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# The eukaryotic initiation factor 2-associated 67-kDa polypeptide (p<sup>67</sup>) plays a critical role in regulation of protein synthesis initiation in animal cells

(translational regulation/peptide chain initiation/protein phosphorylation)

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**ABSTRACT** The eukaryotic initiation factor 2 (eIF-2)-associated 67-kDa polypeptide (p<sup>67</sup>) isolated from reticulocyte lysate protects the eIF-2  $\alpha$  subunit from eIF-2 kinase-catalyzed phosphorylation and promotes protein synthesis in the presence of active eIF-2 kinases. We have now studied the roles of p<sup>67</sup> and eIF-2 kinases in regulation of protein synthesis using several animal cell lysates and an animal cell line (KRC-7) in culture under various growth conditions. The results are as follows. (i) Both p<sup>67</sup> and eIF-2 kinase(s) are present in active forms in all animal cells under normal growth conditions and p<sup>67</sup> protects the eIF-2  $\alpha$  subunit from eIF-2 kinase-catalyzed phosphorylation, thus promoting protein synthesis in the presence of active eIF-2 kinases. (ii) In heme-deficient reticulocyte lysates and in serum-starved KRC-7 cells in culture, p<sup>67</sup> is deglycosylated and subsequently degraded. This leads to eIF-2 kinase-catalyzed eIF-2  $\alpha$ -subunit phosphorylation and thus to protein synthesis inhibition. (iii) Addition of a mitogen (namely, phorbol 12-myristate 13-acetate) to serum-starved KRC-7 cells in culture induces an increase of p<sup>67</sup> and thus increases protein synthesis. These results suggest the following conclusions. (i) Protein synthesis inhibition in a heme-deficient reticulocyte lysate is not due to the activation of an eIF-2 kinase (heme-regulated inhibitor), as is generally believed, but is due to degradation of p<sup>67</sup>. The heme-regulated inhibitor is present in an active form and possibly in equal amounts in both heme-deficient and heme-supplemented reticulocyte lysates but cannot phosphorylate eIF-2  $\alpha$  subunit because of the presence of p<sup>67</sup>. (ii) p<sup>67</sup> is essential for protein synthesis as it protects the eIF-2  $\alpha$  subunit from eIF-2 kinase-catalyzed phosphorylation and promotes protein synthesis in the presence of one or more active eIF-2 kinases present in all animal cells. (iii) p<sup>67</sup> is both degradable and inducible. Only the p<sup>67</sup> level correlates directly with the protein synthesis activity of the cell, indicating that p<sup>67</sup> is a critical factor in protein synthesis regulation in animal cells.

A widely used protein synthesis regulatory mechanism in animal cells involves phosphorylation of the  $\alpha$  subunit of a key peptide chain initiation factor, eukaryotic initiation factor 2 (eIF-2), by one or more eIF-2 kinases, which inhibits protein synthesis. In several cases studied, these inhibitors appear to remain in an inactive form and are activated under certain physiological conditions. Numerous reports indicate that in reticulocyte lysates one inhibitor, heme-regulated inhibitor (HRI), is activated in the absence of hemin and another inhibitor, double-stranded-RNA-activated inhibitor (dsI), is activated in the presence of double-stranded RNA (for reviews, see refs. 1 and 2). In both cases the activated inhibitor specifically phosphorylates the eIF-2  $\alpha$  subunit and thus inactivates eIF-2 activity and inhibits protein synthesis.

As noted earlier, this regulatory mechanism involving eIF-2  $\alpha$ -subunit phosphorylation is widely used in animal cells to regulate protein synthesis under various physiological conditions, which include nutritional deprivation (3, 4), heat shock (4–6), and viral infection (7–14).

