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Activation of Double-Stranded RNA-Activated Protein Kinase in HeLa Cells after Poliovirus Infection Does Not Result in Increased Phosphorylation of Eucaryotic Initiation Factor-2

LYNN J. RANSONE AND ASIM DASGUPTA*

Department of Microbiology and Immunology and Jonsson Comprehensive Cancer Center, UCLA School of Medicine, Los Angeles, California 90024

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Protein kinase activity in general is stimulated at least 5- to 10-fold in ribosomal salt wash preparations from poliovirus-infected HeLa cells compared with those from mock-infected cells. 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. The M_r 68,000 phosphoprotein is structurally identical to the interferon-induced, double-stranded RNA-activated protein kinase (P1) which phosphorylates the α subunit of eucaryotic initiation factor-2 (eIF-2). A similar protein of M_r 68,000 is more phosphorylated in poliovirus-infected cells than in mock-infected cells. Increased phosphorylation of P1 protein in poliovirus-infected cells, however, does not result in an increased phosphorylation of the α subunit of endogenous or exogenously added eIF-2, both in vitro and in vivo. These results suggest that a mechanism must exist in poliovirus-infected HeLa cells which prevents further phosphorylation of eIF-2 by the activated kinase.

Infection of animal cells with picornaviruses leads to several changes in cellular metabolism (9, 14, 15). The early metabolic changes after virus infection may conceivably favor efficient expression of the viral genome compared with that of cellular genes. These cellular changes may be triggered by a viral component synthesized early in infected cells or could simply be due to infection of cells with the virus. One of the most well-understood reversible changes that leads to regulation of several cellular enzymatic activities is the phosphorylation-dephosphorylation of proteins (3). Earlier studies from this laboratory have shown that an M_r 67,000 to 68,000 cellular protein (host factor) needed for in vitro replication of poliovirus (6) is a phosphoprotein with a possible protein kinase activity (16). Previous results also suggested that protein phosphorylation plays an important role in viral RNA replication (16). In many respects, the 67-kilodalton (kDa) host factor isolated from uninfected HeLa cell ribosomes appeared similar to a previously described 67- to 72-kDa ribosomal protein involved in a protein kinase system (16). This protein kinase activity dependent on double-stranded (ds) RNA is enhanced in mouse and human cells after treatment with interferon (IFN) (1). The kinase activity is manifested by phosphorylation of an endogeneous 68,000- to 72,000-M_r protein (called P1) in human cells and an endogenous or exogenous 38,000-M_r protein which is the α subunit of eucaryotic initiation factor-2 (eIF-2). The role of this protein kinase is considered to be phosphorylation of the α subunit of eIF-2, thus mediating inhibition of initiation of protein synthesis in cell-free systems (7, 8, 29). 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 (10, 17, 21).

Because our previous studies have suggested that a protein involved in the IFN-induced, dsRNA-activated protein kinase system is similar to one (host factor) involved in in vitro replication of poliovirus RNA, we studied phosphorylation of P1 protein in poliovirus-infected cells. We show here that protein kinase activity in general is greatly stimulated in the ribosomal salt wash (RSW) fraction from poliovirus-infected cells. One of the proteins whose phosphorylation is stimulated in poliovirus-infected cells appears to be similar to IFN-induced, dsRNA-activated P1 protein. In vivo ^{32}P-labeling experiments indicated that a similar 68-kDa protein is more phosphorylated in infected cells than in mock-infected cells. Phosphorylation of the α subunit of eIF-2, however, was not stimulated in infected cells compared with that in mock-infected cells both in vivo and in vitro. We suggest that a mechanism must exist in poliovirus-infected cells which prevents further phosphorylation of the α subunit of eIF2 by the activated kinase compared with its basal level of phosphorylation observed in mock-infected cells.

MATERIALS AND METHODS

All chemicals, unless specifically stated, were purchased from Sigma Chemical Co. (St. Louis, Mo.). All radioisotopes were purchased from New England Nuclear Corp. (Boston, Mass.). β-IFN was obtained from Lee Biomolecular Research Laboratories (San Diego, Calif.).

Cells and virus. HeLa cells were grown in Spinner cultures with Jokliks modified essential medium supplemented with 6% newborn calf serum and infected with poliovirus type 1 (Mahoney strain) as previously described (5). Treatment of cells with β-IFN at a concentration of 200 reference units per ml was done for 17 h as described previously (17).

Labeling cells in vivo with $^{32}P_i$. HeLa cells (4×10^8) were pelleted by centrifugation, and the cell pellet was washed three times with medium minus phosphate. After a 30-min virus adsorption, cells were suspended in medium (minus phosphate) containing 5% dialyzed fetal calf serum at a concentration of 4×10^6 cells per ml. After incubation at 37°C for 15 min, cells were supplemented with 100 μCi of

* Corresponding author.

