JOURNAL OF VIROLOGY, Jan. 1979, p. 301-311
0022-538X/79/01-0301/11$02.00/0

Vol. 29, No. 1

Specific Protein Phosphorylation in Interferon-Treated Uninfected and Virus-Infected Mouse L929 Cells: Enhancement by Double-Stranded RNA

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Received for publication 26 June 1978

The enhanced phosphorylation of specific protein(s) observed in extracts from interferon-treated cells (in the presence of ATP and double-stranded [ds] RNA) was also seen in intact mouse L929 cells upon treatment with dsRNA, polyriboinosinic·polyribocytidylic acid [poly(rI·rC)] or reovirus dsRNA, using $^{32}P_i$ as radiolabel. Labeling of a 65,000-dalton protein(s) with ^{32}P was greatly increased in interferon-treated cells in the presence of added dsRNA, suggesting that the expression in vivo of the kinase activity involved is regulated by dsRNA. This was used as a test system to investigate whether the activity of interferon-induced enzyme(s) is stimulated following virus infection, possibly owing to the accumulation of dsRNA. No obvious increase in ^{32}P-labeling of 65,000-dalton protein(s) was observed upon infection of interferon-treated cells with mengovirus or vesicular stomatitis virus. A basal level of ^{32}P-labeling of the 65,000-dalton protein(s) was detected in interferon-treated cells in the absence of added dsRNA, indicating a basal level of expression of the kinase activity involved. The possible implications of these results are discussed.

In mammals and other higher eucaryotes, replication of a variety of viruses is strongly inhibited in cells pretreated with interferon. There is a volume of evidence showing that in the case of lytic viruses, viral mRNA and/or protein accumulation is inhibited in interferon-treated cells (10). On the other hand, cellular protein synthesis goes on in interferon-treated uninfected cells and, at least in certain systems, virus-infected cells (12, 41). Impairment of mRNA translation is also observed in cell-free systems prepared from interferon-treated cells, which is apparently due to the presence of certain inhibitors (8, 11, 13, 32) and is overcome to an extent by the addition of tRNA (6, 13, 14). However, the selectivity observed in intact cells is not faithfully represented in cell-free systems (8, 12, 13). Cell-free translation systems prepared from interferon-treated cells in a conventional manner (preincubated and passed through Sephadex) also inactivate certain species of tRNA (14, 33); however, such tRNA inactivation is not detected in intact cells (5, 33).

In cell-free translation systems prepared from interferon-treated cells, the inhibition of mRNA translation is greatly enhanced upon the addition of double-stranded (ds) RNA (22, 23). Three enzymatic activities, which are stimulated by dsRNA, have been identified in interferon-treated cell extracts. They are as follows: (i) The first is phosphorylation of at least two proteins (a 64,000- to 67,000-dalton protein[s] and a 35,000- to 37,000-dalton protein[s]) in the presence of ATP and dsRNA (26, 31, 42). Their identity has not been established. However, this activation seems to be analogous to that of a protein kinase enzyme which is seen in rabbit reticulocyte lysates upon addition of dsRNA (or deprivation of hemin). This kinase phosphorylates the small subunit (35,000 to 38,000 daltons) of initiation factor eIF-2, which leads to the inhibition of protein synthesis (9, 27). (ii) The second is an endonuclease activity (endonuclease$_{INT}$) which is also activated by dsRNA and ATP and degrades added viral and cellular mRNA (29, 34). (iii) The third is an enzyme which synthesizes an unusual oligonucleotide from ATP with 2′,5′-phosphodiester bonds in the presence of dsRNA. This oligonucleotide is a potent inhibitor of mRNA translation in cell-free systems prepared from untreated mouse cells as well as from rabbit reticulocytes (17, 21, 31).

It was recently reported that a similar oligonucleotide made in interferon-treated Ehrlich ascites tumor cell extracts serves as an activator of the above mentioned endonuclease$_{INT}$ (30).

The fact that these enzymes are greatly stim-

ulated by dsRNA suggests a possible role for dsRNA in interferon action. If these enzymes are involved in the antiviral mechanism, two possibilities may have to be considered. (i) The first is that the interferon treatment of cells may induce these enzymes which normally remain latent and are activated only upon virus infection, possibly due to an accumulation of dsRNA. Infection with certain viruses is known to result in an accumulation of dsRNA (4). This would be a two-step mechanism and is attractive because it offers the advantage that the interferon-induced enzymes, which could possibly inhibit cellular functions as well, would remain latent until the cells are infected by a virus. (ii) The second is that these activities are already expressed in cells and that their expression does not depend on the appearance of dsRNA upon virus infection. In this case the operations of the antiviral mechanism might be self-sufficient, independent of an accumulation of dsRNA resulting from virus infection.

The experiments reported here represent an attempt to distinguish between these two possibilities by making use of the interferon-induced protein kinase activity as a test system.

MATERIALS AND METHODS

Cells. Mouse L929 cells (kindly provided by George Bekesi, Mt. Sinai School of Medicine, New York) were grown in monolayers at 37°C using Eagle minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS) in an incubator flushed with 5% CO_2 in air.

Chemicals and radiochemicals. Mouse interferon was produced by infecting Ehrlich ascites tumor cells with Newcastle disease virus and partially purified up to the CM-Sephadex step (20) (specific activity ~1 × 10^7 to 2 × 10^7 reference units/mg of protein). Polyriboinosinic·polyribocytidylic acid [poly(rI·rC)] was purchased from P-L Biochemicals. Radioactive isotopes used were obtained from New England Nuclear.

