Inhibition of Protein Synthesis in Reovirus-infected HeLa Cells with Elevated Levels of Interferon-induced Protein Kinase Activity*

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Protein synthesis was inhibited in one line of interferon-treated HeLa cells (line 2) upon infection with reovirus, but not in different HeLa cells (line 1) treated in the same way. The inhibition resulted in polysome runoff, suggesting that it was due to an impairment of peptide chain initiation. Interferon induces the synthesis of a protein kinase, which is activated in cell-free systems by double-stranded RNA and phosphorylates the a subunit of eukaryotic initiation factor 2, thus inhibiting the initiation of protein synthesis. Therefore, we measured the level of this protein kinase in extracts prepared from the two HeLa cell lines. Cells of line 2 showed about 3-4 times more protein kinase activity than cells of line 1. The inhibition of protein synthesis upon infection with reovirus was correlated with an increased phosphorylation of the a subunit of eukaryotic initiation factor 2 in interferon-treated cells labeled with 32P. The kinase was presumably activated in intact cells by viral double-stranded RNA, but this activation resulted in inhibition of protein synthesis only in cells with elevated levels of the kinase.

Treatment of mammalian cells with interferon induces the synthesis of two enzymes: a protein kinase, which phosphorylates the a subunit of eIF-2, and a (2'-5')oligoadenylate polymerase or synthetase (1). Both enzymes are latent and require activation by dsRNA to inhibit protein synthesis with distinct mechanisms (1). The kinase inhibits peptide chain initiation because the phosphorylated eIF-2 may not cyclize after forming a ternary complex with Met-tRNAf and GTP (2), whereas the (2'-5')oligo(A) activates an endonuclease, which cleaves mRNA (3). These two inhibitory mechanisms have been implicated in the antiviral activity of interferon and convincing evidence was presented for the formation of (2'-5')oligo(A) and the activation of an endonuclease in interferon-treated cells infected either with encephalomyocarditis virus or with reovirus (4, 5). Recently, Gupta et al. (6) reported that the eIF-2 kinase is activated in interferon-treated mouse L929 cells infected with reovirus and proposed a role for the kinase in the antiviral action of interferon against this virus.

In experiments with interferon-treated HeLa cells, we could not detect an inhibition of protein synthesis when the concentration of (2'-5')oligo(A) was elevated by infection with reovirus (7). We proposed that the (2'-5')oligo(A) polymerase was activated in these cells by dsRNA of viral origin and showed that cellular and presumably viral RNA was cleaved by the (2'-5')oligo(A)-activated endonuclease (5). The cleavage of cellular mRNA did not result in an inhibition of protein synthesis in these cells because the loss of mRNA was apparently compensated by the production of new mRNA (7). In these interferon-treated cells, there was sufficient viral dsRNA to activate the (2'-5')oligo(A) polymerase, but apparently either not enough eIF-2 kinase was activated or a negligible inhibition of protein synthesis resulted from such an activation.

In recent experiments with a different HeLa cell line, we observed that protein synthesis was inhibited in interferon-treated cells infected with reovirus. In the present study, we compared these HeLa cells with the other HeLa cell line previously investigated. These two cell lines were found to differ in the relative content of a M, = 72,000 polypeptide which could be phosphorylated by the dsRNA-activated protein kinase. Protein synthesis was inhibited only in the cells which showed elevated levels of this polypeptide upon treatment with interferon. The inhibition of protein synthesis was correlated with an increased phosphorylation of the a subunit of eIF-2.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatment—HeLa cells were obtained from Dr. Sheldon Penman of the Massachusetts Institute of Technology (line 1) and Dr. Thoru Pederson of the Worcester Foundation for Experimental Biology (line 2). The HeLa cells were grown in spinner cultures in Joklik's modified Eagle's medium with 7.5% horse serum. The cells were treated for 18 h with 200 units/ml of human / interferon (100 units/mg of protein; obtained from HEM Research, Inc.) and infected with reovirus as previously described (5).

