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The α Subunit of Eucaryotic Initiation Factor 2 Is Phosphorylated in Mengovirus-Infected Mouse L Cells

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Infection of mouse L cells with mengovirus resulted in the activation of a protein kinase (PK) that selectively phosphorylated the small, 38,000-molecular-weight α subunit of eucaryotic initiation factor 2 (eIF-2) in vitro. The mengovirus-activated kinase was detected in vitro approximately 3 h after virus adsorption. The ratio of phosphorylated to unphosphorylated eIF-2 also increased in vivo between 3 and 7 h after adsorption. The virus-activated kinase fractionated with the ribosomal pellet and had a high affinity for DEAE-cellulose and Mono Q ion-exchange columns. Gel electrophoresis of the kinase activity eluting from the Mono Q column and silver staining of the gel revealed only one protein band with a molecular mass of 70 kilodaltons. The optimal assay conditions for the mengovirus-activated kinase paralleled those of the double-stranded RNA-activated PK (dsRNA-PK). Lysates from infected cells contained elements capable of activating partially purified dsRNA-PK. These elements were identified as double-stranded RNA by their sensitivity to double-stranded RNase. The phosphorylation of the α subunit of eIF-2 coincided with the synthesis of dsRNA in infected cells, suggesting that the mengovirus-activated kinase is the dsRNA-PK. The phosphorylation of the α subunit of eIF-2 correlated with the global inhibition of protein synthesis that occurs at late times after infection.

Infection of cells by the picornavirus mengovirus results in the rapid inhibition of host cell protein synthesis (5, 7, 11, 26, 35). Midway into the infection, the rate of protein synthesis increases because of the synthesis of viral proteins. Shortly thereafter, viral protein synthesis begins to decline as well. The same shutoff profile is found in poliovirus-infected HeLa cells (17). The inhibition of protein synthesis in mengovirus-infected mouse L cells occurs at the level of polypeptide chain initiation (7, 35). Late in infection, ribosomes from infected cells are much less active than ribosomes from uninfected cells because of the presence of an inhibitor that fractionates with them (35).

Lysates from mengovirus-infected cells also contain a protein kinase (PK) activity capable of phosphorylating the α subunit of exogenous initiation factor 2 (eIF-2) (33). The kinase is detected approximately 3 h after infection. In poliovirus-infected cells, there is an increase in the phosphorylation of the α subunit of eIF-2 (eIF-2α) at 3 h after infection because of the activation of the double-stranded RNA-activated PK (dsRNA-PK) (3). Using a poliovirus mutant that fails to inhibit host protein synthesis early in infection, O'Neill and Racaniello (31) observed that the phosphorylation of eIF-2α correlates with the late phase of protein synthesis inhibition.

Interestingly, the activation of the dsRNA-PK in poliovirus-infected cells and the phosphorylation of eIF-2α appear to be an exception rather than the rule. In cells infected by adenovirus (43, 44, 46), influenza virus (21), reovirus serotype 1 (19), and vaccinia virus (49), the dsRNA-PK fails to be activated during the course of infection as a result of the expression of specific viral products that prevent its activation. In the case of adenovirus-infected cells, the VAI RNA prevents activation (43, 44, 46), and in the case of influenza virus (21), reovirus serotype 1 (19), and vaccinia virus (49), specific viral proteins have been hypothesized to carry out this function.

Activation of dsRNA-PK has a detrimental effect on protein synthesis because the activated kinase phosphorylates the 38-kilodalton (kDa) eIF-2α, which is involved in the delivery of Met-tRNA$_i$, along with GTP, to the 40S ribosomal subunit (for reviews, see the work of Pain [32] and Safer [40]). In a subsequent step, GTP is hydrolyzed and eIF-2-GDP must be regenerated to eIF-2-GTP in order for the eIF-2 to function catalytically (32, 40). Another factor, the guanine nucleotide exchange factor, is involved in the reconversion (22, 32). When eIF-2 is phosphorylated, the guanine nucleotide exchange factor presumably binds to phosphorylated eIF-2-GDP with high affinity but fails to release the GDP (34). Thus, guanine nucleotide exchange factor fails to regenerate eIF-2-GTP and is tightly trapped in an inactive complex (32, 34).

In this report, we show that there is an increase in the phosphorylation of eIF-2α in vivo between 3 and 7 h after L cells are infected by mengovirus and that the kinase responsible for the phosphorylation is probably the 67-kDa dsRNA-PK. We discuss the possibility that the phosphorylation of eIF-2α, which occurs long after host protein synthesis has been decreased, may play a regulatory role in the life cycle of the virus. It may serve to clear viral messages of polysomes so that they can assemble into progeny virions.

MATERIALS AND METHODS

Cell culture conditions and virus growth. Mouse L cells were grown as monolayer cultures in RPMI 1640 medium supplemented with 5% horse serum. Mengovirus (strain is$^+$) (47) was obtained from P. I. Marcus via E. Simon. This strain of mengovirus is a poor inducer of interferon (27). It was purified by the method of Abreu and Lucas-Lenard (1), and its titer was 1.5×10^{10} PFU/ml. In some experiments requiring phosphorylated dsRNA-PK and eIF-2 for gel markers, the L(Y) cell line, obtained from P. I. Marcus and M. Sekellick via J. S. Youngner, was used. In L(Y) cells,

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high levels of dsRNA-PK are readily induced with low doses (100 U/ml) of interferon (alpha and beta, Lee Biomolecular).

Preparation of the interferon-induced dsRNA-PK (IFN-dsRNA-PK) from L(Y) cells. The IFN-dsRNA-PK was extracted by the method of Galabru et al. (13) from the ribosomal pellet with which it fractionates. The resulting extract was dialyzed against buffer I (30 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.4], 50 mM KCl, 2.5 mM magnesium acetate, 2 mM dithiothreitol, 0.3 mM phenylmethylsulfonyl fluoride, 0.01% Triton X-100, 10% glycerol) and adsorbed to DEAE-cellulose equilibrated in buffer I. The flowthrough fraction was used for experiments requiring IFN-dsRNA-PK.

