Partial Purification and Characterization of a 90 000-Dalton Peptide Involved in Activation of the eIF-2α Protein Kinase of the Hemin-Controlled Translational Repressor†

Mary H. Wallis, Gisela Kramer, and Boyd Hardesty*

ABSTRACT: In the absence of heme, a negative translational control system is activated in reticulocytes or their lysates that causes the phosphorylation of the smallest subunit of peptide initiation factor 2 and the inhibition of peptide initiation. Two partially purified enzyme fractions are shown to give a concerted effect for phosphorylation of this subunit of initiation factor 2 and binding of methionyl-tRNA$_f$ to 40S ribosomal subunits. One enzyme fraction contains a 90 000-dalton peptide that functions in activation of an enzyme containing a 100 000-dalton peptide of the other fraction. Phosphorylation of the 100 000-dalton peptide is correlated with activation of the kinase for the smallest subunit of initiation factor 2. Antibodies against the 90 000-dalton peptide decrease phosphorylation of both the 100 000-dalton peptide and the subunit of initiation factor 2. The results indicate that at least two components function in a sequence of reactions that inhibits protein synthesis by phosphorylation of the smallest subunit of eucaryotic initiation factor 2. The same sequence may be activated in the presence of heme by a cascade type of reactions initiated by a heat-stable protein, HS [Henderson, A. B., Miller, A. H., & Hardesty, B. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2605-2609].

Protein synthesis in rabbit reticulocytes is controlled by the availability of heme (Kruh & Borsook, 1956; Bruns & London, 1965). In the absence of heme, a translational inhibitor, called the hemin-controlled repressor (HCR),[1] is activated in reticulocytes or their cell-free lysates (Maxwell & Rabinovitz, 1969; Maxwell et al., 1971). Gross & Rabinovitz (1973) isolated what they called a prorepressor, a protein complex of (3-4) $\times 10^5$ daltons that was activated in the absence of heme to give the inhibitory activity of HCR. Preparations of HCR have been shown to cause the following: (a) inhibition of protein synthesis in reticulocyte lysates by blocking peptide initiation (Legon et al., 1973; Balkow et al., 1973); (b) inhibition of binding of methionyl-tRNA$_f$ to reticuloycte 40S ribosomal subunits that is dependent upon peptide initiation factor eIF-2 (Pinphanichakarn et al., 1976; Kramer et al., 1977; Ranu et al., 1978); (c) phosphorylation of the smallest subunit of eIF-2 (Farrell et al., 1977; Kramer et al., 1976; Levin et al., 1976). This subunit has a molecular weight of $\sim$38 000 and is designated eIF-2α. Henderson et al. (1979) described two proteins that lead to the activation of the eIF-2α kinase of the HCR system in the presence of heme. One of these proteins, HS, may be reversibly activated by heat or pressure. Activated HS with ATP causes the irreversible activation of a heat-labile protein of $\sim$40 000 daltons, HL, which in turn promotes activation of the eIF-2α kinase of the HCR system. The mechanism by which HL leads to activation of this eIF-2α kinase is not known; however, there appears to be at least one intervening reaction between HL and activation of either the 90 000- or 100 000-dalton components described here.

The characteristics of the eIF-2α kinase of the HCR system and the proximal reactions by which this kinase is activated are of primary interest in relation to translational regulation of protein synthesis. Maxwell et al. (1971) observed inhibition of protein synthesis in some reticulocyte lysates that was nonlinear with increasing concentrations of the inhibitor. The results might reflect an interaction of multiple components required for expression of inhibition by HCR. Farrell et al. (1977) found that a phosphorylated peptide which migrated with an apparent molecular weight of 96 000 during electrophoresis in NaDodSO$_4$-polyacrylamide gels was associated with inhibitory activity in preparations of HCR that had been purified 3000-4000-fold. These HCR preparations also contained peptides of 86 000 and 91 000 daltons. Gross & Mendelewski (1978) demonstrated a correlation between activation of the prorepressor form of HCR and phosphorylation of a 100 000-dalton peptide. Trachsel et al. (1978) isolated an inhibitor protein that migrated as a single 95 000-dalton peptide during NaDodSO$_4$ gel electrophoresis. In its activated form the protein caused phosphorylation of eIF-2α and inhibition of protein synthesis with the characteristics of HCR. The peptide underwent phosphorylation from ATP during activation in the absence of heme; however, a requirement for other protein components for this activation was not detected.

Here we present evidence that a protein which gives a single 90 000-dalton peptide by NaDodSO$_4$ gel electrophoresis functions in the activation of another component, apparently the 95 000-100 000-dalton peptide, to give the active form of the eIF-2α kinase associated with HCR activity. The results are interpreted to reflect the proximal steps of a sequence of reactions involved in the regulation of protein synthesis through the eIF-2α kinase of the HCR system.

Experimental Procedure

Preparation of HCR. HCR was prepared from the postribosomal supernatant of rabbit reticulocytes. This fraction was obtained as described earlier (Henderson & Hardesty, 1978). Four liters of postribosomal supernatant was chro-

† From the Clayton Foundation Biochemical Institute, Department of Chemistry, The University of Texas, Austin, Texas 78712. *Received June 5, 1979; revised manuscript received November 13, 1979.* Supported in part by Grants No. CA09182 and CA16608 awarded by the National Cancer Institute, Department of Health, Education, and Welfare, to Dr. Boyd Hardesty. A preliminary report of these results has been presented at the FASEB Meeting, Dallas, TX, in April 1979 [cf. Wallis & Kramer (1979)].

[1] Abbreviations used: HCR, hemin-controlled repressor, a fraction containing protein kinase activity for eIF-2α; HS, a heat-stable protein isolated from reticulocyte postribosomal supernatant that activates HL and thereby the eIF-2α kinase of the HCR system as described by Henderson et al. (1979); HL, the heat-labile component of the HS-HL system that can activate the eIF-2α kinase of the HCR system; eIF-2, eucaryotic initiation factor 2; eIF-2α, the smallest subunit of eIF-2 (M_r 38 000); NaDodSO$_4$, sodium dodecyl sulfate; DTE, dithioerythritol; GMP-P(CH$_2$)P, guanosine 5'-(β,γ-methylene)triphosphate.

