The eukaryotic initiation factor 2-associated 67-kDa polypeptide (p$^{67}$) plays a critical role in regulation of protein synthesis initiation in animal cells

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The eukaryotic initiation factor 2-associated 67-kDa polypeptide (p67) 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 (p67) isolated from reticulocyte lysate protects the eIF-2 α 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 p67 and eIF-2 kinases in regulation of protein synthesis using several animal cell lines and an animal cell line (KRC-7) in culture under various growth conditions. The results are as follows. (i) Both p67 and eIF-2 kinase(s) are present in active forms in all animal cells under normal growth conditions and p67 protects the eIF-2 α 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, p67 is deglycosylated and subsequently degraded. This leads to eIF-2 kinase-catalyzed eIF-2 α-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 p67 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 p67. 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 α subunit because of the presence of p67. (ii) p67 is essential for protein synthesis as it protects the eIF-2 α 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) p67 is both degradable and inducible. Only the p67 level correlates directly with the protein synthesis activity of the cell, indicating that p67 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 α 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 heme 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 α subunit and thus inactivates eIF-2 activity and inhibits protein synthesis.

As noted earlier, this regulatory mechanism involving eIF-2 α-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, p67, which remains associated with reticulocyte eIF-2 through several steps of purification, protects the eIF-2 α subunit from eIF-2 kinase-catalyzed phosphorylation, thus promoting protein synthesis in the presence of activated eIF-2 kinases (15). p67 is a glycoprotein and contains multiple O-linked GlcNAc residues (16). There are indications that the glycosyl residues on p67 may be necessary to protect the eIF-2 α 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 p67. eIF-2 kinases phosphorylate the eIF-2 α subunit and thus inhibit protein synthesis, and the increased availability of p67 renders the eIF-2 α 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 p67 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 p67 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 α subunit because of the presence of p67. Under certain physiological conditions, such as during heme deficiency in reticulocyte lysates and also in serum-starved animal cells in culture, p67 is degraded and thus eIF-2 kinase(s) are able to phosphorylate the eIF-2 α subunit and inhibit protein synthesis. p67 is both inducible and degradable. Also, p67 levels in the cells correlate directly with the protein synthesis activity of the cells, suggesting that p67 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 × 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 α or β subunits or against p67 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 NaDodSO4/PAGE followed by immunoblot analysis of ds1 were purified from interferon-β-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 p67 were prepared as described (16). As reported these monoclonal antibodies specifically recognize the GlcNAc moieties on p67 (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 Trevisan 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 μM phorbol 12-myristate 13-acetate (PMA) (Sigma). For analysis, 6 × 10^6 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 Heps, pH 7.5/10 mM KCl/1.5 mM Mg(OAc)2/2 mM dithiothreitol]. Cell lysates were centrifuged at 10,000 rpm in a Beckman Microfuge B centrifuge for 10 min. Supernatants were stored at −70°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 p67 Activities.** To assay both eIF-2 kinase(s) and p67 activities in a mixture, we developed an experimental procedure that measures eIF-2 α-subunit phosphorylation in the mixture before and after inhibition of p67 activity by preincubation with either p67 antibodies or wheat germ agglutinin (WGA). As reported (16), WGA binds to the glycosyl residues on p67 and interferes with the p67 activity to protect the eIF-2 α subunit from eIF-2 kinase-catalyzed phosphorylation.

The results of this assay are shown in Fig. 1. As before, HR1 efficiently phosphorylated the three-subunit eIF-2 (lane 1) and addition of p67 protected the eIF-2 α subunit from phosphorylation (lane 2). However, the ability of p67 to protect the eIF-2 α subunit was almost completely inhibited by preincubation of the mixture of eIF-2 and p67 with either p67 antibodies (lane 3) or WGA (lane 6). Under similar conditions, preincubation of the mixture of eIF-2 and p67 with either eIF-2 α-subunit or β-subunit antibodies had no significant effect (lanes 4 and 5).