Protein Synthesis and Polysome Analysis—1-ml culture aliquots were incubated for 60 min with 20 µCi/ml of 3Hlysine. The cells were centrifuged, and the acid-precipitable radioactivity was counted after addition of 0.5 ml of 0.5 N NaOH and 1 ml of 20% trichloroacetic acid. Cell extracts were prepared by homogenization as described (8). Polysome profiles were analyzed by centrifuging cell extracts for 90 min at 40,000 rpm on 15-40% sucrose gradients and scanning through a recording spectrophotometer (6).

Protein Kinase Assays—The activity of the dsRNA-activated protein kinase was measured as previously described (9). Briefly, ribosome pellets were prepared from cell extracts, resuspended, and incubated for 7 min with 0.1 mM [y-32P]ATP and 1 µg/ml of poly(I)-poly(C) (9). The samples were fractionated by gel electrophoresis and transferred to dry gels autodigested. Phosphorylated polypeptides were quantitated by scanning the autoradiographs at 560 nm, as reported (9). The phosphorylation of the subunit of eIF-2 in intact cells was monitored by transferring HeLa cells to phosphate-free medium with 5% dialyzed horse serum and adding 1 mCi/ml of [32P]phosphate for 90 min. Cell extracts were prepared by homogenization in 1.5 mM Mg(OAc)2, 10 mM KCl, 0.5% Triton X-100, and 20 mM Tris, pH 7.5. The samples were centrifuged 5 min at 30,000 x g, and the supernatants were applied to small phosphocellulose columns equilibrated with 0.2 mM EDTA, 10 mM NaF, 10 mM mercaptoethanol, and 20 mM Tris, pH 7.6 (Buffer A). The columns were washed with 50 ml of 0.1...
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m and 50 ml of 0.3 M KCl in Buffer A and finally eluted with 3 ml of 0.6 M KCl in Buffer A. This procedure follows that published by Wong et al. (10). The NaF is added to inhibit the phosphatase which is known to dephosphorylate eIF-2 (10). To the 0.6 M KCl fraction were added 50 μg of bovine serum albumin and trichloroacetic acid to 20%. The precipitate obtained was washed with 20% trichloroacetic acid, acetone, and ether, dried, and redissolved in sample buffer for gel electrophoresis. The same procedure was followed for the purification of eIF-2 phosphorylated in an incubation of ribosomes from interferon-treated HeLa cells with poly(I)-poly(C). About 1 μg of purified eIF-2 (a gift of Dr. David Shafritz of the Albert Einstein Medical School) was phosphorylated as previously described (9). The phosphorylation of proteins co-sedimenting with ribosomes was monitored by analyzing the fraction prepared from extracts of cells incubated with [32P]phosphate. The cell extracts were layered over 4 ml of 15% sucrose and centrifuged 4 h at 200,000 × g. The pellets were dissolved in sample buffer and analyzed by gel electrophoresis.

### RESULTS

Protein synthesis was monitored in two lines of HeLa cells for 6.5 h after infection with reovirus or mock infection. The incorporation of [3H]lysine was not inhibited in these cells (data not shown), in agreement with previous studies which show that infection with reovirus does not switch off protein synthesis for several hours (11, 12). When the cells were treated with interferon and then infected, protein synthesis was not inhibited (Fig. 1) in the HeLa cells (line 1) previously examined for the presence of (2’-5’)A, during reovirus infection (5). In HeLa cells of line 2, however, protein synthesis was progressively inhibited after 3 h of infection (Fig. 1). This inhibition was greater in cells infected with 20 plaque-forming units of reovirus than in cells infected with 5 plaque-forming units, suggesting that it was correlated with the multiplicity of infection.

To confirm that the decrease in lysine incorporation was due to a change in the rate of protein synthesis, we examined the polysome profile of interferon-treated cells after infections with reovirus (Fig. 2). A decrease in polysomes and a corresponding increase in 80 S ribosomes was observed in HeLa cells of line 2, but not in mock infected cells or in infected cells not treated with interferon. The polysome runoff was maximal at 5 h after infection (shown in Fig. 2). No change in the polysome profile was observed in HeLa cells of line 1 treated in the same way (7). The proteins synthesized by interferon-treated HeLa cells were examined by labeling the cells with [35S]methionine and fractionating the proteins by gel electrophoresis, as described (7). No change was observed in the protein pattern between control and infected cells, as previously reported (7). Subsequent experiments were directed at investigating the mechanism(s) for the inhibition of protein synthesis upon reovirus infections in cells of line 2.