0006-2960/80/0419-0798$01.00/0

matographed on a DEAE-cellulose column (7.5 × 100 cm, DE-52, microgranular, from Whatman, Ltd., Kent, England) equilibrated in 20 mM Tris-HCl (pH 7.5), 20 mM KCl, and 10 mM β-mercaptoethanol. The column was washed extensively, and then protein that eluted with 250 mM KCl in otherwise the same solution was collected, concentrated by the addition of ammonium sulfate in the cold to give 50% saturation, and dialyzed against 10 mM potassium phosphate, pH 7.5. This fraction was then loaded on a column (2.5 × 25 cm) filled with washed phosphocellulose (from Whatman, Ltd., Kent, England) that had been equilibrated with the same buffer solution. The fraction that did not adsorb to the phosphocellulose under these conditions was collected, concentrated, and dialyzed against 10 mM phosphate (pH 8.5), 100 mM KCl, and 10 μM cAMP. This material (~800 mg of protein) was applied to a column (2 × 17 cm) of histone–Sepharose prepared as described below and equilibrated in the same buffer solution. After extensive washing of the column, adsorbed protein was batch eluted in the following steps: (a) 350 mM KCl in 10 mM KH_2PO_4–K_2HPO_4, pH 7.5, and 10 μM cAMP; (b) 500 mM KCl in 10 mM KH_2PO_4–K_2HPO_4, pH 7.0, and 10 μM cAMP; (c) 2 M KCl in 10 mM KH_2PO_4–K_2HPO_4, pH 6.0, and 10 μM cAMP. HCR activity was found in fraction b after dialysis against 20 mM Tris-HCl (pH 7.5) and 100 mM KCl. The fraction was concentrated, dialyzed against this solution and then the same solution containing 50% glycerol, and stored in small aliquots at −80 °C.

Preparation of the 100K Fraction. Protein adsorbed to the phosphocellulose column described above was eluted with a linear gradient of 0–400 mM KCl in 10 mM phosphate, pH 7.5. Fractions eluting slightly behind the main protein peak at ~100 mM KCl exhibited some HCR activity (cf. Figure 3). They are enriched in a 100 000-dalton peptide that can be phosphorylated, as described under Results. These fractions were pooled, concentrated, dialyzed against 20 mM Tris-HCl (pH 7.5) and 100 mM KCl, and then dialyzed against the same solution containing 50% glycerol. This fraction was stored frozen at −80 °C.

Preparation of the 90K Fraction. Protein (150–200 mg) eluted from histone–Sepharose with step a in the preparation of HCR was concentrated and dialyzed against 75 mM potassium phosphate, pH 7.5. This material was then loaded on a hydroxylapatite column (1.5 × 17 cm, containing hapatite C from Clarkson Chemical Co., Williamsport, PA) equilibrated in the same buffer solution. Nonadsorbed protein was washed from the column, and then adsorbed protein was eluted with a linear gradient (75–350 mM potassium phosphate, pH 7.5, 300 mL total). Fractions eluting with ~300 mM phosphate contained a 90 000-dalton peptide of ~80% purity when analyzed by polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ (as described below; cf. Figure 2). These fractions were pooled, concentrated, and dialyzed against 20 mM Tris-HCl (pH 7.5) and 100 mM KCl and then against the same solution containing 50% glycerol. This fraction was kept at −20 °C.

Preparation of the 90K Peptide Used as Antigen. A protein fraction that did not adsorb to phosphocellulose as described above in the preparation of HCR was dialyzed against 75 mM phosphate (pH 7.5) and chromatographed directly on hydroxylapatite. About 600 mg of protein was loaded on a 1.5 × 35 cm column filled with hydroxylapatite and equilibrated in the same buffer. Nonadsorbed protein was washed off, and then additional protein was eluted in two steps: (a) with 250 mM phosphate, pH 7.5; (b) with 400 mM phosphate, pH 7.5. This latter fraction was concentrated and then dialyzed against 20 mM Tris-HCl (pH 7.5) and 50 mM KCl. About 1 mg of this fraction was subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis as described below. The predominant peptide in these fractions had an apparent molecular weight of 90 000 (cf. Figure 5). This band was cut out of the gel, and then the peptide was eluted from the polyacrylamide and concentrated in an ISCO electrophoretic concentrator (Model 1750, Instrumentation Specialties Co., Lincoln, NE) with the same solution as used for the $NaDodSO_4$ gel electrophoresis.

Enzyme Assays. (A) Inhibition of Protein Synthesis in Reticulocyte Lysates. Reticulocyte lysates were prepared as originally described by Adamson et al. (1968). The assay system is slightly modified from that described earlier (Kramer et al., 1976). The reaction mixtures contained the following in a final volume of 50 μL: 10 mM Tris-HCl (pH 7.5), 90 mM KCl, 1.5 mM $MgCl_2$, 5 mM DTE, 0.5 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 26 units/mL creatine phosphokinase, 0.05 mM [^{14}C]leucine (40 Ci/mol), 0.1 mM of the 19 other unlabeled amino acids, 10 μM hemin, and 10 μL of rabbit reticulocyte lysate. Incubation was carried out at 37 °C for 30 min. Reactions were terminated by the addition of 100 μL of 1 N NaOH and 100 μL of 3% H_2O_2 to each assay tube followed by further incubation at 37 °C for 5 min. Protein was precipitated with 5% trichloroacetic acid, incubated in a boiling water bath for 3 min, and then collected and washed with 5% trichloroacetic acid on glass fiber filters. The radioactivity retained on the glass filters was measured by liquid scintillation in 10 mL of a counting fluid containing 5 g of 2,5-diphenyloxazole per L of toluene.

