MATERIALS AND METHODS

Most of the experimental procedures and the materials used in this study were the same as described previously (Datta et al., 1988, 1989; Ray et al., 1992, 1993).

Three subunit eIF-2 and p^{67} were purified according to the procedure described by Datta et al. (1988). 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).

HRI was purified as described by Trachsel et al. (1978). A sample of dsI, prepared according to Szyszka et al. (1989), was generously donated by Dr. Giesla Kramer (Clayton Foundation, Biochemistry Inst., University of Texas, Austin, TX). We determined the specific activities of the eIF-2 kinase preparations by measuring TCA (5%) insoluble eIF-α[P^{32}] synthesis during 10-min incubation under standard incubation conditions. The specific activities were as follows: dsI, 85; HRI, 50. One unit of activity was defined as the amount of protein required to form 1 pmol of eIF-2 α(P) under the standard incubation conditions.

Preparation of Polyclonal and Monoclonal Antibodies. The polyclonal antibodies against different polypeptides eIF-2 α and β, p^{94} (Co-eIF-2A, see Roy et al. (1988)), p^{110}, and p^{180} [polypeptides in Co-eIF-2C preparation, see Roy et al. (1988)] were prepared following the procedure described by Ray et al. (1992). The monoclonal antibodies against p^{67} were prepared as described (Datta et al., 1989). As reported, these monoclonal antibodies specifically recognize the GlcNAc moieties on p^{67} (Datta et al., 1989). All of the antibodies used in the experiments were monospecific and reacted only with their corresponding antigens.

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[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 agglutinine.

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Phosphorylation of eIF-2. Phosphorylation of eIF-2, using purified eIF-2 kinases (HRI, dsI, and casein kinase), was carried out using the procedure described by Datta et al. (1988). Protein phosphorylation in reticulocyte lysate was carried out as described by Ray et al. (1992). A detailed experimental procedure is also described in the figure captions.

Analysis for Glycosylation and Deglycosylation of p^{67} in Reticulocyte Lysate. Two different experimental procedures were used: (i) Using antibodies: Both p^{67} mono- and polyclonal antibodies were used to analyze glycosylated and deglycosylated p^{67}. As reported (Datta et al., 1989), p^{67} monoclonal antibodies recognize only the glycosylated p^{67}. For these experiments p^{67} in the reticulocyte lysate was precipitated using either p^{67} monoclonal antibodies (for glycosylated p^{67}) or p^{67} polyclonal antibodies (for glycosylated + deglycosylated p^{67}) and protein A–agarose. p^{67} in the immunoprecipitate was then analyzed by SDS–PAGE followed by immunoblotting using p^{67} polyclonal antibodies as described (Ray et al., 1992). (ii) Using WGA: The experimental procedures were similar to those described previously (Datta et al., 1989). p^{67} (both glycosylated and deglycosylated) in the reticulocyte lysate was immunoprecipitated using p^{67} polyclonal antibodies and protein A–agarose. Protein in the immunoprecipitate was then separated by SDS–PAGE and electrolytically transferred to nitrocellulose filters. Nitrocellulose filters bearing p^{67} were then incubated with biotinylated WGA (Vector Laboratory), and the bound lectins were subsequently detected following treatment of filters with avidin-conjugated horseradish peroxidase (Vector Laboratory).

RESULTS

Hemin-supplemented reticulocyte lysate actively synthesizes protein over a long period. When hemin is absent, protein synthesis continues for a short period and then abruptly ceases. It has been postulated that a latent protein synthesis inhibitor is activated after a short lag period and this activated inhibitor leads to a subsequent shut-off of protein synthesis.

It is widely believed that the activated inhibitor is an eIF-2 kinase (also called HRI, heme-regulated protein synthesis inhibitor). Upon activation, this eIF-2 kinase phosphorylates eIF-2 α-subunit. This then inactivates eIF-2 activity and inhibits protein synthesis. The precise mechanism of HRI activation is not fully understood, but possibly involves HRI autophosphorylation. Supporting this postulation, several laboratories reported that hemin inhibits *in vitro* autophosphorylation of HRI (Yang et al., 1992) and also HRI-catalyzed phosphorylation of eIF-2 α-subunit (Hronis & Traugh, 1981, 1986; Yang et al., 1992; Mendez et al., 1992). Our laboratory, however, has provided evidence that a heme-regulated eIF-2 kinase is indeed present in active form in reticulocyte lysate (Ray et al., 1992). However, this eIF-2 kinase cannot phosphorylate eIF-2 α-subunit because a 67-kDa glycoprotein, p^{67}, that is also present in reticulocyte lysate protects eIF-2 α-subunit from eIF-2 kinase catalyzed phosphorylation (Datta et al., 1988, 1989). During heme deficiency in reticulocyte lysate, p^{67} is deglycosylated and subsequently degraded, thus leading to inactivation of p^{67} and accompanying eIF-2 α-subunit phosphorylation. We then postulated that the function of hemin is to inhibit p^{67} deglycosylation. The mechanism of such inhibition was not known.

