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Regulation of Interferon-impaired Initiation Factor Activity *in Vitro* by cAMP and dsRNA (40369)

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We have reported that treatment of mouse L cells with homologous interferon results in a reduction in the ability of initiation factor (eIF-2) to bind with initiator tRNA (Met-$tRNA_f$) and GTP (1). In the rabbit reticulocyte lysate-protein synthesizing systems, polypeptide chain initiation is inhibited by a heme-regulated translation inhibitor (HRI) in the presence of ATP. Inhibition is induced also by a low concentration of double-stranded RNA (dsRNA), but prevented by the addition of either excess eIF or cAMP and GTP (2, 3). Recent evidence showed that the inhibition of polypeptide chain initiation induced by HRI involves the phosphorylation of eIF-2 by HRI associated protein kinase (4–9). In the interferon system it has also been reported that protein synthesis in cell-extracts from interferon-treated cells is decreased by low concentrations of dsRNA (10, 11) which activates certain protein kinases, a nuclease and production of a low molecular inhibitor of protein synthesis (11–17).

The present study was undertaken to study the effect of these nucleotides, high and low concentrations of dsRNA, and other control substances on the eIF-2 inhibitory mechanism induced by interferon in mouse L cells. The results provide evidence that substances which affect the activity of different protein kinases strongly influence the level of eIF-2 activity as shown in the rabbit reticulocyte lysate system (4–9).

Materials and Methods. Interferon treatment. Exponentially growing mouse L cells (monolayer strain L929) were treated with 300 reference units/ml of mouse interferon (specific activity: 10^7 units/mg) for 24 hr at 37° in the presence of 2% fetal calf serum. After treatment, the cells were harvested and washed with 10 m*M* Tris–HCl buffer (pH 7.5) containing 10 m*M* KCl, 5 m*M* $MgCl_2$ and 2 m*M* dithiothreitol.

^{35}S-Met-$tRNA_f$ preparation. Initiator tRNA ($tRNA_f$-Met) was purified from rat liver using DEAE-cellulose and BD-cellulose, successively, and then charged with ^{35}S-methionine (22.3 Ci/mmol) using Met-tRNA synthetase purified from *Escherichia coli* as previously reported (1).

Preparation of eIF-2. The cells treated or untreated with interferon (about 4×10^9 cells) were homogenized in 20 m*M* Tris–HCl buffer (pH 7.5) containing 10 m*M* KCl, 5 m*M* $MgCl_2$ and 2 m*M* dithiothreitol (DTT), then centrifuged for 20 min at 15,000 rpm. Ribosomes in this supernatant were further purified by 60% sucrose cushion gradient centrifugation as previously reported (1). The purified ribosomes were suspended in 10 m*M* Tris–HCl (pH 7.5) containing 0.25 *M* sucrose, 1 m*M* EDTA, 2 m*M* DTT and 0.5 *M* KCl, and then gently stirred for 60 min at 4°. After centrifugation (45,000 rpm for 3 hr), solid ammonium sulfate (0.361 g/ml) was added to the supernatant. The precipitate was redissolved in 1.0 ml of 20 m*M* Tris–HCl (pH 7.5) containing 0.1 *M* KCl, 2 m*M* DTT and 5% glycerol. After dialysis against the same buffer, the crude eIF-2 preparation was stored at −20° and used within 1 month.

Assay of eIF-2 activity. Each 0.1 ml of the reaction mixture contained 50 m*M* Tris–HCl (pH 7.5), 0.1 *M* KCl, 2 m*M* dithiothreitol, 50 pmoles of ^{35}S-Met-$tRNA_f$ (4,500 cpm/pmol) and amount of eIF-2 indicated in the text. The solution was mixed before and after the addition of eIF-2, then incubated for 10 min at 37° in the presence of 1 m*M* GTP. The ternary complex formation (^{35}S-Met-$tRNA_f$-eIF-2-GTP) was determined as previously reported (1).

Chemicals. ^{35}S-Methionine (22.3 Ci/mmol) was obtained from Schwarz/Mann, cAMP and derivatives of cAMP from P-L Biochemicals Inc., poly rI and poly rC from Miles Laboratories and crude mouse interferon from the Bionetics Corp.

Results. Effect of cAMP and ATP on the eIF-2 activity from interferon-treated cells.