None of the antibodies (p67 or eIF-2 α or β subunits), when added alone in the absence of p67, inhibited HR1-catalyzed phosphorylation of the eIF-2 α subunit (data not shown). We also analyzed various antibody-treated incubation mixtures for possible degradation of p67 by a standard immunoblot procedure. p67 remained unchanged during incubation (data not shown).

We carried out similar experiments using purified ds1 in place of HR1 and the results were similar. p67 inhibited ds1-catalyzed phosphorylation of eIF-2 α subunits. Also p67 antibodies and WGA inhibited this p67 activity, but eIF-2 α- and β-subunit antibodies had no effect.

**Studies Using Animal Cell Lysates.** We used the above assay to study eIF-2 kinase(s) and p67 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 p67 antibodies, significant eIF-2 α-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 p67 antibodies significant phosphorylation of the eIF-2 α 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-
taining p67 antibodies was observed with the eIF-2 α subunit, one polypeptide of ~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 p67 are present in active forms in all of the cell lysates and p67 inhibits eIF-2 α-subunit phosphorylation by active eIF-2 kinases. Removal of p67 by p67 antibodies facilitated eIF-2 kinase-catalyzed phosphorylation of the eIF-2 α subunit.

The results presented in Fig. 3 show that p67 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 p67 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 immuno blot using p67 polyclonal antibodies. p67 is present in heme-supplemented reticulocyte lysate (Fig. 3A and B, lanes 2) and also in heme-deficient reticulocyte lysate (Fig. 3A and B, lanes 1). However, the level of this polypeptide in heme-deficient reticulocyte lysate was significantly lower. In experiments described in Fig. 3A, p67 present in the lysates was immunoprecipitated using p67 monoclonal antibodies, which reacted only with glycosylated p67. The p67 level in heme-deficient lysate (lane 1) was <20% of that present in heme-supplemented lysate (lane 2), indicating extensive deglycosylation of p67 in heme-deficient lysate.

These results thus provide evidence that p67 is present in both heme-supplemented and heme-deficient reticulocyte lysates. In heme-deficient lysate, p67 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 p67 is present in glycosylated 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 p67 and eIF-2 kinase(s) in regulating protein synthesis under various growth conditions. We grew the cells to confluence, 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 confluence, after serum starvation, and after PMA addition. Protein synthesis rate was measured by incorporation of [35S]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 (p67, dsl, and eIF-2 β and α subunits) at various times, after serum starvation and after PMA addition (Fig. 4). p67 levels were measured using both monoclonal and polyclonal antibodies. dsl and eIF-2 β- and α-subunit levels were measured using polyclonal antibodies.

As shown in Fig. 4, levels of eIF-2 β and α subunits and, more importantly, the level of dsl remained essentially unchanged in confluent (~72 hr), serum-starved, and PMA-stimulated cells. On the other hand, p67 levels changed dramatically; p67 was prominent in confluent cells, disappeared rapidly after serum starvation, and became prominent after PMA addition. A significant observation is that p67 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 p67 and that deglycosylated p67 is subsequently degraded.

eIF-2 Kinase Activity. The results presented in Fig. 4 show that the dsl polypeptide level remains essentially unchanged under various growth conditions. However, the eIF-2 kinases, such as HR1 and dsl, may remain in either active or inactive forms. We have, therefore, analyzed eIF-2 kinase