A mechanism which may cause inhibition of protein synthesis in interferon-treated cells is the activation of the eIF-2 kinase. Gupta et al. (6) recently reported that this kinase is activated in interferon-treated L929 cells infected with reovirus, but did not investigate the effect of the activated kinase on protein synthesis. It seemed possible from our results that protein synthesis was inhibited in HeLa cells of line 2 by the phosphorylation of eIF-2 and that a low level of eIF-2 kinase could explain the lack of inhibition in HeLa cells of line 1. To test this explanation, we measured the eIF-2 kinase level in interferon-treated HeLa cells with the assay previously described, i.e. phosphorylation of a ribosome-associated polypeptide of M, = 72,000 (9). The relative amount of this polypeptide was previously correlated with the inhibition of protein synthesis in a cell-free system upon addition of dsRNA (9).

Ribosomes prepared from the two HeLa cell lines were

**FIG. 1. Inhibition of protein synthesis in interferon-treated HeLa cells infected with reovirus (Dearing type 3).** Cultures of HeLa cells of line 1 (x) or line 2 (other symbols) were treated with interferon and mock infected (○) or infected with 5 (▲) and 20 (x and □) plaque-forming units of reovirus/cell, as described under "Experimental Procedures." A 1-ml aliquot of each culture was incubated for 60 min with 20 μCi of [3H]lysine at the indicated times, and the acid-precipitable radioactivity was measured.

**FIG. 2. Polysome profile of interferon-treated HeLa cells infected with 20 plaque-forming units of reovirus/cell.** A culture of HeLa cells (line 2) was treated with interferon and infected with reovirus as described under "Experimental Procedures." Cell extracts were prepared from 25-ml aliquots of the culture, and the polysomes were fractionated by centrifugation for 90 min at 40,000 rpm on 15-40% sucrose gradients. A260 was monitored with a recording spectrophotometer. A, cells infected for 2 h; B, cells infected for 6 h.

**FIG. 3. Phosphorylation of a M, = 72,000 polypeptide with ribosomes of line 2 (lanes a, b, and c) and line 1 (lanes d and e) HeLa cells.** The ribosomes were prepared by centrifugation of cell extracts obtained from untreated cells (lane a) or from cells treated with interferon as described under "Experimental Procedures." The phosphorylation assay with [γ-32P]ATP, the fractionation by gel electrophoresis, and the detection by autoradiography were described in detail previously (9). Lanes a, c, and e, incubations containing 1 μg/ml of poly(I)-poly(C); Lanes b and d, 20 added dsRNA. The arrow indicates the position of the M, = 72,000 polypeptide.
incubated with [γ-³²P]ATP and poly(I)-poly(C) as an activator of the kinase. The labeled polypeptides were separated by gel electrophoresis and detected by autoradiography (Fig. 3). To quantitate the relative phosphorylation of the M₁ = 72,000 polypeptide, the autoradiographs were scanned in a recording spectrophotometer, as described (9). There was 3-4 times more phosphorylated M₁ = 72,000 polypeptide in HeLa cells of line 2 than in HeLa cells of line 1. This polypeptide was detected only in interferon-treated cells and in incubations with added dsRNA (Fig. 3). We concluded from these data that the presence of elevated levels of dsRNA-activated kinase was correlated with an inhibition of protein synthesis in intact cells.

Control experiments were carried out to exclude that some difference in the activation of the kinase by dsRNA could explain the reduced phosphorylation of the M₁ = 72,000 polypeptide in HeLa cells of line 1. The optimum dsRNA concentration for the activation of the kinase was determined in a series of incubations containing from 10 ng to 5 µg/ml of poly(I)-poly(C). Maximum activation was obtained in both cell lines with 0.5 µg/ml of this dsRNA, in agreement with previous observations (9). Other changes in the composition of the incubations, such as ATP or magnesium concentration, did not eliminate the difference in the relative phosphorylation of the M₁ = 72,000 polypeptide between the two HeLa cell lines (data not shown).