Effects of Hemin and p^{67} on eIF-2 Phosphorylation in Vitro. To gain a better understanding of the roles of hemin and p^{67} on eIF-2 phosphorylation, we studied their effects on *in vitro* eIF-2 phosphorylation using three different eIF-2 kinases: HRI, dsI, and casein kinase (Figure 1). Panel A shows that

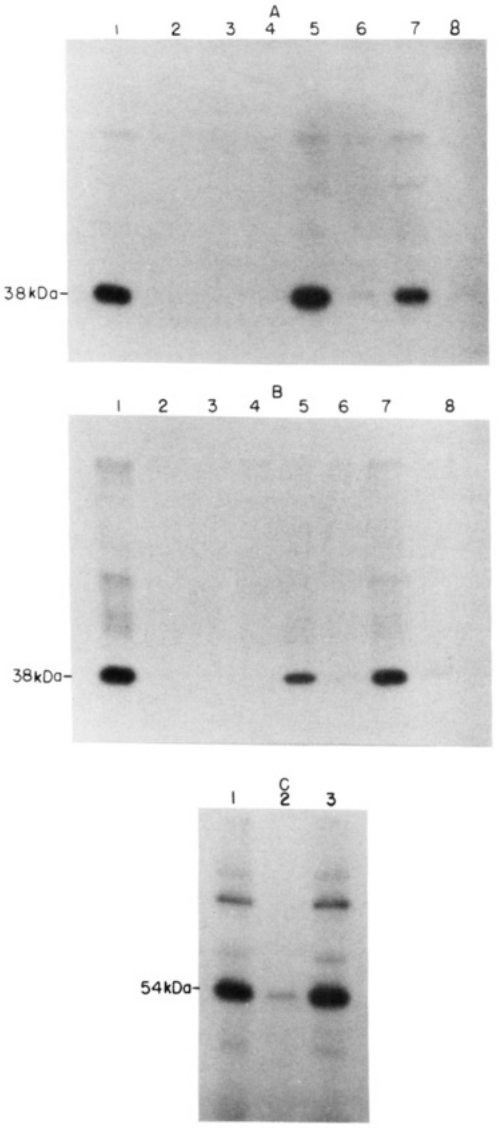

FIGURE 1: Effects of hemin and p^{67} on eIF-2 α-subunit phosphorylation catalyzed by eIF-2 kinases. Panel A: HRI-catalyzed phosphorylation of eIF-2 α-subunit. The reaction mixtures contained (total volume, 25 μL) 20 mM Tris-HCl (pH 7.8), 100 mM potassium chloride, 40 μM ATP, 10 μg of bovine serum albumin, 2 mM dithiothreitol, 2 μg of eIF-2 (fraction V), and, where indicated 1 μg of p^{67} and 5 μg of p^{67} antibodies. The reactions containing p^{67} and p^{67} antibodies were preincubated in ice for 30 min. The reactions were started by addition of 0.05 μg of HRI and 48 μCi of [γ-^{32}P]ATP (sp act. 7000 Ci/mmol; ICN). The reaction mixtures were incubated at 37 °C for 10 min. The radioactively labeled eIF-2 was then analyzed by SDS–PAGE followed by autoradiography (Ray et al., 1992). Lanes: 1, eIF-2 + HRI; 2, eIF-2 + HRI + hemin; 3, eIF-2 + p^{67} + HRI; 4, eIF-2 + p^{67} + HRI + hemin; 5, eIF-2 + p^{67} antibodies + HRI; 6, eIF-2 + p^{67} antibodies + HRI + hemin; 7, eIF-2 + p^{67} + p^{67} antibodies + HRI; and 8, eIF-2 + p^{67} + p^{67} antibodies + HRI + hemin. Panel B: dsI-catalyzed phosphorylation of eIF-2 α-subunit. The reaction conditions in all eight experiments described in lanes 1–8 were the same as described in panel A except that dsI (0.05 μg) was used in place of HRI. Panel C: casein kinase II catalyzed phosphorylation of eIF-2 β-subunit. The reaction conditions were the same as described in panel A except that casein kinase II (6 μg) was used in place of HRI. Lanes: 1, eIF-2 + casein kinase II; 2, eIF-2 + casein kinase II + hemin; 3, eIF-2 + casein kinase II + p^{67}.