(B) Binding of [^{35}S]Methionyl-tRNA$_f$ to Reticulocyte 40S Ribosomal Subunits. The preparation of components for the formation of the 40S initiation complex has been described elsewhere (Odom et al., 1978). The assay conditions were originally described by Gupta et al. (1975) and modified by Spremulli et al. (1977). A three-step assay was performed as described previously (Kramer et al., 1977) to measure HCR-mediated inhibition. Step 1 requires a 5-min preincubation at 37 °C with eIF-2, HCR (in the amounts indicated in the legends), and 0.5 mM ATP under conditions described below for the protein kinase assay. This preincubation is terminated by the addition of 0.35 unit of hexokinase (from yeast, Boehringer/Mannheim, Germany) and glucose to give a final concentration of 1 mM in a final volume of 50 μL. Incubation was continued for 3 min at 37 °C. Finally, in step 3 the preincubated components were mixed with a reaction mixture containing the following in a final volume of 100 μL: 20 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 85 mM KCl, 2.5 mM DTE, 0.2 mM GMP-P(CH_2)P, 75 μM spermine, 25 μg of FII (Odom et al., 1978), 0.1 A_{260} unit of reticulocyte 40S subunits, and 8 pmol of [^{35}S]methionyl-tRNA$_f$ (1.5–2 Ci/mmol). Incubation was continued at 37 °C for 5 min and then at 0 °C for 5 min. Reaction mixtures were diluted with an ice-cold solution containing 20 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, and 85 mM KCl. The samples were filtered slowly through nitrocellulose filters (0.45-μm pore size, type HAWG, Millipore Corp., Bedford, MA), washed with the same buffer, dried, and counted as described above. A blank value (usually ~5–10%) for [^{35}S]methionyl-tRNA$_f$ bound to Millipore filters in the absence of 40S particles served as a control. These counts were subtracted to calculate binding to 40S ribosomal subunits.

(C) Protein Kinase Activity. Incubation mixtures to measure eIF-2 kinase activity contained the following in a final volume of 30 μL: 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2.5 mM DTE, 0.2 mM [γ-^{32}P]ATP (50–500 Ci/mol), 1.5–2

μg of eIF-2 [~80% pure; prepared as described by Odom et al. (1978)]. The reaction mixtures were incubated for 5 min at 37 °C and terminated by the addition of 30 μL of sample buffer containing 50 mM Tris-HCl (pH 6.8), 2% $NaDodSO_4$, 10% glycerol, 0.1 M DTE, and 0.001% bromophenol blue. The samples were analyzed by polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ as described by Laemmli (1970) and modified by Anderson et al. (1973). Then autoradiograms were prepared as detailed previously (Kramer et al., 1977). The stained band was cut out from the dried gel to determine quantitatively the amount of [^{32}P]phosphate incorporated into eIF-2α. The gel pieces were digested with 200 μL of H_2O_2 at 60 °C for ~8 h, and then their radioactivity was measured by liquid scintillation in 10 mL of a counting fluid containing 5 g of 2,5-diphenyloxazole per L of toluene and 10% Biosolve (Beckman Instruments, Inc., Palo Alto, CA).

Preparation of Histone–Sepharose. The conditions for coupling histones (Type IIA from calf thymus, from Sigma Chemical Co., St. Louis, MO) to CNBr-activated Sepharose 4B (Sigma Chemical Co.) were those recomended by *Affinity Chromatography* (1974). The cyanogen bromide activated Sepharose 4B was swollen and washed thoroughly with 1 mM HCl followed by washing with coupling buffer containing 0.1 M $NaHCO_3$, pH 8.5. Equal volumes of coupling buffer and then histones at 5 mg/mL were added to the swollen gel and mixed for 20–24 h at 4 °C on a rotary stirring apparatus. The reaction was stopped by extensive washings with cold, distilled H_2O followed by equilibration for several days in the starting buffer solution used in the chromatography as described.

Preparation of Antibodies. A four-month-old goat was immunized by six monthly injections, each containing ~100 μg of the 90K peptide eluted from $NaDodSO_4$–polyacrylamide gels as described above. The first injection contained an equal volume of complete Freund's adjuvant (Grand Island Biochemical Co., Grand Island, NY). The goat was bled 1 week after the fifth and sixth injections. An IgG fraction was obtained by precipitation of the serum with ammonium sulfate at 50% saturation followed by chromatography on DEAE-cellulose (pH 8.0). Normal IgG was obtained from the goat before the first injection.

Assay for Antibodies. Immunoprecipitation in the Presence of Staphylococcal-A Protein. The procedure described by Kessler (1975) was followed. Protein A from *Staphylococcus aureus* ("Pansorbin") was purchased from Calbiochem-Behring. Just before use the suspension was washed as outlined by Kessler (1975). About 50 μg of antigen was incubated with 200–400 μg of immune or control IgG for ~2 h on ice, and then 50 μL of Pansorbin suspension was added. The incubation was continued for 20 min on ice; then the cell suspension was washed thoroughly. Adsorbed antigen–antibody complexes were eluted and prepared for $NaDodSO_4$–polyacrylamide gel electrophoresis as described by Kessler (1975). Controls were run by omitting one of the interacting components.

