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Interferon-induced inhibition of protein synthesis in L-cell extracts: An ATP-dependent step in the activation of an inhibitor by double-stranded RNA

(encephalomyocarditis virus/cell-free systems)

WALDEN K. ROBERTS*, MICHAEL J. CLEMENS†, AND IAN M. KERR

Division of Biochemistry, National Institute for Medical Research, Mill Hill, London NW7 1AA

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ABSTRACT **The translation of encephalomyocarditis virion RNA in extracts from interferon-treated L-cells is inhibited by the addition of double-stranded RNA (dsRNA) at 400 ng/ml. A similar inhibition in response to dsRNA is seen in control cell extracts supplemented with small amounts of a postribosomal supernatant fraction from interferon-treated cells (interferon cell sap). Neither interferon cell sap nor dsRNA alone is inhibitory in control systems. The inhibition is much reduced if translation is carried out at low ATP concentrations. Conversely, the inhibitory capacity of the interferon cell sap is increased 100-fold if it is preincubated with dsRNA and ATP prior to its addition to the protein-synthesizing system. After this preincubation all detectable dsRNA can be removed without any diminution of the inhibitory activity of the cell sap. These results are compatible with a two-step model for the inhibition in which a pre-inhibitor is activated by dsRNA, the activated inhibitor then interacting with the protein synthesis system to inhibit translation.**

Double-stranded RNA (dsRNA) inhibits protein synthesis in animal cells and cell-free systems (1–4). Interferon treatment of cells renders them more sensitive to the toxic effects of dsRNA (5); and with extracts from interferon-treated L-cells, the translation of encephalomyocarditis virion RNA (EMC RNA) in the cell-free system shows an enhanced sensitivity to inhibition by dsRNA (6). The latter inhibition is specific for dsRNA, and the interferon dose-response curve and heat-inactivation studies show a close correlation between the antiviral activity of interferon and the enhanced sensitivity to dsRNA observed in the cell-free system (6).

Recently we have found that the addition of small amounts of a postribosomal supernatant fraction from interferon-treated cells (interferon cell sap) renders cell-free protein-synthesizing systems from control cells sensitive to inhibition by dsRNA‡. This has permitted the quantitation of a putative dsRNA-dependent inhibitor in the interferon cell sap. Here we show that this inhibitor requires activation and that the activation is dependent upon dsRNA and ATP.

MATERIALS AND METHODS

EMC virus was purchased from G. D. Searle and Co. Ltd., High Wycombe, England and the viral RNA extracted as before (7). The dsRNA was from *Penicillium chrysogenum* phage, a generous gift from Dr. C. Burbidge. The [^{3}H]uridine-labeled poliovirus dsRNA (replicative form) was prepared according to Spector and Baltimore (8).

Preparation of L-Cell Extracts. L-cells were grown in suspension culture and, where indicated, treated with 200–300 International Reference Units of interferon per ml for 17 hr at 37° (9). Ten reference units were equivalent to approximately one effective unit in reducing the yield of EMC by 50% in samples of the L-cells used in the preparation of the cell-free systems. L-cell interferon was from Dr. K. Paucker and had been purified by affinity chromatography to $\geq 5 \times 10^7$ reference units/mg of protein (10). Postmitochondrial supernatant fractions (S10) were prepared from L-cells (9) and preincubated and dialyzed as described (11). Interferon and control cell saps were prepared from S10 that had not been preincubated. These were centrifuged at 100,000 × *g* for 2 hr at 4°, and the supernatant cell saps were dialyzed as above.

Amino Acid Incorporation Assays. Preincubated and dialyzed S10 from control cells provided the ribosomes, enzymes, factors, and tRNA for the amino acid incorporation assays. Interferon and control cell saps and dsRNA were added with and without preincubation, as indicated in the individual experiments. The conditions used in the assay of the incorporation of a mixture of fourteen ^{14}C-labeled amino acids (54 mCi/milliatom of carbon, The Radiochemical Centre, Amersham, England) were as described, with the omission of the reticulocyte initiation factors (11). Incubations were for 120 min at 30° unless otherwise stated, and 10-μl aliquots were assayed for amino acid incorporation.