![Fig. 3.](image-url) Analysis of p67 in heme-deficient and heme-supplemented reticulocyte lysates. Reticulocyte lysates (40 μl) without or with hemin (25 μl) were incubated for 10 min at 37°C. (A) The lysates were mixed with 25 μg of p67 monoclonal antibodies. (B) The lysates were mixed with 25 μg of p67 polyclonal antibodies. The reaction mixtures were incubated at 4°C overnight and antigen-antibody complexes were precipitated by adding 30 μl 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× PBS containing 0.05% Nonidet P-40. The polypeptides in the precipitates were then separated by NaDodSO4/PAGE on 15% gels and subsequently transferred electrophotically to a nitrocellulose sheet. Lanes were excised from the blot and incubated with p67 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.](image-url) Immunoblot analysis of p67, dsl, eIF-2 β subunit (p48), and eIF-2 α subunit (p69) in tumor hepatoma cells (KRC-7) that are confluent, quiescent, or PMA-induced. Cells grown to confluence (lane ~72 hr) were transfected to serum-depleted medium and after 72 hr (lane 0 hr) were treated with PMA. Approximately 120 μg of protein in cell lysates was analyzed on an immunoblot using p67 monoclonal antibodies (row a), p48 polyclonal antibodies (row b), dsl polyclonal antibodies (row c), eIF-2 β-subunit polyclonal antibodies (row d), and eIF-2 α-subunit polyclonal antibodies (row e). The immunoblot procedure for detection of p67 and eIF-2 β and eIF-2 α subunits was the same as described in Fig. 3. The immunoblot procedure for detection of dsl was the same except that goat anti-rabbit IgG conjugated to horseradish peroxidase was used to detect dsl rabbit antibodies.
activities by analyzing eIF-2 α-subunit phosphorylation of endogenous eIF-2 using cell extracts at various times after the assay as described in Fig. 1. eIF-2 α-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 p67 polyclonal antibodies to remove endogenous p67 (Fig. 5B) or with WGA to inhibit the eIF-2–p67 interaction (Fig. 5C), the extent of eIF-2 α-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 kinase may or may not be evident, depending on the variable presence of p67. p67 protects the eIF-2 α subunit from eIF-2 kinase-catalyzed phosphorylation and thus promotes protein synthesis in the presence of active eIF-2 kinase.

In separate experiments we observed that p67 specifically protects the eIF-2 α 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 β subunit (data not shown). However, the results presented in Fig. 2 indicate that p67 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 p67 may bind to these additional proteins and thus inhibit their phosphorylation. On the other hand, the possibility that p67 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 p67 is to protect the eIF-2 α subunit from eIF-2 kinase-catalyzed phosphorylation and thus promote protein synthesis in the presence of active eIF-2 kinase.

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 p67, whereas in heme-deficient lysates p67 appears to be degraded, thus allowing HRI to actively phosphorylate eIF-2 α subunit. Removal of p67 from both heme-supplemented and heme-deficient reticulocyte lysates with p67 antibodies resulted in similar extents of eIF-2 α-subunit phosphorylations. 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 α-subunit phosphorylation, observed in heme-deficient reticulocyte lysate, is not due to activation of HRI, as is widely believed, but rather to degradation of p67. We also provide evidence that p67 degradation involves partial deglycosylation in heme-deficient lysates. It may, therefore, be assumed that hemin, present in heme-supplemented lysates, prevents p67 deglycosylation, possibly by inhibiting the deglycosylating enzymes.

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 p67. The mechanism of eIF-2 α-subunit phosphorylation by dsI in the presence of p67 is not clear. Further work will be necessary to examine p67 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 α subunit even in the presence of equimolar concentrations of p67.

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 p67 and eIF-2 kinase(s) in active forms, and eIF-2 kinase activity becomes evident only after removal of p67 with p67 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 confluence, 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 p67 concentrations.

The roles and requirements for p67 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 p67 level and the protein synthesis activity of the cells, indicating a direct involvement of p67 in protein synthesis. Furthermore, a comparison of the p67 levels studied using monoclonal (Fig. 4, row a) and polyclonal (Fig. 4, row b) antibodies reveals (in agreement with our previous observation with the hem-deficient reticulocyte lysates, Fig. 3) that the initial event in p67 degradation involves deglycosylation of p67 and deglycosylated p67 is unstable and is subsequently degraded. These results, therefore, establish the following facts. (i) p67 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) p67 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 p67 degradation involves deglycosylation of the p67 polypeptide.

An important aspect of p67 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 p67. The deglycosylated p67 is unstable and subsequently degraded. Again, addition of a mitogen, such as PMA, induces increased appearance of p67, presumably by transcription activation of the p67 gene. Thus, p67 level in the cell is regulated both transcriptionally and posttranslationally. This factor is essential for protein synthesis and thus plays a critical role in protein synthesis regulation.

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