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A Heat-Sensitive Inhibitor in Poliovirus-Infected Cells Which Selectively Blocks Phosphorylation of the α Subunit of Eucaryotic Initiation Factor 2 by the Double-Stranded RNA-Activated Protein Kinase

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We partially purified an inhibitor from poliovirus-infected HeLa cells which specifically blocked phosphorylation of the α subunit of eucaryotic initiation factor 2 by the double-stranded RNA-activated protein kinase. The inhibitory activity eluted from a sizing column with an approximate molecular weight of 80,000 to 100,000 and was sensitive to heat, suggesting a protein nature for the inhibitor. No specific virus-encoded protein purified with the inhibitor. The inhibition of phosphorylation of the α subunit of eucaryotic initiation factor 2 was not due to a protein phosphatase associated with the inhibitor. The inhibitor did not seem to prevent phosphorylation of the double-stranded RNA-activated protein kinase but inhibited the phosphorylation of the α subunit of eucaryotic initiation factor 2 by the activated kinase. Double-stranded RNA-induced inhibition of in vitro protein synthesis in reticulocyte lysates could be prevented by the addition of the partially purified inhibitor during preincubation of lysate with double-stranded RNA.

Protein kinase activity in general is stimulated at least 5- to 10-fold in ribosomal salt wash (RSW) preparations from poliovirus-infected HeLa cells compared with those from mock-infected cells (19, 30). The stimulation of kinase activity is manifested by increased phosphorylation of ribosome-associated polypeptides having approximate molecular weights of 135,000, 120,000 85,000 68,000, 65,000, 40,000, 28,000 25,000, and 21,000 (19). Among these phosphoproteins, the M_r 68,000 polypeptide is structurally identical to the interferon (IFN)-induced, double-stranded RNA (dsRNA)-activated protein kinase (P1) that phosphorylates the α subunit (eIF-2α) of eucaryotic initiation factor 2 (eIF-2) (19). This protein kinase activity is enhanced in mouse and human cells after treatment with IFN (1, 12, 28, 32). The kinase activity is manifested by phosphorylation of an endogenous 68,000 to 72,000-M_r protein (P1) in human cells and an endogenous or exogenous 38,000-M_r protein which is eIF-2α. The role of this protein kinase is considered to be phosphorylation of eIF-2α, thus mediating inhibition of initiation of protein synthesis in cell-free systems (5, 7, 32). Phosphorylation of P1 protein has been previously shown in IFN-treated cells during virus infection, but the significance of this observation and its correlation with phosphorylation of eIF-2α remain to be understood (9, 16, 21, 25).

Previous results from our laboratory have shown that increased phosphorylation (also called activation) of P1 protein in cells infected with poliovirus does not result in an increased phosphorylation of eIF-2α of endogenous or exogenously added eIF-2, both in vitro and in vivo (19). This observation led us to suggest that a mechanism must exist in virus-infected cells which prevents phosphorylation of eIF-2α by the activated P1 protein kinase (19). We show here that an activity can be partially purified from poliovirus-infected HeLa cells which selectively inhibits phosphorylation of eIF-2α by the dsRNA-activated protein kinase. The inhibitory activity was heat labile, suggesting that it is most probably due to a protein. The inhibitory activity was not due to a protein phosphatase associated with the inhibitor. dsRNA-induced inhibition of in vitro translation in reticulocyte lysate could be prevented by the addition of partially purified inhibitor during preincubation of the lysate with dsRNA, suggesting that the inhibitor prevents dsRNA-induced inhibition of protein synthesis by blocking phosphorylation of eIF-2 by the dsRNA-activated kinase.

MATERIALS AND METHODS

All chemicals, unless specifically stated, were purchased from Sigma Chemical Co. (St. Louis, Mo.). Phosphocellulose was obtained from Whatman, Inc. (Clifton, N.J.). Radioisotopes were purchased from Amersham Corp. (Arlington Heights, Ill.) and ICN Pharmaceuticals, Inc. (Irvine, Calif.) Reticulocytes were obtained from Pel Freez Biologicals (Rogers, Ariz.).

Cells and virus. HeLa cells were grown in spinner cultures with Joklik modified essential medium supplemented with 6% newborn calf serum and infected with poliovirus type 1 (Mahoney strain) as previously described (3).

Labeling poliovirus proteins with [^{35}S]methionine. For labeling poliovirus proteins, cells were suspended, after adsorption, in Earle saline supplemented with 5% dialyzed fetal calf serum and all amino acids except methionine. [^{35}S]methionine Trans-label (specific activity, 1,000 Ci/mmol; 1 mCi/4 × 10^8 cells; ICN) was added to the culture after 2.5 h of infection. Cells were harvested 5 h after infection by centrifugation, and the pellets were washed with phosphate-buffered saline and stored at −70°C until preparation of the RSWs.