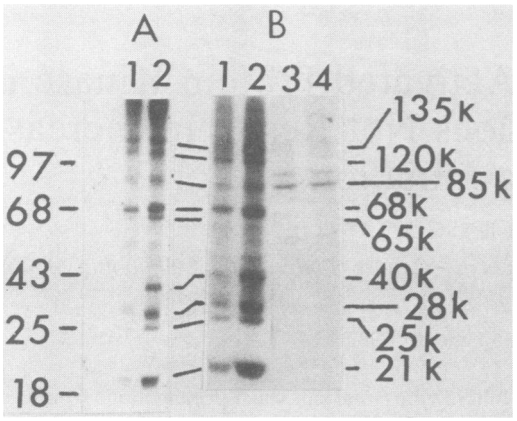

FIG. 1. Stimulation of protein kinase activity in poliovirus-infected HeLa cells. RSW and S100 fractions were prepared from mock- or poliovirus-infected HeLa cells (4×10^8) at 3 h postinfection (or mock infection). Proteins in RSW and S100 fractions were then phosphorylated in vitro as described in Materials and Methods. Phosphorylated proteins were analyzed by SDS-PAGE. Migration of protein markers of defined molecular weights ($\times 10^3$) are shown on the left. (A) Lanes: 1, 2.5 μg of mock RSW; 2, 2.5 μg of infected RSW. (B) Lanes: 1, 5 μg of mock RSW; 2, 5 μg of infected RSW; 3, 5 μg of mock S100; 4, 5 μg of infected S100. k, $\times 10^3$.

carrier-free $^{32}P_i$ per ml. Infection was allowed to proceed for a total of 3 h at which time cells were harvested by centrifugation, and pellets were washed with phosphate-buffered saline and stored at −70°C until preparation of the RSW. Mock-infected ^{32}P-labeled cells were obtained by the same protocol, except that these cells received the same volume of medium instead of virus.

Preparation of RSW. Approximately 4×10^8 HeLa cells were infected (or mock infected) with poliovirus at a multiplicity of infection of 20 as described previously (5). Cells were harvested at 3 h postinfection by centrifugation and washed in phosphate-buffered saline. Cells were disrupted by Dounce homogenization, and cytoplasmic extracts (S100) and RSW were prepared as described previously (16).

In vitro kinase assay. The standard reaction mixture for kinase assays contained (in 25 μl): 50 mM HEPES (*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; New England Nuclear Corp.), and proteins from RSW. Some reactions also contained 1 μg of purified reticulocyte eIF-2 (kindly provided by N. K. Gupta, University of Nebraska, Lincoln) or various amounts of poly(I · C) as indicated, or both. Incubation was for 15 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 (4).

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 to 8. The second dimension was a 15% SDS-polyacrylamide gel. After separation of proteins in the two dimensions, the gel was dried and autoradiographed.

Phosphopeptide mapping. Phosphopeptide mapping by limited proteolytic digestion was done by the method of Cleveland et al. (2) as previously described with 0.5 μg of *Staphylococcus aureus* V8 protease.

Phosphatase assay. To assay for protein phosphatase activity, we incubated ^{32}P-labeled eIF-2 with increasing concentrations of either mock- or poliovirus-infected RSW fractions for 6 min at 30°C as described previously (11). Labeled proteins were analyzed by SDS-PAGE followed by autoradiography.

RESULTS

Stimulation of protein kinase activity in RSW from poliovirus-infected cells. To determine whether protein kinase activity is modified in poliovirus-infected HeLa cells, we prepared RSW fractions from mock- and poliovirus-infected cells. Samples of RSW were phosphorylated with [γ-^{32}P]ATP, and phosphorylated proteins were analyzed by SDS-PAGE. Phosphorylation of several polypeptides was stimulated in infected RSW (Fig. 1A, lane 2; Fig. 1B, lane 2) compared with that in mock-infected RSW (Fig. 1A, lane 1; Fig. 1B, lane 1). These polypeptides had approximate molecular weights of 135,000, 120,000, 85,000, 68,000, 65,000, 40,000, 28,000, 25,000, and 21,000. Phosphorylation of all these polypeptides, with the exception of the M_r 85,000 polypeptide, was limited to the RSW. Phosphorylation of the M_r 85,000 polypeptide and an M_r 100,000 polypeptide was observed in S100 fractions from both mock- and virus-infected cells (Fig. 1B, lane 3). The appearance of the 65-kDa phosphoprotein varied in different preparations of RSW (compare lane 2 between Fig. 1A and B). The results presented in Fig. 1 clearly demonstrate that most of the stimulated protein kinase activity in poliovirus-infected cells is ribosome associated and that this activity can be washed off of ribosomes by extraction with 0.5 M salt. It is also clear that almost all these polypeptides are of cellular origin since they can be detected in mock-infected cells.