The inhibition of protein synthesis in interferon-treated HeLa cells infected with reovirus could be explained by an activity of the dsRNA-activated eIF-2 kinase and the phosphorylation of this initiation factor. To measure kinase activity in intact cells, we incubated the HeLa cells with [³²P]phosphate from 4 to 5.5 h after infection with reovirus at the time of maximal inhibition of protein synthesis. Mock infected cells served as a control. Cell extracts were prepared and centrifuged at high speed to fractionate by gel electrophoresis the proteins co-sedimenting with ribosomes; which include the M₂ = 72,000 polypeptide (9) and initiation factors. The autoradiographs showed a phosphorylated band of M₂ = 72,000, which was present in greater amounts in interferon-treated cells (Fig. 4, lane b) than in mock infected cells (Fig. 4, lane a). This difference was not easily detectable because this polypeptide was poorly separated from other phosphorylated polypeptides. Furthermore, a polypeptide co-migrating with the α subunit of eIF-2 and another polypeptide of about M₁ = 30,000 was more phosphorylated in the sample from infected cells than in that of mock infected cells. To provide additional evidence for an increased eIF-2α phosphorylation, the cell extracts were fractionated by chromatography on phosphocellulose, and the fraction eluted with 0.6 M KCl, which contains eIF-2α, was analyzed by gel electrophoresis. A preparation of eIF-2 phosphorylated in an incubation with the ribosome fraction obtained from interferon-treated cells and purified by chromatography on phosphocellulose served to identify the α subunit of eIF-2 (Fig. 4, lane c). A band corresponding to eIF-2α was detected in both infected and mock infected cells (Fig. 4, lanes d and e). The relative phosphorylation of this band was measured by scanning the autoradiographs, and the infected cells showed a 3-fold greater phosphorylation of this band than mock infected cells. The phosphorylation of this polypeptide therefore appeared to be correlated with the inhibition of protein synthesis in intact cells.

**FIG. 4. Phosphorylation of the α subunit of eIF-2 in interferon-treated HeLa cells infected with reovirus.** A HeLa cell culture (line 2) was treated with interferon and either mock infected (lanes a and d) or infected with reovirus as described under "Experimental Procedures." At 3.5 h after infection, the cells were transferred to phosphate-free medium containing 5% dialyzed horse serum. After 39 min, 1 mCi/ml of [³²P]phosphate was added, and the cultures were incubated for 90 min. At the end of this incubation, the cells were washed with medium minus serum and broken by homogenization (see "Experimental Procedures"). An aliquot of the extracts was chromatographed on phosphocellulose as described under "Experimental Procedures," and the fraction eluting with 0.6 M KCl was precipitated with 5% trichloroacetic acid and analyzed by gel electrophoresis (lanes a and b). Authentic eIF-2 phosphorylated in vitro with the kinase of interferon-treated HeLa cells as described in the legend to Fig. 3 and purified by chromatography on phosphocellulose is shown in lane c. Another aliquot of the cell extracts prepared as described above was centrifuged for 4 h at 200,000 × g. The pellets obtained were dissolved and analyzed by gel electrophoresis (lanes d and e). An authentic sample of pure eIF-2 α, phosphorylated in vivo, is shown in lane f. 1 and 2 indicate the position of the M₁ = 72,000 polypeptide and of the α subunit of eIF-2, respectively.