Results

Isolation of the 90 000-Dalton Peptide. Our first indication that more than one component may be involved in the inhibitory activity of HCR came during attempts to purify the protein kinase for eIF-2α and the associated inhibitory activity for protein synthesis. These activities were inevitably spread through a wide range of the eluant when crude enzyme fractions containing HCR were chromatographed on either gel filtration or ion-exchange columns. In addition, most fractionation procedures gave a large and frequently variable loss in total inhibitory activity. Furthermore, the inhibition

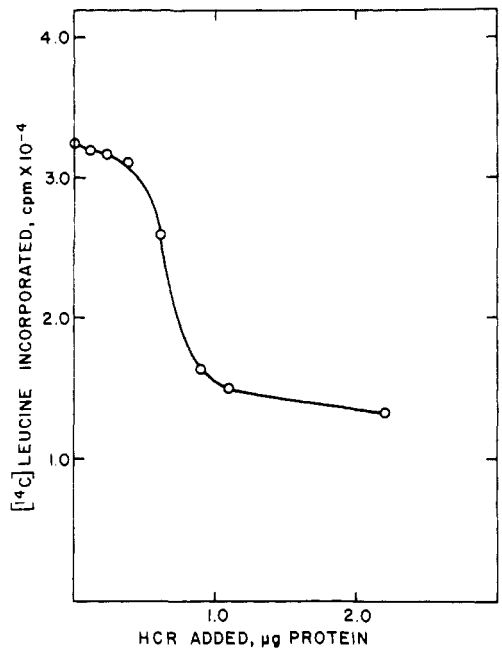

FIGURE 1: Atypical, nonlinear inhibition of protein synthesis in reticulocyte lysates observed with some lysates and HCR preparations. [^{14}C]Leucine incorporation into proteins was measured in the reticulocyte lysate system as described under Experimental Procedure. An HCR fraction that did not adsorb to hydroxylapatite was chromatographed on Sepharose 6B [cf. Kramer et al. (1976)]. Protein from this fraction was added to the assay system in the amounts indicated.

of protein synthesis given by some of these subfractions of HCR in most reticulocyte lysates is not linear with increasing amounts of the inhibitor fractions. An inhibition curve of this type for increasing concentration of an HCR fraction is shown in Figure 1. We have observed considerable variability in the magnitude of the inhibition and shape of the inhibition curve obtained with different lysates and different preparations of HCR. This variability appears to be similar to that noticed by Rabinovitz and his co-workers with certain "atypical lysates" (Maxwell et al., 1971). The bases for these effects are not known; however, they may reflect variable amounts in fractions of HCR and lysates of different components required for inhibition of protein synthesis.

These characteristics of the assay system have seriously compromised our efforts to isolate components responsible for the inhibitory activity of HCR. However, our attention was focused on two peptides seen after $NaDodSO_4$ gel electrophoresis that appeared to be inevitably associated with HCR activity in various subfractions of the inhibitor. One is a peptide of ~100 000 daltons that becomes phosphorylated from ATP in enzyme subfractions that are active for phosphorylation of eIF-2α. This component seems to be identical with the 95 000–100 000-dalton peptide observed in other laboratories, as described above. The second component is detected as a peptide of ~90 000 daltons on stained $NaDodSO_4$–polyacrylamide gels. Incubation of fractions containing the 90 000-dalton peptide but no visible 100 000-dalton component with [γ-^{32}P]ATP followed by $NaDodSO_4$ gel electrophoresis demonstrated that they contained trace amounts of the 100 000-dalton peptide that was discernible only after radioautography of the gel. These results prompted us to undertake the isolation of the proteins containing the 90 000-dalton peptide. A procedure involving chromatography on histone–Sepharose and then hydroxylapatite as described under Experimental Procedure was developed. Chromatography on hydroxylapatite is a critical step in the procedure.

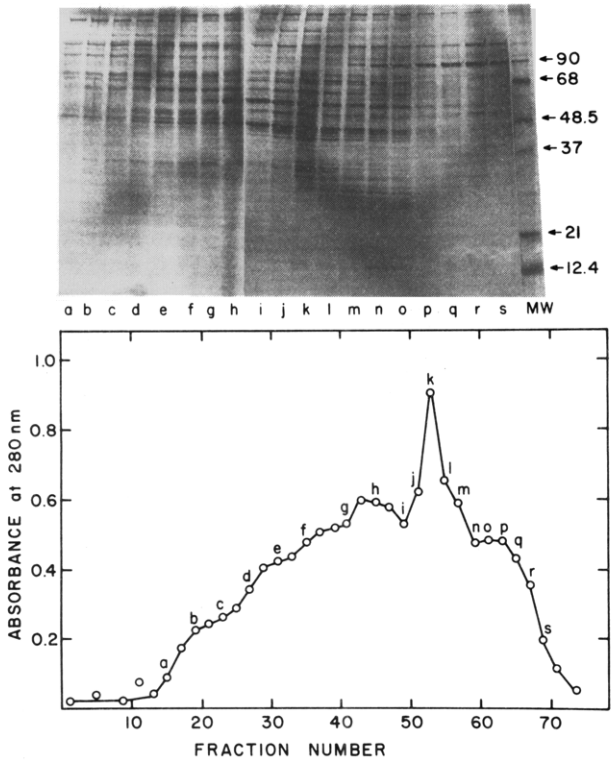

FIGURE 2: Chromatography on hydroxylapatite to purify the 90K fraction. About 190 mg of fraction a protein from histone–Sepharose chromatography was applied to a hydroxylapatite column equilibrated in 75 mM potassium phosphate solution, pH 7.5. Elution of adsorbed protein was carried out with a linear gradient from 75 to 350 mM potassium phosphate solution, pH 7.5. The lower part of the figure shows the concentration profile of protein eluted by this gradient. The upper section exhibits the peptide pattern analyzed by $NaDodSO_4$ gel electrophoresis from 15-μL aliquots (a–f) or 10-μL aliquots (g–s) of individual fractions of the eluted protein. The letters in the graph and under the tracks from the stained gel denote the same fractions.

The elution profile from this column is shown in Figure 2. Fractions collected near the end of the elution gradient contain primarily the 90 000-dalton peptide and are free of detectable amounts of the 100 000-dalton peptide when analyzed by autoradiography after incubation with [γ-^{32}P]ATP. These fractions are pooled and used as a source of the 90 000-dalton peptide in the studies described below. This preparation is called the 90K fraction in the following text. The activity of the 90K fraction appears to be relatively unstable in that it has been lost upon storage in 50% glycerol at −20 °C or while frozen at −80 °C and during further fractionation.