Similarity of M_r 68,000 phosphoprotein to IFN-induced, dsRNA-activated protein P1. To determine whether the M_r 68,000 phosphoprotein detected in mock- and poliovirus-infected RSW fractions (Fig. 1A, lanes 1 and 2) is similar to the IFN-induced, dsRNA-activated protein P1 (12, 13, 22, 25), we treated uninfected HeLa cells with β-IFN, and an RSW was prepared from these cells. Portions of RSW were phosphorylated in the absence (Fig. 2A, lane 1) and presence (Fig. 2A, lanes 2 to 7) of increasing concentrations of poly(I · C), a synthetic dsRNA. Phosphorylation of a 68-kDa protein was stimulated at lower concentrations of dsRNA, whereas higher concentrations of dsRNA inhibited its phosphorylation (Fig. 2A). This was consistent with previously published results from a number of laboratories that phosphorylation of a 68-kDa protein from IFN-treated cells was stimulated at lower concentrations of dsRNA but was inhibited at higher dsRNA concentrations (5, 12, 13, 17, 22, 25). Among other polypeptides whose phosphorylations were stimulated by added dsRNA, one of the polypeptides migrating at 38,000 daltons was found to be the α subunit of eIF-2. The M_r 68,000 phosphoprotein found in mock- and poliovirus-infected RSW fractions (Fig. 2A, lanes 8 to 11) comigrated with the dsRNA-activated P1 protein from IFN-treated cells. We were unable to detect phosphorylated α subunit of endogenous eIF-2 in mock- or poliovirus-infected RSW fractions (Fig. 2A, lanes 8, 9, 10, and 11). Instead, a phosphoprotein (indicated as a 40-kDa protein in lanes 8 and 10) mistaken to be the α subunit of eIF-2 during the initial course of this investigation, actually migrated slower than eIF-2α (compare lanes 8 and 10 with lane 7). This result was surprising because increased phosphorylation of the 68-kDa (P1) protein is usually accompanied by an increase in eIF-2α

phosphorylation (1, 7, 8, 12, 13, 17, 22, 29). It should be pointed out that stimulation of phosphorylation by dsRNA of polypeptides in RSW from IFN-treated cells was not limited to the 68-kDa protein (Fig. 2A). The reason for this is not apparent and could be due to use of RSW rather than intact ribosomes from HeLa cells in the in vitro reaction. To ascertain that the 68-kDa phosphoprotein found in mock- and poliovirus-infected RSW was similar to the IFN-induced, dsRNA-activated P1 protein, appropriate ^{32}P-labeled bands were excised from the gel (Fig. 2A, lanes 2, 10, and 11) and digested with V8 protease. The resulting phosphopeptides were analyzed by SDS-PAGE. The phosphopeptide profile of IFN-induced, dsRNA-activated P1 protein was identical to those of 68-kDa protein isolated from mock- and poliovirus-infected RSW fractions (Fig. 2B, lanes 1 to 3).

To further characterize the M_r 68,000 polypeptide, we compared phosphorylated 68-kDa protein from mock- and poliovirus-infected RSW fractions with the dsRNA-activated P1 protein from IFN-treated cells by two-dimensional gel electrophoresis. The phosphorylated form of the 68,000-M_r polypeptide from both mock-infected and infected RSW migrated between pI values of 7.2 and 7.8. Again, a stimulation of phosphorylation of this protein was observed in poliovirus-infected RSW fraction (compare Fig. 3A and B). Two barely visible tiny spots migrating at an approximate pI value of 6.2 to 6.4 were observed only in poliovirus-infected RSW. The majority of phosphorylated 68-kDa protein from IFN-treated HeLa cells migrated between pI values of 7.2 and 7.8; however, a faint streak of radioactivity stretching up to pI 6.4 was observed. The two ^{32}P-labeled spots at pI 6.2 to 6.4 previously detected in poliovirus-infected RSW were also observed in the IFN-treated RSW fraction.

