Phosphorylation of Eukaryotic Initiation Factor 2 during Physiological Stresses Which Affect Protein Synthesis*

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Kathleen A. Scorsone*, Richard Panniers, Anne G. Rowlands, and Edgar C. Henshaw
From the Cancer Center and Department of Biochemistry, University of Rochester, Rochester, New York 14642

Phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF-2) is a major mechanism regulating protein synthesis in rabbit reticulocytes. To determine whether phosphorylation of eIF-2α is a likely regulatory mechanism in the Ehrlich cell, we have measured the percent of cellular eIF-2α which is phosphorylated in cells exposed to heat shock, 2-deoxyglucose, or amino acid deprivation, conditions which rapidly decrease the concentration of 40 S initiation complexes and inhibit protein synthesis. eIF-2α and eIF-2α(P) were separated by isoelectric focusing and were detected by immunoblotting with a monoclonal antibody we developed for this purpose.

Under the above three inhibitory conditions, phosphorylation of eIF-2α increased rapidly, and this increase correlated in time with the rapid inhibition of protein synthesis. In heat-shocked cells which were returned to 37°C, both phosphorylation and protein synthesis remained unchanged for 10 min and then returned toward control values slowly and in parallel. The close temporal correspondence between changes in protein synthesis and phosphorylation supports an important regulatory role for phosphorylation in protein synthesis.

An increase of 25–35 percentage points, to 50–60% phosphorylation from control levels of 20–30% phosphorylation, correlated with an 80–100% inhibition of protein synthesis. This steep curve of inhibition is consistent with a mechanism in which eIF-2α(P) saturates and inhibits the guanine-nucleotide exchange factor.

EXPERIMENTAL PROCEDURES

RESULTS

Regulation of Phosphorylation of eIF-2α in Response to Physiological Stimuli in the Ehrlich Cell

We have used the IEF immunoassay to assess the possibility that increased phosphorylation of eIF-2α is responsible for...
**FIG. 4.** Phosphorylation of eIF-2α in heat-shocked cells. Cells were incubated with [14C]leucine at 37 °C (□) or 43 °C (■). Fifty-μl samples were removed at indicated times to determine the amount of leucine incorporated into protein (A), separate and detect eIF-2α and eIF-2a(P) by isoelectric focusing and immunoblot analysis (B) (paired lanes represent 15-μl and 30-μl cell samples, respectively), and quantitate the percent eIF-2 phosphorylated from the autoradiographs in B by laser densitometry (C).

**FIG. 5.** Recovery of protein synthesis and dephosphorylation of eIF-2α in heat-shocked cells returned to 37 °C. Cells were incubated as described in Fig. 4, but one culture (■) was transferred from 43 to 37 °C after 20 min at the higher temperature. Analyses of protein synthesis (A) and quantitation of levels of eIF-2(P) (B) are described in the legend to Fig. 4.

**FIG. 6.** Correlation of rate of protein synthesis and increase in eIF-2α(P) in amino acid-deprived cells. Fed cells (□) or cells deprived of glutamine (■) were incubated at 37 °C with [14C]leucine. Samples were analyzed for uptake of leucine into protein (A) and level of eIF-2α(P) (B) as described.

**TABLE I**

<table>
<thead>
<tr>
<th>Protein synthesis</th>
<th>eIF-2 phosphorylated</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpm</td>
<td>%</td>
</tr>
<tr>
<td>Control</td>
<td>9060</td>
</tr>
<tr>
<td>-Glucose</td>
<td>7000</td>
</tr>
<tr>
<td>+2-Deoxyglucose</td>
<td>1500</td>
</tr>
<tr>
<td>-Glucose</td>
<td></td>
</tr>
</tbody>
</table>

5B), the recovery in eIF-2 showing a similar lag and perhaps slightly preceding the recovery of protein synthesis. This figure also illustrates the reproducibility of the pattern of response of eIF-2 phosphorylation.

the inhibition of protein synthesis under three conditions in which the concentration of 40 S initiation complexes is known to be depressed. On the basis of known eIF-2 characteristics, increased phosphorylation would be expected to reduce 40 S complexes.