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Previously, we have reported that treatment of cells with interferon results in reduction of the activity of eIF-2 which interacts with Met-tRNA$_f$ and GTP to form a ternary complex (Met-tRNA$_f$-eIF-2-GTP) (1). When mouse L cells were exposed to mouse interferon (300 units/ml) for 24 hr at 37°, the eIF-2 activity was reduced about 60–70% as compared with that from the untreated control (1). Experiments similar to those performed with rabbit reticulocyte lysates (2, 3) were done to test the effects of cAMP, cAMP derivatives and ATP on the activity of eIF-2 from interferon treated cells. Figure 1 shows that the activity of eIF-2 from interferon treated cells is increased by cAMP at concentrations between 10 μM and 50 μM. The optimum concentration of cAMP was 12 μM and this dose increased eIF-2 activity 2.8 times. cGMP (data not shown) and derivatives of cAMP (Table I) did not substitute for cAMP. High concentrations of cAMP (higher than 1 mM) significantly inhibited the eIF-2 activity from both interferon treated and untreated cells (about 49%). ATP (1–3 mM) partly reversed the impaired eIF-2 activity from interferon treated cells. The increase of activity of eIF from interferon treated cells by 1 mM ATP was less than that obtained with cAMP. The effect of cAMP and ATP was further increased in the presence of Mg^{2+} (1 mM) as compared with its absence (Table II). The enhancing effect of Mg^{2+} also occurred with Mn^{2+}, but not with Ca^{2+} (data not shown) reversal of interferon-impaired eIF-2 activity by cAMP, ATP and either Mg^{2+} or Mn^{2+} strongly suggests an enzymatic control of the eIF-2 activity related to phosphorylation by protein kinases.

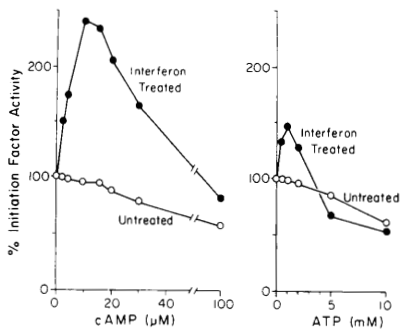

FIG. 1. Effect of cAMP and ATP on the eIF-2 activity from interferon-treated and untreated cells. The eIF-2 from cells treated with interferon (300 units/ml) and untreated cells prepared as described in Materials and Methods. The activity of eIF-2 (10 μg protein) was assayed in the presence of either cAMP (left) or ATP (right). The eIF-2 activity was plotted as % of initial activity. 100% of eIF-2 activity corresponds to the activity to 10 μg of eIF-2 from interferon-treated and untreated cells, respectively. The eIF-2 activity from interferon-treated (○) and untreated cells (○).

TABLE I. EFFECT OF cAMP AND ITS DERIVATIVE COMPOUNDS ON eIF-2 ACTIVITY FROM INTERFERON TREATED AND UNTREATED CELLS.[a]

Compound tested	^{35}S-Met-tRNA$_f$ in ternary complex eIF-2 from: Untreated cells	^{35}S-Met-tRNA$_f$ in ternary complex eIF-2 from: Interferon treated cells
	pmol	pmol
None	15.0	4.9
Adenosine 3′:5′-cyclic phosphate (cAMP)	14.4	14.8
8-Bromadenosine 3′:5′-cyclic phosphate	8.7	5.0
8-Methylthioadenosine 3′:5′-cyclic phosphate	12.8	5.1
N^6-Monobutyryladenosine 3′:5′-cyclic phosphate	10.9	5.1
2′-O-Monobutyryladenosine 3′:5′-cyclic phosphate	11.8	5.8
N^6-Benzoyladenosine 3′:5′-cyclic phosphate	11.0	5.7
N^6,O^2-Dibutyryladenosine 3′:5′-cyclic phosphate	10.5	5.8

[a] The complete reaction mixture (0.1 ml) contained 40 mM Tris–HCl (pH 7.5), 2 mM dithiothreitol, 0.1 M KCl, 1 mM $MgCl_2$, 1 mM GTP, 50 pmoles of ^{35}S-Met-tRNA$_f$ (4500 cpm/pmol) and 10 μg of eIF-2 from either interferon treated cells or untreated cells. 12 μM cAMP and the same concentration of its derivative compounds were added to the separated reaction mixtures and incubated for 10 min at 37°. The eIF-2 activity was determined as described in Materials and Methods.