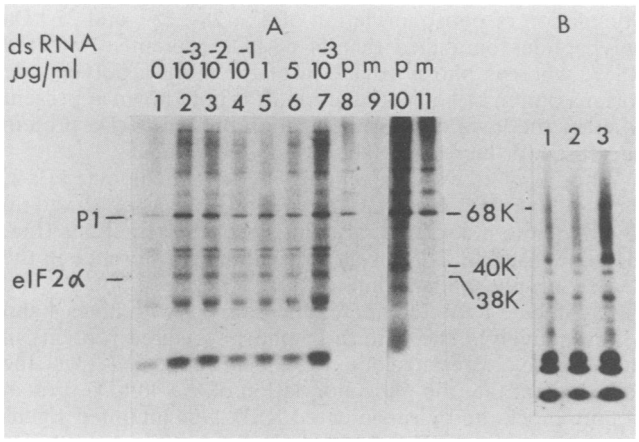

FIG. 2. Comparison of the 68-kDa phosphoprotein with the dSRNA-activated P1 protein from IFN-treated HeLa cells. (A) RSW was prepared from mock- and poliovirus-infected or β-IFN-treated HeLa cells (4×10^8). Samples of RSW from β-IFN-treated cells were phosphorylated in the absence or presence of various concentrations of poly(I · C) with [γ-^{32}P]ATP. Phosphorylation of RSW from mock- and poliovirus-infected cells was as described in the legend to Fig. 1. Lane 1, 5 μg of RSW from IFN-treated cells minus dsRNA; lanes 2 to 7, 5 μg of RSW from IFN-treated cells plus indicated concentrations of dsRNA, respectively; lane 8, 2.5 μg of infected RSW; lane 9, 2.5 μg of mock-infected RSW; lane 10, 5 μg of infected RSW; lane 11, 5 μg of mock-infected RSW. Migration of dsRNA-activated P1 protein and of the α subunit of eIF-2 are shown on the left. (B) The M_r 68,000 phosphoprotein bands (panel A) were excised from lanes 2 (IFN), 10 (poliovirus infected), and 11 (mock infected) and were digested with 0.5 μg of V8 protease. The resulting phosphopeptides were analyzed on a 15% SDS-polyacrylamide gel. Lane 1, 68-kDa band from panel A, lane 2; lane 2, 68-kDa band from panel A, lane 11; lane 3, 68-kDa band from panel A, lane 10. K, Kilodaltons.

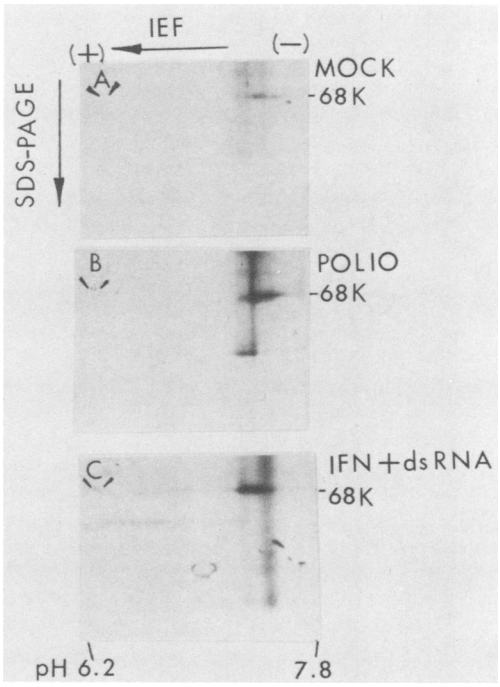

FIG. 3. Two-dimensional gel electrophoresis of 68-kDa phosphoprotein from mock- and poliovirus-infected RSW and of P1 protein from IFN-treated cells. Samples of RSW containing 5 μg of protein from mock-infected, poliovirus-infected, and IFN-treated cells were phosphorylated in vitro with [γ-^{32}P]ATP. The reaction mixture containing RSW from IFN-treated cells contained 10^{-3} μg of dsRNA per ml. Phosphorylated proteins were then subjected to two-dimensional gel electrophoresis as described in Materials and Methods. (A) RSW from mock-infected cells; (B) RSW from poliovirus-infected cells; (C) RSW from IFN-treated cells. K, Kilodaltons; IEF, isoelectric focusing.

Phosphorylation of α subunit of eIF-2 is not stimulated in RSW from infected cells. We were unable to detect phosphorylated α subunit of endogenous eIF-2 in mock- or poliovirus-infected RSW fractions under the conditions used for one-dimensional gel electrophoresis (Fig. 1 and 2A). However, we were able to detect phosphorylated eIF-2α by two-dimensional gel electrophoresis (Fig. 4). This was achieved by loading the two-dimensional gels with two to three times the amount of phosphorylated proteins normally used for loading one-dimensional gels. Under similar conditions, autoradiograms of one-dimensional gels are difficult to interpret because of high backgrounds. A comparison between mock (Fig. 4A)- and poliovirus (Fig. 4B)-infected RSW showed that there was no increase in phosphorylation of the α subunit of eIF-2 in infected RSW compared with that in mock-infected RSW. In fact, phosphorylation of eIF-2α in infected RSW was approximately 40% less than that in mock RSW. The radioactive spots indicated by the upward-pointing arrowheads (Fig. 4) were indeed eIF-2α-P since purified reticulocyte eIF-2α-P comigrated with these spots on two-dimensional gels (data not shown). Phosphorylation of other polypeptides, however, was significantly stimulated in infected RSW. Two-dimensional gel electrophoresis of