Preparation of RSWs. Approximately 2 × 10^9 HeLa cells were infected (or mock infected) with poliovirus at a multiplicity of infection of 20 as described previously (2, 4). Cells were harvested at 3 h postinfection (unless otherwise indicated) by centrifugation and washed in phosphate-buffered

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saline. Cells were disrupted by Dounce homogenization, and cytoplasmic extracts (S100) and RSWs were prepared as described previously (15). The RSW was dialyzed overnight against buffer A (50 mM Tris hydrochloride [pH 8.0], 10% glycerol, 5 mM 2-mercaptoethanol, 50 mM KCl) with two changes. The dialyzed RSW was considered the fraction 1 inhibitor.

Purification of the inhibitory activity. A phosphocellulose column (1.5 by 10 cm) was equilibrated with buffer A overnight, and fraction 1 was applied. The column was then thoroughly washed with buffer A, and bound protein was eluted with three step gradients of buffer A containing 250 mM, 500 mM, and 1 M KCl, respectively. Each fraction was concentrated against solid sucrose overnight and dialyzed against buffer A with one change. The dialyzed 250 mM salt cut contained over 90% of the total eIF-2α phosphorylation-inhibitory activity and was considered fraction 2. Further purification of the inhibitor was carried out by gel filtration of the fraction 2 inhibitor through an AcA 44 column (fractionation range, 30,000 through 132,000). An AcA 44 column (1.5 by 30 cm; void volume, 10 ml) was equilibrated with buffer A, and fraction 2 inhibitor was loaded onto the column at a flow rate of 0.5 ml/min. The column was then developed with 100 ml of buffer A. Fractions of 1 ml were collected and assayed for the ability to inhibit phosphorylation of eIF-2α. Active fractions were stored at −70°C (fraction 3).

In vitro kinase assay. The standard mixture for kinase assays contained (in 25 μl) 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 8.0), 5 mM magnesium acetate, 4 mM dithiothreitol, 1 μCi of [γ-^{32}P]ATP (specific activity, 1,000 to 3,000 Ci/mmol; Amersham), and proteins from RSW (5 to 20 μg) or column fractions (1 to 6 μg). Where indicated, RSW was inactivated by heating at 65°C for 10 min, followed by centrifugation to remove debris. When testing for inhibitory activity, reactions were supplemented with 0.2 to 0.5 μg of reticulocyte eIF-2 (kindly provided by N. K. Gupta, University of Nebraska, Lincoln) or 1 μg of phosphocellulose-purified RSW from HeLa cells treated with IFN. Standard incubations were for 10 min at 30°C. Reactions were stopped by adding 2× sodium dodecyl sulfate (SDS)-gel sample buffer, and proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) as previously described (2).

Two-dimensional gel electrophoresis. Two-dimensional gel analysis of ^{32}P-labeled proteins was performed as described by O'Farrell (18). The first dimension was an isoelectric focusing gel in the pH range of 4 through 8. The second dimension was a 15% SDS–polyacrylamide gel. After separation of proteins in the two dimensions, the gel was dried and autoradiographed.

Phosphatase assay. Protein phosphatase activity was assayed by incubating ^{32}P-labeled, partially purified HeLa eIF-2 with AcA 44 fractions derived from either mock- or poliovirus-infected cells for 6 min at 30°C as described previously (11). Labeled proteins were analyzed by SDS-PAGE followed by autoradiography.

Protein synthesis in reticulocyte lysates. Rabbit reticulocytes (Pel Freez Biologicals) were washed and lysed in an equal volume of water as described previously (6, 7), and protein synthesis was performed (6). The stroma was removed by centrifugation at 20,000 × *g* for 15 min, and the supernatant was frozen in small samples at −70°C. The assay mixture contained 20 μM hemin, 10 mM Tris hydrochloride (pH 7.3), 70 mM KCl, 1 mg of creatine kinase per ml, 6.5 mM creatine phosphate, 1 mM ATP, 0.2 mM GTP, amino acids without methionine, 10 μCi of [^{35}S]methionine (1,000 Ci/mmol), reticulocyte lysate, and, where indicated, 10 ng of poly(I:C) per ml and fraction 3 inhibitor. The complete assay mixture was incubated at 30°C, and 5-μl samples were removed at the indicated times and pipetted into 1 ml of 1.0 N NaOH. These samples were incubated for 15 min at 37°C, and the protein was precipitated with 5% trichloroacetic acid before filtration on Whatman GF/C glass-fiber filters. After drying, filters were counted to Filtron X liquid scintillation fluid (National Diagnostics, Somerville, N.J.).

