1979

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Ralston, R. O.; Das, A.; Grace, M.; Das, H.; and Gupta, N. K., "Protein synthesis in rabbit reticulocytes: Characteristics of a postribosomal supernatant factor that reverses inhibition of protein synthesis in heme-deficient lysates and inhibition of ternary complex (Met-tRNA\(^\text{Met}\)\(\text{f}\)Met•eIF-2•GTP) formation by heme-regulated inhibitor" (1979). Faculty Publications -- Chemistry Department. 40.
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Protein synthesis in rabbit reticulocytes: Characteristics of a postribosomal supernatant factor that reverses inhibition of protein synthesis in heme-deficient lysates and inhibition of ternary complex (Met-tRNA_{Met}·eIF-2·GTP) formation by heme-regulated inhibitor*

(translational regulation/Met-tRNA_{Met} binding factor/eukaryotic initiation factors)

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Communicated by Myron K. Brakke, July 27, 1979

ABSTRACT During heme deficiency in reticulocyte lysates, a translational inhibitor (heme-regulated inhibitor, HRI) that blocks polypeptide chain initiation is activated. HRI is a protein kinase that specifically phosphorylates the 38,000-dalton subunit of the Met-tRNA_{Met} binding factor, eIF-2. Phosphorylation of eIF-2 by HRI prevents its interaction with at least two additional factors, resulting in a net reduction in formation of ternary complex (Met-tRNA_{Met}·eIF-2·GTP) and AUC-dependent transfer of Met-tRNA_{Met} to 40S ribosomal subunits. A factor (sRF) that reverses protein synthesis inhibition in heme-deficient lysates has been purified from reticulocyte postribosomal supernatant. sRF also reverses the inhibition of ternary complex formation by HRI in a fractionated system. The ternary complex inhibition reversal activity and the protein synthesis inhibition reversal activity co sediment at 12.5 S upon glycerol density gradient centrifugation, and both activities are sensitive to heat or N-ethylmaleimide. Purified sRF does not dephosphorylate eIF-2 whose phosphorylation has been catalyzed by HRI, nor does the sRF prevent the phosphorylation of eIF-2 by HRI in a fractionated system. sRF stimulates ternary complex formation by both phosphorylated and nonphosphorylated eIF-2. These observations suggest that the sensitivity of protein synthesis to phosphorylation of eIF-2 by HRI may be modulated by the concentration and activity of sRF.

During heme deficiency in reticulocyte lysates, protein synthesis shuts off abruptly due to activation of a latent protein synthesis inhibitor, the heme-regulated inhibitor (HRI) (9–19). HRI is a protein kinase that specifically phosphorylates the 38,000-dalton subunit of the Met-tRNA_{Met} (tRNA_{Met}) will be abbreviated to tRNA_{Met} binding factor, eukaryotic initiation factor 2 (eIF-2), producing eIF-2(P) (20–24). Phosphorylation of eIF-2 does not inhibit its activity per se but prevents its interaction with at least two factors: Co-eIF-2B (or ternary complex dissociation factor, TDF) (25, 26) and Co-eIF-2C (1, 7). Co-eIF-2B is required for AUC-dependent Met-tRNA_{Met} binding to 40S ribosomal subunits (4). Co-eIF-2C relieves the Mg^{2+} inhibition of ternary complex formation, resulting in an apparent stimulation (1, 7). Factors similar to Co-eIF-2C have been reported by deHaro et al. (27, 28) and Ranu and London (29). Phosphorylation of eIF-2 by HRI thus results in a net reduction of ternary complex formation and loss of efficient Met-tRNA_{Met} transfer to 40S ribosomal subunits.

The inhibition of protein synthesis in heme-deficient lysates can be overcome by the addition of the ribosomal salt (0.5 M KCl) wash or a partially purified preparation of eIF-2 (30–33). We have reported (8) that this activity (protein synthesis inhibition reversal factor from ribosomes, rRF) is not due to eIF-2 nor is it correlated with eIF-2 activity or concentration. rRF was resolved from the bulk of eIF-2. Also, homogeneous eIF-2 was inactive in reversal of protein synthesis inhibition in heme-deficient lysates.