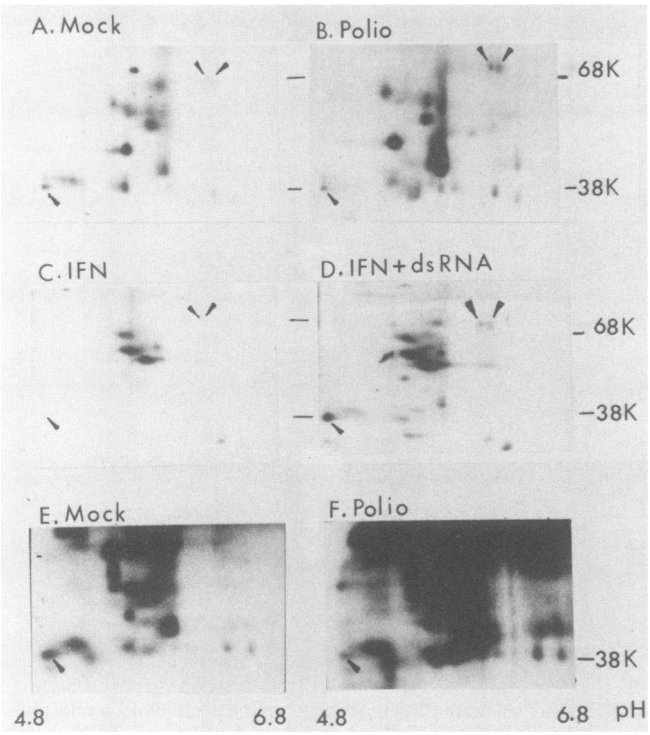

FIG. 4. Analysis of relatively acidic phosphoproteins of RSW from mock- and poliovirus-infected and IFN-treated cells. Reactions similar to those described in the legend to Fig. 3 were performed, and ^{32}P-labeled phosphoproteins were analyzed by two-dimensional gel electrophoresis. (A) RSW from mock-infected cells; (B) RSW from poliovirus-infected cells; (C) RSW from IFN-treated cells minus dsRNA; (D) RSW from IFN-treated cells plus 10^{-3} μg of dsRNA per ml. Experiments shown in panels A, B, C, and D were performed with the same batch of HeLa cells. (E) RSW (20 μg) from mock-infected cells. (F) RSW (20 μg) from poliovirus-infected cells. Experiments shown in panels E and F were done with another batch of cells. The upward-pointing arrowhead indicates the position of the α subunit of eIF-2. The downward-pointing arrowheads indicate two spots migrating at pH 6.2 to 6.4 and slightly slower than the 68-kDa protein.

RSW prepared from IFN-treated cells showed a significantly higher level of phosphorylation of eIF-2α in the presence of added dsRNA (Fig. 4D). In the absence of dsRNA, very little phosphorylation of eIF-2α was detected (Fig. 4C). Low levels of phosphorylation of polypeptides other than the α subunit of eIF-2 were detected in RSW from IFN-treated cells in the presence of dsRNA (Fig. 4D). Among these were the two spots migrating slightly slower than the 68-kDa protein with pI values of 6.2 to 6.4 as previously detected (Fig. 3C). Similar spots can be clearly observed in poliovirus-infected RSW fraction (Fig. 4B). The experiments presented in Fig. 4A, B, C, and D were performed with the same batch of HeLa cells so that results could be compared in a quantitative manner. To determine whether the observed difference in the level of phosphorylation of eIF-2 between mock- and virus-infected RSW fractions (Fig. 4A and B) was reproducible, RSW fractions were prepared from a different batch of mock- and poliovirus-infected cells, and phosphorylated proteins were analyzed by two-dimensional gel electrophoresis. As is evident from the results presented in Fig. 4E and F, phosphorylation of the α subunit of eIF-2 in RSW from mock-infected cells was approximately threefold greater than that in RSW from poliovirus-infected cells. As observed earlier, phosphorylation of other polypeptides was significantly stimulated in infected RSW compared with that in mock-infected RSW. A number of experiments performed in our laboratory indicate that the degree of phosphorylation of eIF-2α in infected RSW varies from 25 to 75% of that of mock-infected RSW, depending on different batches of HeLa cells used in these experiments. Nevertheless, it was clear from results presented in Fig. 2, 3, and 4 that although phosphorylation of a polypeptide structurally identical to IFN-induced, dsRNA-activated P1 protein was stimulated in poliovirus-infected RSW, there was no increase in phosphorylation of the α subunit of endogenous eIF-2 in infected cells.

Stimulation of kinase activity in poliovirus-infected cells is an early response. To determine whether phosphorylation of the 68-kDa protein occurs early during infection of cells with poliovirus, portions of infected (or mock-infected) cells were removed after different times of infection (or mock infection); RSW were prepared and phosphorylations were performed as described above. The pattern of phosphorylation of 68-kDa protein as well as those of 40-, 28-, 25-, and 21-kDa polypeptides did not change significantly during mock infection of cells of up to 4 h (Fig. 5A, lanes 1, 3, 5, and 7). Stimulation of phosphorylation of the 68-kDa protein was detected as early as 1 h postinfection (Fig. 5A, lane 2), after which the degree of phosphorylation of the 68-kDa protein was increased, reaching a maximum at 3 h postinfection (Fig. 5A, lane 6). At 4 h postinfection the amount of phosphorylated 68-kDa protein in infected RSW was even less than that in mock-infected cells (compare lanes 7 and 8). Stimulation of phosphorylation of 85-, 28-, 25-, and 21-kDa polypeptides paralleled that of 68-kDa protein in infected RSW, whereas phosphorylation of the 40-kDa polypeptide was maximum at 4 h postinfection. It is not known at present whether the level of phosphorylation of the 40-kDa protein decreases (or increases) late during infection.