RESULTS

Poliovirus-infected cell extracts contain a heat-labile inhibitor which blocks phosphorylation of eIF-2α. To determine whether poliovirus encodes or induces a factor which inhibits phosphorylation of eIF-2 by the dsRNA-activated protein kinase, RSW fractions were prepared from mock- and poliovirus-infected cells after 3 h of infection. We then examined the ability of these RSWs to inhibit phosphorylation of eIF-2α by the reticulocyte dsRNA-activated protein kinase. The dsRNA-activated protein kinase is constitutively expressed in rabbit reticulocytes and does not require IFN treatment for induction. Reaction mixtures containing dsRNA-activated protein kinase, eIF-2, and RSWs were incubated in the presence of [γ-^{32}P]ATP, and phosphorylated proteins were analyzed by two-dimensional gel electrophoresis followed by autoradiography. When purified reticulocyte dsRNA-activated protein kinase was incubated with purified reticulocyte eIF-2, phosphorylation of both the kinase and eIF-2α was evident (Fig. 1A). The addition of a RSW from mock-infected cells to the reaction reduced phosphorylation of the dsRNA-activated protein kinase and eIF-2α to some extent (Fig. 1B). The addition of a RSW fraction from virus-infected cells to the reaction resulted in approximately fourfold reduction of eIF-2α phosphorylation (Fig. 1E) compared with that observed in the reaction containing RSW from mock-infected cells (Fig. 1B). The degree of phosphorylation of the dsRNA-activated protein kinase, however, did not change significantly between reactions containing mock- and virus-infected RSWs (compare dsI phosphorylation in Fig. 1B and E). At the concentration tested, no endogenous eIF-2α phosphorylation could be detected in mock- and virus-infected RSW fractions (Fig. 1C and F, respectively). The addition of reticulocyte eIF-2 to these extracts revealed no difference in the degree of phosphorylation of exogenously added eIF-2 between mock- and poliovirus-infected RSW fractions (Fig. 1D and G), as observed in our earlier studies (19). These results suggest that RSW prepared from poliovirus-infected cells contains an activity which inhibits phosphorylation of eIF-2α by the dsI without affecting significantly the phosphorylation of the dsRNA-activated protein kinase.

To examine the nature of the inhibitory material present in virus-infected extracts, we determined the effect of heat on its ability to inhibit phosphorylation of eIF-2 by the reticulocyte dsRNA-activated kinase. Experiments were performed in which unheated or heated (65°C for 10 min) RSWs for mock- and poliovirus-infected cells were incubated with reticulocyte protein kinase, eIF-2, and [γ-^{32}P]ATP. Phosphorylation of eIF-2α was greatly reduced in the presence of unheated RSW from virus-infected cells compared with that in the presence of unheated RSW from mock-infected cells as previously observed (Fig. 2A and B, respectively). In the presence of heated, mock-infected RSW, phosphorylation of eIF-2α was slightly reduced (Fig. 2C) compared with that