*Heat Shock*—Fig. 4A shows the rapid onset of severe progressive inhibition of protein synthesis in Ehrlich cells incubated at 43 °C. A significant difference in phosphorylation of eIF-2α is apparent within 8 min between control (26%) and heat-shocked cells (34%) in the autoradiograph (Fig. 4B) and laser densitometer scan of the autoradiograph (Fig. 4C). By the next time point, experimental cells have reached 60%, while controls remain steady at 26%. At 2.5 min, however, there is little difference in degree of phosphorylation and no inhibition of protein synthesis is visible. Fig. 5 presents data from a similar experiment in which one culture was returned to 37 °C after 20 min of heat shock. As we have shown previously (6), protein synthesis recovered after a lag (Fig. 5A). Over the time period of protein synthesis recovery, the phosphorylation state of eIF-2α returned toward normal (Fig.
Amino Acid Deprivation—Protein synthesis falls very quickly in Ehrlich cells deprived of glutamine, and the concentration of 40 S initiation complexes is reduced (2, 25). Fig. 6A shows the rate of protein synthesis in such an experiment. Fig. 6B shows that within 10 min the phosphorylation of eIF-2a increased to a greater extent (37%) compared to 22% in control cells. In this experiment, phosphorylation in control cells rose slowly over an hour from 22 to 30% even though the rate of protein synthesis appeared to remain constant over the same period.

Glucose Deprivation—In glucose-deprived cells, the inhibition of protein synthesis was initially less severe than in amino acid-deprived cells. Although the time course was somewhat variable from experiment to experiment, inhibition was generally only 20–35% at 1 h, presumably because of the availability of other energy sources such as glycogen (Table I). Inhibition became progressively more severe with time, reaching 40–60% at 5 h (not shown). The energy deficit and the inhibition of protein synthesis could be made much more rapid and dramatic by the addition of 2-deoxyglucose, an inhibitor of glycolysis, to the glucose-free medium. In the experiment shown in Table I, protein synthesis was inhibited 25% by glucose deprivation for 1 h and was inhibited 84% with the addition of 2-deoxyglucose. After 1 h, the level of phosphorylation of eIF-2 in control cells was 38% compared to 48% in the 2-deoxyglucose-treated cells, but did not increase perceptibly in cells deprived only of glucose. In 2-deoxyglucose-treated cells, inhibition of protein synthesis and phosphorylation of eIF-2 increased in parallel and were at a plateau level by 20 min (not shown). More prolonged deprivation of glucose alone led to more severe inhibition of protein synthesis and to some increase in phosphorylation of eIF-2. The increase in phosphorylation was not, however, as great as in heat-shocked cells at comparable level of inhibition of protein synthesis.

Emetine—We have also treated the cells with emetine, an inhibitor of polypeptide chain elongation (33) which would not be expected to produce a decrease in 40 S initiation complexes. While protein synthesis was inhibited 96%, in contrast to the results under the previous conditions, phosphorylation of eIF-2a did not rise, and, in fact, fell from 23 to 16% within 10 min (data not shown).