Test for protein phosphorylation. (i) Intact cells. Mouse L929 cells were grown to confluence in 100-mm tissue culture dishes (Falcon) using MEM with 10% FCS. One group was treated with interferon (200 to 250 reference units/ml for 16 to 18 h in MEM with 10% FCS), while another group served as a control. Then the medium was aspirated, and the cells were washed once with 5 ml of MEM without serum. The cells were labeled with ^{32}P by adding 4 ml of labeling medium (phosphate-free MEM supplemented with 10 mM HEPES [*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid]-KOH buffer [pH 7.2] and 25 μCi of carrier-free $^{32}P_i$ per ml) with or without poly(rI·rC) (25 μg/ml). In some experiments, the labeling medium also contained 2% dialyzed FCS. After incubation for 1 h at 37°C in a CO_2 incubator, the medium was aspirated, the cells were washed with 10 ml of chilled phosphate-buffered saline (PBS, Ca^{2+}, Mg^{2+}-free), and lysed in the cold by adding 0.4 ml of buffer (10 mM Tris-hydrochloride [pH 7.4], 50 mM KCl, and 5 mM $MgCl_2$) containing 0.2% Triton X-100 and spreading it uniformly. After 5 min, the cells were scraped with a rubber policeman, and the suspension was collected and kept on ice for 5 min. The lysate was centrifuged at 600 × *g* for 10 min, and the resultant supernatant was centrifuged at 10,000 × *g* for 15 min in the cold. The supernatant (S-10) was collected and stored in portions at −70°C. ^{32}P-labeling of proteins was determined by spotting small portions (10 μl) on Whatman 3 MM disks, and hot trichloroacetic acid-insoluble counts were determined (1).

Portions of S-10 preparations (20 to 40 μl) were fractionated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) in slab gels (18 cm long) under conditions described by Laemmli (25), except that the stacking gel consisted of 4.5% acrylamide and the SDS concentration in samples was 2.5%. Electrophoresis was carried out initially at 16 mA for 90 min and then at 22 mA at constant current until the voltage reached about 140, when it was changed to constant power (3 W) until the dye marker reached the other end. The following proteins were run in parallel as molecular weight markers: bovine serum albumin (67,000), catalase (60,000), bovine γ-globulin heavy chain (55,000) and light chain (25,000), aldolase (40,000), and bovine RNase A (13,700). The gels were stained in Coomassie brilliant blue (0.05%) to identify the markers, destained, dried on a filter paper under vacuum, and autoradiographed with Kodak XRP-5 film to identify radioactive bands.

In experiments with mengovirus or vesicular stomatitis virus (VSV) infection, some of the control and interferon-treated culture plates were infected with the virus, whereas some others served as uninfected controls. The virus was added at a multiplicity of infection of 20 in 1- to 2-ml of medium and adsorbed for 1 h with frequent tilting of the plates. The unadsorbed virus was aspirated, and the cells were incubated in 8 ml of MEM with 10% FCS. These cells were labeled with $^{32}P_i$ at various times after virus infection as above. The labeling medium also contained 2% dialyzed FCS. S-10 extracts were prepared and analyzed on slab gels as above.

(ii) Cell extracts. Cells were grown in 150-mm tissue culture dishes to confluence. One group (10 plates) was treated with mouse interferon (200 reference units/ml, 18 h) and another served as a control. The cells were washed three times with buffer containing 35 mM Tris-hydrochloride (pH 7.5) and 146 mM NaCl. To each plate was added 1 ml of hypotonic buffer (10 mM Tris-hydrochloride [pH 7.5], 15 mM KCl, 1.5 mM Mg-acetate, and 7 mM β-mercaptoethanol) and spread uniformly. The cells were allowed to swell in the cold for 5 min, and the buffer was sucked off. The cells were scraped with a rubber policeman, collected in a Dounce homogenizer cup and lysed by 30 strokes of a tight piston. The ionic concentration was raised by adding buffer containing 0.25 M Tris-hydrochloride (pH 7.5), 0.8 M KCl, and 0.04 M Mg-acetate (70 μl per ml of lysate). The lysate was centrifuged at 800 × *g* for 10 min and then at 10,000 × *g* for 15 min. The resultant supernatant was collected and

dialyzed in the cold against 300 ml of buffer containing 10 mM HEPES-KOH (pH 7.5), 80 mM KCl, 1.5 mM Mg-acetate, and 7 mM β-mercaptoethanol for 5 h with one buffer change. The dialyzed extract was centrifuged at 10,000 × *g* for 5 min, and the supernatant (S-10) was stored in portions in liquid nitrogen.

To test for protein phosphorylation, incubation mixtures (50 μl) containing 20 mM HEPES-KOH (pH 7.5), 120 mM KCl, 3.5 mM Mg-acetate, 7 mM β-mercaptoethanol, S-10 extract (absorbancy at 260 nm of 0.6), [γ-^{32}P]ATP (116 μM, specific activity 1,350 mCi/mmol) and, when added, poly(rI·rC) (200 ng/ml) were incubated at 30°C for 10 min. A 20-μl portion of each sample (containing ~100,000 hot trichloroacetic acid-precipitable counts) was fractionated by SDS-PAGE in slab gels and autoradiographed as above.

RESULTS

Poly(rI·rC) is cytotoxic to interferon-treated L929 cells. It has been reported that interferon treatment of various cell types makes them susceptible to dsRNA which causes them to lyse (37, 38). The L929 cells used for this study are very sensitive to this effect of interferon. Cells treated with moderate levels of interferon (100 to 200 reference units/ml) developed strong cytotoxicity within 2 h when exposed to 10 or 25 μg of poly(rI·rC) per ml. When cells were treated with lower levels of interferon (25 reference units/ml), higher levels of poly(rI·rC) (50 μg/ml) or longer intervals were required for cytotoxicity to develop. Cells not treated with interferon did not show any cytotoxicity upon similar treatment with poly(rI·rC).