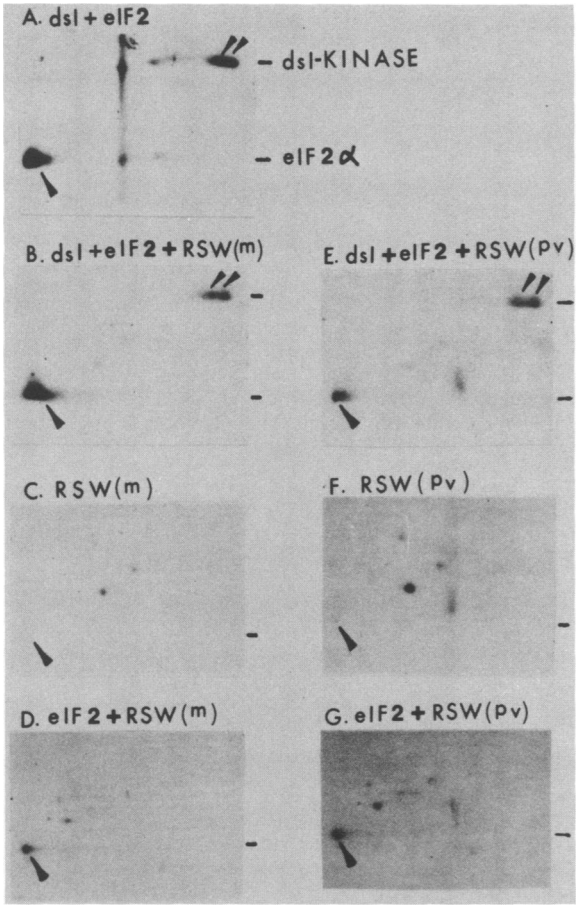

FIG. 1. RSW from poliovirus-infected cells inhibits phosphorylation of eIF-2 by the dsRNA-activated protein kinase (dsI). Proteins were phosphorylated in vitro with [γ-^{32}P]ATP, and phosphorylated proteins were analyzed by two-dimensional gel electrophoresis as described in Materials and Methods: (A) 0.5 μg each of purified reticulocyte-derived eIF-2 and dsRNA-activated protein kinase; (B) same as in A plus 5 μg of RSW from mock-infected cells; (C) 5 μg of RSW from mock-infected cells alone; (D) same as in C plus 0.5 μg of eIF-2; (E) 0.5 μg each of purified eIF-2 and dsRNA-activated protein kinase plus 5 μg of RSW from poliovirus-infected cells; (F) 5 μg of RSW from infected cells alone; (G) same as in F plus 0.5 μg of eIF-2. The double arrowheads and the single arrowhead indicate the position of migration of dsRNA-activated protein kinase and eIF-2, respectively.

with unheated RSW (Fig. 2A). Heating the RSW from infected cells at 65°C for 10 min, however, almost completely destroyed its ability to inhibit phosphorylation of eIF-2α (Fig. 2D). These results suggest that the inhibitory activity present in the RSW derived from poliovirus-infected HeLa cells is most probably due to one or more proteins.

Further purification of the inhibitor. To purify the inhibitory activity that blocks phosphorylation of eIF-2 by the dsRNA-activated protein kinase, a RSW from virus-infected cells was passed through a phosphocellulose column as described in Materials and Methods. The inhibitory activity bound to the column and was eluted with a step gradient containing 250 mM KCl (data not shown). Further purification of the inhibitor was achieved by gel filtration through an AcA 44 column. Figure 3 shows elution of the inhibitory activity from the AcA 44 column. The majority of the inhibitory activity was included in the column and eluted

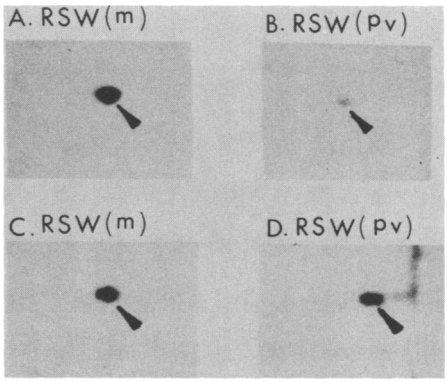

FIG. 2. The activity in the RSW from poliovirus-infected cells which inhibits phosphorylation of eIF-2α is heat labile. RSWs prepared from mock-infected (A and C) and poliovirus-infected (B and D) cells were either heated at 65°C for 10 min (C and D) or not heated (A and B). These RSWs were then mixed with reticulocyte dsRNA-activated protein kinase, eIF-2, and [γ-^{32}P]ATP, and the mixtures were incubated at 37°C for 10 min. The phosphorylated eIF-2α (arrowheads) was analyzed by two-dimensional gel electrophoresis and visualized by autoradiography.

between fractions 14 and 17 (Fig. 3). We estimated an approximate molecular weight of the inhibitor to lie between 80,000 and 100,000 judging from the elution patterns of several standard molecular weight marker proteins from this column (data not shown). A ribosomal salt wash preparation from IFN-treated HeLa cells was used as the source of the dsRNA-activated kinase in this experiment. The assay for endogenous eIF-2 phosphorylation included 100 ng of dsRNA per ml as described earlier (19). The identity of the HeLa phosphorylated eIF-2α was confirmed by comigration of the 38-kilodalton band with the reticulocyte phosphorylated eIF-2α by two-dimensional gel electrophoresis (data not shown).

To determine whether the activity which inhibits phosphorylation of eIF-2α, can be detected in mock-infected HeLa cells, we fractionated RSW from mock- and poliovirus-infected cells in parallel by phosphocellulose and AcA 44 column chromatography. Figure 4A shows analysis of peak fractions containing the inhibitory activity from AcA 44 column derived from mock-infected and poliovirus-infected cells. Fractions 14 through 17 from the column derived from fractionation of mock-infected RSW were found to contain some inhibitory activity in that the degree of phosphorylation of eIF-2α in the presence of these fractions was significantly lower than that in the control (Fig. 4A, lane C). The same fractions derived by AcA 44 chromatography of the RSW from infected cells, however, showed a greater degree of inhibition of eIF-2α phosphorylation.

The inhibition of phosphorylation of eIF-2α by the partially purified inhibitor was concentration dependent (Fig. 4B). Almost complete inhibition of eIF-2α phosphorylation was evident at higher concentrations of the fraction containing the inhibitor (Fig. 4B).

