SPECIFIC PHOSPHORYLATION IN VITRO OF A PROTEIN ASSOCIATED WITH RIBOSOMES OF INTERFERON-TREATED MOUSE L CELLS

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Received 19 July 1976

1. Introduction

Cultures of L cells treated with mouse interferon yield cell extracts with a reduced activity to translate exogenously added mRNAs into proteins [1–5]. There is a strong correlation between the interferon-induced antiviral state in intact cells and the development of the in vitro translational inhibition [3]. The selectivity of interferon's effect against viral, but not cellular, protein synthesis in intact cells [6–8] is, however, partially lost in the cell-free systems at high doses of interferon [3].

The reduced translational activity in vitro results from a dominant inhibitor(s), loosely associated with the ribosomes, whose activity increases with the dose of interferon used to treat the cells [3], but which has been only partially purified and characterized [3–5,9]. The inhibition affects both initiation and elongation of the polypeptide chains [9–11], and can be reduced by supplementing the extracts with excess amounts of some tRNA species [11–15]. In contrast, the addition of double stranded RNA (ds RNA), as poly I:C [16] or the replicative form of Mengo RNA [11], increases the translation inhibition seen in extracts of interferon-treated cells.

Recent work with reticulocyte lysates [17–19] has indicated that the inhibitory activity of ds RNA on translation may be mediated by protein kinase activities. This observation prompted us to examine whether the increased sensitivity of extracts from interferon treated cells to ds RNA, is also mediated by protein phosphorylation. We show, here, that in the subcellular fraction which contains the interferon-induced inhibitor(s) of translation, there is strong phosphorylation of a 67 000 mol. wt. (67K) polypeptide.

2. Materials and methods

2.1. *Cell cultures and cell-free protein synthesis*

Mouse interferon, induced in L cells by NDV, was prepared and purified by ammonium sulfate fractionation and carboxymethyl cellulose chromatography [3,12]. Suspension cultures of L cells (CCL1), of 1–2 liters and 10^6 cells/ml, were used as before [3,12] to prepare the cell-free extracts. The antiviral state was induced by adding 200 U/ml of interferon (measured by plaque reduction with VSV and titrating 5×10^6 IU/mg protein). After 24–36 h at 37°C, cells were harvested and post-mitochondrial supernatants (S10) prepared [3,12]. Aliquots of S10 were preincubated [3], filtered through Sephadex G-25 in Buffer B (20 mM Hepes buffer, pH 7.4, 5 mM $MgCl_2$, 120 mM KCl, 7 mM 2-mercaptoethanol, 10% glycerol), and stored in liquid nitrogen. To prepare the ribosomal wash fraction (RWF), non-preincubated and non-dialyzed crude extracts were centrifuged for 2.5 h at 150 000 × *g*, 4°C. The high-speed supernatant (S100) was filtered through Sephadex G-25 in Buffer B. The microsomal pellet was rinsed, resuspended in Buffer B (one fifth of the original S10 volume) and adjusted to 0.5 M KCl. After shaking 45 min, the suspension was centrifuged as above, the supernatant (RWF) filtered through Sephadex G-25 in Buffer B and stored in liquid nitrogen. The A_{280}/A_{260} ratio of RWF was about 1.1. Ribosomes were obtained from the pellet after

incubation with puromycin and treatment by 0.8% Na deoxycholate [9].

Mengo virus RNA and rabbit reticulocyte globin mRNA were prepared and used for cell-free translation in L cell S10 as detailed previously [11]. When indicated, ^{35}S-methionine (200 Ci/mmol) was used at 5 μCi per 0.025 ml reaction mixture. Poly I:C (from PL Biochemicals) was stored at 1 mg per ml in Buffer B and diluted as indicated. The degree of double strandedness was checked by RNase resistance.