RESULTS

Throughout this work incorporation of ^{14}C-labeled amino acids in response to EMC RNA was measured in cell-free systems using preincubated and dialyzed extracts (S10) from control L-cells. With this as the basic assay, the inhibitory effects of the addition of interferon cell sap and dsRNA on the translation of EMC RNA was investigated.

Requirement for both interferon cell sap and dsRNA for inhibition of translation

Under the assay conditions used for this investigation, no significant inhibition of the translation of EMC RNA was observed when interferon cell sap alone was added to the reaction mixture. Some slight inhibition was occasionally observed on the addition of dsRNA alone, but this was never greater than 10–15%. The addition of small amounts of both interferon cell sap and dsRNA, however, resulted in a 65–85% inhibition, depending upon the preparation and concentrations used in the assay (Fig. 1).

Abbreviations: dsRNA, double-stranded RNA; EMC virus, encephalomyocarditis virus; S10, postmitochondrial supernatant fractions.

* Permanent address: Department of Microbiology, University of Colorado Medical School, Denver, Colo. 80220.
† Present address: Department of Biochemistry, St. George's Hospital Medical School, Tooting, London SW17 0QT.

‡ I. M. Kerr, R. E. Brown, M. J. Clemens, and C. S. Gilbert, manuscript submitted for publication.

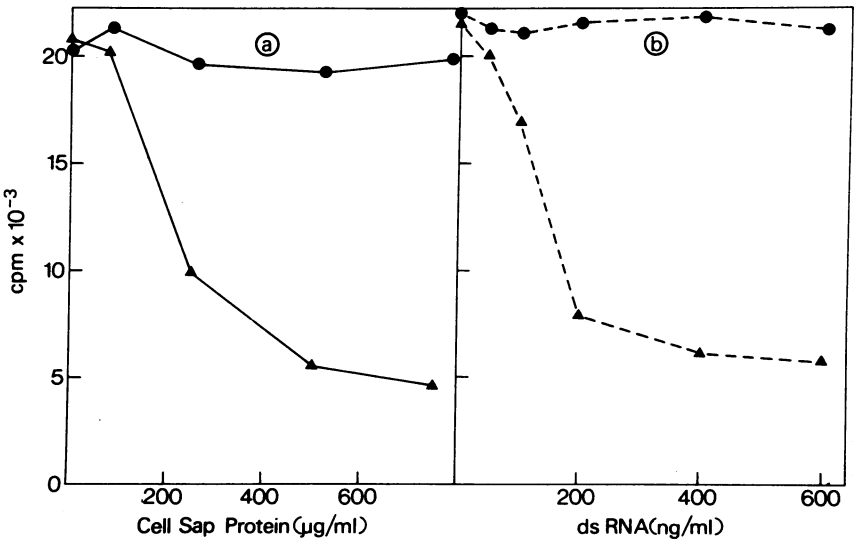

FIG. 1. Inhibition of the translation of EMC RNA in the presence of interferon cell sap and dsRNA. The translation of EMC RNA was assayed in cell-free systems using preincubated and dialyzed S10 from control cells. (a) Incorporation of ^{14}C-labeled amino acids with dsRNA (400 ng/ml) and varying concentrations of cell sap from (▲) interferon-treated or (●) control cells. (b) Incorporation of ^{14}C-labeled amino acids with (▲) interferon cell sap (500 μg of protein per ml) or (●) control cell sap (520 μg of protein per ml) and varying concentrations of dsRNA.

ATP requirement for inhibition of translation

A number of studies have suggested that protein kinases might be involved in control of protein synthesis in animal cells (reviewed in ref. 12). If a protein kinase is involved in the inhibition illustrated in Fig. 1, and if this kinase has a Michaelis constant (K_m) for ATP significantly larger than the K_m values of the aminoacyl-tRNA synthetases, then limiting the ATP concentration in the protein synthesis assay should reduce the inhibition while still permitting limited protein synthesis to occur. To test this possibility, we investigated the translation of EMC RNA and its inhibition by interferon cell sap and dsRNA at varying ATP concentrations (Fig. 2). For these assays, protein synthesis was carried out both in systems in which the Mg^{++} concentration was held constant at 2 mM and in systems in which the change in ATP concentration was compensated for by an equimolar change in the Mg^{++} concentration. In both types of experiments there was only a 12% inhibition by interferon cell sap with dsRNA when the assays were performed in the absence of any added ATP. (The limited amount of protein synthesis that occurs under these conditions is presumably supported by residual endogenous ATP in the dialyzed S10 and cell sap preparations, this being maintained by the energy-generating system included in the assays.) The inhibition increased from 12% to greater than 80% on the addition of ATP, which appears, therefore, to be required for the full development of the inhibition (Fig. 2a and b).