The kinetics of phosphorylation of eIF-2 in the absence of the inhibitor showed that eIF-2α was phosphorylated in a time-dependent manner during a 10-min period (Fig. 4C). In the presence of the inhibitor, some eIF-2 was phosphorylated early during the reaction; however, its phosphorylation did not increase significantly with time as was observed in the reactions lacking the inhibitor (Fig. 4C).

No particular virus-specific protein purifies with the inhibitory activity. To examine whether any particular virus-

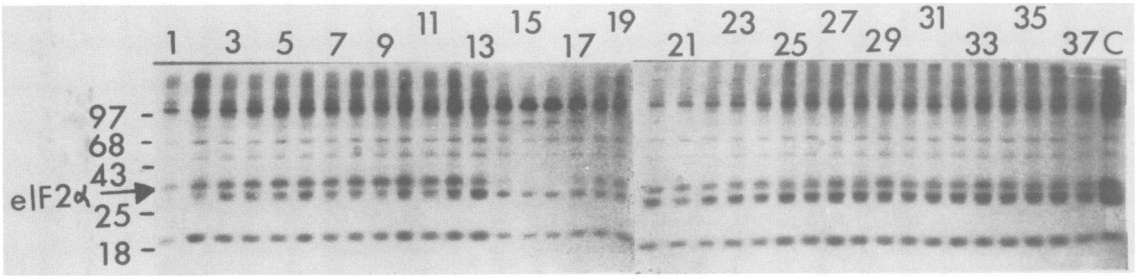

FIG. 3. Purification of the eIF-2α phosphorylation-inhibitory activity from virus-infected cells by gel filtration. An RSW prepared from HeLa cells (2×10^9) infected with poliovirus was purified by phosphocellulose and AcA 44 column chromatography as described in Materials and Methods. Fractions (1 through 37) eluting from the AcA 44 column were incubated with 1 μg of an RSW prepared from IFN-treated (uninfected) HeLa cells, 100 ng of poly(I:C) per ml, and 1 μCi of [γ-^{32}P]ATP. Phosphorylated proteins were analyzed by SDS-PAGE and visualized by autoradiography. Lanes 1 through 37 contained RSW from IFN-treated cells, 100 ng of dsRNA per ml, plus AcA 44 fractions 1 through 37, respectively. Lane C contained RSW from IFN-treated cells plus 100 ng of dsRNA per ml. The migration of phosphorylated eIF-2α is indicated.

specific protein copurified with the inhibitory activity, virus-specific proteins were labeled with [^{35}S]methionine during infection of cells with poliovirus. Because poliovirus shuts off cellular protein synthesis soon after infection (2.5 h), the addition of labeled methionine after 2.5 h of infection results in specific labeling of viral proteins. The inhibitory activity was purified from [^{35}S]methionine-labeled infected cells by phosphocellulose and AcA 44 column chromatography. Figure 5 shows the elution pattern of viral proteins in fractions recovered from AcA 44 column chromatography. No particular virus-specific protein appeared to copurify with the inhibitory activity.

Inhibition of phosphorylation of eIF-2 is not due to a phosphatase. Increased activity of a phosphorylated eIF-2α phosphatase in poliovirus-infected cells could explain why phosphorylation of eIF-2 is inhibited in poliovirus-infected cells compared with that in mock-infected cells. To determine whether a protein phosphatase activity copurified with the inhibitory activity, prephosphorylated eIF-2 was incubated in the absence or in the presence of the AcA 44-purified inhibitor isolated from mock- or virus-infected cells. If the inhibition of eIF-2α phosphorylation was due to a phosphatase, one would expect disappearance of labeled phosphates from eIF-2α. Incubation of the partially purified inhibitor from mock-infected (Fig. 6, lane 7) or virus-infected (lane 8) cells with prephosphorylated eIF-2 did not significantly reduce the amount of phosphorylated eIF-2α band as compared with that in the control (lane 6). Inhibition of eIF-2α phosphorylation was evident when the inhibitor isolated from infected cells was present during the phosphorylation reaction (lane 3). Significant inhibition of eIF-2α was also observed in the presence of the inhibitor isolated from mock-infected cells (lane 2) compared with that in the control (lane 1). Phosphorylation of the fraction 3 (AcA 44 purified) inhibitor alone from mock-infected (lane 4) or poliovirus-infected (lane 5) cells showed that both preparations contained a phosphoprotein of approximate M_r 100,000. In addition, the partially purified inhibitor from poliovirus-infected cells contained a phosphoprotein of approximate M_r 65,000. It should be noted that the fraction 3 inhibitor preparation contained several polypeptides as judged by silver staining of the gel (data not shown), and we do not know at present whether the 100- and 65-kilodalton phosphoproteins play any role in the inhibition of eIF-2 phosphorylation. These results suggest that the inhibition of phosphorylation of eIF-2α is not due to a phosphatase present in the inhibitor fraction.