DISCUSSION

Inhibition of eIF-2 function in Ehrlich cells exposed to heat shock, amino acid deprivation, and 2-deoxyglucose has been implied previously by the depression of 40 S initiation complexes. The increase in phosphorylation of eIF-2a under these three conditions provides an explanation for the impaired function of eIF-2 because the guanine-nucleotide exchange factor, GEF, is able to release GDP only from eIF-2, not eIF-2a(P). Thus, phosphorylated eIF-2 is unable to function cyclically in protein synthesis. However, the fractional loss in rate of protein synthesis is much greater than the fractional increase in phosphorylated eIF-2, so that an increase in phosphorylation from 26 to 63% during heat shock, for instance, caused a 96% fall in the rate of protein synthesis. Thus, small changes in phosphorylation are correlated with large inhibitions of protein synthesis. This phenomenon has been clearly demonstrated in the reticulocyte lysate protein-synthesizing system (30–32) and is due to a large excess of eIF-2 over GEF in the lysate, and to the fact that eIF-2a(P)–GDP binds GEF much more effectively than eIF-2–GDP, thus competing for GEF and effectively inhibiting its GDP exchange function. Similarly, even though phosphorylation of eIF-2 reaches only a very low basal state may explain the inhibition of protein synthesis in the heat-shocked cells (21). In the Ehrlich cell, a crude estimate, based on the inhibition of protein synthesis during heat shock (Fig. 4), suggests that an increase from 25% to 60% in percent of eIF-2 which is phosphorylated is sufficient to cause complete inhibition of protein synthesis. This implies that the GEF concentration should be roughly 50–60% of the total eIF-2 concentration. This is much higher than has generally been reported in rabbit reticulocytes (34) and possibly in HeLa cells. However, our recent measurements of GEF concentration suggest that the molar GEF concentration is in fact about 50% of the eIF-2 concentration in Ehrlich cells. It is not clear why the concentration is so much higher in Ehrlich cells than in reticulocytes or HeLa cells, but there is no a priori reason why it should not be so, and a wide range of concentrations may be revealed as more tissues are tested.

The sharp elevation of eIF-2 phosphorylation in heat-shocked cells offers an explanation for the marked depression in protein synthesis rate in these cells. Using the cell-free protein-synthesizing system prepared from Ehrlich cell lysates, we have shown previously that exogenously added eIF-4F, a cap-binding protein, restores control rates of synthesis to lysates prepared from heat-shocked cells but does not stimulate control lysates, implying an impairment of eIF-4F function as well (35). In our cell-free system, unlike the reticulocyte cell-free system, eIF-2 function is not limiting over the early time course, explaining the ability of eIF-4F to restore complete activity despite increased phosphorylation of eIF-2a. In the absence of better information concerning which steps are rate-limiting in intact control and heat-shocked cells, it is not possible to determine how much the impairment of eIF-4F activity contributes to the inhibition of protein synthesis in vivo, compared to phosphorylation of eIF-2. However, the data in this paper are compatible with a strong dependence of protein synthesis upon eIF-2 function in intact cells, and a clear effect of impaired eIF-4F is to cause preferential translation of mRNAs (36) for heat shock protein (35), as they have less dependence upon eIF-4F. We favor the hypothesis that phosphorylation of eIF-2 is primarily responsible for the overall inhibition of protein synthesis in heat-shocked cells and impairment of eIF-4F is responsible for the preferential synthesis of heat shock proteins.

We have shown previously that initiation in the cell-free protein-synthesizing system from Ehrlich cells is very sensitive to the GDP/GTP ratio, over a narrow range of values (37). This is expected on the basis of our knowledge of the competition between GDP and GTP in the GEF-catalyzed GDP/GTP exchange reaction. The in vivo GDP/GTP ratio falls over the same narrow range in glucose-deprived Ehrlich cells and, to a lesser extent, in amino acid-deprived cells (3). We are unable to assess with precision the relative contributions of fall in energy charge and increase in eIF-2 phosphorylation to the inhibition of protein synthesis in amino acid-deprived cells. A simple assumption is that both contribute. However, in the glucose-deprived cells early during the time course (1 h), the change in phosphorylation was often imperceptible (Table I) and could not explain the inhibition of protein synthesis (Table I). In this circumstance, the fall in GTP/GDP ratio appears to be the major regulatory mechanism. With the more severe energy deficit caused by 2-deoxyglucose, phosphorylation of eIF-2 is increased to a greater extent (Table I), but the increase is still less than that in heat-shocked cells (Fig. 5) in which inhibition of
synthesis is comparable. It is perhaps not surprising that the GTP/GDP ratio would fall and would be the major regulator of protein synthesis in energy-deprived cells. It is not clear why this ratio should fall in cells deprived of one essential amino acid. We have shown, however, that glucose utilization actually falls in amino acid-deprived Ehrlich cells (5).