**DISCUSSION**

Protein synthesis was inhibited upon infection with reovirus in interferon-treated HeLa cells with elevated levels of dsRNA-activated protein kinase, whereas it was not inhibited in a different HeLa cell line with low levels of this kinase. We suggest that this inhibition resulted from the activation of the kinase by viral dsRNA. The activation of the (2'-5')-oligo(A) polymerase in these cells previously indicated that dsRNA was present during the infection with reovirus (5). Moreover, activation of the kinase was demonstrated in interferon-treated L929 cells infected with this virus by measuring the phosphorylation of a M₁ = 67,000 polypeptide (which corresponds (14) to the human M₁ = 72,000 polypeptide) in intact cells and the phosphorylation of eIF-2α in cell extracts (6). The activation of the protein kinase by dsRNA results in an increase in the first rate of the eIF-2 phosphorylation (13). Since the M₁ = 72,000 polypeptide was not well separated from other phosphorylated polypeptides in the present experiments, we obtained evidence for the activation of the kinase by monitoring the phosphorylation of eIF-2 in intact cells. The M₁ subunit of this initiation factor appeared to be significantly more phosphorylated in cells infected with reovirus than in mock infected cells (Fig. 4).

These experiments established a correlation between inhibition of protein synthesis and phosphorylation of eIF-2α in intact cells. It should be pointed out that prior to this report there was no evidence for a role of the dsRNA-activated protein kinase in the inhibition of protein synthesis in intact cells. In spite of extensive work in cell-free systems on the inhibition of peptide chain initiation resulting from eIF-2α phosphorylation, a translational inhibition had not been demonstrated in vivo until recently, when Leroux and London (15) reported that treatment of intact reticulocytes with an inhibitor of heme synthesis resulted in inhibition of protein synthesis and increased phosphorylation of eIF-2, presumably
due to a kinase which is activated in cell-free systems in the absence of hemin. A relatively small fraction eIF-2 was phosphorylated in intact cells (15). Protein synthesis might be inhibited, however, because a recycling factor present in limited amounts forms an inactive complex with phosphorylated eIF-2 (16). Therefore, the phosphorylation of a small fraction of cellular eIF-2 may be responsible for the inhibition of protein synthesis observed in the present experiments.

We have no explanation for the presence of different levels of dsRNA-activated kinase in the two lines of HeLa cells treated with interferon. These cells were equally responsive to interferon, as shown by a comparable increase in (2'→5')oligo(A) polymerase and by the protection against infection with reovirus and encephalomyocarditis virus in HeLa cells infected with the same interferon concentrations.2 We measured the level of dsRNA-activated protein kinase in HeLa cells of line 2 over a period of 2 years and found that after several months in culture gradually less protein kinase activity was induced upon treatment with interferon. The reasons for this decline in kinase inducibility are not clear, but we used for the present experiments HeLa cells kept in culture for less than a month.

An increase in dsRNA-activated kinase is apparently not required for the establishment of an antiviral state against encephalomyocarditis virus in HeLa cells (17), and it remains to be shown that the kinase plays an important role in the antiviral action of interferon. Gupta et al. (6) suggested that the kinase is involved in the antiviral action of interferon because this enzyme was elevated in NIH 3T3 cells chronically infected with Moloney leukemia virus, which were reported to be deficient in (2'→5')oligo(A)-activated endonuclease (18), and it seemed unlikely that the inhibitory pathway which leads to the cleavage of RNA could be activated in these cells. We have recently reported, however, that the (2'→5')oligo(A)-activated endonuclease can be detected in extracts of these NIH 3T3 cells prepared in the presence of a protease inhibitor (19). Therefore, the antiviral activity of interferon in these cells cannot be attributed solely to the dsRNA-activated protein kinase. Since different molecular mechanisms participate in the antiviral activity induced by interferon, the contribution of each component may vary with different viruses and in different cells. The use of cell lines defective in one component, like the HeLa cells of line 1, might help in some cases in establishing the relative importance of different mechanisms in the inhibition of virus replication.

After this manuscript had been submitted for publication, Vaquero et al. (20) reported that a decrease in the amount of methionyl-tRNA-40 S ribosomal subunit complexes in extracts prepared from interferon-treated L-cells infected with Mengo virus might account for the inhibition of protein synthesis observed in these cells. It seems possible that this inhibition is due to the dsRNA-activated eIF-2 kinase, which may be responsible for this effect on protein synthesis in interferon-treated cells infected by other viruses beside reovirus.

REFERENCES