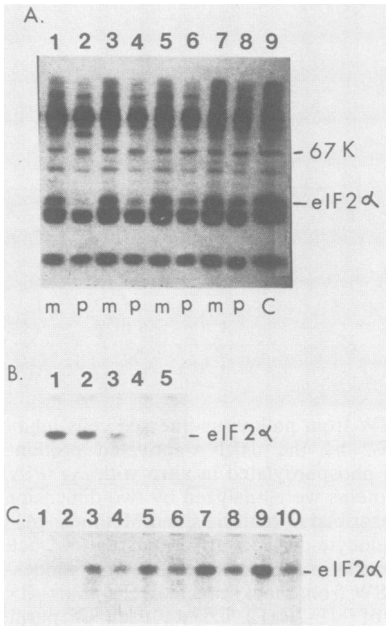

FIG. 4. (A) Comparison of eIF-2α phosphorylation-inhibitory activities from mock- and poliovirus-infected cells; (B and C) concentration- and time-dependent inhibition of eIF-2 phosphorylation by the partially purified inhibitor. (A) RSWs prepared from mock- and poliovirus-infected HeLa cells were fractionated in parallel by phosphocellulose and AcA 44 column chromatography. Fractions eluting from two AcA 44 columns were assayed for the eIF-2α phosphorylation-inhibitory activity as described in the legend of Fig. 3, except that 0.5 μg of purified reticulocyte eIF-2 was added to each reaction. From both AcA 44 columns, the inhibitory activities eluted in the same fractions. The figure shows the assays of the inhibitory material only of those fractions containing the inhibitory activity. Lanes 1, 3, 5, and 7: AcA 44 fractions 15, 16, 17, and 14, respectively, derived from fractionation of a RSW from mock-infected cells. Lanes 2, 4, 6, and 8: AcA 44 fractions 15, 16, 17, and 14, respectively, derived from fractionation of a RSW from virus-infected cells. Lane 9: control. (B and C) AcA 44-purified inhibitor derived from poliovirus-infected cells was assayed as described in the legend to Fig. 1 with reticulocyte dsRNA-activated protein kinase and eIF-2. The phosphorylated eIF-2α band was analyzed by SDS-PAGE. (B) Lanes 1 through 5: 0, 1.5, 3, 6, and 12 μg of the inhibitor, respectively. (C) Time of eIF-2α phosphorylation in the absence (lanes 1, 3, 5, 7, and 9) and in the presence (lanes 2, 4, 6, 8, and 10) of 3 μg of AcA 44-purified inhibitor. Lanes: 1 and 2, 0 min; 3 and 4, 2 min; 5 and 6, 4 min; 7 and 8, 6 min; 9 and 10, 8 min.

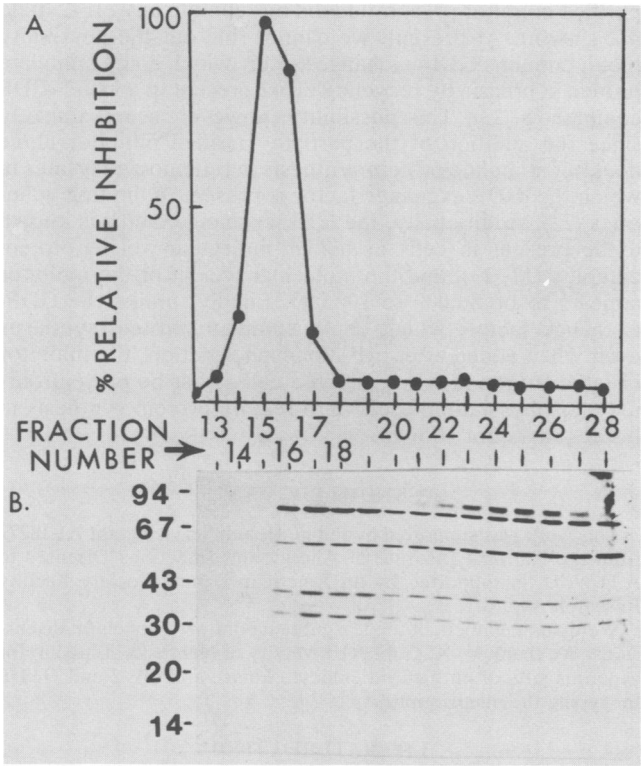

FIG. 5. No particular virus-specific protein copurified with the inhibitor. RSW was prepared from cells infected with poliovirus in which virus-specific proteins were labeled with [^{35}S]methionine. The inhibitor was purified from labeled extracts by phosphocellulose and AcA 44 column chromatography. Fractions 13 through 28 eluting from AcA 44 column were assayed for inhibition of eIF-2α phosphorylation (A), and [^{35}S]methionine-labeled viral proteins in those fractions were analyzed by SDS-PAGE and fluorography (B). Migration of several standard molecular weight markers is shown on the left.