Effect of preincubation of interferon cell sap on kinetics of the inhibition

The inhibition of EMC RNA translation by interferon cell sap and dsRNA was seldom greater than 80% when assayed by the usual procedure. This incomplete inhibition could result from (*i*) a partial inactivation of the protein synthesis system that occurs immediately and persists throughout the course of protein synthesis, (*ii*) a complete inactivation of the system that requires an incubation period to develop, or (*iii*) an immediate complete inactivation of the system that is partially reversed upon prolonged incubation. To distinguish between these possibilities we examined the kinetics of inhibition of the translation of the viral RNA by interferon cell sap and dsRNA.

In the uninhibited system in the presence of interferon cell sap alone, after a short lag period incorporation proceeds linearly for 40 min, then gradually declines until the termination of the assay at 120 min (Fig. 3, filled circles). Addition of both dsRNA

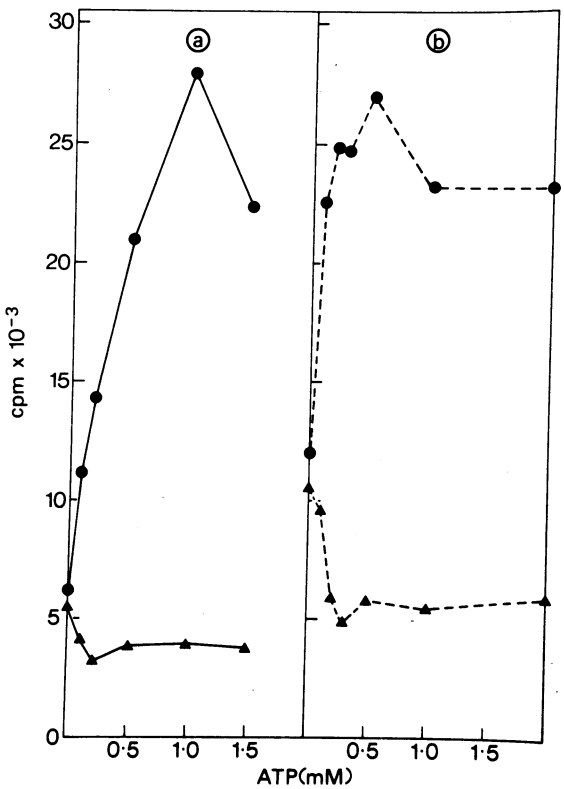

FIG. 2. Effect of ATP concentration on the inhibition of translation. Incorporation assays of ^{14}C-labeled amino acids were carried out as in the legend of Fig. 1. ATP was added as indicated. (a) At 2 mM Mg^{++}; (b) at 1 mM Mg^{++} plus concentrations of Mg^{++} equal to that of the added ATP. (▲) Interferon cell sap (500 μg of protein per ml) plus 400 ng/ml of dsRNA; (●) control cell sap (520 μg of protein per ml) plus 400 ng/ml of dsRNA.