Infection of cell monolayers. Nearly confluent monolayers of L cells were infected with mengovirus at a multiplicity of infection of 25 PFU per cell in RPMI 1640 medium supplemented with 2% horse serum and 10 mM HEPES. After a 45-min adsorption period at 37°C, the inoculum was removed and replaced with growth medium. The infection was continued for the time periods indicated in the figure legends.

Preparation of cell lysates. Mouse L-cell monolayers were washed with ice-cold phosphate-buffered saline and then lysed by addition of 200 μl (for 100-mm plates) of buffer I containing 0.4% Triton X-100. The lysates were scraped from the plates and centrifuged at 4°C in a microcentrifuge for 3 min at 12,000 × *g* for small preparations or in a Sorvall SS-34 rotor at 14,500 × *g* for 15 min for larger preparations (S-15 fractions). These S-15 fractions and all subsequent fractions were stored at −70°C and used for various assays or processed further as described below.

Ribosome isolation and salt wash preparation. Ribosomes (40S subunits and greater) were isolated by centrifuging S-15 fractions at 160,000 × *g* for 2.5 h. The resulting ribosomal pellet was first rinsed with buffer I, then suspended in salt wash buffer (buffer I containing 500 mM KCl and 1% Triton X-100) for 30 min at 4°C, and finally centrifuged at 160,000 × *g* for 2.5 h. The ribosomal salt wash was dialyzed against buffer I (two changes) overnight at 4°C and stored at −70°C or used directly for the in vitro kinase assay. The salt-washed ribosomes were resuspended in buffer I and stored or assayed directly as above.

Protein kinase assay. Reaction mixtures contained the following in a final volume of 15 μl: 20 mM HEPES (pH 7.4), 20 to 46 mM KCl, 1 mM magnesium acetate, 1.5 mM dithiothreitol, 25 μM ATP, 2 μCi of [γ-^{32}P]ATP (specific activity, 1,500 to 3,000 Ci/mmol), and 5 μg of protein from the various cellular fractions. Reticulocyte eIF-2 (0.5 μg) and poly(I)-poly(C) (1.5 ng) were included in the assay as indicated in the figure legends. In some assays, 1.8 μg of crude IFN-dsRNA-PK from DEAE-cellulose, prepared as described above, was included.

The mixtures were incubated at 30°C for 30 min, and the reactions were terminated by the addition of an equal volume of 2× sodium dodecyl sulfate sample buffer (23). The heated samples (100°C for 2 min) were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels (23). The gels were dried and autoradiographed.

Labeling conditions. For in vivo protein synthesis analysis, L cells were pulse-labeled for 30 min with 25 μCi of [^{35}S]methionine (specific activity, approximately 1,000 Ci/mmol) per ml in minimal essential medium minus amino acids and containing 2% horse serum. Samples from S-15 fractions were either precipitated with trichloroacetic acid for radioactivity determinations or assayed for their protein content.

Detection of eIF-2 and phosphorylated eIF-2. Lysates from control and infected cells were lysed with buffer I containing 0.4% Triton X-100 and 25 mM NaF at the times indicated in the figure legends. The lysates were centrifuged for 30 s in a microcentrifuge, and the resulting supernatant fraction was analyzed to determine the percentage of eIF-2α in the phosphorylated form as described below. In some experiments, whole cell lysates were prepared by lysing cells in saturated urea buffer (9.2 M urea, 2% 2-mercaptoethanol, 4% ampholyte mixture [pH 3 to 5 and 5 to 7] at a 4:1 ratio). The phosphorylated and unphosphorylated forms of eIF-2α were separated by slab gel isoelectric focusing in the presence of 9.2 M urea as previously described (45). eIF-2 separated in this manner was transferred to nitrocellulose and detected by using an eIF-2α-specific monoclonal antibody as described by Scorsone et al. (45).

Purification of the mengovirus-activated kinase. The mengovirus-activated kinase was purified from salt-washed ribosomes derived from approximately 3 × 10^9 L cells (about 300 100-mm-diameter plates) as described below, with all procedures performed at 4°C. The salt-washed ribosomes were resuspended in 8 ml of buffer I for 1 h and repelleted as described above. This procedure was repeated three times, with 8 ml of fresh buffer I added to the ribosomes each time. The kinase activity in the pooled supernatant fractions represented only a fraction of the kinase activity originally pelleting with the ribosome fraction.

The pooled supernatant fractions were applied to a DEAE-cellulose column (5 ml) that had been previously equilibrated with buffer I. The column was washed with 15 ml of buffer I and then step eluted by the successive addition of 15 ml each of buffer I containing 0.2, 0.3, 0.4, 0.5, 0.6, and 1 M KCl. The resulting fractions were dialyzed overnight against buffer I, and then 8-μl samples of each fraction were assayed for kinase activity. Fractions containing activity (those eluting at 0.4 and 0.5 M KCl) were combined and concentrated by membrane ultrafiltration with a UM10 Diaflo ultrafiltration membrane. The concentrate (about 3 ml) was applied to a Pharmacia Mono Q HR 5/5 column that had been equilibrated with buffer I. After the column was washed with 5 ml of buffer I, a 20-ml linear gradient of 0.05 to 0.8 M KCl in buffer I was applied. The flow rate was maintained at 1 ml/min, and 1-ml fractions were collected. The fractions were dialyzed against buffer I overnight, and 8-μl samples were assayed for kinase activity. The kinase activity eluted between 0.6 and 0.67 M KCl. The activity in individual fractions was assayed by the methods described above. After gel electrophoresis and autoradiography of the gel, the relative kinase activity was quantitated with an LKB Ultrascan densitometer.