It may be mentioned that the two other lines of mouse L-cells which were tested for this effect of interferon showed much slower development of cytotoxicity after treatment with poly(rI·rC) (50 μg/ml). Therefore it seems that the sensitivity of various cell lines to this effect is variable. Whether this is due to differences in the permeability to dsRNA is not known. The L929 cell line which showed high sensitivity to poly(rI·rC) after interferon treatment was chosen for studies presented here with the assumption that these cells may be more sensitive to the effect of dsRNA added exogenously or appearing endogenously.

Enhanced phosphorylation of specific protein(s) in interferon-treated cells and in their extracts in the presence of dsRNA, poly(rI·rC), or reovirus dsRNA. Various groups have reported that phosphorylation of at least two proteins is greatly enhanced in extracts prepared from interferon-treated cells in the presence of added dsRNA (26, 31, 42). A similar result was obtained with extracts prepared from interferon-treated L929 cells used here (Fig. 1). Phosphorylation of two protein bands (indicated by arrows) was enhanced in extracts prepared from interferon-treated cells when poly(rI·rC) was included in the reaction mixture (tracks 3 and 4). These proteins migrated in SDS-gels with mobilities corresponding to molecular weights ~65,000 (65K) and ~38,000 (38K). These estimates compare well with those reported by

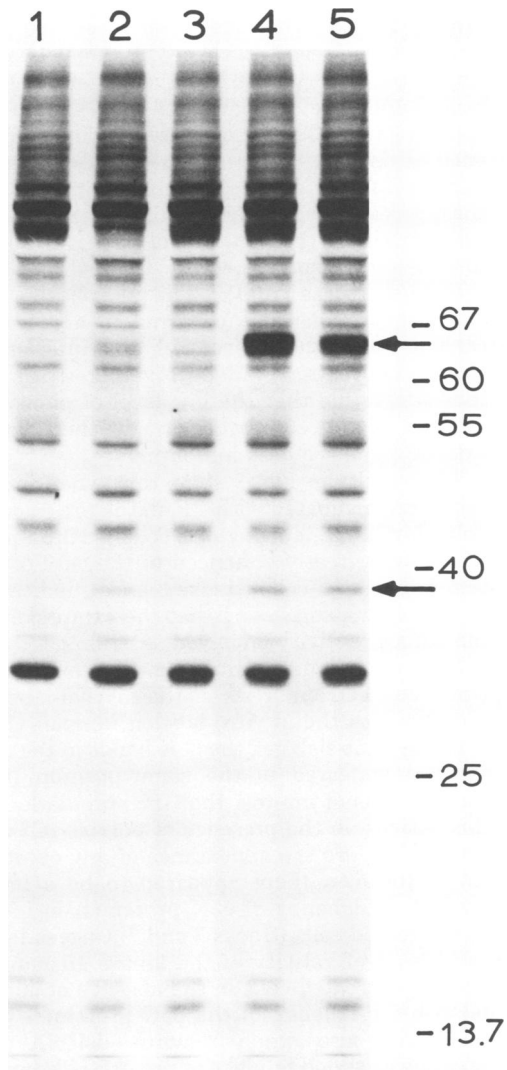

FIG. 1. *Protein phosphorylation in control and interferon-treated L929 cell extracts in vitro. Incubations were carried out with either control (tracks 1 and 2) or interferon-treated cell extracts (tracks 3 and 4) as described in the text in the presence of [γ-^{32}P]ATP in the absence (tracks 1 and 3) or presence (tracks 2 and 4) of poly(rI·rC) (200 ng/ml). Track 5 represents a reaction carried out with control and interferon-treated cell extracts added together (absorbancy at 260 nm of 0.3 each) in the presence of poly(rI·rC).*

others (26, 42). The identity of these two proteins has not been established. By analogy of a similar phosphorylation observed in reticulocyte lysates upon addition of dsRNA (9, 27), the small component (35-38K) phosphorylated in the presence of dsRNA could be the small subunit of initiation factor eIF-2. The phosphorylation of these two bands was also enhanced in control cell extracts in the presence of poly(rI·rC) but to a lesser extent, especially that of the 65K protein(s) (tracks 1 and 2). The phosphorylation of these proteins also occurred in a reaction carried out with the control and interferon-treated cell extracts added together (track 5), thus suggesting that the low level of phosphorylation of the 65K protein(s) in the control extract was not due to inhibitors.

The results in Fig. 2 show that when intact cells were incubated with $^{32}P_i$ in the presence of poly(rI·rC), ^{32}P-labeling of a 65,000-dalton protein(s) was greatly enhanced in the case of interferon-treated cells (tracks 3 and 4, indicated by an arrow), suggesting that the expression of the kinase activity involved is stimulated by poly(rI·rC) under physiological conditions. Upon co-electrophoresis of the in vivo-labeled samples with the in vitro-labeled samples (Fig. 1) in the same slab gel, the 65K bands in the two samples appeared in the same position (not shown). It is of interest that the stimulation of this activity in the presence of poly(rI·rC) was detected before the appearance of any cytotoxicity. However, there appeared to be a basal level of ^{32}P-labeling of 65K protein(s) in interferon-treated cells (tracks 3 and 7), suggesting a basal level of activity of the kinase enzyme in interferon-treated cells in the absence of added dsRNA. ^{32}P-labeling of the 65K protein(s) was stimulated also when reovirus dsRNA (25 μg/ml) was added in place of poly(rI·rC) during labeling (data not shown).