When assayed with exogenously added reticulocyte eIF-2, the basal level of phosphorylation of eIF-2 was observed up to 4 h in both mock- and virus-infected RSW fractions (Fig. 5B, lanes 1 to 8). There was no significant difference in the degree of phosphorylation of eIF-2 between mock- and virus-infected RSW (compare lanes 3 and 5 with lanes 4 and 6, respectively). The pattern of phosphorylated proteins in the absence or presence of exogenously added eIF-2 was the same except that the phosphorylation of the 40-kDa protein in both mock- and virus-infected RSW was inhibited significantly in the presence of added eIF-2 (compare the 40-kDa band in lanes 5, 6, 7, and 8 between Fig. 5A and B). Reactions similar to those shown in Fig. 5B, lanes 5 and 6, were repeated without exogenously added eIF-2, and the results are shown in lanes 1 and 2 of Fig. 5C.

The appearance of the 65-kDa phosphoprotein (in Fig. 5B) which could not be detected in Fig. 5A was not due to the addition of eIF-2. The appearance of the 65-kDa protein varied from one reaction to the other even when the same preparation of RSW was used (compare Fig. 1A, lane 2, with Fig. 2A, lane 10).

Phosphorylation of eIF-2 is not stimulated in poliovirus-infected cells. To determine whether a stimulation of phosphorylation of the 68-kDa protein could be detected in vivo, we labeled HeLa cells with $^{32}P_i$ during infection with poliovirus. RSW was prepared, and ^{32}P-labeled proteins were analyzed by SDS-PAGE. A phosphoprotein with an apparent molecular weight of 68,000 was detected in poliovirus-infected cells (Fig. 6A, lane 4). This polypeptide,

which comigrated with the in vitro-phosphorylated 68-kDa protein (Fig. 6A, lanes 1 and 2), was not detected in mock-infected cells (Fig. 6A, lane 3). A labeled polypeptide at 38,000 molecular weight was also detected in both mock- and poliovirus-infected cells (Fig. 6A, lanes 3 and 4). This polypeptide comigrated with the α subunit of eIF-2 (data not shown). Two-dimensional gel electrophoresis showed that the polypeptide of M_r 38,000 was indeed the α subunit of eIF-2 (Fig. 6B and C). Quantitation of radioactivity in these spots showed that the level of phosphorylated α subunit of eIF-2 in infected cells was approximately twofold less than that in mock-infected cells. We were unable to detect in vivo-labeled phosphorylated 68-kDa protein on two-dimensional gels because of low amounts of radioactivity incorporated in the 68-kDa molecule.

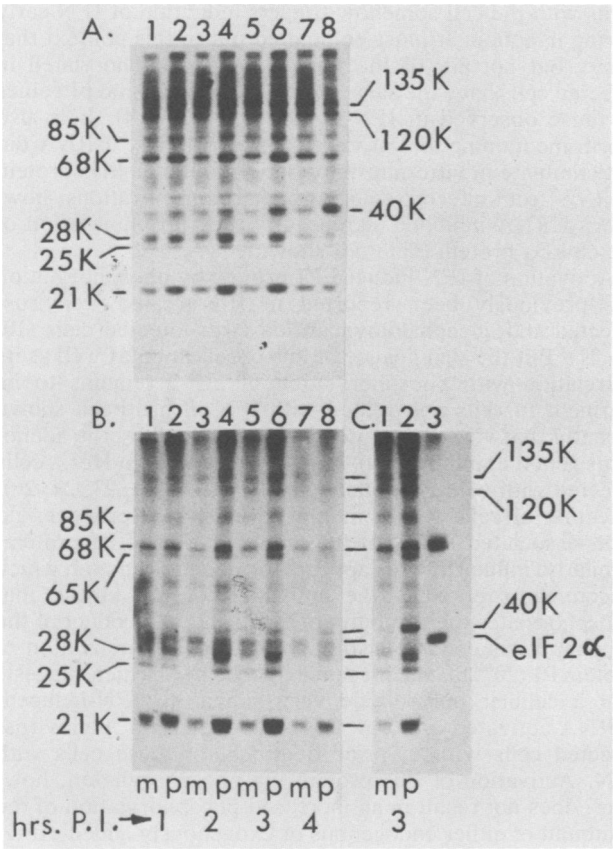

FIG. 5. Time course of activation of 68-kDa protein during mock and virus infection of HeLa cells. RSW were prepared from 4×10^8 cells infected (or mock infected) with poliovirus for 1, 2, 3, and 4 h. Protein (5 μg) was phosphorylated in vitro with [γ-^{32}P]ATP in the absence (panels A and C) or presence (panel B) of added reticulocyte eIF-2 (1 μg). Phosphorylated proteins were analyzed by SDS-PAGE. (A) Lanes 1, 3, 5, and 7, Mock-infected RSW prepared at 1, 2, 3, and 4 h after mock infection, respectively; lanes 2, 4, 6, and 8, poliovirus-infected RSW prepared at 1, 2, 3, and 4 h after infection, respectively. (B) Same as in panel A except that phosphorylations were done in the presence of 1 μg of purified eIF-2. (C) Lane 1, Same as in lane 5 of panel B minus eIF-2; lane 2, same as in lane 6 of panel B minus eIF-2; lane 3, 1 μg of purified eIF-2 was phosphorylated with 0.5 μg of purified reticulocyte dsRNA-activated kinase. Mock- and poliovirus-infected RSW fractions are denoted by the letters m and p, respectively. The numbers at the bottom indicate hours of infection or mock infection. K, Kilodaltons.