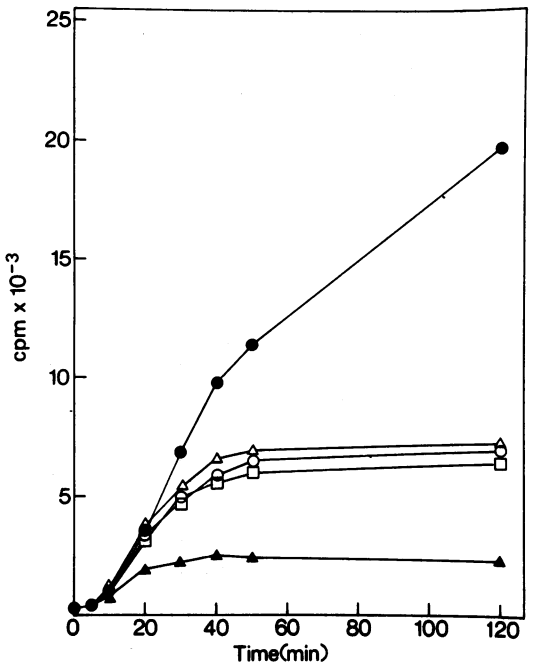

FIG. 3. Activation of interferon cell sap by preincubation with dsRNA and ATP: effect on the kinetics of the inhibition of the translation of EMC RNA in the cell-free system. Incorporation assays of ^{14}C-labeled amino acids were carried out as in the legend of Fig. 1 with the following additions: interferon cell sap, no preincubation, (●) minus and (□) plus dsRNA; interferon cell sap preincubated with (△) ATP, (○) dsRNA, (▲) ATP and dsRNA. Preincubation of the cell sap (500 μg of protein per ml) was for 10 min at 30° under the salt conditions for the amino acid incorporation assay with 1 mM ATP and 400 ng/ml of dsRNA, where indicated. Translation was initiated by the addition of the missing components of the amino acid incorporation system, dsRNA (if not already present), and EMC RNA; and incubation at 30° was continued. At the times indicated, 10-μl aliquots were removed and analyzed for acid-insoluble radioactivity.

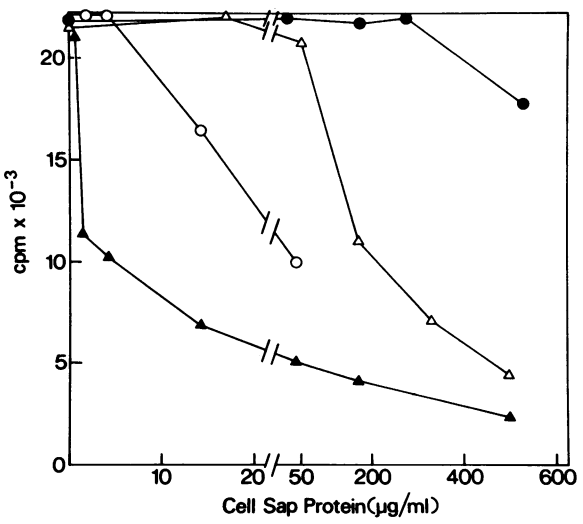

FIG. 4. Concentration of activated cell sap required for inhibition of the translation of EMC RNA. Cell saps were activated by preincubation for 40 min at 30° in 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) (pH 7.5), 90 mM KCl, 1.5 mM magnesium acetate, 7 mM 2-mercaptoethanol, with or without 400 ng/ml of dsRNA and 1 mM ATP, as indicated. After preincubation, samples were removed, diluted in the above buffer as appropriate, and assayed for their effect on the translation of EMC RNA in the cell-free system as in the legend of Fig. 1. (●) Control cell sap preincubated with ATP and dsRNA, dsRNA (400 ng/ml) added to the assays. Interferon cell sap preincubated with (△) ATP but no dsRNA, dsRNA (400 ng/ml) added to the assay; (○) dsRNA but no added ATP, no additional dsRNA added to the assays; (▲) ATP and dsRNA, no additional dsRNA added to the assays.

and interferon cell sap to the system results in little inhibition of incorporation for the first 20 min of incubation followed by an increasing inhibition which is virtually complete by 50 min (Fig. 3, squares). Thus, the inhibition is apparently expressed by mechanism (*ii*) above, a lag period followed by a virtually complete inactivation of the system.

These results suggested that the initial lag in inhibition might be eliminated and the inhibition increased by preincubation of the complete system with interferon cell sap and dsRNA prior to the addition of the EMC RNA. This proved to be the case (data not shown). In fact, preincubation of the interferon cell sap alone with dsRNA and ATP prior to the addition of the S10 and EMC RNA was sufficient to eliminate most of the lag in the inhibition (Fig. 3, filled triangles). Incubation in the presence of ATP, therefore, resulted either in the activation of an interferon cell sap factor by dsRNA or the activation of the dsRNA by a component of the interferon cell sap. Neither protein synthesis nor ribosomes were required for this activation.