We have observed that a 67-kDa polypeptide, p<sup>67</sup>, which remains associated with reticulocyte eIF-2 through several steps of purification, protects the eIF-2  $\alpha$  subunit from eIF-2 kinase-catalyzed phosphorylation, thus promoting protein synthesis in the presence of activated eIF-2 kinases (15). p<sup>67</sup> is a glycoprotein and contains multiple O-linked GlcNAc residues (16). There are indications that the glycosyl residues on p<sup>67</sup> may be necessary to protect the eIF-2  $\alpha$  subunit from eIF-2 kinase-catalyzed phosphorylation. Based on our results, we postulated that protein synthesis in animal cells may be regulated by eIF-2 kinases and by the availability of p<sup>67</sup>. eIF-2 kinases phosphorylate the eIF-2  $\alpha$  subunit and thus inhibit protein synthesis, and the increased availability of p<sup>67</sup> renders the eIF-2  $\alpha$  subunit resistant to eIF-2 kinase-catalyzed phosphorylation, which promotes protein synthesis in the presence of eIF-2 kinases. Two reports from our laboratory are in agreement with this hypothesis (17, 18).

In the present study, we have examined the roles of p<sup>67</sup> and the eIF-2 kinases in regulation of protein synthesis using several animal cell lysates and also an animal cell line (KRC-7) in culture under various growth conditions. We provide evidence that p<sup>67</sup> and one or more eIF-2 kinases are present in active forms in all animal cells under normal growth conditions. However, eIF-2 kinases cannot phosphorylate the eIF-2  $\alpha$  subunit because of the presence of p<sup>67</sup>. Under certain physiological conditions, such as during heme deficiency in reticulocyte lysates and also in serum-starved animal cells in culture, p<sup>67</sup> is degraded and thus eIF-2 kinase(s) are able to phosphorylate the eIF-2  $\alpha$  subunit and inhibit protein synthesis. p<sup>67</sup> is both inducible and degradable. Also, p<sup>67</sup> levels in the cells correlate directly with the protein synthesis activity of the cells, suggesting that p<sup>67</sup> plays a critical role in regulation of protein synthesis.

## MATERIALS AND METHODS

Most of the materials used in this study were as described (15–17). The cloned cell line KRC-7 derived from Reuber H35 rat hepatoma cells was kindly provided by John Koontz (University of Tennessee, Knoxville). A sample of dsI prepared according to Kudlicki *et al.* (19) was generously

Abbreviations: eIF-2, eukaryotic initiation factor 2; HRI, heme-regulated inhibitor; dsI, double-stranded-RNA-activated inhibitor; WGA, wheat germ agglutinin; PMA, phorbol 12-myristate 13-acetate.

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donated by Gisela Kramer (Clayton Foundation, Biochemistry Institute, Austin, TX). A 10,000  $\times$  *g* cell supernatant from rat liver and from rat brain was prepared as described by Pain *et al.* (20).

**Preparation of Antibodies.** Polyclonal antibodies against eIF-2  $\alpha$  or  $\beta$  subunits or against  $p^{67}$  were prepared using a modification of the procedure described (15, 16). The isolated polypeptides were injected into mice. When the antibody titer in the serum was  $>1:1000$ , the mouse was injected with TG-180 sarcoma cells (obtained from American Type Culture Collection) for generation of ascites fluid. Antibodies from the ascites fluid were purified on DEAE-Affi-Gel Blue, according to the procedure supplied by the manufacturer (Bio-Rad), and were characterized by NaDodSO<sub>4</sub>/PAGE followed by immunoblot analysis. dsI was purified from interferon- $\beta$ -induced HeLa cells by the procedure of Ray *et al.* (21) and was injected into rabbit. Immune serum from the rabbit was purified on DEAE-Affi-Gel Blue and characterized as above. The monoclonal antibodies against  $p^{67}$  were prepared as described (16). As reported these monoclonal antibodies specifically recognize the GlcNAc moieties on  $p^{67}$  (16). All the antibodies were monospecific and reacted only with their corresponding antigens.

**Cell Culture.** KRC-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM), containing 5% (vol/vol) fetal calf serum and 5% (vol/vol) calf serum. The experimental procedures were essentially similar to those described by Trevillyan *et al.* (22). Serum-starved cells were prepared by washing cell monolayers with Hanks' balanced salt solution followed by culturing in serum-free DMEM for an additional 3 days. The serum-starved cells were stimulated by addition of 1.5  $\mu$ M phorbol 12-myristate 13-acetate (PMA) (Sigma). For analysis,  $6 \times 10^5$  cells were seeded onto 100-mm tissue culture dishes containing 10 ml of medium.