Another factor that reverses protein synthesis inhibition in heme-deficient lysates has been isolated from reticulocyte postribosomal supernatant (34–36). In this paper, we describe further purification and characterization of this factor, protein synthesis inhibition reversal factor from supernatant (sRF).

sRF efficiently restores the protein synthesis activity of heme-deficient lysates to the level observed in the presence of hemin and also reverses the inhibition of ternary complex formation by HRI in a fractionated system. The sRF activities for ternary complex inhibition reversal and for protein synthesis inhibition reversal coseediment at 12.5 S upon glycerol density gradient centrifugation, and both activities are sensitive to N-ethylmaleimide and heat. sRF neither dephosphorylates eIF-2(P) nor blocks phosphorylation of eIF-2 by HRI in a fractionated system. sRF stimulates ternary complex formation by both phosphorylated and nonphosphorylated eIF-2.

MATERIALS AND METHODS

Materials. The materials were obtained from the following sources: [14C]leucine [289–355 mCi/mmol (1 Ci = 3.7 × 10^{10} becquerels)] from New England Nuclear; [35S]methionine (900 Ci/mmol) from Amersham; [γ-33P]ATP (2.7–3.1 Ci/mmol) from ICN; PM-10 ultrafiltration membranes from Amicon, Lexington, MA; GTP-agarose from Sigma. The sources of most of the other materials used in these studies have been described (8).

Preparations of Rabbit Reticulocyte Lysates and Assay of Protein Synthesis. The procedures for the preparation of reticulocytes and reticulocyte lysates and the incubation mixtures for protein synthesis have been described (14, 20). Protein synthesis was assayed by the incorporation of [14C]leucine into hot trichloroacetic acid-insoluble protein. The specific activity

Abbreviations: The present nomenclature for the peptide chain eukaryotic initiation factors (eIFs) (1), their past nomenclature (shown in parentheses) (2) as used by our laboratory, and their characteristic functions are: eIF-2 (EIF-1), Met-tRNA_{Met} binding factor (3, 4), eIF-2(P), phosphorylated eIF-2 (phosphorylation catalyzed by heme-regulated translational inhibitor); Co-eIF-2A (Co-EIF-1), stimulates Met-tRNA_{Met} binding to eIF-2 (5, 6); Co-eIF-2B (EIF-2, TDF), promotes dissociation of ternary complex at high Mg^{2+} (4); Co-eIF-2C (EIF-2B), reverses Mg^{2+} inhibition of ternary complex formation (7); HRI, heme-regulated translational inhibitor; rRF, ribosomal salt wash factor that reverses protein synthesis inhibition in heme-deficient lysates; tRNA_{Met}, tRNA_{Met} binding factor, N-dodecyl sodium dodecyl sulfate, MalNEI, N-ethylmaleimide.

* This paper is no. 25 in a series. Paper no. 24 is ref. 1.
of the [14C]leucine used is shown in the figure legends. The KCl concentration in the lysates was 80 mM in all cases. Incubation was at 30°C for 30 min unless otherwise indicated. Three different reaction volumes were used (12, 25, and 50 μl) for convenience in particular experiments. Specific activities of [14C]leucine and aliquot size were adjusted so that the amounts of [14C]leucine incorporated in the aliquots were comparable in all three systems.

Sodium Dodecyl Sulfate (NaDodSO4) Gel Electrophoresis and Autoradiography. Electrophoresis of proteins in NaDodSO4 polyacrylamide gels was performed according to the method of Laemmli (37). The gels contained 10% acrylamide (10% acrylamide, 0.2% N,N'-methylenebisacrylamide). The separating gels were formed as 1.5 × 100 × 150-mm slabs, with 1.5-cm stacking gels (5% acrylamide). Electrophoresis was performed at 150 V for 3/2 hr in a Bio-Rad model 220 electrophoresis apparatus. The gels were stained with Coomassie brilliant blue. Autoradiography was performed as described with Kodak X-OMat R film (21).

Protein Determination. Protein concentration was determined according to the method of Bradford (38) as modified by Spector (39) with bovine serum albumin as the standard.

Preparation of sRF. The sRF was prepared as described by Gross (35), except that 10 mM Tris-HCl, pH 7.8 was used instead of Hapes, and the DEAE-cellulose column was eluted by using 0.1 M, 0.2 M, 0.3 M, and 0.5 M KCl. The bulk of the sRF activity eluted with the 0.2 M KCl wash. The active fractions were pooled and concentrated by ultrafiltration. The concentrated sRF (1.5 ml, 14 mg/ml) was loaded on a 1.2 × 4.8-cm GTP-agarose column equilibrated with buffer A (10 mM Tris-HCl, pH 7.8/1 mM dithiothreitol/0.1 mM EDTA/0.1 M KCl). The bulk of the sRF activity does not bind to the column and elutes in the void volume. The active fractions were pooled and concentrated by dialysis against 80–90% saturated ammonium sulfate as described by Schreier et al. (40). The concentrated sRF was dissolved in buffer A containing 10% (vol/ vol) glycerol at a protein concentration of 4–8 mg/ml and stored at −96°C.