The specific involvement of interferon was established as follows. Using an eIF-2 preparation from untreated cells, no effect of cAMP and ATP on the eIF-2 activity was observed (Fig. 1). Similarly, eIF-2 preparations from cells treated with heterologous human leukocyte interferon (300 units/ml) at levels which did not induce antiviral activity in mouse L cells (18), or treated with both mouse interferon (300 units/ml) and either actinomycin D (98% inhibition of cellular RNA synthesis) or cycloheximide (93% inhibition of cellular protein synthesis) which inhibit the action of interferon on the cells (19, 20) were not affected by cAMP and ATP (data not shown).

TABLE II. EFFECT OF ATP, cAMP, dsRNA AND AMINOPURINE ON eIF-2 ACTIVITY IMPAIRED BY INTERFERON.[a]

Addition	^{35}S-Met-tRNA$_f$ in ternary complex	
	$+Mg^{2+}$	$-Mg^{2+}$
	pmol	pmol
None	4.9	5.2
1 m*M* ATP	7.9	5.9
12 μ*M* cAMP	12.8	8.1
12 μ*M* cAMP + 1 m*M* ATP	14.8	8.7
5 μg/ml dsRNA	4.7	4.8
5 μg/ml dsRNA + 12 μ*M* cAMP + 1 m*M* ATP	5.1	5.0
50 ng/ml dsRNA	4.9	4.9
50 ng/ml dsRNA + 1 m*M* ATP	2.0	4.5
50 ng/ml dsRNA + 12 μ*M* cAMP + 1 m*M* ATP	2.4	4.6
50 ng/ml dsRNA + 2 m*M* Aminopurine + 1 m*M* ATP	4.7	5.0
2 m*M* Aminopurine	5.1	5.0
2 m*M* Aminopurine + 12 μ*M* cAMP + 1 m*M* ATP	5.5	5.3

[a] The complete reaction mixture (0.1 ml) contained 40 m*M* Tris–HCl (pH 7.5), 2 m*M* dithiothreitol, 0.1 *M* KCl, 1 m*M* GTP, 50 pmoles of ^{35}S-Met-tRNA$_f$ (4500 cpm/pmol) and 10 μg of eIF-2 from interferon treated or untreated cells. The reaction mixture was incubated for 10 min at 37°. The effect of ATP, cAMP, dsRNA and aminopurine on the eIF-2 activity was examined in the absence or presence of 1 m*M* $MgCl_2$. Poly rI:poly rC was prepared (22) and used as a dsRNA.

Effect of dsRNA and aminopurine on the eIF-2 activity from interferon treated cells. The cAMP and ATP requirements for the increase of interferon-impaired eIF-2 activity suggest the possibility that the reversal reaction may be associated with protein kinase(s). To test this possibility the effect of protein kinase inhibitors (aminopurine and high concentrations of dsRNA (7, 21)) on the reversion of interferon-impaired eIF-2 activity by both cAMP and ATP was examined. When the impaired eIF-2 from interferon treated cells was incubated with both cAMP (12 μM) and ATP (1 m*M*) in the presence of Mg^{2+} (1 m*M*), the increased level of eIF-2 activity was close to that obtained from untreated cells without these chemicals (Tables I and II). This increase of activity was not observed under conditions of inhibition of protein kinase activity (dsRNA 5 μg/ml) or aminopurine (2 m*M*). The concentration of dsRNA and aminopurine used inhibited more than 95% of the protein kinase activity (histone phosphorylation with γ-^{32}P-ATP) which was also present in the eIF-2 preparation from interferon-treated cells but did not significantly affect the eIF-2 activity (Table II). However, the activity of eIF-2 from interferon-treated cells was further reduced when incubated with a low concentration of dsRNA (50 ng/ml which activates certain protein kinases) in the presence of both ATP and Mg^{2+} (about 78% inhibition). The effect of a low level of dsRNA was not observed in the eIF-2 preparation from untreated cells and was prevented by the addition of aminopurine (2 m*M*).

These results suggest that the increase and decrease of activity of eIF-2 from interferon-treated cells by cAMP and low levels of dsRNA in the presence of ATP, respectively, may involve protein kinases which are sensitive to both aminopurine and high concentrations of dsRNA, and requires Mg^{2+} for optimum enzyme activity.