**Cell Lysate Preparation.** Cells were harvested, washed twice with phosphate-buffered saline (PBS, GIBCO), and lysed with lysis buffer [20 mM Hepes, pH 7.5/10 mM KCl/1.5 mM Mg(OAc)<sub>2</sub>/2 mM dithiothreitol]. Cell lysates were centrifuged at 10,000 rpm in a Beckman Microfuge B centrifuge for 10 min. Supernatants were stored at  $-70^\circ\text{C}$ . The concentration of proteins in the lysate was measured by Bio-Rad protein assay kit using a bovine serum albumin standard.

## RESULTS

**Assays for eIF-2 Kinase and  $p^{67}$  Activities.** To assay both eIF-2 kinase(s) and  $p^{67}$  activities in a mixture, we developed an experimental procedure that measures eIF-2  $\alpha$ -subunit phosphorylation in the mixture before and after inhibition of  $p^{67}$  activity by preincubation with either  $p^{67}$  antibodies or wheat germ agglutinin (WGA). As reported (16), WGA binds to the glycosyl residues on  $p^{67}$  and interferes with the  $p^{67}$  activity to protect the eIF-2  $\alpha$  subunit from eIF-2 kinase-catalyzed phosphorylation.

The results of this assay are shown in Fig. 1. As before, HRI efficiently phosphorylated the three-subunit eIF-2 (lane 1) and addition of  $p^{67}$  protected the eIF-2  $\alpha$  subunit from phosphorylation (lane 2). However, the ability of  $p^{67}$  to protect the eIF-2  $\alpha$  subunit was almost completely inhibited by preincubation of the mixture of eIF-2 and  $p^{67}$  with either  $p^{67}$  antibodies (lane 3) or WGA (lane 6). Under similar conditions, preincubation of the mixture of eIF-2 and  $p^{67}$  with either eIF-2  $\alpha$ -subunit or  $\beta$ -subunit antibodies had no significant effect (lanes 4 and 5). None of the antibodies ( $p^{67}$  or eIF-2  $\alpha$  or  $\beta$  subunits), when added alone in the absence of  $p^{67}$ , inhibited HRI-catalyzed phosphorylation of the eIF-2  $\alpha$  subunit (data not shown). We also analyzed various antibody-treated incubation mixtures for possible degradation of  $p^{67}$  by a standard immunoblot

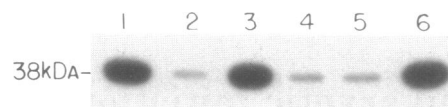


FIG. 1. Assay for eIF-2 kinases and  $p^{67}$  activities. Reaction conditions for HRI-catalyzed phosphorylation of eIF-2  $\alpha$  subunit were as described (15). A 25- $\mu$ l reaction mixture contained 20 mM Tris-HCl (pH 7.8), 100 mM KCl, 10  $\mu$ g of bovine serum albumin, 2 mM dithiothreitol, 3  $\mu$ g of eIF-2 (fraction V), 1.6  $\mu$ g of  $p^{67}$ , and where indicated 10  $\mu$ g of polyclonal antibodies against  $p^{67}$  or against eIF-2  $\alpha$  or  $\beta$  subunits or 10  $\mu$ g of WGA. Reaction mixtures were incubated on ice for 1 hr, then 0.02  $\mu$ g of HRI and 10  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP were added, and the mixtures were further incubated at  $37^\circ\text{C}$  for 10 min. The radioactively labeled eIF-2  $\alpha$  subunit was then analyzed by NaDodSO<sub>4</sub>/PAGE followed by autoradiography. Lanes: 1, eIF-2 alone; 2, eIF-2 plus  $p^{67}$ ; 3, eIF-2,  $p^{67}$ , plus  $p^{67}$  antibodies; 4, eIF-2,  $p^{67}$ , plus eIF-2  $\alpha$ -subunit antibodies; 5, eIF-2,  $p^{67}$ , plus eIF-2  $\beta$ -subunit antibodies; 6, eIF-2,  $p^{67}$ , plus WGA.

procedure.  $p^{67}$  remained undegraded during incubation (data not shown).

We carried out similar experiments using purified dsI in place of HRI and the results were similar.  $p^{67}$  inhibited dsI-catalyzed phosphorylation of eIF-2  $\alpha$  subunits. Also  $p^{67}$  antibodies and WGA inhibited this  $p^{67}$  activity, but eIF-2  $\alpha$ - and  $\beta$ -subunit antibodies had no effect.