It should be pointed out that the enhancement in phosphorylation of 38K component observed when extracts from interferon-treated cells are incubated with [γ-^{32}P]ATP and dsRNA was not observed upon ^{32}P-labeling in vivo. Whether this was due to a lower specific activity of labeling obtained under in vivo labeling conditions which made it undetectable or whether the phosphorylation of the 38K component was not enhanced in vivo upon such treatment with poly(rI·rC) is not clear. Even under in vitro labeling conditions when the 65K protein(s) is (are) very heavily labeled, the enhancement in the labeling of 38K protein(s) is much smaller. This may also depend on the amount of endogenous 38K protein(s) available for phosphorylation in situ.

Characteristics of ^{32}P-labeled 65K and other bands identified in slab gels. Various tests were carried out to analyze the characteristics of the ^{32}P-labeled components identified in autoradiographs. The pattern of labeling of the 65K band, and most other bands, was similar when ^{32}P-labeling was done in the presence of actinomycin D (2 μg/ml) to block the incorporation of ^{32}P into RNA (data not shown). Furthermore, the 65K and several other bands were resistant to RNase treatment but were sensitive to treatment with Pronase (Fig. 3a). These results and the fact that the ^{32}P label was not dissociated upon solubilization in hot SDS indicates that the label is covalently associated with proteins. (Pronase-treated samples often displayed some faint bands in the lower portion of the autoradiograms below 30,000 molecular weight but showed no bands in the region of larger proteins. These might be due to RNA.) The ^{32}P-labeled protein bands were labile to treatment with alkali (1 N NaOH, 50°C, 15 min, not shown). These properties are consistent with the 65K band and several others being phosphoproteins.

Upon high-speed centrifugation, the bulk of the ^{32}P-labeled 65K protein(s) was pelleted with the ribosomes when it appeared as the strongest band in autoradiograms (Fig. 3b). Similar distribution was observed by Zilberstein et al. (42) while testing for phosphorylation in vitro.

If the interferon treatment was given in the presence of actinomycin D (2 μg/ml), the ^{32}P-labeling of the 65K band was diminished and little, if any, enhancement was obtained in the presence of poly(rI·rC) (data not shown), suggesting that one or more of the components involved in this kinase activity may be synthesized de novo after interferon treatment. Actinomycin D is known to block the establishment of the interferon-induced antiviral state (39).

Phosphorylation of 65K protein(s) in interferon-treated uninfected and virus-infected cells. The fact that the phosphorylation of 65K protein(s) is enhanced in intact cells upon addition of poly(rI·rC) indicates that the intracellular expression of the kinase activity involved is subject to a similar effect of dsRNA as that observed in vitro. This should allow us to question whether the expression of this enzyme activity in interferon-treated cells is enhanced upon virus infection, which should be the case if the antiviral mechanism is activated upon virus infection due to the accumulation of dsRNA. Thus an increase in dsRNA after virus infection should be reflected in enhanced activity of this and other interferon-induced enzymes which are stimulated by dsRNA. Figure 4 shows the results

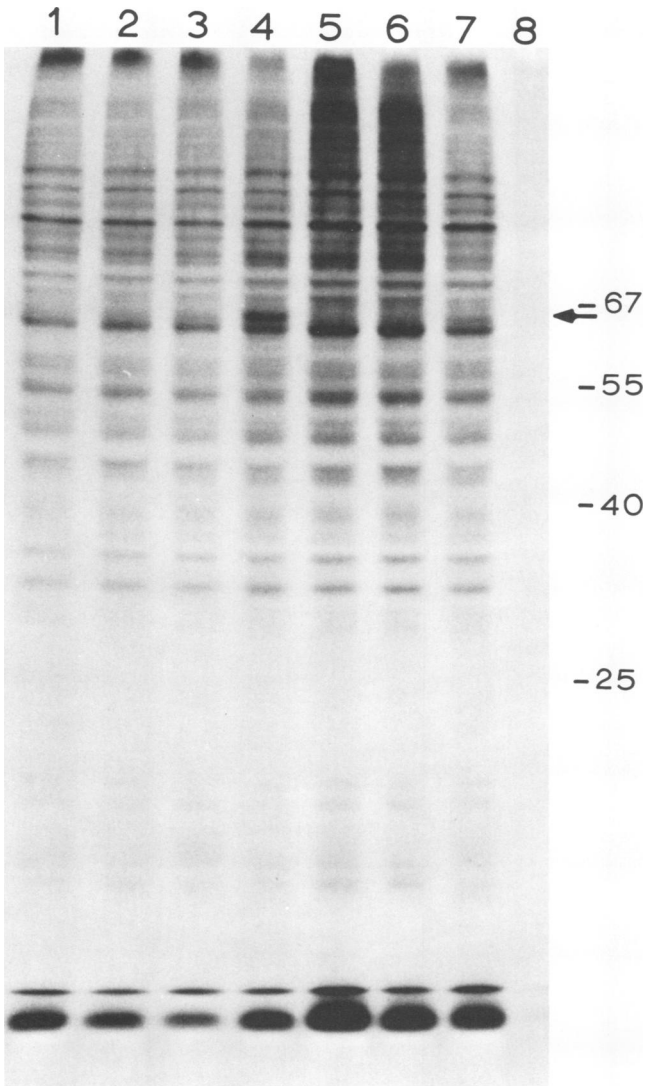

FIG. 2. *^{32}P-labeling of proteins in untreated and interferon-treated L929 cells and the effect of poly(rI·rC). Control and interferon-treated L929 cells in 100-mm plates were washed and incubated with phosphate-free MEM without serum (4 ml). poly(rI·rC) (25 μg/ml final) was added to four plates in each group, whereas another four plates served as control. One set of two plates in each subgroup was labeled with $^{32}P_i$ for 1 h right after the addition of poly(rI·rC) (0 to 1 h of labeling). The other set of plates was labeled with ^{32}P 2 h after treatment with poly(rI·rC) (2 to 3 h of labeling). Cell extracts were prepared after labeling and 40 μl of each was analyzed on SDS-slab gels as described in the text. Tracks 1–4, 0 to 1 h of labeling; tracks 5–8, 2 to 3 h of labeling. Control cells, tracks 1, 2, 5, and 6; interferon-treated cells, tracks 3, 4, 7, and 8. The even numbers represent samples from cells treated with poly(rI·rC); the odd numbers represent samples not treated with poly(rI·rC). Sample number 8 contained very few ^{32}P counts in hot trichloroacetic acid-precipitable material, which reflects the fact that these cells were already lysed within 2 h of poly(rI·rC) treatment.*