Activation of the inhibitor of translation

Additional evidence was sought for the activation of an inhibitor during the preincubation of interferon cell sap, dsRNA, and ATP. It was obtained in an experiment in which interferon cell sap was incubated in the presence and absence of dsRNA and ATP and the amount of inhibitor in each incubation mixture subsequently assayed by determining the dilution at which a 50% inhibition of translation was observed in the cell-free system. Preincubation of control cell sap with dsRNA and ATP did not result in the development of inhibitory activity (Fig. 4, filled circles). Preincubation of interferon cell sap in the absence of dsRNA did not increase its inhibitory effect on subsequent assay in the cell-free system in the presence of dsRNA (Fig. 4, open triangles). If, however, the preincubation was carried out in the presence of both dsRNA and ATP, a 100-fold increase in the inhibitory activity of the interferon cell sap was obtained (Fig. 4, filled triangles). Additional dsRNA was not required in the subsequent assay for the inhibition to be effective. Preincubation of the interferon cell sap in the presence of dsRNA but in the absence of added ATP gave a preparation with intermediate inhibitory activity (Fig. 4, open circles). These results provide additional evidence that full activation of an inhibitor of translation occurs only when interferon cell sap, dsRNA, and ATP are incubated together.

Evidence that dsRNA does not participate directly in inhibition of translation

The results presented in Figs. 3 and 4 show the activation of an inhibitor upon preincubation, but do not distinguish between an activation of the dsRNA or of a cell sap component(s). An experiment was, therefore, performed to test for any requirement for a putative activated dsRNA for the inhibition of translation to occur. Interferon cell sap was preincubated with ATP and with ^{3}H-labeled poliovirus dsRNA (800 ng/ml) and passed through a small DEAE-cellulose column. This procedure removed all detectable (over 99%) ^{3}H-labeled dsRNA from the mixture. Despite the removal of the dsRNA from the activated cell sap, the inhibitory activity of the latter was apparently unchanged (compare Figs. 4 and 5). Moreover, it can be calculated that after passage through DEAE-cellulose, the activated interferon cell sap contained no more than 0.008 ng/ml

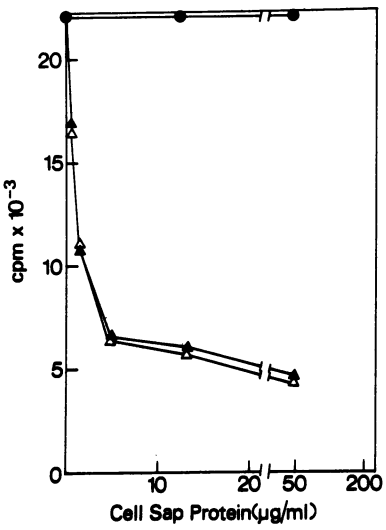

FIG. 5. Inhibition of EMC RNA translation by activated interferon cell sap: absence of requirement for dsRNA. Cell saps (50 μl) were preincubated as in the legend of Fig. 4 with 1 mM ATP and 800 ng/ml of [^{3}H]uridine-labeled poliovirus dsRNA (300 cpm/ng). After preincubation, the KCl concentration was adjusted to 0.3 M and each sample was passed through a 0.1-ml column of DEAE-cellulose (Whatman DE 32) equilibrated with 0.35 M KCl. The first 25 μl of eluate were discarded; the remaining eluate was collected together with that from a 75-μl 0.35 M KCl wash. Aliquots were analyzed for radioactivity to ensure that all of the dsRNA had been retained on the column and assayed for protein concentration. Further samples were assayed at a range of dilutions for their ability to inhibit the translation of EMC RNA in the cell-free system. (●) Preincubated control cell sap, dsRNA removed; (▲) preincubated interferon cell sap, dsRNA removed; (△) preincubated interferon cell sap, dsRNA removed, dsRNA (400 ng/ml) added to the cell-free system assay.

of residual ^{3}H-labeled dsRNA when assayed at a concentration of 2 μg/ml of protein (sufficient to produce a 50% inhibition of the translation of EMC RNA in the cell-free system, Fig. 5, filled triangles). Addition of 400 ng/ml of dsRNA to the dsRNA-depleted system produced no increase in the inhibitory activity (Fig. 5, open triangles), and no inhibitory activity was associated with control cell sap that had been incubated and passed through the column in a similar manner (Fig. 5, circles). We conclude that dsRNA is only required for the activation of an inhibitor and plays no role in the inhibition itself.