RESULTS

The sRF was purified by using DEAE-cellulose and GTP-agarose column chromatography. The bulk of sRF activity was not retained on the GTP-agarose column, whereas almost all of eIF-2 activity was bound and was later eluted by using 0.1 M GTP (Fig. 1). The GTP-agarose-purified sRF preparation was stored at −96°C. Under this condition, the preparation remained active for at least a month.

GTP-agarose-purified sRF efficiently reversed protein synthesis inhibition in heme-deficient lysates (Fig. 2). The amount of sRF giving maximum reversal (86 μg) (Fig. 2) bound only 0.31 pmol of [35S]Met-tRNAf in the presence of GTP in the Millipore filtration assay. NaDodSO4 gel electrophoresis result (data not shown) also confirmed the removal of nearly all eIF-2 from sRF by GTP-agarose chromatography.

Phosphorylation of eIF-2 by HRI inhibits its interaction with Co-eIF-2C, resulting in a net reduction in ternary complex formation when assayed in the presence of Mg2+ (1, 7). We investigated the effect of sRF on inhibition of ternary complex formation by HRI. In these experiments we used partially purified eIF-2 preparations (eIF) [fraction II (41)] that contained Co-eIF-2C activity.

As shown in Table 1, phosphorylation of eIF-2 by HRI inhibited its ability to form the ternary complex. sRF stimulated ternary complex formation by both phosphorylated and non-phosphorylated eIF-2. This stimulation of eIF-2 activity by sRF was observed even if sRF was added after eIF-2 had been phosphorylated by HRI (Table 1, experiment 2).
TABLE 1. Reversal of HRI inhibition of ternary complex formation by sRF

<table>
<thead>
<tr>
<th>Additions</th>
<th>ATP</th>
<th>[35S]Met-tRNA&lt;sub&gt;f&lt;/sub&gt; bound, pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eIF</td>
<td>-</td>
<td>2.11 ( \pm ) 2.30</td>
</tr>
<tr>
<td>sRF</td>
<td>-</td>
<td>0.51 ( \pm ) 0.49</td>
</tr>
<tr>
<td>eIF + sRF</td>
<td>-</td>
<td>3.30 ( \pm ) 3.42</td>
</tr>
<tr>
<td>eIF</td>
<td>+</td>
<td>2.39 ( \pm ) 1.00</td>
</tr>
<tr>
<td>sRF</td>
<td>+</td>
<td>0.39 ( \pm ) 0.44</td>
</tr>
<tr>
<td>eIF + sRF</td>
<td>+</td>
<td>3.08 ( \pm ) 2.95</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eIF</td>
<td>+</td>
<td>1.75 ( \pm ) 0.94</td>
</tr>
<tr>
<td>eIF + sRF (stage I)</td>
<td>+</td>
<td>2.35 ( \pm ) 2.11</td>
</tr>
<tr>
<td>eIF + sRF (stage II)</td>
<td>+</td>
<td>2.25 ( \pm ) 2.25</td>
</tr>
</tbody>
</table>

Standard Millipore filtration assay conditions were used. The incubation mixtures in Exp. 1 contained, in a total volume of 50 \( \mu \)l: 30 mM Tris-HCl at pH 7.8; 130 mM KCl; 15 \( \mu \)g of bovine serum albumin; 3 mM dithiothreitol; 1.5 mM magnesium acetate; and, where indicated, 0.2 mM ATP, 15 \( \mu \)g of eIF [fraction II (41)], 0.8 \( \mu \)g of HRI, and 16 \( \mu \)g of sRF. The reaction mixtures were incubated at 37°C for 10 min (stage I). Ten microliters (16, \( \mu \)g) of [35S]Met-tRNA<sub>f</sub>; (1). Used bound [35S]Met-tRNA<sub>f</sub>; (1). The reaction mixtures were assayed by Millipore filtration as described (41). The sRF preparation (16 \( \mu \)g) used in this experiment bound 0.3 pmol of [35S]Met-tRNA<sub>f</sub> in the absence of ATP. The reaction conditions in Exp. 2 were the same as above except that sRF (16 \( \mu \)g) was added at either stage I or stage II.