HRI efficiently phosphorylated eIF-2 α-subunit (lane 1). Hemin (lane 2), p^{67} (lane 3), and also a mixture of hemin and p^{67} (lane 4) inhibited almost completely this phosphorylation reaction. p^{67} antibodies alone had no effect on the HRI-catalyzed phosphorylation reaction (lane 5) or on hemin inhibition of HRI-catalyzed phosphorylation (lane 6). How-

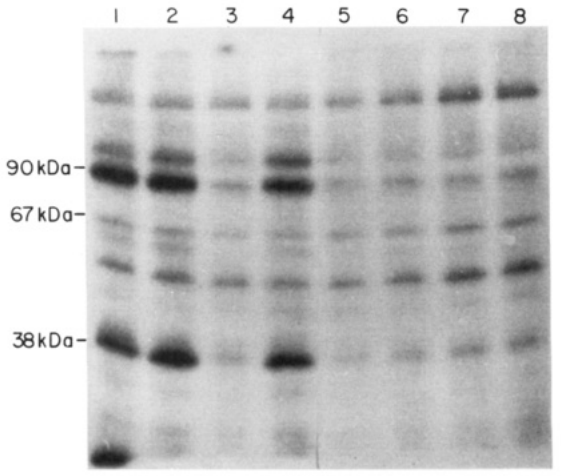

FIGURE 2: Effects of hemin and different antibody preparations on eIF-2 α-subunit phosphorylation in reticulocyte lysates. The reaction conditions were the same as described (Ray et al., 1992). The reaction mixtures contained (total volume 25 μL) 20 mM Tris-HCl (pH 7.8), 100 mM KCl, 10 μg of bovine serum albumin, 2 mM dithiothreitol, 40 μM ATP, 10 μL of reticulocyte lysate, and, where indicated, 20 μM hemin and 10 μg of different antibody preparations. The reactions were incubated with the antibodies in ice for 30 min. The reactions were then started by addition of 48 μCi of [γ-P^{32}]ATP (7000 Ci/mmol; ICN) and were incubated at 37 °C for 10 min. The radioactively labeled polypeptides were then analyzed by SDS–PAGE followed by autoradiography (Ray et al., 1992). Lanes: 1, –hemin; 2, –hemin + p^{67} antibodies; 3, +hemin; 4, +hemin + p^{67} antibodies; 5, +hemin + eIF-2 α-subunit antibodies; 6, +hemin + 94-kDa polypeptide antibodies; 7, +hemin + 110-kDa polypeptide antibodies; 8, +hemin + 180-kDa polypeptide antibodies.

ever, when these antibodies were added to a reaction mixture containing p^{67} (as described in lane 3), eIF-2 α-subunit was phosphorylated (lane 7), presumably because these antibodies inactivated p^{67}. This effect was not restricted to p^{67} antibodies. WGA which binds to the GlcNAc residues on p^{67} similarly inhibited p^{67} activity and restored eIF-2 α-subunit phosphorylation by HRI (Ray et al., 1992). Also, as shown, hemin inhibition of HRI-catalyzed eIF-2 α-subunit phosphorylation was observed in a reaction containing both p^{67} and p^{67} antibodies (lane 8). Panel B presents the results of identical experiments (as described in panel A), but HRI in these experiments were replaced by another eIF-2 α-subunit kinase, namely, dsI. The results were identical. This would indicate that both hemin and p^{67} inhibit HRI- and dsI-catalyzed eIF-2 α-subunit phosphorylation. The mechanism of each inhibition may be similar in both cases. Panel C shows the effects of hemin and p^{67} on casein kinase II catalyzed phosphorylation of eIF-2 β-subunit. Again, hemin almost completely inhibited casein kinase catalyzed phosphorylation of eIF-2 β-subunit. However, p^{67} had no significant effect on this phosphorylation reaction.

Effects of Hemin and p^{67} on eIF-2 α-Subunit Phosphorylation in Reticulocyte Lysate. We also studied the effects of hemin and p^{67} on eIF-2 α-subunit phosphorylation using reticulocyte lysate. The results are shown in Figure 2. As reported (Ray et al., 1992), eIF-2 α-subunit was efficiently phosphorylated in hemin-deficient lysate (lane 1). The presence of hemin prevented this phosphorylation reaction (lane 3). However, when the same lysate was preincubated with p^{67} antibodies to inactivate endogenous p^{67}, eIF-2 α-subunit was efficiently phosphorylated, both in the absence (lane 2) and in the presence of hemin (lane 4). Other antibodies tested, such as eIF-2 α-subunit antibodies (lane 5) and three peptide chain initiation factor [94 kDa (lane 6), 110 kDa (lane 7), and 180 kDa (lane 8), see Roy et al. (1988)] antibodies, had no significant effect on eIF-2 α-subunit phosphorylation in hemin-supplemented lysate.