Synergism for Inhibition and Phosphorylation of eIF-2α. The 90K fraction has little or no inhibitory activity for eIF-2-mediated binding of methionyl-$tRNA_f$ to 40S ribosomal subunits, as shown by the data presented in Figure 3. It does not carry out phosphorylation of eIF-2α, as shown by the data presented in Figure 4 and Table I. However, it effectively increases the activity of certain enzyme fractions for these reactions. The critical step for preparation of these enzyme fractions is chromatography on phosphocellulose, as described under Experimental Procedure. This fraction contains a number of components, as detected by $NaDodSO_4$ gel electrophoresis. However, it is enriched in the 100 000-dalton peptide but is apparently deficient in the 90 000-dalton components. This preparation is called the 100K fraction. As shown in Figure 3, this 100K fraction is quite inhibitory for eIF-2-mediated binding of methionyl-$tRNA_f$ to 40S ribosomal subunits. Fifty percent inhibition is obtained with ~1 μg of protein in this assay system. However, this level of inhibition

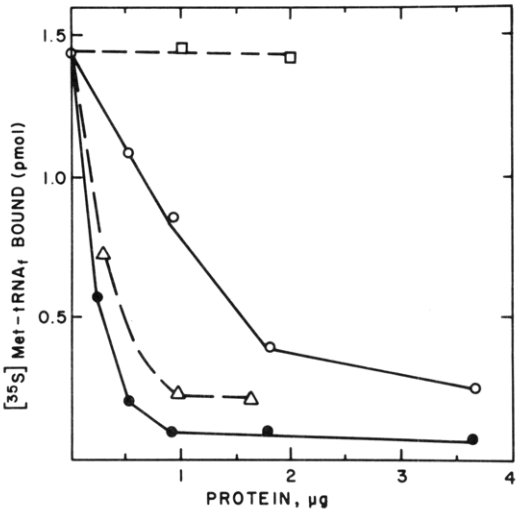

FIGURE 3: Synergism between the 90K and 100K fractions for inhibition of methionyl-$tRNA_f$ binding to reticulocyte 40S ribosomal subunits. Binding of [^{35}S]Met-$tRNA_f$ to 40S subunits was determined as described under Experimental Procedure. About 2 μg of eIF-2 and different HCR or protein fractions were added in the amounts indicated during the first step of incubation. (□) 90K fraction; (○) 100K fraction; (●) 100K fraction in amounts given on the abscissa plus 1 μg of 90K fraction; (△) fraction b from the histone–Sepharose chromatography described under Experimental Procedure.

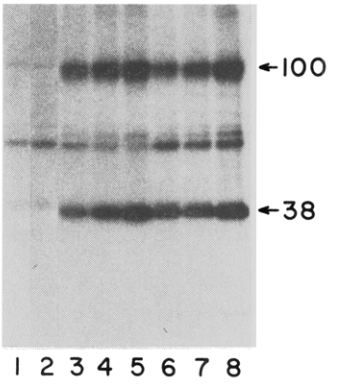

FIGURE 4: Synergism for phosphorylation of eIF-2α between the 90K and 100K fractions. A protein kinase assay as described under Experimental Procedure was carried out. About 2 μg of eIF-2 was incubated with [γ-^{32}P]ATP (193 Ci/mol) (track 1) and 1 μg of the 90K fraction (tracks 2 and 6–8). Tracks 3–8 also contained the 100K fraction in the following amounts: tracks 3 and 6, 0.3 μg; tracks 4 and 7, 0.5 μg; tracks 5 and 8, 1.0 μg. Incorporation of [^{32}P]phosphate into peptides was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis followed by autoradiography. The autoradiogram is shown.

is obtained with less than 0.25 μg of protein from the 100K fraction if 1 μg of protein from the 90K fraction also is added to the assay reaction mixture. This amount of the 90K fraction causes no detectable inhibition in the absence of the 100K material. An inhibition curve for an active HCR fraction containing both the 90 000-dalton and the 100 000-dalton peptides is shown for comparison.

The phosphorylation of eIF-2α by the 100K fraction (tracks 3–5) is increased by the 90K fraction (tracks 6–8), as shown in Figure 4. eIF-2α is not phosphorylated to an appreciable extent when incubated alone or in the presence of only the 90K fraction. Phosphorylated 100 000-dalton peptide is not detectable. Quantitative data for phosphorylation of eIF-2α under these conditions are given in Table I. For these determinations, the eIF-2α band was cut from $NaDodSO_4$–polyacrylamide gels and then counted as described under Experimental Procedure. It is evident from these data that the 90K fraction does not appear to contain an active eIF-2α

Table I: Quantitative Determination of eIF-2α Phosphorylation[a]

additions	[32P]phosphate in eIF-2α (pmol)	
	−90K fraction	+90K fraction
none	0.76	0.73
100K fraction, 0.3 μg	1.54	2.12
100K fraction, 0.5 μg	2.35	2.90
100K fraction, 1.0 μg	4.17	6.28

[a] The stained eIF-2α peptide from the experiment described in Figure 4 was cut out from the gel and its radioactivity determined.

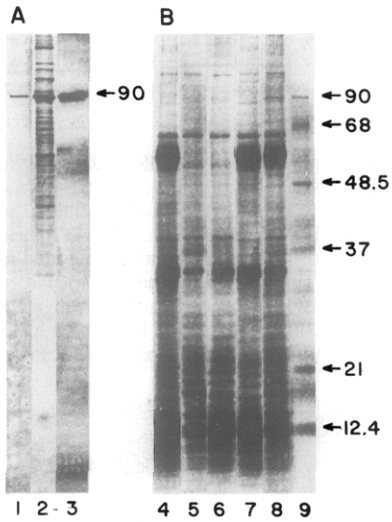

FIGURE 5: (A) $NaDodSO_4$ gel electrophoresis of the 90 000-dalton peptide antigen preparation. A stained gel is shown separating the 90 000-dalton peptide from contaminating protein by $NaDodSO_4$–polyacrylamide gel electrophoresis. Tracks 1 and 2: 1 and 15 μg, respectively, of the hydroxylapatite fraction (described under Experimental Procedure) containing the 90 000-dalton peptide. Track 3: 10 μg of the isolated 90 000-dalton peptide. (B) Immunoprecipitation of the 90 000-dalton peptide with staphylococcal-A protein. Adsorption of the antigen–antibody complexes to staphylococcal-A protein was carried out as described under Experimental Procedure. Controls are shown in tracks 4–7. Tracks 4–8 contain the staphylococcal-A protein: nonimmune IgG in track 4; no addition in track 5; with antigen only in track 6; with immune IgG only in track 7; with both antigen and immune IgG in track 8. Track 9 shows the following molecular weight markers: isolated 90 000-dalton peptide, marked 90; 68 is bovine serum albumin; 48.5 is fumarase; 37 is glyceraldehyde-3-phosphate dehydrogenase; 21 is trypsin inhibitor; 12.4 is cytochrome *c*. The numbers denote their molecular weight × 10^{-3}.