HRI and ATP, correlated well with RF activity. The downward trend of the RF activity profile was due to inhibition of protein synthesis by increasing concentrations of glycerol. In this case and as reported previously (8), amino acid incorporation decreased linearly with glycerol concentration when blank gradients were used. Addition of 5 \( \mu \)l of solution containing 30% glycerol (concentration at the bottom of the gradient) lowered [14C]leucine incorporation to approximately 60%. The amino acid incorporation activity of gradient fractions 1–6 (Fig. 3) was the same as the control within experimental error.

In order to investigate whether sRF contained phosphatase activity, the effect of sRF on phosphorylation of eIF-2 by HRI was analyzed by NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis and autoradiography. The results are shown in Fig. 4. Comparison of the kinetics of phosphorylation of eIF-2 in the absence (tracks 6–8) and presence (tracks 9–11) of sRF clearly shows that sRF did not reduce the amount of phosphorylation of the 38,000-dalton subunit of eIF-2. When eIF-2 was phosphorylated by preincubation with HRI, the extent of phosphorylation was not affected by addition of sRF (tracks 12–14). When HRI was preincubated with sRF, its ability to phosphorylate eIF-2 was unaffected (tracks 15–17). These results clearly demonstrate that sRF did not dephosphorylate eIF-2(P) or block phosphorylation of eIF-2 by HRI.

Another experiment, the effects of sRF and Co-eIF-2C on ternary complex formation by purified eIF-2(2P) were compared. The results are shown in Table 2. As in the case of eIF-2, ternary complex formation by eIF-2(2P) was strongly inhibited by Mg<sup>2+</sup>. sRF stimulated ternary complex formation by eIF-2(2P). As expected, Co-eIF-2C did not stimulate ternary complex formation by eIF-2(2P) even at saturating concentrations (1). These results clearly demonstrate that reversal of ternary complex inhibition of sRF was not due to Co-eIF-2C.

Another factor, Co-eIF-2A, stimulates ternary complex formation by both eIF-2 and eIF-2(2P). The molecular weight of the purified factor is 25,000. However, a heavy form of this
DISCUSSION

The results presented here clearly demonstrate that the supernatant factor, sRF, that reverses inhibition of protein synthesis in heme-deficient lysates also reverses inhibition of ternary complex formation by HRI. sRF stimulates ternary complex formation under experimental conditions in which eIF-2 is phosphorylated by HRI. sRF also stimulates ternary complex formation by purified eIF-2(P) as well as by the non-phosphorylated form. This stimulation is not due to dephosphorylation of eIF-2(P) by sRF. Also, sRF does not prevent phosphorylation of eIF-2 by HRI. These results suggest that sRF can promote protein synthesis even in the presence of inhibitory concentrations of HRI, with eIF-2(P) as an active initiation factor.

The supernatant factor sRF described in this paper appears similar to another factor that we now term rRF. rRF is purified from the ribosomal 0.5 M KCl wash and reverses protein synthesis inhibition in heme-deficient lysates in a manner similar to sRF (8). rRF has been purified from the bulk of eIF-2 activity by using GTP-agarose chromatography, and there are indications that rRF also reverses ternary complex inhibition by HRI. Like sRF, rRF sediments at 12–13 S upon glycerol density gradient centrifugation.

These observations raise important questions regarding the basic mechanism of peptide chain initiation and the regulation of protein synthesis in eukaryotic cells. Despite numerous reports from several laboratories, the detailed mechanism of peptide chain initiation and characteristics of the protein factors involved are not clearly understood. The first step in peptide chain initiation is the formation of a ternary complex, Met-tRNAf-eIF-2-GTP. Reports from our laboratory (4–7) and elsewhere (27–29) indicate that ternary complex formation by eIF-2 is regulated by several protein factors, although the precise roles of these factors in protein synthesis initiation are not known. Formation of the ternary complex by eIF-2 is strongly affected by Mg\(^{2+}\). Ternary complex formation by homogeneous preparations of eIF-2 is optimal in the absence of Mg\(^{2+}\) and is strongly inhibited by Mg\(^{2+}\). However, once the ternary complex is formed, the complex is stable to further addition of Mg\(^{2+}\). Co-eIF-2C relieves Mg\(^{2+}\) inhibition of ternary complex formation, producing an apparent effect of stimulation (7). This factor would, therefore, appear to be required for protein synthesis initiation at physiological Mg\(^{2+}\) concentrations.

HRI phosphorylates the 38,000-dalton subunit of eIF-2. Ternary complex formation by eIF-2(P) is not stimulated by Co-eIF-2C (1, 7, 27–29). It has been suggested that loss of interaction of eIF-2(P) with Co-eIF-2C is responsible for the inhibition of ternary complex formation by HRI.