2.2. *Protein phosphorylation*

Phosphorylation of proteins in crude extracts was carried out under conditions of protein synthesis in 0.025 ml, with 20 mM Hepes buffer, pH 7.5, 125 mM K acetate, 3 mM Mg acetate, 6 mM 2-mercaptoethanol, 1 mM [γ-^{32}P]ATP (final specific activity, 100 mCi/mmol), 0.07 mM GTP, 5 mM creatine phosphate, 5 μg creatine kinase, 40 μM each of 19 unlabeled amino acids, 40 μCi [^{3}H]leucine (57 Ci/mmol), 0.15 A_{260} units preincubated S10 extract, 0.5 μg rabbit globin mRNA or Mengo RNA. With globin mRNA, 0.5 μg crude DEAE-cellulose fraction of rabbit reticulocytes initiation factors [20] was added. When only the ribosomal wash fractions (RWF) were assayed for phosphorylation, the S10, mRNA and reticulocyte initiation factors were omitted from the reaction. Unless otherwise indicated, incubation was for 60 min at 30°C. After a further 10 min incubation with 10 μg/ml pancreatic ribonuclease in 0.01 M EDTA, 1 mM Na phosphate buffer, pH 7.4, the samples were heated to 100°C for 5 min in 0.5 volumes of 0.15 M Tris-HCl, pH 6.8, 3% Na dodecyl sulfate (SDS), 3% 2-mercaptoethanol and 30% glycerol. Electrophoresis at 150 V for 3 h was carried out as before [11] in 10 cm slab gels made up of a 10–20% polyacrylamide gradient prepared as described by Maizel [21]. Gels were stained with Coomassie blue, dried and exposed to X-ray film (Kodak, RP 54). The areas of the bands were determined after scanning at 560 nm in a Gilford recording spectrophotometer. All radioactive compounds were from The Radiochemical Center, Amersham.

3. Results

3.1. *Effect of ds RNA on the inhibition of mRNA translation in extracts of interferon treated cells*

Crude extracts from uninfected L cells, pretreated by interferon (Interferon S10), translate Mengo RNA and globin mRNA much less efficiently than similar extracts from untreated cells (Control S10). Over a series of twenty pairs of control and interferon S10, our data show that the degree of inhibition, measured by aminoacid incorporation in response to Mengo RNA, can vary from 30–95%. Addition of poly I:C (1–100 ng/ml) to partially inhibited extracts, markedly potentiated in all cases the inhibition of Mengo RNA and globin mRNA translation due to interferon treatment of the cells. Control extracts could only be inhibited by much higher amounts of ds RNA or prolonged incubation periods.

Since fractionation of the crude extracts had shown that the translational defect in interferon S10 results from an inhibitory factor present amount the

Table 1
Translational inhibitor in ribosomal wash fractions from interferon treated L cells

Poly I:C added	Mengo RNA dependent incorporation of ^{35}S-methionine by L cell S10			
ng/ml	Control RWF cpm	(Inhibition) %	Interferon RWF cpm	(Inhibition) %
0	239 000	(0)	209 500	(13)
10	230 000	(4)	109 500	(55)
30	207 500	(13)	99 000	(59)

Incorporation without mRNA, about 18 000 cpm, was subtracted. The value without RWF, 230 000 cpm, was not reduced by ds RNA. Reactions of 0.025 ml as in Methods, with 0.2 A_{260} units S10 from untreated L cells and 5 μg proteins from ribosomal wash fractions from control or interferon treated cells. After incubation, an aliquot of 0.01 ml was used to determine hot-TCA insoluble radioactivity.

proteins washed-off ribosomes by 0.5 M KCl buffers [3,9,12], we examined the effect of poly I:C on this fraction. The inhibitory factor in the ribosomal wash fraction (RWF) is measured by its ability to reduce Mengo RNA translation in extracts of cells which have not been treated by interferon. Table 1 shows that the translational inhibitory activity of interferon RWF is potentiated by ds RNA. In the presence of 10–30 ng/ml poly I:C, one can detect a clear inhibition of Mengo RNA translation with amounts of RWF from interferon treated cells which are too small to produce by themselves a significant effect.

The cell sap (S100) did not contain a similar ds RNA-sensitive inhibitory activity, at least when the extracts were not pre-incubated. Ribosomes from interferon treated cells, recovered after high salt treatment, were also fully active for Mengo RNA translation [3,9].