kinase but causes an increase in phosphorylation of this component in the presence of the 100K fraction.

Antibodies against the 90 000-Dalton Peptide Affect Biological Activity and Phosphorylation of eIF-2α. The results of experiments employing antibodies against the 90 000-dalton peptide also indicate that it functions in the reactions leading to the phosphorylation of eIF-2α and the inhibition attributed to HCR. For these experiments a fraction enriched in the 90 000-dalton peptide prepared as described under Experimental Procedure was electrophoresed on $NaDodSO_4$–polyacrylamide gels with the results shown in tracks 1 and 2 of Figure 5. For track 2, the sample was heavily overloaded to demonstrate the heterogeneity of this fraction. The stained band of the 90 000-dalton protein was carefully cut from a number of $NaDodSO_4$ gels similar to that shown in track 2 in order to obtain a highly homogeneous sample of the 90 000-dalton peptide as an antigen. The peptide was separated from the polyacrylamide and concentrated by electrophoresis as described under Experimental Procedure. An

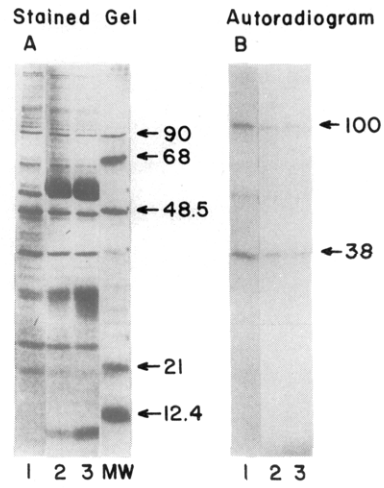

FIGURE 6: Effect of the immune IgG fraction on the phosphorylation of eIF-2 and of the 100 000-dalton peptide. An HCR fraction from the phosphocellulose column (3.75 μg of protein) was preincubated without or with the anti-90K IgG fraction for 20 min on ice, and then its activity in the phosphorylation of eIF-2 was measured as described under Experimental Procedure. (A) Stained gel; (B) autoradiogram. About 2.4 μg of eIF-2 was used with [γ-^{32}P]ATP (43 Ci/mol). Track 1, no immune IgG; tracks 2 and 3, 9 and 18 μg of the anti-90K IgG fraction, respectively, were used. MW = molecular weight markers as described for Figure 5.

aliquot of the resulting preparation of the 90 000-dalton peptide is shown in track 3. This peptide preparation was injected into a goat at 4-week intervals for 5 months, at which time the goat was bled. A partially purified IgG fraction was prepared by ammonium sulfate fractionation and chromatography on DEAE-cellulose. Staphylococcal-A protein was used to detect the formation of the IgG·90 000-dalton peptide complex, as described by Kessler (1975). The precipitates obtained were analyzed by $NaDodSO_4$ gel electrophoresis (Figure 5B). The 90 000-dalton peptide is seen only in track 8. This is from the reaction mixture in which the immune IgG was incubated with the 90K fraction. Tracks 5–7 represent controls in which at least one of the interacting components was omitted. Nonimmune IgG is used as another control with the result shown in track 4.

The effect of the anti-90 000-dalton peptide antibodies on phosphorylation of both the 100 000-dalton peptide and eIF-2α are shown in Figure 6. A fraction of active HCR was preincubated in the amounts indicated with the anti-90K immune IgG, and then eIF-2 and [γ-^{32}P]ATP were added and the reaction mixtures were incubated under the conditions of the protein kinase assay. Decreased phosphorylation of both the 100 000-dalton peptide and eIF-2α is observed with the immune IgG.

The data presented in Figure 7 demonstrate that the anti-90 000-dalton peptide antibodies do not cross-react with the 100K peptide that is phosphorylated in active HCR fractions. An HCR fraction isolated after chromatography on histone–Sepharose was incubated with [γ-^{32}P]ATP under the protein kinase assay conditions and separated from the nucleotides by chromatography on Sephadex G-25. This [^{32}P]phosphate-labeled fraction is then assayed with different IgG fractions for immune precipitation. Neither immune IgG raised against the 90 000-dalton peptide nor nonimmune IgG precipitates the 10 000-dalton peptide to a level above that seen in the control where no IgG fraction was added. However, immune IgG raised against a highly purified HCR fraction [cf. Kramer et al. (1976)] used as a control in these experiments efficiently precipitates the radioactively labeled 100K peptide.

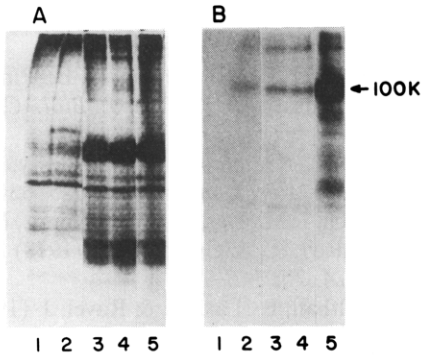

FIGURE 7: No immunological cross-reactivity between 90 000-dalton and 100 000-dalton peptides. A preparation of [^{32}P]phosphate-labeled 100 000-dalton peptide as described in the text (sp act. 587 Ci/mol) was subjected to immunoprecipitation as detailed under Experimental Procedure with different IgG preparations. Controls are shown in track 1 (Pansorbin alone) and track 2 (only antigen). Track 3, plus anti-90 000-dalton peptide antibodies; track 4, nonimmune IgG; track 5, anti-HCR IgG. (A) Stained gel; (B) autoradiogram.