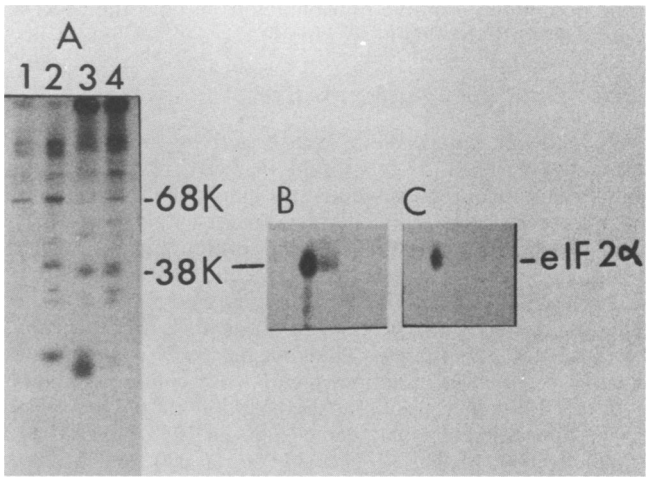

FIG. 6. Comparison between in vitro and in vivo ^{32}P-labeled proteins from mock- and poliovirus-infected cells. (A) RSW were prepared from unlabeled or $^{32}P_i$-labeled mock- and poliovirus-infected cells. Portions of unlabeled RSW were phosphorylated with [γ-^{32}P]ATP, and in vitro-phosphorylated proteins were compared with in vivo-labeled proteins by SDS-PAGE. (A) lane 1, In vitro ^{32}P-labeled mock-infected RSW; lane 2, in vitro ^{32}P-labeled poliovirus-infected RSW; lane 3, in vivo ^{32}P-labeled mock-infected RSW; lane 4, in vivo ^{32}P-labeled poliovirus-infected RSW; (B and C) Two-dimensional gel electrophoresis of the α subunit of eIF-2 from in vivo ^{32}P-labeled, mock- and poliovirus-infected cells, respectively. K, Kilodaltons.

Lack of increased phosphorylation of eIF-2 in RSW from infected cells is not due to a specific phosphatase. To determine whether a specific protein phosphatase is induced in poliovirus-infected cells which dephosphorylates the phosphorylated α subunit of eIF-2, we incubated prephosphorylated reticulocyte eIF-2 with increasing concentrations of mock- or poliovirus-infected RSW fractions. No specific dephosphorylation of eIF-2α-P could be detected in infected RSW compared with that in mock-infected RSW (Fig. 7). Under similar conditions, however, alkaline phosphatase

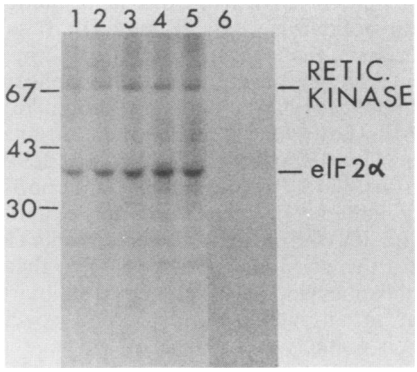

FIG. 7. Assay for phosphatase activity in RSW from mock- and poliovirus-infected cells. Reticulocyte eIF-2 (1 μg) was phosphorylated with the reticulocyte kinase. Prephosphorylated eIF-2 was used as the substrate for a specific phosphatase in mock- or poliovirus-infected RSW. Lane 1, Control; lanes 2 and 4, same as in lane 1 plus 4 and 8 μg of mock-infected RSW, respectively; lanes 3 and 5, same as in lane 1 plus 4 and 8 μg of poliovirus-infected RSW, respectively; lane 6, same as in lane 1 plus 5 U of alkaline phosphatase. Numbers on left are in kilodaltons.

could completely remove phosphates from both the 68-kDa protein and eIF-2α-P (Fig. 7, lane 6).

DISCUSSION

We showed that protein kinase activity in general is stimulated at least 5- to 10-fold in RSW prepared from poliovirus-infected HeLa cells compared with that from mock-infected cells. This is in agreement with a previously published report by Tershak (27) that protein kinase activity is stimulated in HeLa cells during infection with poliovirus. This protein kinase activity (or activities) associated with the cellular ribosomes can be washed off of these structures by 0.5 M salt (27; this report). The kinase activity is stimulated as early as 1 h after infection of cells with poliovirus and is manifested by increased phosphorylation of 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. All these polypeptides appear to be of cellular origin since the basal level of phosphorylation of these polypeptides can be detected in mock-infected cells.