The radioactively labeled 38-kDa protein, as shown in Figure 2, precipitated with the eIF-2 α-antibodies and was therefore identified as eIF-2 α-subunit.

Deglycosylation of p^{67} in Heme-Deficient Reticulocyte Lysate. Our previous reports indicated that eIF-2 α-subunit phosphorylation, observed in hemin-deficient reticulocyte lysate (Figure 2, lane 1), is due to deglycosylation and consequent inactivation of p^{67} (Ray et al., 1992). We have now studied the detailed mechanism of this p^{67} deglycosylation reaction. We used two different experimental procedures.

(i) Using p^{67} antibodies: We used both p^{67} monoclonal antibodies and p^{67} polyclonal antibodies to analyze both glycosylated and deglycosylated p^{67} (Figure 3). The experimental procedures were the same as described under Materials and Methods. In the experiments described in panels A and

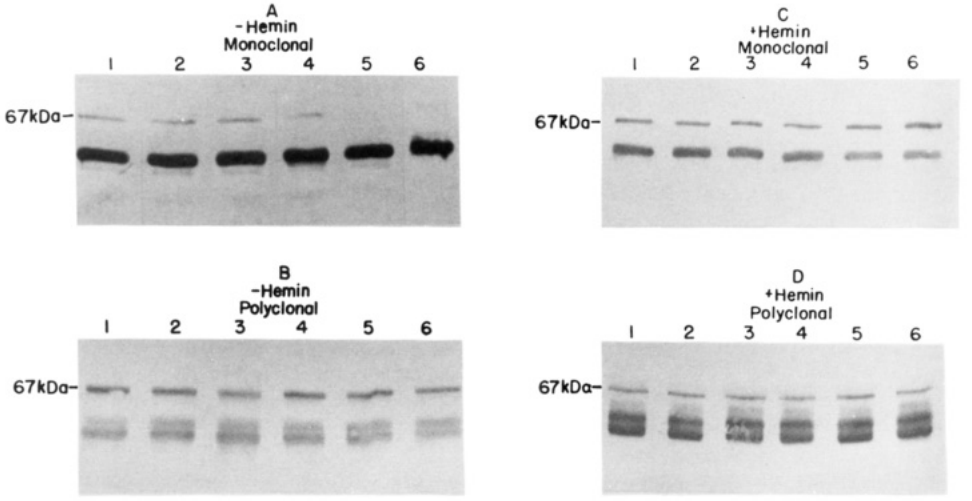

FIGURE 3: Assay for p^{67} deglycosylation in reticulocyte lysate using p^{67} antibodies. The reaction mixtures contained (total volume, 40 μL) 15 μL of reticulocyte lysate, 10 mM Tris-HCl, pH 7.8, 1 mM $Mg(OAc)_2$, 100 mM KCl, 0.2 mM GTP, 5 mM creatine phosphate, and 0.4 μg of creatine phosphokinase. Hemin (20 μM) was added in reactions described in panels C and D. The reactions were incubated at 30 °C. At the indicated times, p^{67} in the reaction mixtures was immunoprecipitated using either p^{67} monoclonal (panels A and C) or polyclonal (panels B and D) antibodies and protein A–agarose and was subsequently analyzed by immunoblotting using p^{67} polyclonal antibodies (as described under Materials and Methods). Different lanes indicate different incubation times: Lanes: 1, 0 min; 2, 1 min; 3, 3 min; 4, 5 min; 5, 10 min; 6, 20 min. The lower molecular mass bands represent the IgG heavy chain.

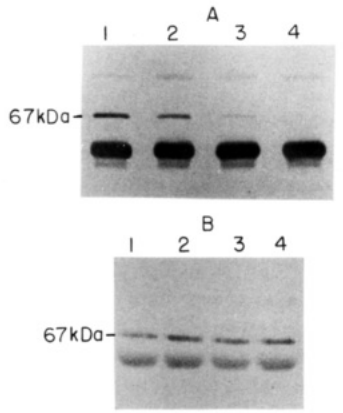

FIGURE 4: Assay for p^{67} deglycosylation in reticulocyte lysate using biotinylated WGA. The reaction conditions were the same as described in Figure 3. The reactions described in panel B contained 20 μM hemin. At the indicated times, p^{67} in the reaction mixtures was immunoprecipitated using p^{67} polyclonal antibodies and protein A–agarose. The immunoprecipitate was subsequently analyzed for WGA binding using the procedure described under Materials and Methods. Different lanes indicate different incubation times. Lanes: 1, 0 min; 2, 5 min; 3, 10 min; 4, 20 min.

C, p^{67} was precipitated with p^{67} monoclonal antibodies, and in the experiments described in panels B and D, p^{67} was precipitated with p^{67} polyclonal antibodies. The immunoprecipitates containing antigen antibody complexes were then analyzed by SDS–PAGE followed by immunoblotting using p^{67} polyclonal antibodies.