**Studies Using Animal Cell Lysates.** We used the above assay to study eIF-2 kinase(s) and  $p^{67}$  activities in four cell lysates; heme-deficient rabbit reticulocyte lysates, heme-supplemented rabbit reticulocyte lysates, and also cell lysates prepared from rat liver and rat brain (Fig. 2). In the absence of  $p^{67}$  antibodies, significant eIF-2  $\alpha$ -subunit phosphorylation was observed only with heme-deficient reticulocyte lysate. In contrast, the heme-supplemented reticulocyte lysate and also the lysates from liver and brain showed very little or no phosphorylation. However, when the cell lysates were preincubated with  $p^{67}$  antibodies significant phosphorylation of the eIF-2  $\alpha$  subunit was observed with all of the cell lysates. The extent of phosphorylation, with both heme-deficient and heme-supplemented reticulocyte lysates, was comparable. However, it should be noted that, although significant changes in the phosphorylation pattern in all of the cell lysates con-

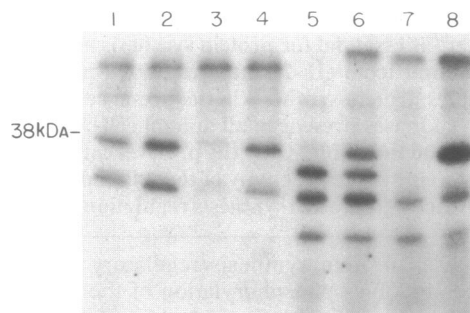


FIG. 2. Analysis of eIF-2 kinases and  $p^{67}$  activities in various cell lysates. Heme-deficient and heme-supplemented reticulocyte lysates and also cell lysates from rat brain and rat liver were used. Heme-deficient and heme-supplemented reticulocyte lysates prepared as described (15) were preincubated at  $37^\circ\text{C}$  for 10 min before use. Approximately 20  $\mu$ l of reticulocyte lysate or 120  $\mu$ g of protein in brain or liver extract was used. eIF-2  $\alpha$ -subunit phosphorylation by endogenous eIF-2 kinase(s) in the presence or absence of  $p^{67}$  antibodies was carried out as described in Fig. 1 and phosphorylated proteins were analyzed by NaDodSO<sub>4</sub>/PAGE and autoradiography. Lanes: 1, heme-deficient reticulocyte lysate; 2, heme-deficient reticulocyte lysate-preincubated with  $p^{67}$  antibodies; 3, heme-supplemented reticulocyte lysate; 4, heme-supplemented reticulocyte lysate preincubated with  $p^{67}$  antibodies; 5, lysate from rat brain; 6, lysate from rat brain preincubated with  $p^{67}$  antibodies; 7, lysate from rat liver; 8, lysate from rat liver preincubated with  $p^{67}$  antibodies.

taining p<sup>67</sup> antibodies was observed with the eIF-2  $\alpha$  subunit, one polypeptide of  $\approx$ 200 kDa or more polypeptides present in reticulocyte lysates and in rat brain and rat liver lysates showed a similar change in phosphorylation pattern. The significance of this observation will be discussed later.

These results, however, show that both eIF-2 kinase(s) and p<sup>67</sup> are present in active forms in all of the cell lysates and p<sup>67</sup> inhibits eIF-2  $\alpha$ -subunit phosphorylation by active eIF-2 kinases. Removal of p<sup>67</sup> by p<sup>67</sup> antibodies facilitated eIF-2 kinase-catalyzed phosphorylation of the eIF-2  $\alpha$  subunit.