of ^{32}P-labeling in uninfected and mengovirus-infected cells (untreated or pretreated with 200 reference units/ml of interferon). It is evident that there was a basal level of ^{32}P-labeling of the 65K protein(s) in interferon-treated cells (tracks 6 and 7). Moreover, there was no distinct enhancement in labeling of this band upon infection with mengovirus at any of the time points analyzed (tracks 8, 9, and 10). Under the conditions of interferon treatment employed, the yield of mengovirus was reduced by more than 2 logs. The overall incorporation of ^{32}P into hot trichlo-

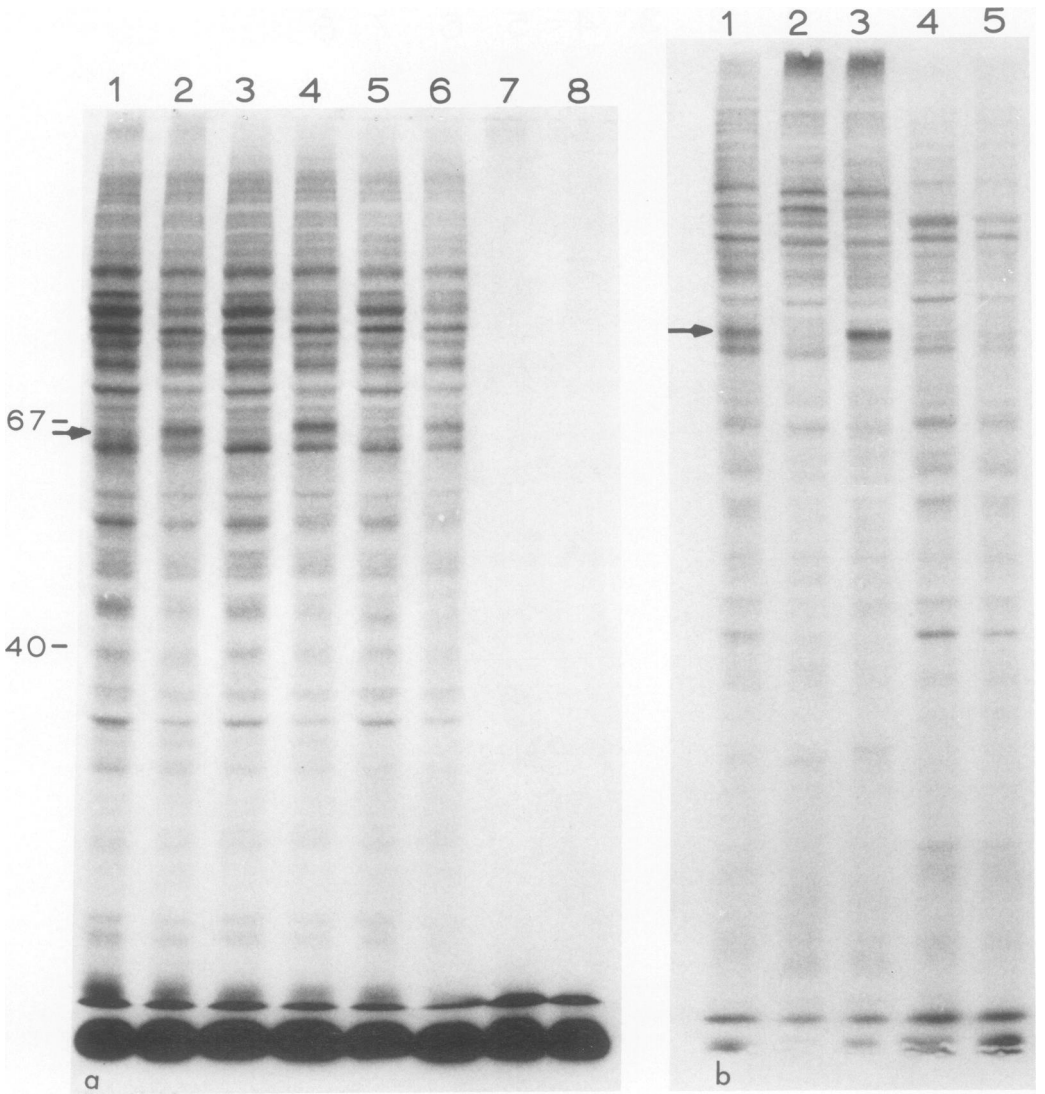

FIG. 3. *Characteristics of ^{32}P-labeled 65K and other bands identified in SDS-slab gels. (A) Effect of RNase and Pronase. Control and interferon-treated L929 cells were labeled with $^{32}P_i$ for 1 h in the presence of poly(rI·rC) (25 μg/ml), and extracts were prepared. Portions of these extracts were analyzed on SDS-slab gels as such (tracks 1 and 2), or after treatment with pancreatic RNase (100 μg/ml, in presence of 15 mM EDTA) either at 0 to 4°C for 1 h (tracks 3 and 4) or at 37°C for 10 min (tracks 5 and 6), or after Pronase treatment (250 μg/ml, Calbiochem, 45 U/mg of protein) at 37°C for 10 min (tracks 7 and 8). The even numbers represent sample from interferon-treated cells; and odd numbers represent sample from control cells. (B) Subcellular distribution of ^{32}P-labeled 65K protein(s). Portions of the two extracts were centrifuged at 150,000 × g for 90 min. The ribosomal pellets were suspended in a small volume of buffer (10 mM Tris-hydrochloride, pH 7.4, 10 mM KCl, and 1 mM $MgCl_2$). Portions of ribosomal and supernatant fraction (~30,000 hot trichloroacetic acid-insoluble cpm) were analyzed on SDS-slab gels in parallel with S-10 from interferon- and poly(rI·rC)-treated cells (track 1). Ribosomal and supernatant fractions from control cells (tracks 2 and 4, respectively), and from interferon-treated cells (tracks 3 and 5, respectively).*

