EXTRACTS OF INTERFERON-TREATED CELLS CAN INHIBIT RETICULOCYTE LYSATE PROTEIN SYNTHESIS

Jonathan A. Cooper,[1] Department of Biological Sciences, University of Warwick, Coventry, England

Paul J. Farrell, Department of Biochemistry, University of Cambridge, Cambridge, England

Received May 16,1977

SUMMARY

Cytoplasmic extracts of interferon-treated L929 cells are capable of forming an inhibitor of reticulocyte lysate protein synthesis when incubated with double stranded RNA and ATP. It appears that this inhibition arises through phosphorylation of the methionyl-tRNA$_f$ binding factor, causing a defect in polypeptide chain initiation.

INTRODUCTION

Low concentrations of dsRNA[2] inhibit protein synthesis in rabbit reticulocyte lysates (1) and in extracts of interferon-treated cells (2). The dsRNA does not inhibit reticulocyte lysates directly but causes the formation of an inhibitor of peptide chain initiation known as DAI (3). This inhibitor appears to prevent the binding of methionyl-tRNA$_f$ to 40S ribosomal subunits, a process mediated by initiation factor eIF-2 (which is the new agreed name for IFE2 (4) and IF-MP (5)). A de novo phosphorylation of the small subunit of eIF-2 correlates with the inhibition of protein synthesis in this system, but the exact nature of the lesion is not yet clear (3).

The dsRNA promoted inhibition of protein synthesis in extracts of interferon-treated cells may involve inactivation of eIF-2 (6), concomitant with changes in protein phosphorylation (6-8), but there are also changes in ribonuclease activity (9). This paper describes the effects of the crude dsRNA-dependent inhibitor from interferon-treated L929 cells on protein

[1]Now at National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014.

[2]Abbreviations: dsRNA, double stranded RNA; HCR, haem controlled repressor; DAI, double stranded RNA activated inhibitor; SDS, sodium dodecyl sulphate.

ISSN 0006-291X

synthesis in the reticulocyte lysate. This approach has allowed a fuller analysis of the mechanism of inhibition than has been possible in L cell extracts, and is also the first demonstration that an interferon-dependent inhibitor from mouse cells can inhibit protein synthesis in extracts of cells from another species.

MATERIALS AND METHODS

Mouse L cell interferon was induced with Newcastle Disease virus (strain F) into serum-free medium and purified by the pH 2 ammonium sulphate procedure (10). It contained about 10^6 reference research units/mg protein. 'Mock' interferon was induced with uninfected allantoic fluid and purified similarly. Confluent monolayers of L929 cells in 140 mm plastic petri dishes were treated for 16-18 hours with 200 reference units/ml interferon or an equivalent concentration of 'mock' interferon. Cytoplasmic extracts were prepared by washing three times with ice-cold phosphate-buffered saline, once with cold 90mM KCl, 30mM N-2-hydroxyethylpiperazine-N'-2-ethane sulphonate pH 7.4, 4mM Mg acetate, 1mM dithiothreitol, and lysing with five 100µl aliquots of 0.1% Triton N101 in the same buffer. After 3 min the fluid was collected, leaving the nuclei attached to the dish (11), and either assayed directly or stored in liquid nitrogen.

Inhibitor was activated at 30^o for 30min in incubations containing (per 100µl) 70µl of L cell extract (1.1-1.4mg protein), 20µl of 0 or 500ng/ml dsRNA (from Penicillium chrysogenum, a kind gift of Dr. C. Burbidge, Beecham Research Laboratories), and 10µl of an energy supply mixture giving final concentrations of 1mM ATP, 0.1mM GTP, 0.6mM CTP, 10mM creatine phosphate, and 50µg/ml creatine kinase (E.C.2.7.3.2). It was then assayed for inhibition of protein synthesis in rabbit reticulocyte lysate incubations (3) in the presence of 10µg/ml dsRNA. Protein synthesis was measured by the incorporation of either L-[^{35}S]-methionine (200Ci/mmole, 36µCi/ml final) or L-[4,5-^{3}H]-leucine (206Ci/mole, 16µCi/ml final) into acid insoluble radioactivity. γ[^{32}P]ATP was prepared and used to label proteins for analysis on SDS polyacrylamide gels as described previously (3).