3.2. *Protein phosphorylation in extracts from interferon treated cells*

In reticulocyte lysates, the translational inhibitory mechanism induced by ds RNA appears to involve protein kinase activities [17–19]. We therefore studied the pattern of protein phosphorylation in extracts from L cells, pretreated or untreated by interferon, in the presence or absence of ds RNA. Incubation of the extracts with [γ-^{32}P]ATP was first carried out under conditions of protein synthesis. The radioactivity incorporated into hot-TCA acid and alcohol-ether precipitable material was determined. The reaction mixture was incubated with ribonuclease and the proteins separated by high resolution polyacrylamide gel electrophoresis in SDS. The autoradiography in figure 1 shows that many proteins were labeled by ^{32}P in the crude extracts, either in the presence or absence of ds RNA. A

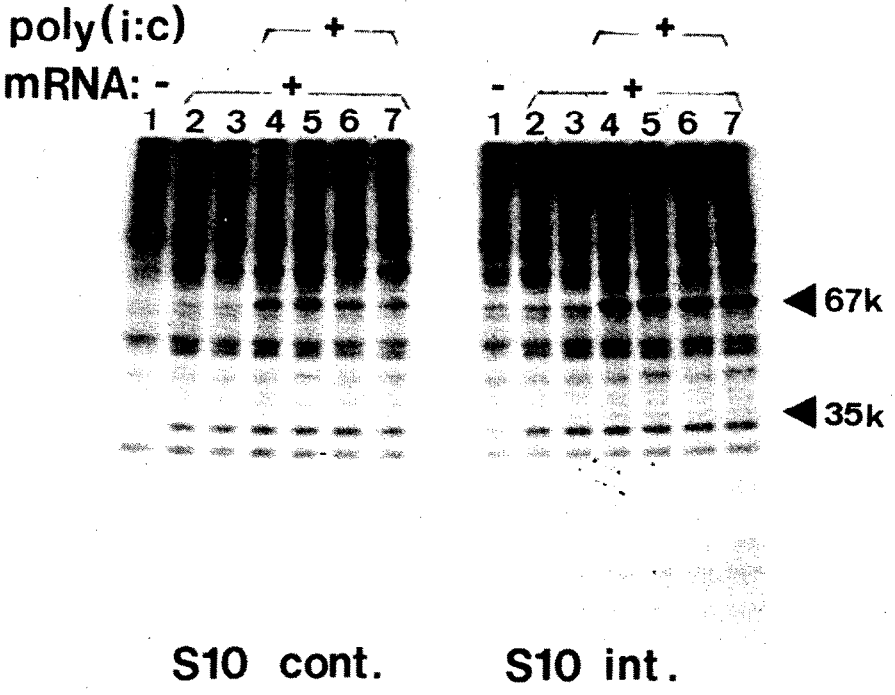

Fig.1. ^{32}P-Labeled proteins of S10 extract from control and interferon treated L cells. Incubation with [γ-^{32}P]ATP under conditions of protein synthesis as in Methods, for 1 h at 30°C. Samples were digested with RNase and submitted to electrophoresis and autoradiography for 2 days. Proteins from control S10 are on the left and of interferon treated S10 on the right side. Slot 1: no mRNA; slot 2: with globin mRNA; slot 3: with mRNA and 0.4 ng/ml poly I:C; slot 4: same but 80 ng/ml poly I:C; slot 5: same as 4 with 10 μM cyclic AMP; slot 6: same as 4 with 0.2 mM theophyline; slot 7: same as 4 with both cyclic AMP and theophyline.

comparison with the protein pattern obtained by Coomassie blue staining showed, however, that only certain protein bands were labeled.

In extracts from interferon treated cells, the intensity of one ^{32}P-labeled polypeptide band is selectively increased (fig.1). The molecular weight of this polypeptide in SDS, determined by comparison with known markers, is 67 000 daltons. ^{32}P-labeling of this 67K polypeptide measured by scanning the autoradiography is stimulated about 6-fold in the crude extracts by the addition of 80 ng/ml poly I:C (slots 4–7), the radioactive band being much weaker with 0.4 ng/ml poly I:C (slot 3). Nevertheless, even in the absence of ds RNA, there was more than a 2-fold increased labeling of the 67K polypeptide in interferon S10 as compared to control S10 (slots 1–2). No other major phosphorylated band was increased in interferon treated L cell extracts or by addition of poly I:C, but a minor 35 K band, whose labeling is ds RNA dependent, was visible. Another polypeptide was phosphorylated only in the presence of mRNA (compare slots 1 and 2), but was the same in control and interferon S10. Phosphorylation of the 67K band did not require the presence of mRNA (not shown); it was not modified when poly I:C was added at the end of the incubation period. Addition of cyclic AMP, with or without theophyline, did not significantly alter the phosphorylation of the 67K protein in interferon S10, unlike what has been reported in the reticulocyte system [18].