roacetic acid-insoluble material was reduced, particularly in interferon-treated cells, at 3 or 6 h after infection with mengovirus. Therefore, approximately equal hot trichloroacetic acid-insoluble counts in each sample were loaded on gels. It was established in a separate experiment that the enhanced labeling of 65K protein(s) with ^{32}P observed in interferon-treated cells in

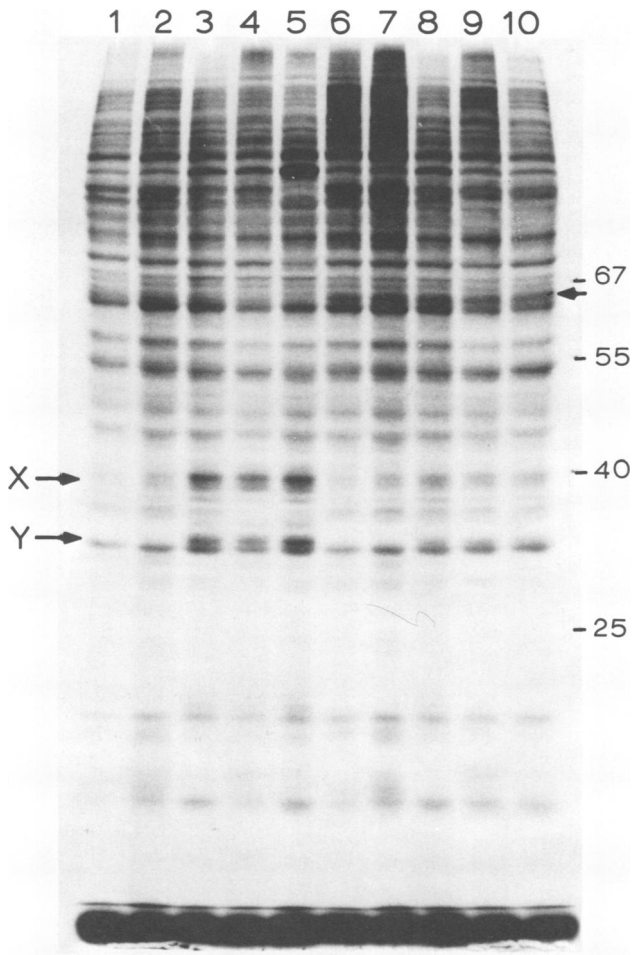

FIG. 4. *^{32}P-labeling of proteins in control and interferon-treated, uninfected and mengovirus-infected cells. Interferon-treated (200 reference units/ml) and control L929 cells in 100-mm plates were divided into two groups. One group was infected with mengovirus at a multiplicity of infection of ≃20. The virus was adsorbed for 1 h with frequent tilting of the plates and the unadsorbed virus was aspirated off. One plate from each group was used immediately for labeling with $^{32}P_i$ as described in the text. Other plates were incubated with 8 ml of MEM containing 10% heat-inactivated FCS and used for labeling at different intervals. Extracts were prepared and analyzed on SDS-slab gels. Samples 1–5 are from control cells and 6–10 are from interferon-treated cells. Samples 1, 2, 6, and 7 are derived from uninfected cells, whereas samples 3–5 and 8–10 are from virus-infected cells labeled either at 0 to 1 h after virus infection (samples 1, 3, 6, and 8), 3 to 4 h after virus infection (samples 4 and 9), or 6 to 7 h after infection (samples 2, 5, 7, and 10). Approximately equal hot trichloroacetic acid-insoluble ^{32}P counts (45,000 to 55,000 cpm) in each sample were analyzed.*

the presence of poly(rI·rC) was not inhibited in mengovirus-infected cells (data not shown).

It is noticeable that mengovirus infection of control cells led to an enhanced labeling with ^{32}P of a band with a molecular weight of approximately 38,000 and occasionally another with a molecular weight of about 32,000 (Fig. 4). Whether this represents labeling of viral proteins or of host proteins, and whether this 38,000-dalton component is related to the small subunit of eIF-2, which is phosphorylated under various conditions, is not known.

Figure 5 shows a similar experiment carried out with VSV infection. This is a negative-strand RNA virus and carries its own transcriptase enzyme which is apparently responsible for the production of viral mRNA after infection (40). ^{32}P-labeling of proteins in untreated and interferon-treated cells was analyzed at various time intervals after infection with VSV. Once