Treatment of the inhibitor (100µl) with micrococcal nuclease (E.C. 3.1.4.7) was by incubation with 50µl of 400µg/ml nuclease, 15mM $CaCl_2$ at 30^o for 30min before the addition of excess ethylene glycol bis (βaminoethylether)N,N'-tetraacetic acid and dilution into the lysate. Similarly, treatment with trypsin (E.C.3.4.4.4) was accomplished by the addition of 50µl of 200µg/ml trypsin at 30^o for 30min, followed by excess trypsin inhibitor.

Binding of [^{35}S]methionine to 40S subunits was determined after 15min at 30^o as described (12) using 15-40% sucrose gradients centrifuged at 50,000 rpm for 2.25 hours at 2^o in the SW 50.1 rotor. Gradient fractions were precipitated with cetyl trimethylammonium bromide and counted (12). Purified initiation factors were the generous gift of T. Staehelin and H. Trachsel, Basel Institute for Immunology. HCR, purified 4000 fold from N-ethyl maleimide treated reticulocyte lysates, was given by T.Hunt and R.Jackson.

RESULTS AND DISCUSSION

Since protein synthesis in reticulocyte lysates is not inhibited by high concentrations (10µg/ml or greater) of dsRNA, it is possible to assay inhibitors of protein synthesis which happen to contain dsRNA by testing

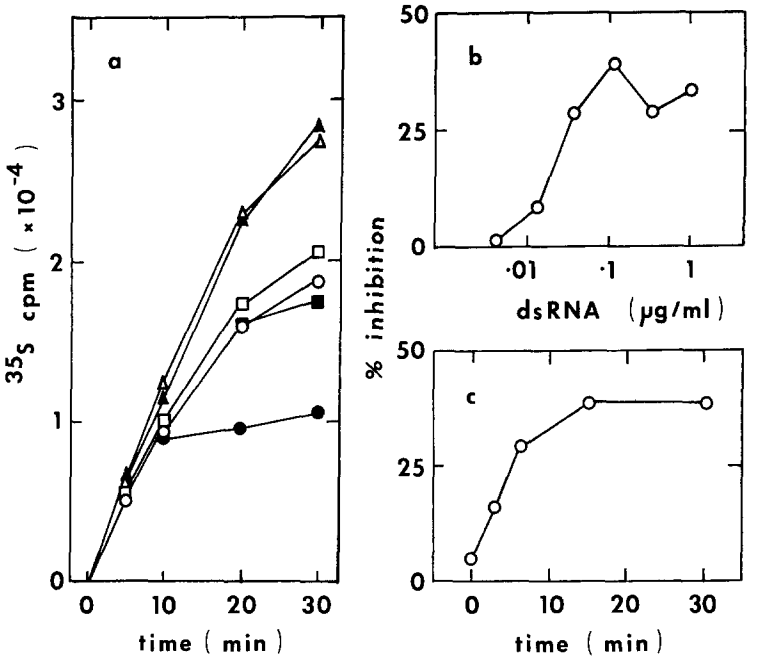

Figure 1: Assay for protein synthesis inhibitor and requirements for formation. Extracts of interferon-treated (circles) or control cells (squares) were incubated for 30 min (parts a and b) or for various times (part c) with energy supply mixture (see Methods) and dsRNA as required. Aliquots were then assayed after diluting ten-fold into reticulocyte lysate containing 10μg/ml dsRNA and L-[^{35}S]-methionine.
a) Kinetics of lysate protein synthesis. Preincubations contained Δ , ▲ no cell extract; o , • interferon treated cell extract; □, ■ control cell extract, and Δ , o , □ no dsRNA or ▲ , • , ■ 100 ng/ml dsRNA.
b) Effect of concentration of dsRNA present in preincubation.
c) Effect of time of preincubation with 100ng/ml dsRNA.

In parts (b) and (c) the incorporation in the lysate assay is expressed as a percentage inhibition relative to a control which contained interferon treated cell extract preincubated in the absence of dsRNA.