When instead of the complete translation system, only the ribosomal wash fraction was incubated with [γ-^{32}P]ATP, phosphorylation of the same 67K polypeptide is seen. Figure 2 shows that in RWF the number of phosphorylated proteins is much smaller than in crude extracts. The 67K band, which co-migrated on the same gel with the interferon-increased band of crude extracts, is much more prominent. Phosphorylation of this band is, again, strongly increased in the fraction from interferon treated cells (table 2). It is striking that in the ribosome-free RWF, ^{32}P-labeling of the 67K mol. wt. protein takes place in the absence of ds RNA as well as with 40 ng/ml poly I:C, in contrast to what is seen in the complete translation system. The increased phosphorylation of the 67K protein in the ribosomal wash fraction from interferon treated cells is, therefore, clearly observed also in the absence of added

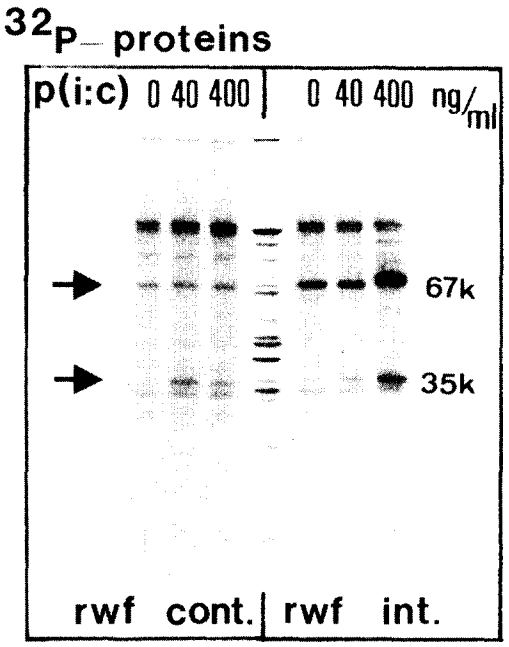

Fig.2. ^{32}P-Labeled proteins from ribosomal wash fraction of control and interferon treated L cells. Incubation as in fig.1, but with only 5 μg proteins of the ribosomal wash fraction (RWF) from untreated cells (left) and interferon treated cells (right). Poly I:C added as indicated. A 2-days autoradiography is shown and quantitative data obtained by scanning are given in table 2. The strong upper band is 100 000 mol. wt. Center slot: Coomassie blue stained gel pattern of RWF from interferon treated cells; the control pattern was identical.

Table 2
Intensity of the ^{32}P-labeled proteins from the ribosomal wash fraction

	Control RWF			Interferon RWF		
Poly I:C, ng/ml	0	40	400	0	40	400
Molecular weight	Area of the peak cm^2					
100 000	4.7	4.4	6.7	4.7	4.2	2.9
67 000	2.1	1.9	2.3	6.8	5.8	11.6
35 000	0.2	1.9	1.7	0.2	1.0	3.5

The autoradiography, as fig.2, was scanned and the area of the major peaks determined.

ds RNA. Larger amounts of poly I:C (400 ng/ml), nevertheless stimulate phosphorylation in the interferon RWF, but not in the control fraction (fig.2). With this high dose of ds RNA, the 67K band appears broader and there is some increase also in other bands.

Addition of 40 ng/ml poly I:C to the RWF stimulates (fig.2), the phosphorylation of a 35K polypeptide. This reaction occurs in both control and interferon RWFs and is clearly ds RNA-dependent. The higher dose of poly I:C stimulates phosphorylation of both the 35K and of a 17K band in interferon RWF. At lower dose of ds RNA, the difference between control and interferon RWF for these two bands, was small.