Table II: Effect of Immune IgG for the 90 000-Dalton Peptide on Inhibition of Methionyl-tRNA$_f$ Binding to 40S Ribosomal Subunits[a]

additions	[^{35}S]methionyl-tRNA$_f$ bound to 40S subunits (pmol)
none	0.92
100K fraction (0.2 μg)	0.92
90K fraction (1.0 μg)	0.92
90K + 100K	0.69
(90K + 2 μg of immune IgG) then + 100K	0.68
(90K + 4 μg of immune IgG) then + 100K	0.76
(90K + 6 μg of immune IgG) then + 100K	0.89
(90K + 16 μg of immune IgG) then + 100K	0.91
(90K + 15 μg of nonimmune IgG) then + 100K	0.67

[a] About 1 μg of the 90K fraction was preincubated on ice for 50 min in the absence or presence of the indicated amounts of the IgG fraction. Then the three-step assay as described under Experimental Procedure was started by the addition of eIF-2, the 100K fraction (0.2 μg), and ATP.

The immune IgG raised against the 90 000-dalton peptide also blocks inhibition of eIF-2-dependent binding of methionyl-tRNA$_f$ to 40S ribosomal subunits by HCR, as shown by the data of Table II. For these experiments a low concentration of the 100K fraction was chosen that is not inhibitory by itself but reduces methionyl-tRNA$_f$ binding to 40S ribosomal subunits upon addition of the 90K fraction. Preincubation of this 90K fraction with the immune IgG preparation abolishes its effect on the 100K fraction to cause inhibition of methionyl-tRNA$_f$ binding. Inhibition is observed when the 90K fraction is preincubated with nonimmune IgG. These results indicate that antibodies against the 90 000-dalton peptide prevent inhibition of the eIF-2-dependent step of peptide initiation in addition to their effect on phosphorylation of eIF-2α.

Discussion

We believe the data presented here justify the conclusion that the 90 000-dalton peptide described above plays a role in inhibition of peptide initiation resulting from phosphorylation of eIF-2α by the kinase that is activated in the absence of heme and in translational regulation of protein synthesis by this mechanism. The results indicate that an enzyme system with multiple components is involved. The 90 000-dalton peptide itself does not phosphorylate eIF-2α. Probably phosphorylation of eIF-2α is carried out by an enzyme containing the 100 000-dalton peptide. This kinase appears to be identical with the enzyme isolated by Trachsel et al. (1978). The nature of the reaction leading to the activation of the eIF-2α kinase is not known. We have been unable to detect any effect of cAMP (Grankowski et al., 1979), Ca^{2+}, calmodulin, or EGTA on the activation process or the activity of the eIF-2α kinase (unpublished experiments). Activation of the eIF-2α kinase appears to be associated with phosphorylation of the 100 000-dalton peptide, as shown here and in other studies (Trachsel et al., 1978; Gross & Mendelewski, 1978). However, our results indicate that autophosphorylation may not be the primary mechanism of this reaction. The activity for autophosphorylation is low in the partially purified fractions of the 100 000-dalton peptide described above. Is the 90 000-dalton peptide part of a protein kinase for the 100 000-dalton component? The results presented here do not establish this point. Antibodies against the 90 000-dalton peptide block phosphorylation of both eIF-2α and the 100 000-dalton peptide. However, we have been unable to clearly demonstrate increased phosphorylation of this peptide that is dependent upon addition of the 90K fraction. This may reflect traces of the 90 000-dalton peptide that seem inevitably to be present in preparations of the 100 000-dalton peptide. Possibly yet another factor is involved in this reaction. In any event, the proteins from which the 90 000-dalton and 100 000-dalton peptides are derived appear to be part of an enzyme system known as HCR.

The relationship, if any, of the 90 000-dalton peptide to the heat-stable and heat-labile components, HS and HL (Henderson et al., 1979), is not clear. In the presence of heme, these proteins lead to phosphorylation of the 100 000-dalton peptide and activation of the eIF-2α kinase of the HCR system in what appears to be a cascade-type sequence of reactions. It may be that HS and HL are the distal components and the 90 000-dalton peptide is a proximal component in an enzyme sequence for activation and control of this eIF-2α kinase.

Acknowledgments

The authors express their appreciation to M. Hardesty, M. Rodgers, and K. Pham for their excellent technical assistance, M. A. Henderson for preparation of the typescript, and F. Hoffman for photography and art work for the figures.

References

Adamson, S. D., Herbert, E., & Godchaux, W. (1968) *Arch. Biochem. Biophys. 125*, 671–683.

Affinity Chromatography (1974) Pharmacia Fine Chemicals, Inc., Uppsala, Sweden.

Anderson, C. W., Baum, P. R., & Gesteland, R. F. (1973) *J. Virol. 12*, 241–252.

Balkow, K., Mizuno, S., & Rabinovitz, M. (1973) *Biochem. Biophys. Res. Commun. 54*, 315–323.

Bruns, G. P., & London, I. M. (1965) *Biochem. Biophys. Res. Commun. 18*, 236–242.

Farrell, P. J., Balkow, K., Hunt, T., & Jackson, R. (1977) *Cell 11*, 187–200.

Grankowski, N., Kramer, G., & Hardesty, B. (1979) *J. Biol. Chem. 254*, 3145–3147.

Gross, M., & Rabinovitz, M. (1973) *Biochem. Biophys. Res. Commun. 50*, 832–838.

Gross, M., & Mendelewski, J. (1978) *Biochim. Biophys. Acta 520*, 650–663.

Gupta, N. K., Chatterjee, B., Chen, Y. C., & Majumdar, A. (1975) *J. Biol. Chem. 250*, 853–862.