The results presented in Fig. 3 show that p<sup>67</sup> is indeed present in heme-supplemented reticulocyte lysate; and this polypeptide is partially deglycosylated in heme-deficient reticulocyte lysate. For these experiments the lysates were preincubated with p<sup>67</sup> monoclonal (Fig. 3A) or polyclonal (Fig. 3B) antibodies and the antigen-antibody complexes were precipitated with protein A-agarose (23). The precipitates were then assayed on an immunoblot using p<sup>67</sup> polyclonal antibodies. p<sup>67</sup> is present in heme-supplemented reticulocyte lysate (Fig. 3 A and B, lanes 2) and also in heme-deficient reticulocyte lysate (Fig. 3 A and B, lanes 1). However, the level of this polypeptide in heme-deficient reticulocyte lysate was significantly lower. In experiments described in Fig. 3A, p<sup>67</sup> present in the lysates was immunoprecipitated using p<sup>67</sup> monoclonal antibodies, which reacted only with glycosylated p<sup>67</sup>. The p<sup>67</sup> level in heme-deficient lysate (lane 1) was  $<20\%$  of that present in heme-supplemented lysate (lane 2), indicating extensive deglycosylation of p<sup>67</sup> in heme-deficient lysate.

These results thus provide evidence that p<sup>67</sup> is present in both heme-supplemented and heme-deficient reticulocyte lysates. In heme-deficient lysate, p<sup>67</sup> is partially deglycosylated. This allows eIF-2 kinases to phosphorylate eIF-2 and thus inhibit protein synthesis. Using a similar immunoblot experiment, we also observed that p<sup>67</sup> is present in glycosy-

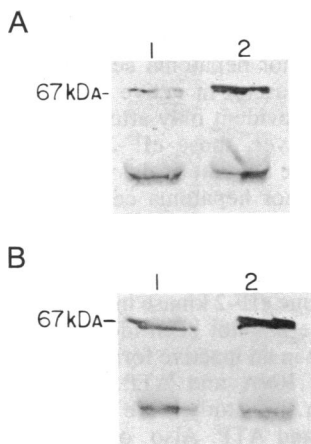
lated form in both rat liver and rat brain extracts (data not shown).

**Studies Using a Tumor Hepatoma Cell Line (KRC-7).** We used a tumor hepatoma cell line (KRC-7) and analyzed the roles of p<sup>67</sup> and eIF-2 kinase(s) in regulating protein synthesis under various growth conditions. We grew the cells to confluency, then added serum-depleted medium for 3–4 days, and stimulated the serum-starved cells with a mitogen, PMA. We measured the protein synthesis rate, the levels of various polypeptides, and also activation states of eIF-2 kinase(s) at various times during confluency, after serum starvation, and after PMA addition. Protein synthesis rate was measured by incorporation of [<sup>35</sup>S]methionine into cellular proteins as described (17). The protein synthesis rate was maximum in confluent cells and was reduced to 30% of the maximum level after 100 hr of serum starvation. Upon PMA addition the serum-starved cells regained 70% of the original protein synthesis activity of the confluent cells within 4 hr.

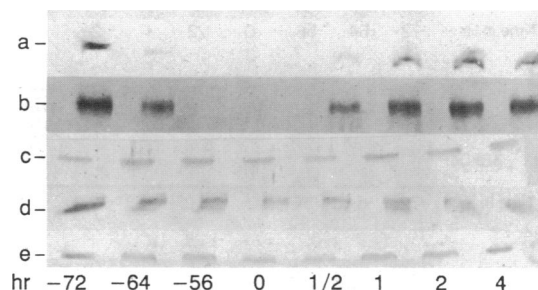
**Levels of Various Polypeptides.** Immunoblot analysis was used to measure levels of various polypeptides (p<sup>67</sup>, dsI, and eIF-2  $\beta$  and  $\alpha$  subunits) at various times, after serum starvation and after PMA addition (Fig. 4). p<sup>67</sup> levels were measured using both monoclonal and polyclonal antibodies. dsI and eIF-2  $\beta$ - and  $\alpha$ -subunit levels were measured using polyclonal antibodies.

As shown in Fig. 4, levels of eIF-2  $\beta$  and  $\alpha$  subunits and, more importantly, the level of dsI remained essentially unchanged in confluent (–72 hr), serum-starved, and PMA-stimulated cells. On the other hand, p<sup>67</sup> levels changed dramatically; p<sup>67</sup> was prominent in confluent cells, disappeared rapidly after serum starvation, and became prominent after PMA addition. A significant observation is that p<sup>67</sup> level decreased faster after serum depletion and appeared more slowly after PMA addition when measured using monoclonal antibodies than when measured using polyclonal antibodies. These results, in agreement with our observation with heme-deficient reticulocyte lysates (Fig. 3), suggest that the first step after serum depletion may be deglycosylation of p<sup>67</sup> and that deglycosylated p<sup>67</sup> is subsequently degraded.