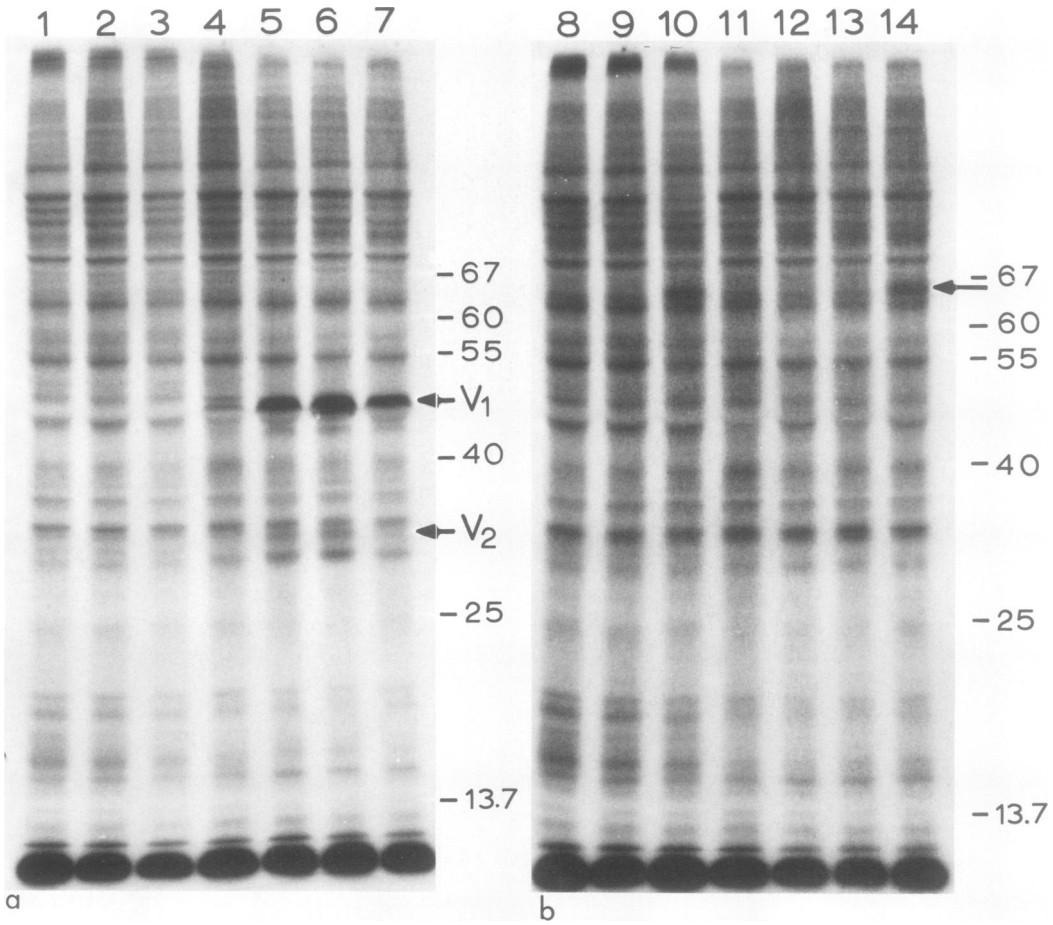

FIG. 5. *^{32}P labeling of proteins in control and interferon-treated, uninfected and VSV-infected L929 cells. Interferon-treated (250 reference units/ml) and control L929 cells in 100-mm plates were divided into two groups. One group in each was infected with VSV at a multiplicity of infection of 20 as in Fig. 4. The uninfected and VSV-infected cells were labeled with $^{32}P_i$ at various time intervals, and extracts were prepared and analyzed on slab gels. Samples 1–7 are from cells not treated with interferon, and samples 8–14 are from cells treated with interferon. Samples 1–3 and 8–10 are from uninfected cells, whereas samples 4–7 and 11–14 are from VSV-infected cells. Samples 1, 4, 8, and 11 were labeled with ^{32}P 30 min after virus infection (0.5 to 1.5 h of labeling), samples 2, 3, 5, and 7 and 9, 10, 12, and 14 at 3 h after infection (3 to 4 h of labeling), and samples 6 and 13 at 6 h after infection (6 to 7 h of labeling). Samples 3, 7, 10, and 14 were labeled with ^{32}P in the presence of 25 μg of poly(rI·rC) per ml.*

again a basal level of ^{32}P-labeling of 65K protein(s) was observed in interferon-treated uninfected cells (Fig. 5b, tracks 8 and 9), and there was no visible enhancement upon VSV infection at any of the time points analyzed (tracks 11, 12, and 13). In this case also the incorporation of ^{32}P into hot trichloroacetic acid-insoluble material was lower at 3 and 6 h after virus infection, and approximately equal counts were analyzed on gels. Under the conditions of interferon treatment, the yield of VSV was reduced by 98% or more.

The lack of an obvious enhancement in ^{32}P-labeling of 65K protein(s) in interferon-treated cells upon virus infection was apparently not due to an inhibitory effect of virus infection on stimulation by dsRNA. ^{32}P-labeling of 65K protein(s) was stimulated by poly(rI·rC) in interferon-treated uninfected or virus-infected cells (Fig. 5b, tracks 10 and 14). It appears that this band moved slightly slower in the case of samples that were labeled in the presence of poly(rI·rC) (tracks 10 and 14).

It may be argued that the levels of interferon used in the above experiments are high enough to cause a basal level of expression of the kinase

activity which may be enough for the inhibition of viral replication, and that if lower levels of interferon are used, the kinase activity may indeed be stimulated upon virus infection. To test this possibility, L929 cell cultures were treated with 25 reference units of interferon per ml and ^{32}P-labeling of 65K protein(s) was analyzed in uninfected or VSV-infected cells. At this level of interferon, there was little, if any, basal level of ^{32}P-labeling of 65K protein(s); it was stimulated by 25 μg of poly(rI·rC) per ml, but again no distinct increase was observed upon infection with VSV as determined right after or 4 h after virus infection (data not shown).

The samples from control cells infected with VSV gave two new bands, one of them labeled more strongly with ^{32}P than the other (Fig. 5a, V_1 and V_2). These migrated with mobilities corresponding to molecular weights between 44,000 and 46,000 and between 30,000 and 31,000, respectively. These were probably due to VSV-specific proteins NS and M, respectively, which are known to be phosphorylated in VSV-infected cells (18, 28, 35). It is not surprising that these bands did not appear in samples obtained from interferon-treated cells infected with VSV (Fig. 5b, tracks 11–14).