As shown in panel A, p^{67} in the reticulocyte lysate was extensively deglycosylated after incubation in the absence of hemin for 5 min (lane 4). This deglycosylation reaction was almost complete after 10 min of incubation (lane 5). It should be emphasized that p^{67} in these experiments was deglycosylated only during initial incubation in the absence of hemin and not during subsequent processing. All of the reactions were similarly processed by overnight incubation in ice. However, as shown, p^{67} remained fully glycosylated after incubation for 1 (lane 2) and 3 min (lane 3) as control (0 min, lane 1). Under identical experimental conditions, p^{67} was increasingly deglycosylated during longer time incubations (5 → 20 min, lanes 4–6) in the absence of hemin. The results presented in panel B show that under similar incubation conditions in the absence of hemin, the p^{67} level remained essentially unchanged after 10 min of incubation when assayed using p^{67} polyclonal antibodies (lane 5). Some degradation was, however, observed after 20 min of incubation (lane 6).

Panels C and D show that glycosylated p^{67}, assayed using either monoclonal (panel C) or polyclonal (panel D) antibodies, remained intact during a 20-min incubation in the presence of hemin. These results suggest that reticulocyte lysate contains a p^{67}-deglycosylase activity and this activity deglycosylates p^{67} in the absence of hemin.

It should be noted that deglycosylated p^{67} produced in reactions, described in panels A and B, is expected to have a slightly lower molecular weight than native p^{67}. This difference was not apparent under the experimental conditions used. However, this difference could be demonstrated using a longer electrophoretic time.

(ii) Using WGA: WGA binds specifically to the GlcNAc moieties and was conveniently used to detect these glycosyl residues on p^{67} (Datta et al., 1989). We used a similar procedure to assay for p^{67} deglycosylation in heme-deficient reticulocyte lysate (Figure 4). As shown in panel A, p^{67} rapidly lost WGA reactive glycosyl residues when incubated in hemin-deficient reticulocyte lysate. Under similar incubation conditions, p^{67} in the hemin-supplemented reticulocyte lysate (panel B) retained most of the WGA reactive glycosyl residues.

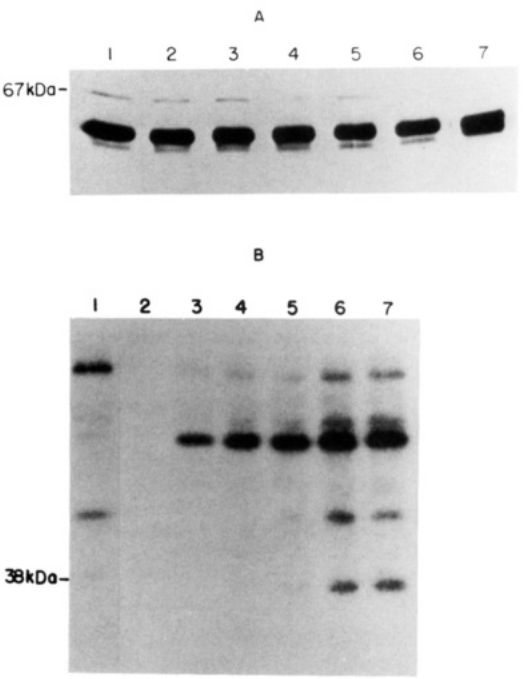

FIGURE 5: Kinetics of p^{67} deglycosylation and eIF-2 α-subunit phosphorylation in a mixture containing preincubated hemin-deficient lysate and fresh lysate. p^{67}-deglycosylase was activated by incubation of hemin-deficient reticulocyte lysate at 30 °C for 20 min. A 20-μL aliquots of preincubated hemin-deficient reticulocyte lysate was then added to 20 μL of fresh lysate, and the hemin concentration of the final solution was adjusted to 20 μM. The reactions were incubated at 30 °C and were then assayed for p^{67} deglycosylation at different intervals following the procedure described in Figure 3 (panel A). eIF-2 α-subunit phosphorylation was assayed using 10-μL aliquots of the same reaction mixture following the procedure described in Figure 2 (panel B). Panel A: Kinetics of p^{67} deglycosylation. Lane 1 shows p^{67} level in the control experiment (containing fresh lysate without preincubated hemin-deficient lysate) incubated for 20 min. Preincubated hemin-deficient lysate was added to fresh lysate in the other experiments, and the glycosylated p^{67} level was measured at different intervals. Lanes: 2, 0 min; 3, 1 min; 4, 3 min; 5, 5 min; 6, 10 min; 7, 20 min. Panel B: Kinetics of eIF-2 α-subunit phosphorylation. The reaction conditions were the same as described in panel A except that the reactions contained radioactively labeled [γ-^{32}P]ATP (48 μCi, sp act. 7000 Ci/mmol). Lane 1 shows eIF-2 α-subunit phosphorylation in the control experiment (containing fresh lysate without preincubated hemin-deficient lysate) incubated for 10 min. Preincubated hemin-deficient lysate was added to fresh lysate in the other experiments, and eIF-2 α-subunit phosphorylation was measured at different intervals. Lanes: 2, 0 min; 3, 1 min; 4, 2 min; 5, 3 min; 6, 5 min; 7, 10 min.