RESULTS

Phosphorylation of endogenous eIF-2α in mengovirus-infected L cells. Earlier we reported that lysates from mengovirus-infected L cells contain a PK activity capable of phosphorylating exogenous eIF-2α (33). To understand the role of the kinase in infected cells, we deemed it crucial to determine whether endogenous eIF-2α is also phosphorylated during the course of infection. For this study, the cellular content of phosphorylated and unphosphorylated eIF-2α at different times during infection was quantitated. The two forms of eIF-2α were separated by isoelectric focusing of cell lysates and identified by using antibody against the α subunit of eIF-2 (see Materials and Methods). The results of a typical experiment are shown in Fig. 1.

It is obvious from the autoradiogram in Fig. 1 that in vivo

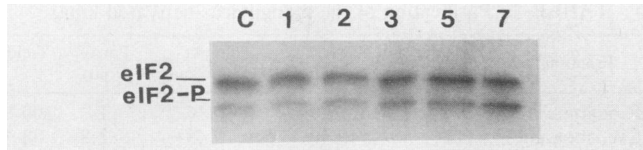

FIG. 1. Autoradiogram of phosphorylated and unphosphorylated eIF-2 in infected L cells. The phosphorylated (P) and unphosphorylated forms of eIF-2 were resolved and identified on one-dimensional isoelectric-focusing gels as described in Materials and Methods. The percents eIF-2 phosphorylated in mock-infected (7 h) (C) and virus-infected (1, 2, 3, 5, and 7 h, indicated above each lane) samples were 17, 23, 28, 36, 31, and 51%, respectively.

eIF-2 phosphorylation was not significantly increased until late in the infection. Quantitation of the data from several experiments by densitometric analysis of the autoradiograms revealed (Fig. 2) that in some instances, eIF-2α phosphorylation was not enhanced until about 5 h after infection. In other cases, enhanced phosphorylation was detected as early as 3 h after infection.

Superimposed on the data in Fig. 2 is a host protein synthesis shutoff curve, which demonstrates the very rapid decrease of host translation (54% within the first h), followed by a rise in viral protein synthesis at 4 h after infection, and a fall in viral protein synthesis shortly thereafter. The inset in Fig. 2 documents the increase in viral protein synthesis at 4 h after infection and the decline in viral protein products soon thereafter. Figure 2 also shows the time course of

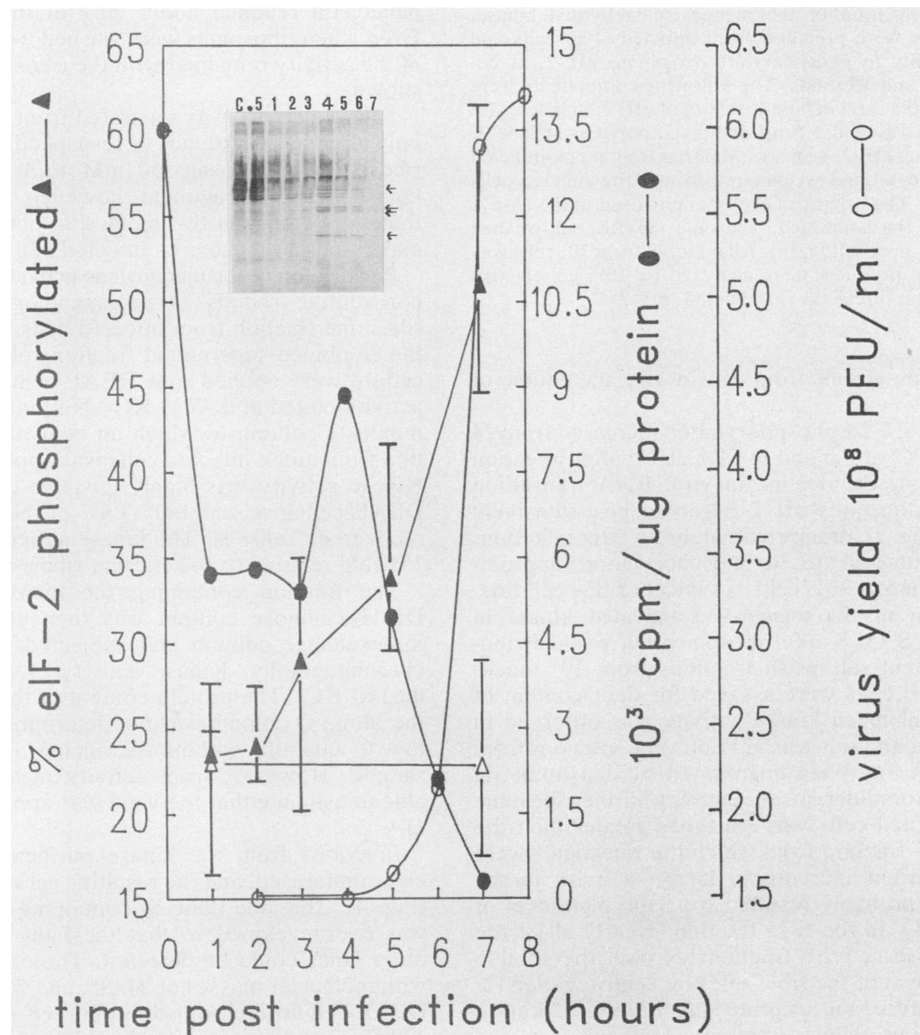

FIG. 2. Phosphorylation of eIF-2 in mengovirus-infected L cells and its correlation to late protein synthesis inhibition. Lysates were prepared from mock-infected (1 and 7 h [△]) or virus-infected (1, 2, 3, 5, and 7 h [▲]) cells, and the percent eIF-2 in the phosphorylated form was determined by scanning the autoradiograms as described in Materials and Methods. Data for the mock-infected (1 h) and virus-infected (1 and 2 h) samples were obtained from three different experiments. All other data were obtained from four different experiments. Error bars represent the highest and the lowest values obtained in the different experiments, and curves are drawn through the average values. The amount of [^{35}S]methionine incorporated into protein as a function of time was determined as described in Materials and Methods. The virus yield at each time point was determined by plaque assay. Inset: Samples from the protein synthesis assay was dissolved in 2× sample buffer (23) and subjected to gel electrophoresis as described in Materials and Methods; the numbers above each lane represent the time (in hours) after infection at which the samples were analyzed; C, mock-infected.