DISCUSSION

The fact that the enzymes that are induced in cells after treatment with interferon are strongly activated by dsRNA (26, 29, 31, 34, 42) suggests a possible role for dsRNA in interferon action. The possibility that the antiviral mechanism induced by interferon is a latent mechanism to be activated only upon virus infection, e.g., due to accumulation of dsRNA, is very attractive. Biosynthesis of cellular and viral macromolecules have common features and share common components. Thus any mechanism induced to block a replicative function common to a wide variety of viruses sensitive to interferon may be expected to have undesirable effects on cellular functions as well. A two-step mechanism such as above would overcome this problem if the antiviral mechanism would be operative only upon virus infection.

Interferon-induced protein kinase activity was used as a test system to question whether virus infection of interferon-treated cells leads to an activation of interferon-induced enzymes (due to an accumulation of dsRNA, or otherwise), which might be expected if the antiviral mechanism operated in two steps. The results show that the labeling of a specific protein band of ~65,000 daltons with $^{32}P_i$ in interferon-treated intact cells was greatly enhanced in the presence of added dsRNA, poly(rI·rC) (Fig. 2–5), or reovirus dsRNA (data not shown) similar to that observed in extracts (Fig. 1 [26, 31, 42]). This suggests that the kinase activity involved was stimulated by dsRNA in an intracellular environment. However, significant ^{32}P-labeling of the 65K protein(s) did occur in interferon-treated cells, suggesting a basal level of activity for the kinase enzyme.

If the antiviral mechanism is activated upon virus infection through accumulation of dsRNA, a similar enhancement in ^{32}P-labeling of the 65K protein(s) should be expected upon virus infection of interferon-treated cells. It was somewhat disappointing therefore to find that infection of interferon-treated cells with two different types of viruses (mengovirus and VSV) did not reveal any obvious enhancement of this kinase activity as determined by ^{32}P-labeling of 65K protein(s) in vivo. Since, under the conditions employed, the yield of either virus was reduced by about 2 logs in interferon-treated cells, the results suggest that infection of interferon-treated cells with either of these viruses does not lead to a gross change in this kinase activity expressed in vivo.

The experiments presented here need not imply that dsRNA may not have a role in the antiviral mechanism. The fact that some phosphorylation of 65K protein(s) was detectable in interferon-treated cells in the absence of added dsRNA suggests that the kinase activity is expressed at a basal level in vivo. It is conceivable that there is endogenous dsRNA in the cell which maintains a basal level of expression of this (and possibly other interferon-induced) enzyme(s). Double-stranded regions are known to be present in nuclear RNA (7, 24); dsRNA also has been isolated from various animal cells and tissues (15, 36).

Other arguments can be made in favor of the results obtained. If the antiviral mechanism depended entirely on the contribution of dsRNA from virus infection, this would require that all interferon-sensitive viruses should lead to the accumulation of dsRNA upon infection of interferon-treated cells (preferably at an early stage since an inhibition of viral mRNA and/or protein accumulation is detectable early after infection of interferon-treated cells [10]). Any viruses which did not do so would be able to bypass the interferon-mediated antiviral mechanism. Moreover, such a requirement might serve as a driving force for interferon-resistant viruses to be evolved.

It should be emphasized that the findings reported here using interferon-induced kinase activity as a test system will have to be verified for other interferon-induced enzymes (i.e., the

2′,5′-oligoA synthesizing enzyme [17] and the endonuclease$_{INT}$ [29, 30]) to test whether their activity is increased upon virus infection of interferon-treated cells. The role and relative significance of various interferon-induced enzymes in the inhibition of various different viruses are presently unclear. The possibility that the two viruses used in this study cause an activation of other interferon-induced enzymes upon infection and not the protein kinase cannot be excluded.

The stimulation of the 65K protein phosphorylation in interferon-treated cells upon incubation with poly(rI·rC) can be detected before the appearance of any cytotoxicity. One wonders whether the stimulation of this and/or other dsRNA-activated enzyme(s) has a role to play in the cell-killing effect of dsRNA observed with certain cell lines following interferon treatment. The interferon-induced protein kinase, endonuclease$_{INT}$ and the 2′,5′-oligoA seem to be active on viral as well as cellular mRNA in vitro (17, 29; Sen et al., personal communication). It is of interest that interferon-treated mouse L-cells are lysed more rapidly than untreated cells upon infection with vaccinia virus (16, 19, 38), which is known to produce dsRNA both in vivo and in vitro (3).

In brief, the fact that the activity of the interferon-induced protein kinase enzyme is stimulated by dsRNA but apparently not upon infection with mengovirus or VSV leaves a number of possibilities open, e.g.: (i) that under the conditions employed the effect of mengovirus or VSV infection on the interferon-induced kinase activity is below the limits of detection, although added poly(rI·rC) can still cause an enhancement; (ii) that the antiviral mechanism induced after interferon treatment does not depend greatly on the appearance of dsRNA as a signal after virus infection; (iii) that interferon-induced enzyme(s) other than the protein kinase might be activated by these two viruses; and (iv) that the dsRNA activation of protein kinase (and other interferon-induced enzymes) is related to the cell-killing effect of dsRNA on interferon-treated cells. Whether any of these possibilities is correct remains to be determined.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants NCI-CA-08748-12 and in part by CA-20490-02 from the National Cancer Institute.

I am thankful to Leota L. Mehra, M. Yee, and A. Hibbs for skilled technical assistance, J. Davis for typing the manuscript, P. Lengyel (Yale University) for a generous gift of mouse interferon and M. Krim for encouragement.

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