dsRNA-mediated inhibition of protein synthesis is prevented by the inhibitor. Incubation of a lysate derived from rabbit reticulocytes with dsRNA leads to inhibition of initiation of protein synthesis (6, 7). It is generally accepted that this inhibition is due to phosphorylation of eIF-2α by the dsRNA-activated protein kinase present in the lysates (7). We examined whether inhibition of protein synthesis in reticulocyte lysate by dsRNA could be prevented by the partially purified inhibitor recovered from poliovirus-infected cells. If the inhibitor blocks phosphorylation of eIF-2 by the dsRNA-activated kinase, one would expect restoration of protein synthesis in reactions where the inhibitor is added along with dsRNA during preincubation of the lysate.

Preincubation of the lysate with low concentration of dsRNA and ATP resulted in approximately 75% inhibition of protein synthesis compared with that in the control lacking dsRNA (Fig. 7). Preincubation of the inhibitor alone with the lysate did not affect significantly the rate or the extent of protein synthesis. Inclusion of the inhibitor during preincubation of the lysate with dsRNA and ATP resulted in approximately 70% recovery of protein synthesis. However, when the inhibitor was heated before incubation with dsRNA and lysate, it was no longer able to restore protein synthesis. This result was consistent with our previous observation that heating the inhibitor destroyed its ability to prevent phosphorylation of eIF-2 by the reticulocyte

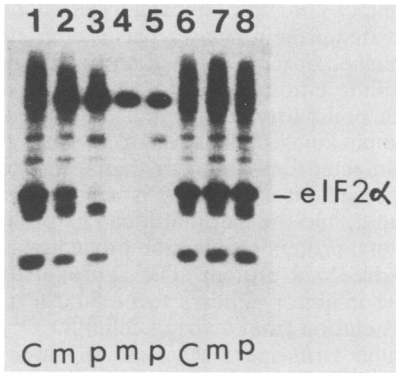

FIG. 6. The inhibition of eIF-2α phosphorylation is not due to a protein phosphatase associated with the inhibitor. AcA 44-purified inhibitor isolated from either mock-infected cells (m) or poliovirus-infected cells (p) was added to reactions containing RSW from uninfected cells (pretreated with IFN) and dsRNA during phosphorylation of eIF-2 (lanes 2 and 3) or to reactions in which eIF-2 was already phosphorylated (lanes 7 and 8). Each reaction contained 0.5 μg of purified reticulocyte eIF-2. The reactions were further incubated at 37°C for 15 min, and the phosphoproteins were analyzed by SDS-PAGE. Lanes: RSW from uninfected cells (treated with IFN) plus dsRNA; 2, same as in lane 1 plus 6 μg of mock-infected cell-derived inhibitor added during eIF-2 phosphorylation; 3, same as in lane 1 plus 6 μg of poliovirus-infected cell derived inhibitor added during eIF-2 phosphorylation; 4, 6 μg of inhibitor from mock-infected cells alone; 5, 6 μg of inhibitor from virus-infected cells alone; 6, prephosphorylated eIF-2 further incubated with buffer alone; 7, prephosphorylated eIF-2 further incubated with mock-infected cell-derived inhibitor (6 μg); 8, prephosphorylated eIF-2 further incubated with 6 μg of infected cell derived inhibitor.

dsRNA-activated protein kinase (Fig. 2). The addition of an equivalent amount of bovine serum albumin during preincubation of lysate with dsRNA could not restore protein synthesis, indicating that the effect seen with the inhibitor was not due to nonspecific interaction between the inhibitor and dsRNA.

DISCUSSION

We have shown that RSWs derived from HeLa cells infected with poliovirus contain an inhibitory activity which

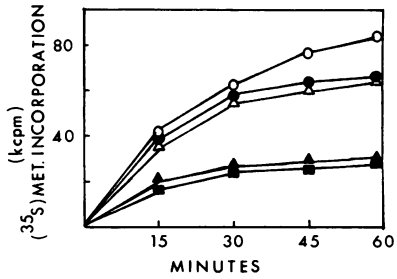

FIG. 7. dsRNA-mediated inhibition of protein synthesis is prevented by the inhibitor. Protein synthesis in reticulocyte lysates was performed as described by Ehrenfeld and Hunt (6). Samples of the reaction mixture were withdrawn at the indicated times. Symbols: (○), no addition; ■, lysate preincubated with 10 ng of dsRNA per ml and ATP; △, lysate preincubated with 1.5 μg of AcA 44-purified inhibitor from infected cells or 5 μg of bovine serum albumin; ●, lysate preincubated with 10 ng of dsRNA per ml and 1.5 μg of inhibitor; ▲, lysate preincubated with 10 ng of dsRNA per ml and 1.5 μg of heat-killed inhibitor.