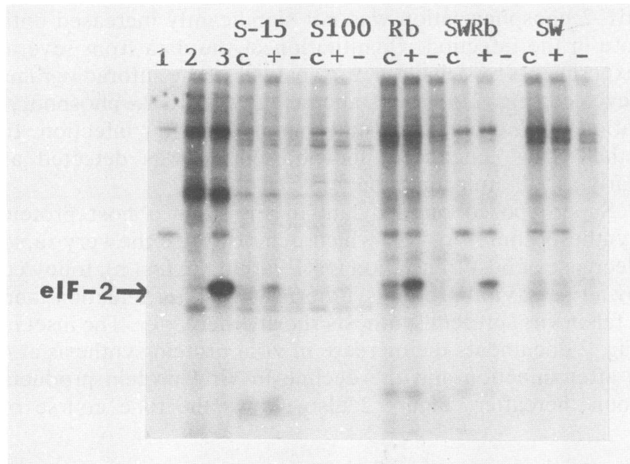

FIG. 3. Cellular location of the mengovirus-activated kinase. Subcellular fractions were prepared from infected (4 h) cells and assayed for the ability to phosphorylate exogenous eIF-2, as described in Materials and Methods. The following components were added to the standard assay: in lane 1, 0.5 μg of eIF-2; in lane 2, 1.8 μg of crude dsRNA-PK and 1.5 μg of poly(I)-poly(C); in lane 3, same as in lane 2, plus eIF-2. Lanes c, Material from mock-infected cells plus eIF-2; lanes + and −, material from virus-infected cells plus or minus eIF-2. The origins of the fractions used in the assays are indicated above the lanes (Rb, ribosome; SWRb, salt-washed ribosome; SW, ribosomal salt wash). All material from 10^5 cells was loaded. The reaction products were analyzed by 10% gel electrophoresis. The position of eIF-2α is indicated (eIF-2).

release of infectious virions from cells during the course of infection.

On the average, eIF-2α phosphorylation increased from 24 to 29% at 3 h to 33% at 5 h and to 51% at 7 h after infection (Fig. 2). In general, the decline in viral RNA translation occurred after endogenous eIF-2 began to be extensively phosphorylated (Fig. 2), or approximately 5 h after infection. The high background of eIF-2α phosphorylation (approximately 24%) in uninfected L cells is typical of this cell line.

Cellular location of the mengovirus-activated kinase in L-cell lysates. The S-15, S-100, ribosome, salt-washed ribosome, and ribosomal salt wash fractions from 10^5 mock-infected or infected cells were assayed for their content of kinase activity. Enhanced kinase activity was observed in the S-15, ribosome, and salt-washed ribosome fractions from infected cells (Fig. 3). When quantitated by densitometric analysis of the autoradiogram, the activity in the ribosome fraction from infected cells was 2.6 times greater than the activity in the S-15 fraction from which the ribosomes were derived. The apparent increase in kinase activity in the ribosome fraction probably resulted from the high level of phosphatase activity in the S-15 fraction. Nearly all of the cellular phosphatase activity fractionates with the soluble fraction after removal of the ribosomes by centrifugation (2, 15, 41, 42). Therefore, an accurate quantitation of kinase activity in the various cellular fractions is difficult.

Only a small amount of eIF-2α kinase activity (about 13% of that found in the S-15 fraction) was detected in the S-100 fraction after fractionation, as determined by densitometric analysis of the autoradiogram in Fig. 3. The bulk of the kinase activity fractionated with the ribosomal pellet. Only 10% of the kinase activity pelleting with ribosomes was extracted from this fraction by washing the ribosomes with 0.5 M KCl–1% Triton X-100. The salt-washed ribosomal

TABLE 1. Purification of the mengovirus-activated kinase[a]

Fraction[b]	Amt (mg) of protein	Activity (AU · mm)[c]	Sp act (AU · mm/μg)	Purity (fold)	Yield (%)
Ribosome	399	62,320	0.16	1	100
SW ribosome	121	54,840	0.45	2.8	88
SWRb sup	8.6	8,500	0.99	6.2	13.6
DEAE	0.1	2,420	24.2	151	3.9
Mono Q	ND[d]	605	ND	ND	1.0

[a] Purified from virus-infected cells as described in Materials and Methods.
[b] SW-ribosome, Salt-washed ribosome; SWRb sup, salt-washed ribosome supernatant; DEAE, DEAE-cellulose-purified material (data obtained from the combined 0.4 and 0.5 M KCl-eluted material); Mono Q, Mono Q-purified material.
[c] AU · mm, Absorbance units times millimeters. Determined by assaying each fraction for eIF-2 kinase activity as described in Materials and Methods and scanning the autoradiograms with a densitometer.
[d] ND, Not determined.

pellet still retained about 90% of the recovered activity. Even when ribosomes were washed with 1 M KCl, nearly all of the activity remained with the ribosome fraction (data not shown).

Kinase activity was separated from the ribosome fraction only after several rounds of resuspending and repelleting the ribosomes in low-salt (50 mM KCl) buffer. The yield of kinase after this treatment, however, was very low. Nevertheless, we utilized this method to purify further the kinase expressed in mengovirus-infected cell lysates.

Purification of the mengovirus-activated kinase. After three consecutive rounds of resuspending and repelleting the ribosome fraction from infected cells to extract the kinase, the combined supernatant fractions obtained from this procedure were applied to a DEAE-cellulose column. Kinase activity eluted at 0.45 M KCl. No kinase was eluted from a duplicate column to which an equivalent supernatant fraction from mock-infected cells was applied (data not shown). Kinase activity was highly unstable during purification on DEAE-cellulose, and only 3.9% of the original activity was recovered (Table 1). The kinase activity was purified about 151-fold relative to the starting ribosome fraction.