The increased phosphorylation of eIF-2 helps explain the decreased 40 S initiation complexes in heat-shocked and 2-deoxyglucose-treated or amino acid-deprived cells, and the excellent correlation between the time course of increased phosphorylation and decreased protein synthesis suggests that the increased phosphorylation is responsible for the inhibition of protein synthesis. However, other explanations for the correlation are possible, as suggested by the result with emetine. In this case, an inhibition of chain elongation caused a decrease in eIF-2 phosphorylation. A possible explanation is that a backup of 40 S subunits, with a shift in proportion of various intermediates, causes accumulation of eIF-2 in a form that has an increase in susceptibility to phosphorylation, favoring dephosphorylation as an event secondary to inhibition of protein synthesis. At this time, it cannot be excluded that the rise in phosphorylation during heat shock and nutrient deprivation are also secondary, for instance to loss of eIF-4F activity and fall in energy charge, respectively.

Acknowledgments—We thank Dr. Rosemary Jagus for a generous gift of sheep antiserum against reticulocyte eIF-2 and Dr. Edith Lord and Lee Harwell for assistance in preparing monoclonal anti-eIF-2. We would also like to thank Kathleen Montine for useful discussions and Eileen Stewart for excellent technical assistance. The word processing expertise of Mary LeRoy-Jacobs and Lynne Palmiere is greatly appreciated.

REFERENCES
**Supplemental Material to**

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**EXPERIMENTAL PROCEDURES**

**Cell Culture**

Ehrlich ascites tumor cells were grown in spinner culture at 37°C in Eagle's minimal essential medium supplemented with 20 mM HEPES (pH 7.5) and 10% calf serum (Microbiological Assoc.). Microbes diluted daily to maintain the cell number between 2 x 10^9 and 8 x 10^9 cells/ ml.

**Measurement of Cellular Protein Synthesis**

Anino Acid Starvation: exponentially growing Ehrlich ascites tumor cells were centrifuged at 500 x g for 7 minutes; washed once with PBS and resuspended with prewarmed serum-free minimal essential medium minus serum at a density of 1 x 10^7 cells/ml. Samples (1-2 ml) were placed in snap-cap tubes which contained either glutamine (final concentration, 20 mM) or the appropriate concentration of other Codistituents.

**Measurement of Cellular Protein Synthesis**

Preparation of crude cell extracts, isolation of nuclear ribosomes, and radiolabeling of RNA were performed as described in materials and methods.

**Autoradiographic Scoring**

 Autoradiographic Scoring was performed as described in materials and methods.

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**RESULTS**

**Validation of the measurement of eIF-2 phosphorylation**

Small changes in phosphorylation can cause large changes in the rate of protein synthesis. Therefore, we have determined the validity of the measurements of the phosphorylation state of eIF-2 in Ehrlich ascites tumor cells. We have utilized the phosphorylation in vivo in these cells as a probe for the phosphorylation state of eIF-2 in vivo. The phosphorylation state of eIF-2 in vivo in Ehrlich ascites tumor cells is shown in Figure 1A. The data show that phosphorylation of eIF-2 in vivo is increased in the presence of hemin and HRI, and decreased in the absence of hemin and HRI. The increase in phosphorylation in vivo is consistent with the increase in phosphorylation in the Ehrlich ascites tumor cell in vivo in Figure 1A.

**Immunoblotting**

 Autoradiographic Scoring was performed as described in materials and methods.

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**Fig. 1**

Immunodetection of eIF-2a and eIF-2a(32P) in isoelectric focusing gels

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**Fig. 2**

Standard curve for eIF-2a and eIF-2a(32P)

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**Fig. 3**

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**Fig. 3** Phosphorylation of eIF-2α in reticulocyte lysate in the absence of hemin or presence of HRI

A. Protein synthesis was followed in the reticulocyte lysate by the incorporation of 15N leucine into TCA precipitable material in 10 μl samples as described (14). Reactions contained 20 μM hemin ( ), no hemin ( ), hemin plus HRI ( ). Samples (10 μl) removed at indicated times were used for the separation and immunodetection of eIF-2α and eIF-2αP as described. Autoradiograms shows 125I secondary antibody labelled eIF-2.

B. Bands representing eIF-2α and eIF-2αP in B, were quantitated by laser densitometry and are expressed as percent eIF-2αP relative to total.