**eIF-2 Kinase Activity.** The results presented in Fig. 4 show that the dsI polypeptide level remains essentially unchanged under various growth conditions. However, the eIF-2 kinases, such as HRI and dsI, may remain in either active or inactive forms. We have, therefore, analyzed eIF-2 kinase



**FIG. 3.** Analysis of p<sup>67</sup> in heme-deficient and heme-supplemented reticulocyte lysates. Reticulocyte lysates (40  $\mu$ l) without or with hemin (25  $\mu$ l) were incubated for 10 min at 37°C. (A) The lysates were mixed with 25  $\mu$ g of p<sup>67</sup> monoclonal antibodies. (B) The lysates were mixed with 25  $\mu$ g of p<sup>67</sup> polyclonal antibodies. The reaction mixtures were incubated at 4°C overnight and antigen-antibody complexes were precipitated by adding 30  $\mu$ l of protein A-agarose (GIBCO) followed by gentle tapping of the suspension for 1 hr at room temperature. The precipitates were then washed three times with 1 $\times$  PBS containing 0.05% Nonidet P-40. The polypeptides in the precipitates were then separated by NaDodSO<sub>4</sub>/PAGE on 15% gels and subsequently transferred electrophoretically to a nitrocellulose sheet. Lanes were excised from the blot and incubated with p<sup>67</sup> antibodies as described by Bio-Rad (Bio-Rad immunoblot assay kit). The antigen-antibody complexes were then detected using goat anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad). (A) Lanes: 1, heme-deficient lysate; 2, heme-supplemented lysate. (B) Lanes: 1, heme-deficient lysate; 2, heme-supplemented lysate. The lower molecular mass bands represent the IgG heavy chain.



**FIG. 4.** Immunoblot analysis of p<sup>67</sup>, dsI, eIF-2  $\beta$  subunit (p<sup>54</sup>), and eIF-2  $\alpha$  subunit (p<sup>38</sup>) in tumor hepatoma cells (KRC-7) that are confluent, quiescent, or PMA-induced. Cells grown to confluency (lane –72 hr) were transferred to serum-depleted medium and after 72 hr (lane 0 hr) were treated with PMA. Approximately 120  $\mu$ g of protein in cell lysates was analyzed on an immunoblot using p<sup>67</sup> monoclonal antibodies (row a), p<sup>67</sup> polyclonal antibodies (row b), dsI polyclonal antibodies (row c), eIF-2  $\beta$ -subunit polyclonal antibodies (row d), and eIF-2  $\alpha$ -subunit polyclonal antibodies (row e). The immunoblot procedure for detection of p<sup>67</sup> and eIF-2  $\alpha$  and eIF-2  $\beta$  subunits was the same as described in Fig. 3. The immunoblot procedure for detection of dsI was the same except that goat anti-rabbit IgG conjugated to horseradish peroxidase was used to detect dsI rabbit antibodies.

activities by analyzing eIF-2  $\alpha$ -subunit phosphorylation of endogenous eIF-2 using cell extracts at various times after the assay as described in Fig. 1. eIF-2  $\alpha$ -subunit phosphorylation was low with extracts from confluent and PMA-induced cells but was significantly increased when a similar extract from quiescent cell was used (Fig. 5A). However, as described in Fig. 1, when the same cell extracts were first preincubated with  $p^{67}$  polyclonal antibodies to remove endogenous  $p^{67}$  (Fig. 5B) or with WGA to inhibit the eIF-2- $p^{67}$  interaction (Fig. 5C), the extent of eIF-2  $\alpha$ -subunit phosphorylation using all the cell extracts was significantly increased to approximately the same level.

## DISCUSSION

Data presented in this report provide evidence that all animal cells contain one or more eIF-2 kinases in the active form and the activities of these eIF-2 kinases may or may not be evident, depending on the variable presence of  $p^{67}$ .  $p^{67}$  protects the eIF-2  $\alpha$  subunit from eIF-2 kinase-catalyzed phosphorylation and thus promotes protein synthesis in the presence of active eIF-2 kinase(s).