---

them in the presence of a high concentration of dsRNA. When extracts from interferon-treated L cells were incubated with 100ng/ml dsRNA and tested in this way, an inhibitor was detected (Fig. 1a). This inhibitor failed to form in extracts from control cells treated similarly and was also absent when extracts from interferon-treated cells were incubated without added dsRNA. It is likely that this inhibitor is the same as that reported by other workers (13), in that its formation requires both dsRNA and cytoplasm from interferon-treated cells. Moreover, the

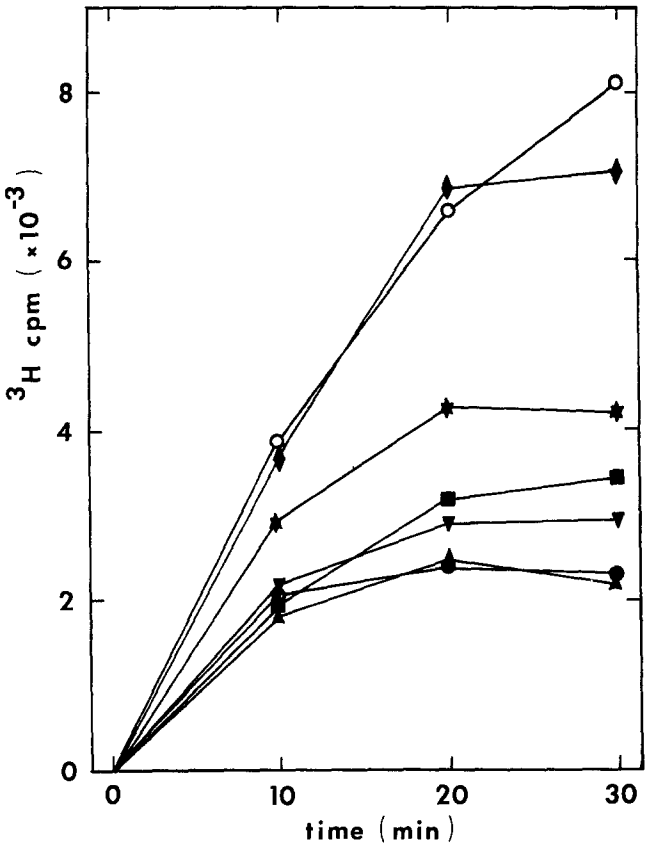

Figure 2: Effects of various compounds on inhibition of the lysate. A preincubation containing interferon treated cell extract, 100ng/ml dsRNA and an energy supply was diluted 20 fold into lysate containing 20µg/ml dsRNA and [^{3}H]leucine. Protein synthesis was then assayed in the presence of • no other additions, ▲ 5mM dithiothreitol, ▼ 2mM glucose, ■ 2.5mM fructose-1,6-diphosphate, ★ 100µg/ml eIF-2, ◆ 5mM cAMP, o no cell extract. Such additions had no effect on assays containing control cell extract.

inhibitor described here is able to inhibit *in vitro* translation in a system derived from mouse cells (I.M. Kerr and J.A.C. results not shown).

Formation of the inhibitor was temperature and time-dependent and it could be formed with 30 ng/ml to 1 µg/ml dsRNA (Fig. 1b and c). Its formation was dependent on the presence of ATP, and neither GTP, CTP, nor a creatine phosphate/creatine kinase couple were active if ATP was omitted (data not shown). Other workers have found similar requirements for the

formation of inhibitors of protein synthesis in L cell and ascites cell free systems (6,13).

The kinetics of inhibition (Fig. 1a) were similar to those seen in reticulocyte lysates during haem deficiency or in the presence of oxidised glutathione or dsRNA. The inhibition was prevented by the addition of 5mM cAMP (Fig. 2) or by 7mM 2-aminopurine (data not shown), both of which prevent inhibition by the haem deficiency inhibitor (HCR), dsRNA and oxidised glutathione (14). Addition of purified eIF-2 greatly reduced the inhibition (Fig. 2), as has been found for haem deprivation (15) and for dsRNA (16,P.J.F. unpublished).