The fraction containing the kinase activity from the DEAE-cellulose column was then applied to a Mono Q ion-exchange column and subjected to fast-protein liquid chromatography. Kinase activity eluted at approximately 0.65 M KCl. The protein content of the kinase eluting from the Mono Q column was not determined because it was too low to quantify without sacrificing a large portion of the sample. However, from activity measurements, we were able to estimate that the yield was approximately 1% (Table 1).

Fractions from the kinase purification procedure were electrophoresed, and the resulting gel was stained with silver (Fig. 4). The lane (lane S) containing the ribosome extract was overdeveloped so that the faint protein bands in the other lanes could be detected. Three major protein bands, with molecular masses of 94, 85, and 70 kDa, remained after DEAE-cellulose chromatography. Of these bands, only the 70-kDa band remained after Mono Q chromatography. Whether this protein band was the kinase could not be ascertained. However, the estimated molecular mass of this band was very close to the molecular mass (67 kDa) of the dsRNA-PK from L cells (2, 41).

Comparison of DEAE-purified, mengovirus-activated kinase and IFN-dsRNA-PK. In additional attempts to determine whether the mengovirus-activated kinase and the IFN-dsRNA-PK were the same enzymes, we compared their

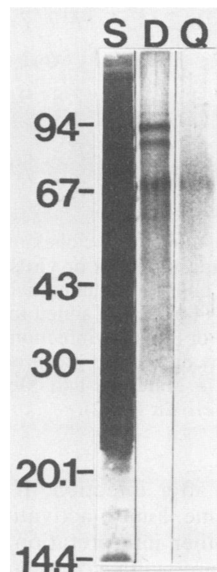

FIG. 4. Silver staining of fractions from mengovirus-activated kinase purification. Fractions from the purification of the mengovirus-activated kinase were electrophoresed on a 10% polyacrylamide gel, and the gel was subsequently stained with a Rapid-Ag-Stain kit (ICN Radiochemicals) by instructions provided by the manufacturer. Protein from the following samples was analyzed: 7.5 μg of salt-washed ribosome supernatant (lane S), 0.75 μg of kinase activity from the DEAE-cellulose column fraction (lane D), and activity from the Mono Q fraction (quantity not determined) (lane Q). The positions of the molecular mass markers (in kilodaltons) are indicated on the left.

optimal assay conditions (Table 2). Both kinases were most active at the lowest magnesium concentration (0.6 mM) tested, and both were inhibited at high (20 mM) concentrations of magnesium. Both kinases were most active at low (40 to 50 mM) concentrations of KCl, and both were inhibited at high (200 mM) concentrations of KCl. The activity of the mengovirus-activated kinase was more sensitive to high KCl concentrations than was the activity of the IFN-dsRNA-PK. At 100 mM KCl, the activity of the mengovirus-activated kinase was inhibited 77% (compared with its activity at 40 mM KCl), while the IFN-dsRNA-PK was inhibited by only 33%.

The activity of both kinases was inhibited in vitro by 10 mM 2-aminopurine but not by 1 mM 2-aminopurine. Although there have been several reports showing that 2-aminopurine is a potent inhibitor of the dsRNA-PK and the hemin-controlled translational repressor of reticulocytes (6, 12, 24), the specificity of this inhibitor in vitro has not been unequivocally demonstrated. The strong similarities between the mengovirus-activated kinase and the IFN-dsRNA-PK under these assay conditions suggest that they may be the same enzyme.

TABLE 2. Comparison of DEAE-purified, mengovirus-activated kinase and IFN-dsRNA-PK[a]

Kinase	Activity[b] (%) with: KCl at (mM):		MgAc$_2$ at (mM):		2-Aminopurine at (mM):		Elution (mM) from DEAE with KCl	Approx molecular size (kDa)
	100	200	5	20	1	10		
Mengovirus-activated	33	5	59	20	100	18	400–500	70
IFN-dsRNA-PK	77	15	59	22	100	8	>400	67

[a] For both kinases, optimum concentrations of KCl and magnesium acetate were 40 to 50 and 0.6 mM, respectively. Both kinases also had eluted from phosphocellulose in the flowthrough.

[b] Assayed as described in Materials and Methods. MgAc$_2$, Magnesium acetate.

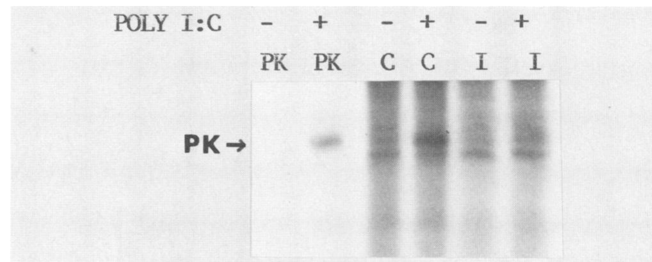

FIG. 5. Level of unactivated dsRNA-PK in uninfected and infected L cells. Ribosome fractions from mock-infected (C) or virus-infected (5 h) (I) cells were isolated and assayed for kinase activity as described in Materials and Methods, except that magnesium was omitted from the lysing medium and 1 mM EDTA was added. Magnesium was present during the kinase assay. Approximately 20 μg of protein from these fractions was assayed in the presence (+) or absence (−) of poly(I)-poly(C) as indicated to determine whether these fractions contained latent dsRNA-PK. The position of the dsRNA-PK (PK) is indicated.