In separate experiments we observed that  $p^{67}$  specifically protects the eIF-2  $\alpha$  subunit from both HRI- and dsI-catalyzed phosphorylation but does not inhibit dsI-catalyzed phosphorylation of histones or casein kinase catalyzed phosphorylation of the eIF-2  $\beta$  subunit (data not shown). However, the results presented in Fig. 2 indicate that  $p^{67}$  also inhibits phosphorylation of one or more additional proteins (30 and 110 kDa) present in reticulocyte lysates and also in rat brain and rat liver lysates. It may be that under *in vitro* conditions, the glycosyl residues of  $p^{67}$  may bind to these additional proteins and thus inhibit their phosphorylation. On the other hand, the possibility that  $p^{67}$  may inhibit phosphorylation of a select group of closely related proteins cannot be ruled out. However, abundant evidence reported previously and also presented in this paper suggests that a major function of  $p^{67}$  is to protect the eIF-2  $\alpha$  subunit from eIF-2 kinase-catalyzed phosphorylation and thus to promote protein synthesis in the presence of active eIF-2 kinases.

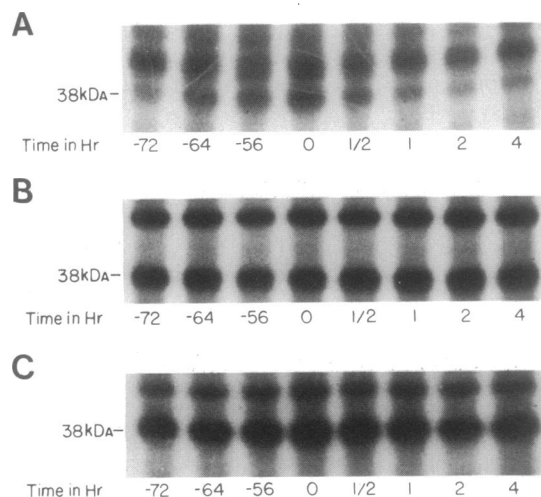


FIG. 5. eIF-2  $\alpha$ -subunit phosphorylation using a lysate from KRC-7 cells grown under various conditions. (A) Approximately 60  $\mu$ g of protein in lysates from confluent (lanes -72 hr), serum-starved (lanes -64 hr through 0 hr), and PMA-induced (lanes 1/2, 1, 2, and 4 hr) KRC-7 cells was used and phosphorylation of the endogenous eIF-2  $\alpha$  subunit by endogenous eIF-2 kinases was carried out as described in Fig. 1. (B) The cell lysates were preincubated with  $p^{67}$  polyclonal antibodies. (C) The lysates were preincubated with WGA and were then phosphorylated.

Two widely studied eIF-2 kinases are HRI and dsI. In heme-deficient reticulocyte lysates, only HRI is active, as dsI requires double-stranded RNA and ATP for activation. In this report, we have provided evidence that, in both heme-supplemented and heme-deficient lysates, HRI is present in active form and possibly in equal amounts. In heme-supplemented lysate, this HRI activity is not evident due to the presence of  $p^{67}$ , whereas in heme-deficient lysates  $p^{67}$  appears to be degraded, thus allowing HRI to actively phosphorylate eIF-2  $\alpha$  subunit. Removal of  $p^{67}$  from both heme-supplemented and heme-deficient reticulocyte lysates with  $p^{67}$  antibodies resulted in similar extents of eIF-2  $\alpha$ -subunit phosphorylation. This indicates that the eIF-2 kinase in active form may be present at the same level in both systems. These results suggest that eIF-2  $\alpha$ -subunit phosphorylation, observed in heme-deficient reticulocyte lysate, is not due to activation of HRI, as is widely believed, but rather to degradation of  $p^{67}$ . We also provide evidence that  $p^{67}$  degradation involves partial deglycosylation in heme-deficient lysates. It may, therefore, be assumed that hemin, present in heme-supplemented lysates, prevents  $p^{67}$  deglycosylation, possibly by inhibiting the deglycosylating enzyme(s).