The inhibited step was characterised by labelling lysate with L-[^{35}S]-methionine in the presence of L cell inhibitor or HCR and analysing the incubations on sucrose density gradients. Table 1 shows that both HCR and the L cell inhibitor caused a reduction in the radioactivity associated with 40S subunits when compared to uninhibited controls. In addition, there was a large increase in the amount of 80S ribosomes in the inhibited incubations, suggesting that polysomes had run off. The increase in radioactivity associated with the 80S ribosomes is probably due to

TABLE 1 Inhibition of Methionyl-tRNA$_f$ Binding by the Interferon-Dependent Inhibitor

Conditions	relative A260 in 40S region*	realtive ^{35}S cpm in 40S region*
Control	0.13	1.64
+ 5µg/ml HCR	0.12	0.26
+ 90µg/ml L cell inhibitor and 10ug/ml dsRNA	0.10	0.29

*Ratio peak height in 40S region to peak height in 80S region.

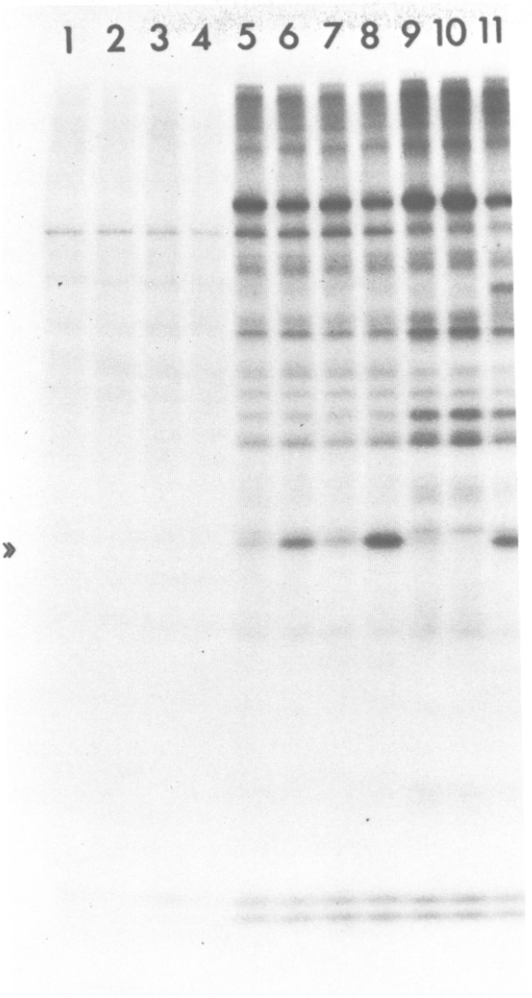

Figure 3: Effect of inhibitor on phosphorylation of reticulocyte ribosomes (made by gel filtration on Sepharose 6B (3)).
Preincubations containing control (tracks 1,2,5,6) or interferon treated (tracks 3,4,7,8) cell extract and 0 (tracks 1,3,5,7) or 100ng/ml dsRNA (tracks 2,4,6,8) and an energy supply were performed at 30° for 30 min. These were diluted 20 fold into phosphorylation assays which all contained γ [^{32}P]ATP at 50μM. Tracks 1-8, 10 contained 20μg/ml dsRNA, tracks 5-11 contained reticulocyte ribosomes (15μg), tracks 9-11 have no cell extract and track 11 contained 50ng/ml dsRNA. After incubation at 30° for 20 min, the proteins were analysed on a 15% polyacrylamide SDS gel (3).

residual synthesis in the inhibited state. It thus seems that the L cell inhibitor prevents initiation by inhibiting the binding of methionyl-$tRNA_f$ to 40S subunits, as has been shown for HCR and DAI (3).