specifically blocks in vitro phosphorylation of eIF-2α by dsRNA-activated protein kinase. This is consistent with our recently published observation that no significant increase in eIF-2α phosphorylation is observed in the RSW from cells infected with poliovirus despite the fact that the dsRNA-activated protein kinase activity is 5 to 10 times greater in the RSW from infected cells than in that from mock-infected cells (19). The inhibitory activity is sensitive to heating at 65°C for 10 min, and the fact that it can be partially purified by conventional protein purification procedures suggest that it could be due to a protein. The approximate molecular weight of the inhibitor appears to be 80,000 to 100,000 as judged by its elution from a sizing column.

No particular virus-specific protein appeared to be associated with the eIF-2α phosphorylation-inhibitory activity (Fig. 5). It is possible that a viral protein in very low quantities is associated with the inhibitory activity and is not detectable by the method used. The viral protein may contain very few methionines, thus making detection difficult. Alternatively, the activity could be cellular and induced by infection of cells with poliovirus. We did detect a cellular activity, which eluted from the AcA 44 column in the same fractions as the inhibitor from virus-infected cells, that was capable of inhibiting eIF-2α phosphorylation of the dsRNA-activated kinase. It is clear that the eIF-2α phosphorylation-inhibitory activity isolated from either mock- or virus-infected cells is not due to one or more of protein phosphatases associated with the inhibitor preparation (Fig. 6). It is difficult, however, to conclude whether the inhibitory activities detected in mock- and poliovirus-infected cells are due to the same protein. Further purification of these inhibitory activities is required before any valid conclusion can be drawn.

How the inhibitor present in cells infected with poliovirus specifically blocks phosphorylation of the α-subunit of eIF-2 is not known at present. The inhibitor does not seem to prevent autophosphorylation of the dsRNA-activated protein kinase (Fig. 1B and E) but can inhibit phosphorylation of eIF-2 by the activated kinase. This observation is quite different from those observed in other virus-host systems. Adenovirus, vaccinia virus, and influenza virus all seem to inhibit eIF-2α phosphorylation by inhibiting the activation (autophosphorylation) of the host cell dsRNA-activated kinase (11, 20, 22, 26, 27, 29, 31). In adenovirus-infected cells this is achieved by the accumulation of very high concentrations of the virus-associated RNA, which physically interacts with the dsRNA-activated protein kinase (10). In vaccinia-virus-infected cells, the virus appears to code for an inhibitor which specifically blocks phosphorylation of the kinase (31). Our preliminary studies suggest that phosphorylation of eIF-2 by the heme-regulated protein kinase, which is activated in reticulocytes by heme deficiency (13, 14) and is known to phosphorylate the same site on eIF-2α as that phosphorylated by the dsRNA-activated kinase (17, 24), is also blocked by the inhibitor isolated from poliovirus-infected cells. We therefore believe that the inhibitor most probably physically interacts with eIF-2 in blocking its phosphorylation by the kinase. Clearly, further careful studies will be necessary before the mechanism of inhibition of eIF-2α phosphorylation by the inhibitor can be determined with certainty.

The partially purified inhibitor from poliovirus-infected cells was able to prevent inhibition of translation in vitro induced by dsRNA (Fig. 7). We believe that the inhibitor does so by inhibiting phosphorylation of eIF-2α by the endogenous kinase present in the lysate. The AcA 44-purified inhibitor preparation did not contain any eIF-2 (data not shown). At present, we cannot rule out the possibility that it contains GDP-exchange factor, which could stimulate protein synthesis by recycling eIF-2 present in a eIF-2–GDP complex (8, 23). This possibility, however, seems unlikely since the addition of the partially purified inhibitor alone does not stimulate protein synthesis in reticulocyte lysates in which this GDP-exchange factor is present in limiting quantities (23). Additionally, the GDP-exchange factor is known to be present in cells as a high-molecular-weight protein complex (17, 23), and the molecular weight of the inhibitor appears to be 80,000 to 100,000. Finally, unlike the GDP-exchange factor, which could stimulate protein synthesis even when added after eIF-2 phosphorylation, the inhibitor isolated from poliovirus-infected cells must be present during phosphorylation of eIF-2 to restore protein synthesis in lysates (data not shown).

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