Activation of p^{67}-Deglycosylase in the Absence of Hemin. The results presented in Figure 5 (panels A and B) provide evidence that a p^{67}-deglycosylase activity remains in latent form in hemin-supplemented reticulocyte lysate. During heme deficiency this deglycosylase activity becomes activated. In these experiments we activated endogenous p^{67}-deglycosylase by incubation of reticulocyte lysate in the absence of hemin. We then mixed 20-μL aliquots of the preincubated hemin-deficient lysate with 20 μL of fresh lysate and incubated the mixture in the presence of hemin. In one series of experiments (described in panel A) we assayed for deglycosylation of endogenous p^{67} in fresh lysate in the presence of hemin. In another series of similar experiments (described in panel B) we used [γ-P^{32}]-ATP and measured eIF-2 α-subunit phosphorylation in the mixture. As shown in panel A, lane 1, p^{67} in the control experiment (containing fresh lysate without added preincubated hemin-deficient lysate) remained fully glycosylated during the entire 20-min incubation in the

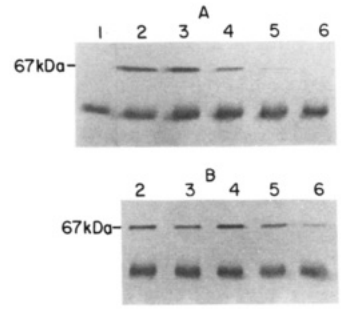

FIGURE 6: Kinetics of deglycosylation of free and eIF-2 bound p^{67}. The reaction conditions were the same as described in Figure 3. The reactions were incubated in the absence of hemin at 30 °C for 20 min. p^{67} (1 μg) (panel A) or 4 μg of eIF-2 containing p^{67} (panel B) was then added in experiments described in lanes 2–6, and the incubation continued at 30 °C. The reactions were terminated at different intervals and analyzed for glycosylated p^{67} as described in Figure 3. Panel A: lane 1, lysate only (control). Lanes 2–6: panel A, $+p^{67}$; panel B, eIF-2 + p^{67}; lane 2, 1 min; lane 3, 3 min; lane 4, 5 min; lane 5, 10 min; lane 6, 20 min.

presence of hemin. However, addition of hemin-deficient reticulocyte lysate, containing preactivated p^{67}-deglycosylase, progressively deglycosylated p^{67} in the fresh lysate even in the presence of hemin (lanes 4 → 7). This deglycosylation reaction was evident after 3-min incubation (lane 4) and was complete after 10-min incubation (lane 6). Similarly, the results presented in panel B show that eIF-2 α-subunit was not phosphorylated in the control experiment during the entire 10-min incubation of the fresh lysate (without added preincubated hemin-deficient lysate) in the presence of hemin (lane 1). However, addition of a 20-μL aliquot of preincubated hemin-deficient reticulocyte lysate to a 20-μL aliquot of fresh lysate caused a progressive increase in eIF-2 α-subunit phosphorylation even in the presence of inhibitory hemin concentration (lanes 6 and 7). In the mixed solution, appreciable deglycosylation of p^{67} occurred after a 3-min incubation (lane 4, panel A). In identical reactions, eIF-2 α-subunit phosphorylation was evident after a 5-min incubation (lane 6, panel B). These results thus show that a p^{67}-deglycosylase is present in reticulocyte lysate in inactive form and is activated in the absence of hemin. Once activated, the p^{67}-deglycosylase deglycosylates p^{67}, even in the presence of hemin. p^{67} deglycosylation leads to p^{67} inactivation and consequent eIF-2 α-subunit phosphorylation.