Three lines of evidence suggest that the M_r 68,000 polypeptide is very similar, if not identical, to the IFN-induced, dsRNA-activated protein P1 which is believed to phosphorylate the α subunit of protein synthesis IF-2 (1). First, phosphorylated 68-kDa protein from poliovirus-infected RSW fraction comigrated with the IFN-induced, phosphorylated P1 protein on an SDS-polyacrylamide gel (Fig. 2). Second, phosphorylated 68-kDa protein from poliovirus-infected RSW had the same isoelectric pH as that of protein P1; and finally, the phosphopeptides generated from the 68-kDa protein by digestion with V8 protease were identical to those generated from P1 protein (Fig. 2). The pI value of the P1 protein from HeLa cells is in good agreement with the previously reported pI value of this IFN-induced protein from another human cell line (13).

The stimulation of phosphorylation of the 68-kDA protein in the RSW fraction from cells infected with poliovirus does not result in an increased phosphorylation of the α subunit of endogenous HeLa eIF-2. This is clearly not due to the presence of limiting concentrations of eIF-2 in HeLa cells because the level of phosphorylation of eIF-2 in the presence of dsRNA in RSW prepared from IFN-treated cells is much greater than that in RSW from the same number of cells infected with poliovirus (Fig. 4B and D). It is logical to assume, therefore, that the majority of eIF-2 in RSW from poliovirus-infected HeLa cells remains unphosphorylated. The level of phosphorylation of 68-kDa protein in poliovirus-infected RSW, however, is comparable to that in IFN-treated cells (Fig. 3B and C). The fact that RSW fractions from poliovirus-infected cells do not phosphorylate exogenously added eIF-2 to a greater extent than that observed with RSW from mock-infected cells (Fig. 5) also argues against this possibility. The possibility that the lack of increased phosphorylation of eIF-2 in poliovirus-infected cells is due to an increased level of a specific eIF-2α phosphatase is unlikely (Fig. 7).

The observation that exogenously added eIF-2 inhibits phosphorylation of a 40-kDa protein in infected-RSW in vitro (Fig. 5) could explain why eIF-2 is not further phosphorylated in infected RSW. Although the phosphopeptide profiles of the 40-kDa protein and eIF-2α are quite different (data not shown), it is still conceivable that this protein somehow competes with eIF-2 for the kinase that phosphorylates both. The 40-kDa protein could simply be a better substrate than eIF-2 in vitro. Further careful studies must be performed before any valid conclusions can be drawn. It must be pointed out that this protein is not as much phosphorylated in vivo as in vitro (Fig. 6).

It is not clear what causes stimulation of kinase activity and consequently phosphorylation of several cellular proteins during infection of HeLa cells with poliovirus. The possibility that viral stocks used for infection may contain IFN which stimulates this kinase activity is not likely because purified poliovirus prepared by density gradient centrifugation is still able to elicit this cellular response. It is also unlikely that viral dsRNA in infected cells is responsible for stimulation of kinase activity through production of IFN since this appears to be a very early response at which time no dsRNA is detectable in infected cells. Also, in the presence of guanidine, which selectively blocks viral RNA replication, stimulation of kinase activity could still be detected (27). However, it is possible that interaction of virus with the cell somehow triggers induction of IFN early during infection. It must be pointed out in this context that some, but not all, of the polypeptides phosphorylated in infected cells have the same molecular weights and pI values as those observed in IFN-treated cells (Fig. 4). It is also worth mentioning that at lower concentrations, dsRNA did not stimulate in vitro phosphorylation of the 68-kDa protein in RSW from infected cells; at higher concentrations, however, dsRNA inhibited significantly the phosphorylation of the 68-kDa protein (data not shown).

Activation of IFN-induced P1 protein by phosphorylation has previously been reported in IFN-treated, reovirus-infected and encephalomyocarditis virus-infected cells (10, 17, 21). But the significance of this observation as well as its correlation with phosphorylation of eIF-2 remains to be clarified. In cells not treated with IFN, it has been shown recently that virus-associated RNA_1 encoded by the adenovirus genome, inhibits activation of P1 protein in HeLa cells infected with wild-type adenovirus (type 5) (19, 23, 24, 26). Infection of cells with a mutant virus, which produces no virus-associated RNA_1, results in activation of P1 protein. Similarly, influenza virus appears to encode a function which selectively suppresses the activation of the kinase that phosphorylates the α subunit of eIF-2 (11). A product of the vaccinia virus genome also appears to inhibit activation of protein P1 (20, 28). We present evidence here which suggests that a cellular polypeptide very similar to IFN-induced, dsRNA-activated protein P1 is activated in poliovirus-infected cells without prior treatment of these cells with IFN. Activation of this protein via phosphorylation, however, does not result in an increased phosphorylation of the α subunit of either endogenous or exogenously added eIF-2. It is our speculation that rather than inhibiting the activation of P1 protein, the phosphorylation of which may be necessary for initiation of viral RNA replication (16), the virus encodes (or induces) a factor which interferes with phosphorylation of eIF-2 by the activated kinase. Our preliminary results indicate that indeed an inhibitor is present in RSW from infected cells which inhibits phosphorylation of exogenously added eIF-2 by the reticulocyte dsRNA-dependent kinase without inhibiting phosphorylation of the kinase itself. In the future, this assay system would enable us to purify the putative inhibitory factor from infected cells and to study the mechanism of inhibition of phosphorylation of eIF-2.

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