Levels of unactivated dsRNA-PK in uninfected and infected L cells. Dactinomycin did not prevent expression of the mengovirus-activated kinase (33), suggesting that a kinase already present in L cells was being activated. Since cells contain different levels of unactivated dsRNA-PK (18), it was crucial to compare the levels in uninfected and infected cells. The addition of poly(I)-poly(C) to lysates from mock-infected (5 h) cells led to the phosphorylation of a 67-kDa protein that comigrated with the IFN-dsRNA-PK (Fig. 5). No phosphorylation was detected in the absence of the activator. These results suggest that even if cells are not pretreated with interferon, they contain a basal level of latent dsRNA-PK that is capable of being activated.

In contrast, the addition of poly(I)-poly(C) to lysates from infected (5 h) cells (Fig. 5) did not result in the phosphorylation of the dsRNA-PK, indicating either that the kinase was already activated by virus-specific elements or that its activity was waning during the course of infection. Black et al. (3) have recently shown that in poliovirus-infected HeLa cells, the dsRNA-PK is activated, but the amount of the kinase progressively decreases during infection as measured with specific human dsRNA-PK antibody.

To prevent any adventitious activation of the dsRNA-PK during preparation of the lysates, we prepared the lysates in the experiments for Fig. 5 in the absence of magnesium (which is necessary for kinase activation) and in the presence of 1 mM EDTA to chelate the cellular magnesium. The optimal magnesium concentration for the experiments in Fig. 5 was restored during the kinase assay.

Presence of elements in lysates from infected cells that can activate the IFN-dsRNA-PK. If our inability to detect latent dsRNA-PK by autophosphorylation in infected-cell lysates results from its having been activated previously in vivo, then these lysates should contain substances that can activate the kinase. For these experiments, the latent IFN-dsRNA-PK was isolated from interferon-treated L(Y) cells and partially purified by passage through a column of DEAE-

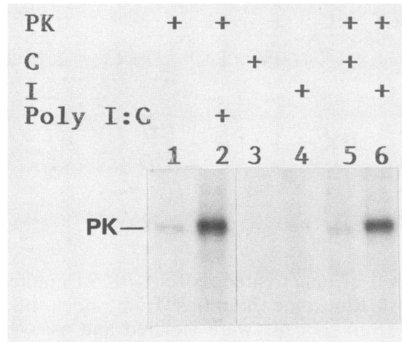

FIG. 6. Presence of elements in lysates from infected cells that can activate IFN-dsRNA-PK. S-15 fractions from mock-infected (C) (lanes 3 and 5) or virus-infected (I) (lanes 4 and 6) cells were prepared at 5 h after infection, and 2 μg of protein from these fractions was incubated with the IFN-dsRNA-PK (lanes 5 and 6). IFN-dsRNA-PK was also incubated with poly(I)-poly(C) (lane 2) or alone (lane 1). PK assay conditions were as described in Materials and Methods. The position of the dsRNA-PK (PK) is indicated.

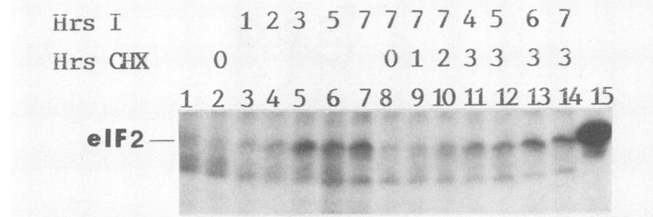

FIG. 7. Kinase activation in cycloheximide-treated cells. Cells were mock-infected (lanes 1 and 2) or virus-infected (I) (lanes 3 to 14) for the number of hours designated above the autoradiogram. Cycloheximide (CHX) (4 μM) was added to the culture medium at 15 min prior to 0, 1, 2, or 3 h after infection as indicated above the autoradiogram. S-15 fractions were prepared and eIF-2 kinase was assayed as described in Materials and Methods. Lane 15 was a marker for identifying eIF-2α (eIF2).

cellulose chromatography as described in Materials and Methods. Incubation of this kinase preparation with an S-15 fraction from infected (5 h) cells resulted in the phosphorylation of the 67-kDa protein kinase (Fig. 6, lane 6). Incubation of the same kinase preparation with an S-15 fraction from uninfected cells did not lead to the phosphorylation of the kinase (Fig. 6, lane 5). These results demonstrate that elements capable of activating the dsRNA-PK exist in lysates from infected cells and that these lysates do not contain inhibitors that prevent dsRNA-PK activation.

In these experiments, we had to rely on kinase activity measurements without the benefit of a specific antibody against the dsRNA-PK. We therefore could not determine whether the quantity of the kinase remained constant during the course of infection. Some of the kinase could have been degraded, as found in poliovirus-infected cells (3), while the remaining kinase was superphosphorylated.

Time course of accumulation of kinase-activating elements in mengovirus-infected cells and their identification. The above-described experiments suggested that elements in virus-infected cells accumulated that could activate the endogenous dsRNA-PK. To determine the minimum time of viral expression necessary for activation, we utilized the inhibitor cycloheximide to stop translation at various points after virus infection. Regardless of the time of addition of the cycloheximide, infection was allowed to proceed for a total of 7 h from the start of adsorption, unless otherwise indicated.

Lanes 5 to 7 of Fig. 7 show the kinase activities in lysates from infected cells harvested at 3, 5, and 7 h after infection. As can be seen, it took at least 3 h of infection to accumulate kinase-activating elements. The addition of cycloheximide to infected cells at 0, 1, and 2 h after infection prevented sufficient accumulation of viral products to activate the kinase even when cell lysates were assayed at 7 h after infection (Fig. 7, lanes 8 to 10).

The addition of cycloheximide at 3 h after infection had no effect on kinase activation when measured at 6 or 7 h after infection (Fig. 7, lanes 13 and 14). When cycloheximide was added at 3 h after infection, the level of kinase activity at 4 and 5 h after infection (Fig. 7, lanes 11 and 12) was reduced compared with the level at 6 and 7 h after infection (Fig. 7, lanes 13 and 14). These results suggest that the viral products necessary to synthesize kinase-activating elements are made during the first 3 h after infection. If protein synthesis is inhibited after that time, kinase-activating elements continue to accumulate. Another interpretation is that once kinase-activating elements are synthesized, continued protein synthesis is not required to maintain the kinase in an activated state.