Kinetics of Deglycosylation of Free and eIF-2 Bound p^{67}. Our previous studies indicated that eIF-2 containing p^{67} promoted protein synthesis in heme-deficient reticulocyte lysate, whereas free eIF-2 and p^{67} alone were almost totally inactive (Datta et al., 1988). We studied the efficiencies of the activated deglycosylase to deglycosylate free and eIF-2 bound p^{67} (Figure 6). In these experiments we preincubated heme-deficient reticulocyte lysate for 20 min to activate p^{67}-deglycosylase and also to deglycosylate endogenous p^{67}. We then added free p^{67} (panel A) and also eIF-2 bound p^{67} (panel B) and studied the rates of deglycosylation during subsequent incubation. As shown, free p^{67} was deglycosylated considerably faster (panel A) than eIF-2 bound p^{67} (panel B). Almost complete deglycosylation of free p^{67} was observed after a 10-min incubation (panel A, lanes 5 and 6), whereas a significant amount of glycosylated p^{67} was present even after a 20-min incubation when eIF-2 bound p^{67} was used (panel B, lane 6). This observation may explain the activity of eIF-2 bound p^{67} to reverse protein synthesis inhibition in heme-deficient reticulocyte lysate over a significantly long period, as previously observed (Datta et al., 1988).

DISCUSSION

Data presented in this paper provide useful information regarding the complex regulation of eIF-2 α-subunit phosphorylation by hemin, HRI, p^{67}, and p^{67}-deglycosylase.

Hemin. Previous reports indicated that hemin inhibited HRI activity *in vitro* and also HRI activation in reticulocyte lysate (Hronis & Traugh, 1981, 1986; Yang et al., 1992; Mendez et al., 1992). We provide evidence that hemin inhibits *in vitro* HRI-catalyzed phosphorylation of eIF-2 α-subunit, as well as dsI-catalyzed phosphorylation of eIF-2 α-subunit and casein kinase II catalyzed phosphorylation of eIF-2 β-subunit (Figure 1). However, hemin inhibition of eIF-2 α-subunit phosphorylation was not observed in reticulocyte lysate: efficient eIF-2 α-subunit phosphorylation was observed when the reticulocyte lysate was treated with p^{67} antibodies to remove endogenous p^{67} (Figure 2). We suggest that hemin inhibition of eIF-2 kinase activities observed *in vitro* is due to unspecific binding of this charged molecule to these proteins. Hemin does not inhibit eIF-2 kinase activities in reticulocyte lysate as the hemin effect is diluted in the presence of the other proteins. A novel observation presented in this paper is that hemin does indeed inhibit activation of a p^{67}-deglycosylase activity in reticulocyte lysate. It may be that hemin inhibition in reticulocyte lysate is determined by the relative affinities of hemin to different protein molecules and hemin may bind more strongly to one or more proteins involved in p^{67} deglycosylation and effectively inhibits their activities.

Heme-Regulated eIF-2 Kinase. Our results reported previously (Ray et al., 1992) and also presented in this paper provide evidence that some heme-regulated eIF-2 kinase is present in active form in reticulocyte lysate. However, this eIF-2 kinase cannot phosphorylate eIF-2 α-subunit as the lysate also contains p^{67}. p^{67} protects eIF-2 α-subunit from the eIF-2 kinase catalyzed phosphorylation and thus promotes protein synthesis in the presence of active eIF-2 kinase. Interestingly, although hemin does not directly inhibit this eIF-2 kinase activity, hemin does indeed regulate this activity by preventing activation of p^{67}-deglycosylase, a potent activator of eIF-2 kinase. This eIF-2 kinase is, therefore, indirectly regulated by hemin and may appropriately be called a heme-regulated inhibitor, HRI.

p^{67}. Our results suggest that p^{67} protects eIF-2 α-subunit from an eIF-2 kinase catalyzed phosphorylation in reticulocyte lysate (Figure 2). However, as reported previously (Ray et al., 1992, 1993), p^{67} appears to inhibit phosphorylation of at least three other proteins (67 kDa, 90 kDa, and 95 kDa) in reticulocyte lysate. The significance of these phosphorylation reactions is unclear. We suggest two possibilities: (a) p^{67} inhibits *mainly* eIF-2 α-subunit phosphorylation. One or more phosphorylated proteins may be eIF-2 kinases, and they are phosphorylated dependent on eIF-2 α-subunit phosphorylation by a previously proposed mechanism (Ray et al., 1992; Gupta et al., 1993). (b) p^{67} also inhibits phosphorylation of one or more key proteins and thus serves other regulatory roles. Further work will be necessary to distinguish between these two possibilities. However, on the basis of several important characteristics of p^{67} reported in this paper and also previously (Datta et al., 1988, 1989; Ray et al., 1992, 1993), we believe that an important role of p^{67} is to protect the eIF-2 α-subunit and promote protein synthesis in the presence of active eIF-2 kinases.

p^{67}-Deglycosylase. We provide evidence that reticulocyte lysate contains a p^{67}-deglycosylase activity in latent form and hemin prevents activation of this deglycosylase. During heme deficiency this deglycosylase is activated after a lag of 2–3