Henderson, A. B., & Hardesty, B. (1978) *Biochem. Biophys. Res. Commun. 83*, 715–723.

Henderson, A. B., Miller, A. H., & Hardesty, B. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2605-2609.
Kessler, S. W. (1975) *J. Immunol. 115*, 1617-1624.
Kramer, G., Cimadevilla, J. M., & Hardesty, B. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3078-3082.
Kramer, G., Henderson, A. B., Pinphanichakarn, P., Wallis, M. H., & Hardesty, B. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1445-1449.
Kruh, J., & Borsook, H. (1956) *J. Biol. Chem. 220*, 905-915.
Laemmli, U. K. (1970) *Nature (London) 227*, 680-685.
Legon, S., Jackson, R., & Hunt, T. (1973) *Nature (London), New Biol. 241*, 150-152.
Levin, D. H., Ranu, R. S., Ernst, V., & London, I. M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3112-3116.
Maxwell, C. R., & Rabinovitz, M. (1969) *Biochem. Biophys. Res. Commun. 35*, 79-85.
Maxwell, C. R., Kamper, C. S., & Rabinovitz, M. (1971) *J. Mol. Biol. 58*, 317-327.
Odom, O. W., Kramer, G., Henderson, A. B., Pinphanichakarn, P., & Hardesty, B. (1978) *J. Biol. Chem. 253*, 1807-1816.
Pinphanichakarn, P., Kramer, G., & Hardesty, B. (1976) *Biochem. Biophys. Res. Commun. 73*, 625-631.
Ranu, R. S., London, I. M., Das, A., Dasgupta, A., Majumdar, A., Ralston, R., Roy, R., & Gupta, N. K. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 745-749.
Spremulli, L., Walthall, B., Lax, S., & Ravel, J. (1977) *Arch. Biochem. Biophys. 178*, 565-575.
Trachsel, H., Ranu, R. S., & London, I. M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3654-3658.
Wallis, M. H., & Kramer, G. (1979) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, 781.

Inhibition of Ribonucleic Acid Accumulation in Mouse L Cells Infected with Vesicular Stomatitis Virus Requires Viral Ribonucleic Acid Transcription†

Fang-Sheng Wu and Jean M. Lucas-Lenard*

ABSTRACT: The accumulation of ribonucleic acid (RNA) in mouse L-929 cells infected with temperature-sensitive mutants of vesicular stomatitis virus or ultraviolet- (UV-) irradiated virus was studied. At the permissive temperature (30 °C) infection by all mutants resulted in an inhibition of cellular RNA accumulation. At the nonpermissive temperature (40 °C) mutants G114 (I) and G22 (II) failed to inhibit RNA accumulation, but mutants G11 (I), O52 (II), G31 (III), G33 (III), G41 (IV), W10 (IV), O45 (V), and O110 (V) were still active in this respect. In most cases the accumulation of 28S and 18S mature rRNA was inhibited to a greater extent than the synthesis of the 45S rRNA precursor. UV irradiation of wild type virus considerably reduced its capacity to inhibit cellular RNA synthesis. The target size for inactivation of this capacity of the virus was ~17% of the viral genome or that corresponding to the N gene. These results indicate that the virion proteins themselves are incapable of inhibiting cellular RNA synthesis and that transcription of ~17% of the genome is required. Expression of RNA synthesis inhibition also requires some function of virion NS protein in addition to its transcriptase activity.

Infection of vertebrate cells with vesicular stomatitis virus (VS virus) causes a marked inhibition of synthesis of cellular macromolecules (Wagner, 1975). Previous reports have suggested that cellular protein synthesis inhibition in L cells infected by VS virus requires virion-associated L protein and transcription and translation of viral genes N and possibly NS (Marvaldi et al., 1977, 1978). The mechanism of cellular RNA synthesis inhibition is not understood. High multiplicities of input intact virions (Huang & Wagner, 1965), defective interfering particles (Baxt & Bablanian, 1976), and large amounts of isolated G (glyco) protein (McSharry & Choppin, 1978) have been reported to inhibit cellular RNA synthesis. The inhibition of RNA synthesis in chicken embryo cells after infection with VS virus is partially a result of a decreased capacity of the infected cells to transport uridine (Genty, 1975). However, in infected L cells, HeLa cells (Genty, 1975), and mouse myeloma cells (Weck & Wagner, 1978), uridine transport remains unaltered.

Protein synthesis inhibition and cell killing appear to be governed by the same gene functions (Marcus & Sekellick, 1975; Marvaldi et al., 1977). We wished to know whether RNA synthesis inhibition is under the control of the same gene functions as protein synthesis inhibition and cell killing. Temperature-sensitive (ts) mutants which are defective in specific gene functions at the restrictive temperature were screened for their ability to inhibit cellular rRNA synthesis at the nonpermissive temperature to investigate this problem. Mutants unable to inhibit cellular rRNA synthesis at the restrictive temperature were considered to be defective in a function needed for the inhibition. Ultraviolet-irradiated wild type VS virus was also used to determine if newly synthesized viral gene products are necessary or whether virion proteins will suffice. The extent of UV irradiation damage was monitored by protein analysis of the residual transcription and translation of the viral genome (Ball & White, 1976; Marvaldi et al., 1978).

Our results suggest that the virion proteins themselves are not capable of inhibiting cellular RNA synthesis. Transcription of at least part of the viral genome is necessary for the inhibition. While our work was in progress, Weck & Wagner (1979) reported the necessity of viral RNA transcription for cellular RNA synthesis inhibition.

† From the Biological Sciences Group, Biochemistry and Biophysics Section, University of Connecticut, Storrs, Connecticut 06268. *Received August 24, 1979.* This research was supported by Grant No. 12144 awarded by the National Cancer Institute, Department of Health, Education, and Welfare, and benefited from use of the Cell Culture Facility supported by National Cancer Institute Grant No. 14733.

0006-2960/80/0419-0804$01.00/0