To determine whether the activator is double-stranded RNA, we tested its sensitivity to a double-stranded RNase recently identified by Meagan and Marcus (28). This nuclease is induced in, and released from, chicken embryo cells when they are induced to produce interferon. If the activating element in lysates from infected cells is double-stranded RNA, then pretreatment of the lysate with the double-stranded RNase prior to assay should abolish its capacity to activate the IFN-dsRNA-PK. On the other hand, treatment of the lysate with single-stranded RNase should have no effect.

The effectiveness of the double-stranded RNase was tested by preincubating poly(I)-poly(C) with the double-stranded RNase. The enzyme completely prevented the poly(I)-poly(C) from subsequently activating the IFN-dsRNA-PK (Fig. 8, lanes 2 and 3). In contrast, preincubation of the poly(I)-poly(C) with RNase A, which degrades primarily single-stranded RNA, had no effect on its ability to activate the kinase (Fig. 8, lane 4).

The addition of lysate from infected cells, but not from uninfected cells, to the IFN-dsRNA-PK resulted in the activation of the kinase (Fig. 8, lanes 7 and 8). Preincubation of the lysate from infected cells with increasing concentrations of the double-stranded RNase abolished its ability to activate the IFN-dsRNA-PK (Fig. 8, lanes 9 to 11). The active ingredient in the double-stranded RNase preparation was the nuclease because preincubation of poly(I)-poly(C) with an equivalent fraction from chicken embryo cells not primed to produce interferon had no effect on the ability of the poly(I)-poly(C) to activate the kinase (Fig. 8, lane 14). Treatment of the infected-cell lysate with RNase had no effect on kinase activation (Fig. 8, lanes 12 and 13).

These results suggest that the activator of the endogenous kinase in mengovirus-infected cells is double-stranded RNA and therefore strongly support the contention that the kinase that is activated in mengovirus-infected cells is the dsRNA-PK.

DISCUSSION

In a report from this laboratory (33), we indicated that lysates from mengovirus-infected cells contain a protein

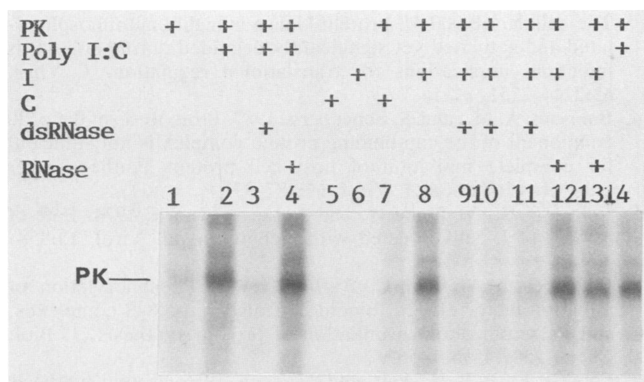

FIG. 8. Double-stranded RNase inactivation of IFN-dsRNA-PK-activating elements present in mengovirus-infected lysates. S-15 fractions were prepared from mock-infected (C) or virus-infected (5 h) (I) cells. The S-15 fractions or poly(I)-poly(C) was pretreated at 37°C for 30 min with 0.25 (lanes 3 and 9), 0.5 (lane 10), or 2 (lane 11) U of double-stranded RNase (dsRNase) per 15 μl of reaction mixture. (One unit is the amount of enzyme that rendered 50% of a poly(I)-poly(^{3}H-C) substrate acid soluble after 30 min at 37°C). Lane 14, Poly(I)-poly(C) pretreated with a fraction from non-interferon-treated cells that was equivalent to the one containing the double-stranded RNase; in lanes 4, 12, and 13, lysates pretreated with 0.1 (lanes 4 and 12) or 1 (lane 13) μg of RNase A per ml. The pretreated fractions were assayed for the ability to activate IFN-dsRNA-PK, as described in Materials and Methods. The kinase was included in all samples except those indicated above the autoradiogram. The position of the dsRNA-PK (PK) is indicated.

kinase that is capable of phosphorylating exogenous eIF-2α in vitro. We report herein that eIF-2α is also phosphorylated in vivo.

Although there was a small increase in eIF-2α phosphorylation as early as 3 h after infection, in most of our experiments, significant phosphorylation did not occur until between 3 and 5 h after adsorption (Fig. 2). This increase in eIF-2 phosphorylation in vivo coincided with a decrease in viral protein synthesis (Fig. 2) and followed an increase in kinase-activating elements, which appear to be double-stranded RNA (Fig. 8), in infected-cell lysates (Fig. 7). The decline in viral protein synthesis preceded the release of infectious virions (Fig. 2).

Rice et al. (38) also found increased eIF-2α phosphorylation, which they attributed to dsRNA-PK activation, in encephalomyocarditis virus-infected HeLa cells. (Encephalomyocarditis virus is a cardiovirus very similar to mengovirus.) The major difference between their results and ours is that they had pretreated their HeLa cells with interferon prior to infection while we had not. Rice et al. (38) did not detect an increase in kinase activity in cells that had not been pretreated with interferon, contrary to the results reported in this paper. In contrast to the results of Rice et al., Gupta (16) did not detect an increase in dsRNA-PK activity in mengovirus-infected, interferon-pretreated L cells. The reasons for the different results may lie in the level of endogenous dsRNA-PK in each cell type.

It is clear from the results presented here that the phosphorylation of eIF-2α in mengovirus-infected L cells cannot explain the early inhibition of host protein synthesis. Protein synthesis in L cells was decreased by about 50% within the first hour after infection (Fig. 2), but eIF-2 was not extensively phosphorylated until much later.