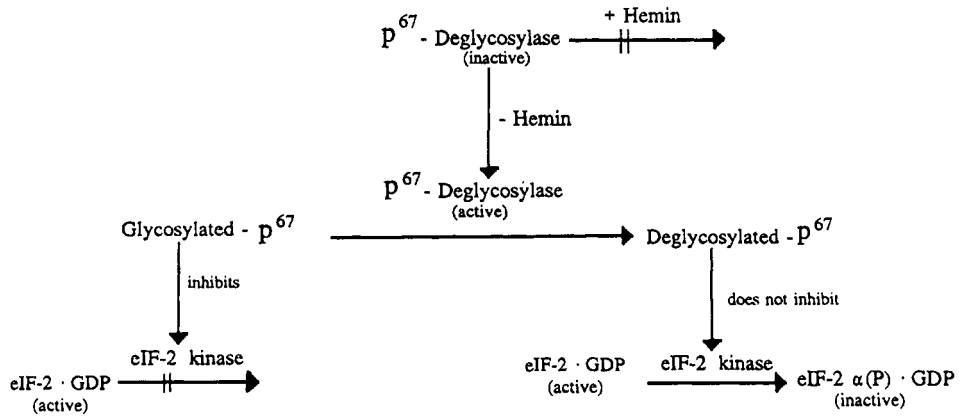

FIGURE 7: A proposed model for the regulation of eIF-2 α-subunit phosphorylation in reticulocyte lysate.

min (Figure 5). However, once activated in the absence of hemin, p^{67}-deglycosylase actively deglycosylates p^{67} even in the presence of hemin (Figure 5, panel A). This deglycosylation reaction leads to eIF-2 α-subunit phosphorylation (Figure 5, panel B).

The mechanism of activation of p^{67}-deglycosylase during heme deficiency is not understood and may involve participation of several protein factors catalyzing a reaction sequence. There are reports that several glycolipid deglycosylases require low molecular weight protein activators (Li et al., 1985). The mechanism of this activation is unknown. Further work will reveal whether a similar mechanism (requiring a protein activator, as operative in glycolipid deglycosylation) is also operative in glycoprotein deglycosylation, as described in this paper, and whether hemin inhibits the activity of this activator protein.

Finally, several laboratories reported (Holt & Hart, 1986; Holt et al., 1987; Schindler et al., 1987; Davis & Blobel, 1987; Hart et al., 1988; Jackson & Tijan, 1989) isolation of glycoproteins containing clusters of O-linked GlcNAc moieties. However, direct experimental evidence for the regulatory roles of these glycosyl residues is still lacking. In this paper we provide evidence that the GlcNAc residues on p^{67} are indeed necessary for p^{67} activity in order to protect eIF-2 α-subunit from eIF-2 kinase(s) catalyzed phosphorylation. Also, reticulocyte lysate contains enzymic activities which can remove these glycosyl residues and inactivate p^{67} activity. We have now partially purified this activity (unpublished observation).

On the basis of our results presented in this paper, we propose a model of regulation of eIF-2 α-subunit phosphorylation involving hemin, HRI, p^{67}, and p^{67}-deglycosylase (Figure 7). According to our proposed model, the role of hemin is restricted to inhibition of activation of p^{67}-deglycosylase activity. Hemin has no direct effect on eIF-2 kinase activity or eIF-2 kinase catalyzed phosphorylation of eIF-2 α-subunit. Also, once activated in the absence of hemin, p^{67}-deglycosylase actively deglycosylates p^{67} and hemin does not inhibit this deglycosylation reaction. Deglycosylation of p^{67} leads to inactivation of p^{67}, allowing eIF-2 kinase(s) to phosphorylate eIF-2 α-subunit and inhibit protein synthesis. As shown in Figure 3, p^{67} deglycosylation starts after a lag of 2–3 min. Addition of hemin within this period inhibits activation of p^{67}-deglycosylase. This prevents subsequent p^{67} deglycosylation and restores protein synthesis activity. However, after this short lag period, hemin addition is not expected to prevent p^{67} deglycosylation and to restore protein synthesis activity. However, there are conflicting reports regarding the effect of late hemin addition to heme-deficient reticulocyte lysate (i.e., after eIF-2 kinase has acted and eIF-2 α-subunit phosphorylation has been switched on) [see Ranu et al. (1976), Gross (1976), and Matts et al. (1986)]. Also, there are reports that, in intact reticulocytes, iron depletion can lead to strong inhibition, followed by reactivation of translation upon addition of iron or heme (Waxman & Rabinovitz, 1965, 1966; Grayzel et al., 1966). The mechanism of heme reactivation of protein synthesis in heme-depleted, intact reticulocytes is unclear. The possibility exists that added hemin inhibits deglycosylation of newly synthesized p^{67} and thus inhibits phosphorylation of newly synthesized eIF-2 and restores protein synthesis.

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