In the reticulocyte lysate some inhibitors of methionyl-$tRNA_f$ binding cause phosphorylation of the small subunit of eIF-2 (3). Figure 3 shows that phosphorylation of this polypeptide (arrowed) also occurs when L cell extracts are incubated with crude reticulocyte ribosomes, provided that the extracts were from cells treated with interferon and had been activated with dsRNA. We conclude that one of the ways that extracts of interferon-treated cells, incubated with dsRNA and ATP, are able to inhibit protein synthesis is by causing phosphorylation of eIF-2 and thus preventing polypeptide chain initiation. The pool of eIF-2 may not be limiting (17), so the low level of phosphorylation found with control cell extracts may be inadequate to affect protein synthesis. In the homologous L cell system there is interferon and dsRNA dependent phosphorylation of a 35,000 dalton protein (6-8), and we find that this has the same mobility on SDS polyacrylamide gels as the small subunit of eIF-2 from Krebs II ascites cells (data not shown).

The inhibition of lysate protein synthesis could be caused by the chelation or degradation of haemin, or by the oxidation of glutathione. However, the inhibition still occurred in the presence of extra haemin (data not shown) and in the presence of glucose, fructose-1,6-diphosphate or dithiothreitol (Fig. 2). The last three compounds would overcome inhibition by oxidised glutathione (R.J. Jackson, unpublished data).

We conclude that the inhibition of protein synthesis caused by dsRNA-activated cytoplasm from interferon-treated cells is probably due to an increased level of a protein kinase, or of a compound capable of activating the reticulocyte protein kinase. Indeed, the inhibitor is destroyed by micrococcal nuclease but is apparently insensitive to trypsin (see Methods, data not shown), suggesting that it may not be an enzyme. However, its relationship to a low molecular weight, heatstable inhibitor described by Roberts et al (13) is not clear. The role of this complex control system

in mediating the biological effects of interferon awaits the characterisation of the inhibitor and its identification in intact cells.

Acknowledgements: We thank Tim Hunt, Richard Jackson and Derek Burke for helpful discussion, and Ian Kerr for assays in the L cell system. We are grateful for a copy of reference (13) prior to publication. JAC was supported by the Cancer Research Campaign, and PJF by the Medical Research Council.

REFERENCES

1) Hunt T., and Ehrenfeld E. (1971) Nature New Biol. 230 91-94
2) Kerr I.M., Brown R.E. and Ball L.A. (1974) Nature 250 57-59
3) Farrell P.J., Balkow K., Hunt T., Jackson R.J., and Trachsel H. (1977) Cell, in the press
4) Schreier M.H., and Staehelin T. (1973) Nature New Biol. 242 35-38
5) Safer B., Anderson W.F. and Merrick W.C. (1975) J. Biol. Chem. 250 9067-9075
6) Zilberstein A., Federman P., Schulman L. and Revel M. (1976) FEBS Letters 68 119-124
7) Roberts W.K., Hovanessian A., Brown R.E., Clemens M.J. and Kerr I.M. (1976) Nature 264 477-480
8) Lebleu B., Sen G.C., Shaila S., Cabrer B. and Lengyel P. (1976) Proc. Nat. Acad. Sci. U.S.A. 73 3107-3111
9) Brown G.E., Lebleu B., Kawakita M., Shaila S., Sen G.C. and Lengyel P. (1976) Biochem. Biophys. Res. Comm. 69 114-122
10) Knight E. Jr. (1975) J. Biol. Chem. 250 4139-4144
11) Tsai R.L. and Green H. (1973) Nature New Biol. 243 168-170
12) Darnborough C., Hunt T. and Jackson R.J. (1972) Biochem. Biophys. Res. Comm. 48 1556-1564
13) Roberts W.K., Clemens M.J. and Kerr I.M. (1976) Proc. Nat. Acad. Sci. U.S.A. 73 3136-3140
14) Legon S., Brayley A., Hunt T. and Jackson R.J. (1973) Nature New Biol. 241 150-152
15) Clemens M.J., Henshaw E.C., Rahamimoff H. and London I.M. (1974) Proc. Nat. Acad. Sci. U.S.A. 71 2946-2950
16) Kaempfer R. (1974) Biochem. Biophys. Res. Comm. 61 591-597
17) Cherbas L. and London I.M. (1976) Proc. Nat. Acad. Sci. U.S.A. 73 3506-3510