However, the precise roles of different factors in the formation of initiation complexes with 40S ribosomal subunits are not known. A high molecular weight factor, Co-eIF-2B, promotes dissociation of the ternary complex in the presence of high Mg\(^{2+}\) concentration (5 mM) and low temperature (0°C). In the presence of eIF-2, Co-eIF-2B preparations give 3-fold stimulation of Met-tRNA\(_{f}\) binding to 40S ribosomes (4). Presumably, some component in this high molecular weight protein complex (Co-eIF-2B) is composed of multiple (16–20) polypeptides and has molecular weight of approximately 450,000. It is necessary for 40S initiation complex formation. However, the relationship of this component to the factor that promotes dissociation of the ternary complex is not clear. The ternary complex formed with eIF-2(P) is not dissociated by Co-eIF-2B and is also inactive in 40S initiation complex formation. This observation suggests that the ternary complex dissociation activity in Co-eIF-2B may be involved in 40S initiation complex formation, possibly releasing eIF-2 after Met-tRNA\(_{f}\) is bound to the 40S ribosomal subunit.

The role of Co-eIF-2C in 40S initiation complex formation is not clear. Co-eIF-2C presumably restores the active conformation of eIF-2 in the presence of Mg\(^{2+}\) and may play an important role in peptide chain initiation. An alternative possibility is that Mg\(^{2+}\) alters the conformation of Met-tRNA\(_{f}\) such that an additional factor (Co-eIF-2C) is required for Met-tRNA\(_{f}\) binding to eIF-2. However, it is conceivable that other factors
besides Co-eIF-2C may perform similar functions. Possibly, sRF is such a factor.

sRF reverses protein synthesis inhibition in heme-deficient lysates and also reverses ternary complex inhibition by HRI. sRF does not dephosphorylate eIF-2(P) or protect eIF-2 from phosphorylation by HRI. sRF stimulates ternary complex formation by both purified eIF-2a and eIF-2P. The characteristics of stimulation of ternary complex formation by sRF are thus clearly different from those observed with Co-eIF-2C. Also, unlike sRF, Co-eIF-2C does not reverse protein synthesis inhibition in heme-deficient lysates. The sRF preparation contains some Co-eIF-2B activity. It is possible that sRF alters the conformation of eIF-2(P) so that it is recognized by Co-eIF-2B and is also active in 40S initiation complex formation.

The results presented in this paper provide another example of complex regulation of protein synthesis initiation in eukaryotic cells. During heme deficiency, protein synthesis in reticulocyte lysate is inhibited due to activation of HRI. However, a careful analysis of the data will reveal that a low but detectable rate of protein synthesis continues over a prolonged period in these heme-deficient lysates. It is conceivable that sRF activity in these lysates is responsible for the continued low rate of protein synthesis in the presence of inhibitory concentrations of HRI. The presence of eIF-2-P (P) phosphatase activity in reticulocyte lysate has also been reported (43) and may provide yet another mechanism of reversal of protein synthesis inhibition in heme-deficient lysates. However, sRF does not contain any eIF-2-P phosphatase activity, and, also, sRF stimulates ternary complex formation even when eIF-2 is phosphorylated. These observations suggest that the sensitivity of protein synthesis to phosphorylation of eIF-2 by HRI may be modulated by the concentration and activity of sRF.

In conclusion, we propose a dual mechanism for regulation of protein synthesis initiation in eukaryotic cells. Both Co-eIF-2C and sRF promote ternary complex formation by eIF-2 at physiological Mg2+ concentrations. We suggest that, in rapidly growing cells, Co-eIF-2C may represent the dominant factor. In such cases, protein synthesis would be expected to be highly sensitive to eIF-2 kinases, as is observed in reticulocyte lysates. In resting cells in which eIF-2 kinases may be active, we suggest that the presence of sRF may ensure a continued low rate of protein synthesis necessary for cell survival.

**Note Added in Proof.** After we submitted our manuscript for review, we noted that Benne et al. [Benne, R., Amseh, H., Goumans, H. & Voorma, H. (1979) *XI International Congress of Biochemistry*, p. 129 (abstr.)] have also observed that a postribosomal supernatant factor, similar to sRF, that reverses protein synthesis inhibition in heme-deficient reticulocytes does not dephosphorylate eIF-2(P) or prevent HRI phosphorylation of eIF-2.

The authors thank Ms. Marcia Berns for the preparation of reticulocyte ribosomes and ribosomal salt wash. This investigation was supported by National Institutes of Health Research Grants GM 15796 and GM 22079. R.O.R. and A.D. were supported by Maude Hammond Fling Fellowships from the University of Nebraska.