Discussion. We have presented indirect evidence which suggests that the interferon-induced mechanisms for regulation of eIF-2 activity have general similarities to those of polypeptide chain initiation induced by HRI in reticulocyte lysates (2–9). These similarities are that: (a) Both inhibitions are specifically overcome in the presence of suitable concentrations of cAMP; (b) the effect of cAMP is stimulated by the addition of either Mg^{2+} or Mn^{2+}; (c) restoration of activity by both cAMP and ATP are prevented by protein kinase inhibitors such as aminopurine (2–5 m*M*) or high concentrations of dsRNA (5–10 μg/ml); and (d) low concentrations of dsRNA (10–200 ng/ml) stimulates both inhibitions. Thus the control of eIF-2 activity in both systems may be due to specific phosphorylations of eIF-2 by different protein kinases.

Dissimilarities of the two systems are that preincubation of impaired eIF-2 from interferon-treated cells with cAMP and GTP, which has an effect in reticulocyte lysates, has no effect in our system (data not shown) and ATP (1 m*M*) which is ineffective in the reticulocyte lysates partly reverses the eIF-2 activity of interferon-treated cells. Moreover, low levels of cAMP which reverse the eIF-2 activity of interferon-treated cells have no effect on the HRI-induced reduction of eIF-2 activity (7). Thus, the two systems have major similarities but may not be entirely comparable.

If protein kinases actually are involved in these processes it is possible that the cAMP and ATP requirements for the reversion of interferon-impaired eIF-2 activity occurs in conjunction with either preexisting protein kinase or with interferon-induced new or increased protein kinase synthesis. Elevation of eIF-2 activity by both cAMP and ATP in the presence of Mg^{2+} does not occur in the eIF-2 preparation from untreated cells, from cells treated with both actinomycin D and interferon, or from cells treated with heterologous human leukocyte interferon (data not shown). Moreover, low concentrations of dsRNA (10–200 ng/ml) also do not enhance the inhibition of eIF-2 activity from untreated cells (data not shown). Therefore, it seems more likely that if protein kinases are involved they are either newly induced or increased by interferon. This aspect is under active study.

Previous reports indicate that the addition of low concentrations of dsRNA (10–200 ng/ml which stimulates certain protein kinases) to cell extracts from interferon-treated cells induces: (a) enhanced inhibition of viral protein synthesis (10); (b) dsRNA-dependent protein kinase mediated synthesis of a low molecular weight inhibitor (LMW-inhibitor) which directly inhibits viral mRNA translation in cell-free system (11–13); (c) phosphorylation of ribosomal and cellular proteins (14–16); (d) activation of uncharacterized protein kinases (14–16); and (e) activation of an endonuclease which digests viral mRNAs faster than those of host mRNAs in interferon treated cells (17). This activation of protein kinase and the protein phosphorylation may explain our finding that the activity of eIF-2 from interferon-treated cells is strongly decreased by the addition of low concentrations of dsRNA (50 ng/ml) in the presence of both ATP (1 m*M*) and Mg^{2+} (1 m*M*). Although it is not clear why the activity of eIF-2 from interferon treated cells is differentially affected by different concentrations of dsRNA (50 ng/ml and 5 μg/ml), there are several possibilities which include activation or inhibition of the same or different protein kinases by the different concentrations of dsRNA (7).

Summary. Interferon treatment of mouse L cells causes the reduction of activity of initiation factor (eIF-2) which forms a ternary complex with Met-$tRNA_f$ and GTP. The activity of eIF from the cells treated with interferon was specifically increased when incubated with 12 μ*M* cAMP, but no effect of cAMP on the eIF-2 activity from untreated cells was observed. ATP (1 mM) also slightly increased the interferon-impaired eIF-2 activity. The restoration of activity of eIF-2 from interferon-treated cells was completely prevented by the addition of inhibitors of protein kinases (either aminopurine (2 m*M*) or a relatively high concentration (5 μg/ml) of dsRNA (poly rI:poly rC)) without a direct effect on normal eIF-2 activity. However, low concentrations of dsRNA (50 ng/ml) which activate certain protein kinases, strongly stimulated the reduction of eIF activity induced by interferon. Taken together, these observations suggest that different protein kinases may be involved in the interferon-induced reduction of eIF-2 activity and the restoration of interferon-impaired eIF-2 activity.

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