The early decrease of cellular protein synthesis in mengovirus- and encephalomyocarditis virus-infected cells is not a result of the degradation of the 220,000-Da subunit of translation initiation factor eIF-4F (cap-binding protein complex) (30), which occurs in poliovirus- and rhinovirus-infected cells (9, 10). The eIF-4F binds to the 5′ m^7 cap structure of all capped mRNAs and is essential for their translation (32). Since poliovirus RNA is uncapped, in theory it does not require the eIF-4F for its translation. Therefore, degradation of the 220,000-Da subunit seems to be a reasonable mechanism for poliovirus to have evolved in order to stop host protein synthesis while leaving the translation of its message unaltered (for a review, see the work of Ehrenfeld [8] and Sonenberg [48]).

In poliovirus-infected cells, proteolysis of the 220-kDa component of the cap-binding complex alone is insufficient for complete inhibition of host cell translation (4). Cellular protein synthesis is reduced to only 30% of control levels. The phosphorylation of eIF-2α together with the degradation of the 220-kDa component appear to be responsible for the total shutoff of host protein synthesis in these cells (3).

The mechanism that mengovirus and encephalomyocarditis virus use to decrease host protein synthesis early in infection is not known. It has been proposed that the cardioviruses inhibit host protein synthesis solely on the basis of the exceptional competitive ability of their mRNAs (which, like poliovirus mRNA, are uncapped) and the large quantity of viral mRNA present during infection (1, 20). Indeed, Rosen et al. (39) have shown that, on a molar basis, mengovirus mRNA competes about 35 times more effectively for a critical component in translation and has a 30-fold higher affinity for eIF-2 than globin mRNA. Perez-Bercoff and Kaempfer (37) have determined the eIF-2-binding site to ^{32}P-labeled mengovirus RNA. Fingerprint analysis of the RNA protected by eIF-2 against RNase T1 digestion yielded three fragments that overlapped entirely with those protected in both 40S and 80S initiation complexes. These results suggest that the eIF-2 binds near or at the translation initiation site of mengovirus RNA and that eIF-2 may be the critical component that allows mengovirus mRNA to be a successful competitor.

The results presented here suggest that the kinase responsible for eIF-2α phosphorylation in mengovirus-infected cells is the dsRNA-PK because of the following reasons. (i) Lysates from uninfected L cells contained measurable amounts of latent dsRNA-PK, which had the potential to be activated by double-stranded RNA. (ii) Elements were present in lysates from infected cells that could activate the dsRNA-PK in uninfected cells (Fig. 6). (iii) Infected-cell lysates no longer contained latent, unactivated dsRNA-PK (Fig. 5). (iv) The optimal conditions for the in vitro assay of the mengovirus-activated kinase and the IFN-dsRNA-PK were nearly identical. (v) The activator of the IFN-dsRNA-PK in lysates from mengovirus-infected cells was double-stranded RNA (Fig. 8). (vi) The only protein band remaining after extensive purification of the mengovirus-activated kinase had a molecular mass of 70 kDa, which approximates closely the molecular mass of L-cell dsRNA-PK (2, 15).

The mengovirus-activated kinase was very difficult to dissociate from the ribosomal pellet with which it cosedimented. One explanation for this observation is that the kinase associates with double-stranded RNA in the viral replicative complex (36), which has a high sedimentation coefficient (25) and would therefore pellet with the ribosomal fraction. Repeated resuspension and repelleting of the ribosome fraction released the activated kinase, probably because the replicative complex began to degrade. However, this method was not effective, because much kinase activity

was lost. The loss of kinase activity may also have been caused by some sort of virus-mediated degradation of the kinase, such as that found in poliovirus-infected cells (3).

Although not shown for mengovirus, earlier studies by Morrow et al. (29) indicated that the dsRNA-PK may be involved in the replication of poliovirus in vitro, in which case the dsRNA-PK might have an advantage to stay tightly bound to the replicative complex or some fraction thereof.

One may speculate about the reasons both mengovirus and poliovirus (and perhaps some other picornaviruses) allow dsRNA-PK activation at a time when viral protein synthesis is maximal. In each of these cases, host protein synthesis is effectively inhibited long before the kinase is activated. Furthermore, in the cases of several other viruses, such as vaccinia virus (49), influenza virus (21), reovirus serotype 1 (19), and adenovirus (14, 43, 44, 46), the virus expresses a product that prevents the activation of the dsRNA-PK. No such anti-dsRNA-PK activity was expressed in mengovirus-infected cells (Fig. 6) or in poliovirus-infected cells (31).

Mengovirus is highly sensitive to the effects of interferon and to activation of the dsRNA-PK (11). Therefore, it is rather interesting that this virus has not evolved a mechanism to defend itself against kinase activity unless the dsRNA-PK itself plays an important regulatory role in the life cycle of the virus. Because of the extraordinarily efficient nature of the viral message and its high affinity for eIF-2, the message may constantly be loaded with ribosomes. Since the viral message is also the viral genome that must be packaged, one wonders how the message is cleared of ribosomes for virus assembly. What more effective way of clearing the viral message of ribosomes is there than inhibiting the rate of polypeptide chain initiation by dsRNA-PK activation and eIF-2α phosphorylation? Indeed, mature viral particles do not begin to be released from cells until after eIF-2 begins to be phosphorylated (Fig. 2). In the cases wherein the virus expresses a product that inhibits kinase activation (vaccinia virus, influenza virus, and adenovirus), the viral mRNA does not also serve as the viral genome.

In conclusion, although it is hard to visualize that activation of the dsRNA-PK is involved in the early decrease of host protein synthesis in mengovirus-infected cells, the second phase of inhibition is likely the result of activation of the dsRNA-PK and phosphorylation of initiation factor eIF-2. The possibilities that kinase activation is a manifestation of a primitive cellular antiviral or defense mechanism (44) or serves as a mechanism to regulate translation and encapsidation in the life cycle of the virus are interesting ideas to pursue.

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