Reticulocyte lysates also contain another eIF-2 kinase, dsI, in inactive form, and this eIF-2 kinase is activated in the presence of double-stranded RNA and ATP (1, 2). Activated dsI inhibits protein synthesis even in the presence of hemin and presumably in the presence of  $p^{67}$ . The mechanism of eIF-2  $\alpha$ -subunit phosphorylation by dsI in the presence of  $p^{67}$  is not clear. Further work will be necessary to examine  $p^{67}$  activity during dsI inhibition of protein synthesis in heme-supplemented reticulocyte lysate. It may be possible, however, that dsI is formed in relatively large excess in the presence of double-stranded RNA and ATP and at a very high concentration can phosphorylate the eIF-2  $\alpha$  subunit even in the presence of equimolar concentrations of  $p^{67}$ .

Like heme-supplemented reticulocyte lysates, the cell lysates prepared from rat brain, rat liver, and confluent and PMA-induced tumor hepatoma cells (KRC-7) contain both  $p^{67}$  and eIF-2 kinase(s) in active forms, and eIF-2 kinase activity becomes evident only after removal of  $p^{67}$  with  $p^{67}$  antibodies. However, these eIF-2 kinases have not been characterized. The amount of dsI polypeptide is essentially unchanged in tumor hepatoma cells under various growth conditions. This includes confluency, serum starvation, and mitogen stimulation (Fig. 4). Again, it is not clear whether this dsI is in an active form. Our results show that these cells contain at least one eIF-2 kinase in an active form; however, this eIF-2 kinase has not been identified as yet as dsI. As noted, dsI exists in an inactive form in the cells and requires double-stranded RNA and ATP for activation. The cell extracts used in this study were not treated with double-stranded RNA and ATP. Also, our present work suggests that the HRI may be a normal cell constituent, as this inhibitor is present in an active form and in approximately equal amounts in both heme-supplemented and heme-deficient reticulocyte lysates. Possibly a HRI-like eIF-2 kinase is present in an active form in all animal cells and dsI activation serves specialized functions under certain physiological conditions. As postulated earlier, this inhibitor (dsI) may be formed in large excess upon activation under certain physiological conditions, such as virus infections, and may inhibit protein synthesis in the presence of normal  $p^{67}$  concentrations.

The roles and requirements for  $p^{67}$  in KRC-7 cells under various growth conditions are clearly evident. This polypeptide is present in high concentrations in confluent cells and disappears rapidly from the cells after serum depletion with an accompanying decrease in the protein synthesis rate in the cells. Also, this polypeptide reappears soon after PMA addition to the serum-starved resting cells as the cells regain

protein synthesis activity. Thus, there is a clear correlation between the  $p^{67}$  level and the protein synthesis activity of the cells, indicating a direct involvement of  $p^{67}$  in protein synthesis. Furthermore, a comparison of the  $p^{67}$  levels studied using monoclonal (Fig. 4, row a) and polyclonal (Fig. 4, row b) antibodies reveals (in agreement with our previous observation with the heme-deficient reticulocyte lysates, Fig. 3) that the initial event in  $p^{67}$  degradation involves deglycosylation of  $p^{67}$  and deglycosylated  $p^{67}$  is unstable and is subsequently degraded. These results, therefore, establish the following facts. (i)  $p^{67}$  is necessary to protect the eIF-2  $\alpha$  subunit from eIF-2 kinase-catalyzed phosphorylation and thus to promote protein synthesis in the presence of active eIF-2 kinase(s). (ii) At least one eIF-2 kinase is present in all of the cells in active form under various growth conditions. (iii)  $p^{67}$  is degraded rapidly in serum-depleted cells thus allowing eIF-2 kinase(s) to phosphorylate eIF-2 and inhibit protein synthesis. (iv) The initial event of  $p^{67}$  degradation involves deglycosylation of the  $p^{67}$  polypeptide.

An important aspect of  $p^{67}$  regulation is that this is the only polypeptide presently studied that is both easily degradable and inducible. The levels of this polypeptide correlate directly to the protein synthesis activities of the cells. As evident in this study, the initial event for degradation involves deglycosylation of  $p^{67}$ . The deglycosylated  $p^{67}$  is unstable and subsequently degraded. Again, addition of a mitogen, such as PMA, induces increased appearance of  $p^{67}$ , presumably by transcription activation of the  $p^{67}$  gene. Thus,  $p^{67}$  level in the cell is regulated both transcriptionally and posttranscriptionally. This factor is essential for protein synthesis and thus plays a critical role in protein synthesis regulation.

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