DISCUSSION

Here we have shown that incubation of cell sap from interferon-treated L-cells with dsRNA and ATP results in the activation of an inhibitor, which on addition to an appropriate cell-free system inhibits the translation of added viral RNA. The interferon-mediate inhibition of translation by dsRNA can, therefore, be divided into an activation step and an inhibitory step, with the corresponding advantage that each can be separately investigated. Using this approach we have shown that dsRNA is apparently required only for the activation of the inhibitor, the nature and site of action of which remain to be determined.

The basis for the requirement for ATP is uncertain. The experiment in Fig. 2 shows that it is required for the development of the inhibitory effect on translation, but does not distinguish between a role in the activation, or the activity, of the inhibitor. The results in Figs. 3 and 4 implicate ATP in the activation step, but do not preclude an additional role in the action of the inhibitor. When the analogue, adenylyl (β,γ-methylene) diphosphate was substituted for ATP during the preincubation of the interferon cell sap with the dsRNA, the concentration of activated inhibitor produced in the incubation mixture was even less than when ATP was omitted (data not shown). This suggests that cleavage of the β,γ-pyrophosphate bond is required for activation to occur. Preliminary experiments in this laboratory have also shown that incubation of interferon cell sap with dsRNA and [γ-^{32}P]ATP, followed by analysis of the ^{32}P-labeled polypeptide products on polyacrylamide gels, results in the accumulation of a new (or greatly increased amount of pre-existing) phosphorylated protein absent from corresponding controls. This suggests that the ATP is being utilized by a protein kinase(s) during the activation step.

The phosphorylation of ribosomal proteins and protein synthesis factors has been reported and the suggestion has been made that this could be important in the control of protein synthesis (reviewed in ref. 12). Recently the phosphorylation of the initiator Met-tRNA binding factor (IF-E2) has been implicated in the inhibition of protein synthesis in the reticulocyte lysate following incubation of the system under different inhibitory conditions, including the presence of dsRNA (P. Farrell, B. Balkow, T. Hunt, and R. J. Jackson, personal communication). Protein kinase activity has also been found associated with a partially purified inhibitor of protein synthesis from reticulocytes (13). It may be that a similar mechanism is involved in the interferon-mediated inhibition. The data clearly suggest the involvement of protein kinases in both systems but at different stages: at the activation step in the interferon-mediated inhibition, and at the inhibitory step in the reticulocyte system. It will be of considerable interest to determine if more than one kinase is involved in both systems.

The relationship between the interferon-mediated inhibition of protein synthesis by dsRNA observed here and the events inhibiting virus replication in the intact interferon-treated, virus-infected cell is not clear (6). Double-stranded RNAs have been reported (14) in extracts of cells infected with vaccinia virus (a DNA virus) and may play a role in the replication of RNA viruses. Moreover, in the intact interferon-treated, vaccinia virus-infected L-cell all protein synthesis is inhibited (15), as would be expected if small amounts of viral dsRNA were produced. A similar inhibition was seen with cell-free systems from such cells (9, 16). On the other hand, whether or not dsRNA exists as such in the intact infected cell, or is only formed during disruption of the cell or subsequent manipulation of the extracts, remains controversial (17). It is possible, however, that even if dsRNA is not produced in the intact cell, the inhibitory events described here could be activated by other stimuli. Whatever the explanation, the point we wish to stress is that the inhibition observed here provides direct evidence for a change in a potential cellular control mechanism in response to interferon treatment and, most important, a means for its investigation. Furthermore, if interferon modulates protein kinase activity in the cell, it is conceivable that such kinases, in addition to any effect they may have on factors involved in protein synthesis, may also play a role in the control of transcription or other viral or cellular functions. This in turn could provide a possible explanation for the diversity of effects that have been observed in response to interferon.

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