The 3- to 5-fold increased ^{32}P-labeling of the 67K polypeptide was observed, with and without ds RNA, in the RWF from several different cultures of L cells treated by interferon. On the Coomassie blue stained gel (fig.2, center) there was no indication of an increase in the absolute amount of this polypeptide in interferon RWF. Like the translational inhibitory activity [9], the 67K phosphorylated band was precipitated between 60 to 85% ammonium sulfate.

The time-course of the phosphorylation was compared to that of the translational inhibition. Table 3 shows that in interferon S10, phosphorylation of the 67K protein takes place within 5 min and is maximum with poly I:C at 15 min. In line with previous kinetic analyses [11,12], protein synthesis goes on at an almost normal rate in the first minutes of incubation, but slows down rapidly with poly I:C, starting at about 15 min. In time, therefore, phosphorylation preceeds the inhibition of translation. In control S10, translation slows down with poly I:C only at a much later time. When translation has completely stopped in interferon S10 with ds RNA, the mRNA (as measured with ^{125}I-9S globin mRNA by gel electrophoresis) is still intact (not shown).

4. Discussion

Translation systems from L cells are not readily inhibited by ds RNA, in contrast to reticulocyte lysates [22]. Extracts from interferon treated cells are, however, much more sensitive to the inhibitory effect of ds RNA [11,16]. As shown here, these cell extracts contain in the RWF an interferon-induced inhibitory factor whose activity is potentiated by ds RNA.

In reticulocyte lysates, ds RNA was shown to inhibit the formation of the met-tRNA$_f$-40S ribosome complex by a mechanism probably involving phosphorylation of the initiation factor which binds met-tRNA$_f$ [19]. This factor, MP or E2, has two subunits of 55 000 and 35 000 mol wt., respectively [23]. Interestingly, formation of the met-tRNA$_f$-40S ribosome complex is also inhibited in translation systems from interferon treated cells [11], and phosphorylation of a 35 000 mol. wt. polypeptide is seen in the RWF when ds RNA is added.

Table 3
Time-course of phosphorylation and translational inhibition

Time Period	[^{3}H]leucine incorporated during the time period			^{32}P-67K protein accumulated at the end of time period		
	Cont.S10 ----	Int.S10 ----	Int.S10 with p (I:C)	Cont.S10 ----	Int.S10 ----	Int.S10 with p (I:C)
minutes	cpm[a]			area, cm^2		
0– 5	780	1300	1100	*	1.3	3.6
5–15	5000	5600	4000	*	1.2	4.4
15–35	7000	3400	900	*	1.2	4.1

[a] Radioactivity incorporated during the indicated time period only.

* Undetectable on the autoradiography scanning.
Globin mRNA translation was carried out as in Methods with S10 extracts from control or interferon treated cells. Poly I:C, 80 ng/ml was added were indicated at time 0. Phosphorylation of the 67 000 mol. wt. polypeptide was measured as in table 2. [^{3}H]Leucine incorporation into protein was measured in a 0.005 ml aliquot at 20% efficiency.

The major effect of interferon treatment, however, is the strongly increased phosphorylation of a 67 000 mol. wt. polypeptide in the RWF, which also contains the translational inhibitor. This reaction is ds RNA-dependent only in the complete translation system, and preceeds in time the inhibition of protein synthesis. It is not clear at present whether the effect of interferon treatment is on a protein kinase activity or represents changes in the phosphorylation of a preexisting protein. Since the interferon-induced change affects only a few polypeptides, among the many phosphorylated proteins in the extracts, the reaction enhanced by interferon treatment must be based on a very specific mechanism. The chemical nature of the ^{32}P-labeled group and the extent at which the 67K protein is phosphorylated in the intact cell, remain to be established. Above all, it will be necessary to demonstrate the relationship between the phosphorylation change and the activity in interferon RWF which inhibits initiation and elongation of protein synthesis [11,12]. This will first require purification of the protein kinase and of the phosphorylated proteins described here.

Acknowledgements

We thank Mr. Z. Katzir for his help in initiating these experiments and Dr. Y. Groner for helpful discussions. We thank Dr. J. Werenne for interesting us several years ago in protein phosphorylation. Work supported by grants from the German Science Fund and the Israel-USA Binational Science Foundation.

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