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Virus-mediated inhibition of the ppp(A2'p)$_n$A system and its prevention by interferon

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SUMMARY: Encephalomyocarditis virus infection of mouse Ehrlich ascites tumour or L-cells or human HeLa cells inhibits the activation of the ppp(A2'p)$_n$A-dependent nuclease and prevents the binding of a radioactive analogue of ppp(A2'p)$_n$A to the ppp(A2'p)$_n$A binding protein. This loss or inactivation of the ppp(A2'p)$_n$A-dependent nuclease in response to virus infection is prevented by interferon pretreatment of the cells.

INTRODUCTION

The oligonucleotides ppp(A2'p)$_n$A (n=2 to ⩾4 collectively known as 2-5A (1)) are synthesised by an enzyme found in a wide variety of cells in amounts which increase in response to interferon and vary with growth and hormone status (2-7). In interferon-treated cells which synthesise 2-5A in response to virus infection (8,9) specific ribosomal RNA (rRNA) cleavages are catalysed by a 2-5A-dependent nuclease (10). Here evidence is presented for an additional level of control in this system. The 2-5A-dependent nuclease appears to be lost with time after infection of mouse L-cells with encephalomyocarditis (EMC) virus. Prior treatment with interferon can partially or completely prevent this loss. Similar results have been obtained with mouse Ehrlich ascites tumour (EAT) and human HeLa cells. No evidence was found for any similar EMC virus-mediated inactivation of the interferon and double-stranded RNA (dsRNA)-dependent protein kinase system.

MATERIALS AND METHODS

The ppp(A2'p)$_n$A, n = 2 or 3 and ppp(A2'p)$_3$A [^{32}P] pCp were prepared as described previously (11,12).

Preparation of cell extracts. The cells were grown in suspension culture in Eagle's medium with 10% newborn calf (HeLa and EAT cells) or foetal calf (L-cells) serum, and infected with EMC virus at a multiplicity of 20 plaque forming units per cell. Interferon treatment was for 17hr at 37°C with 400 reference (20 effective) units per ml of mouse L-cell interferon (specific activity ⩾ 5×10^7 reference units per mg protein). The extracts were prepared by resuspending the cells in 1.5 volumes of 10mM potassium chloride, 1.5mM

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magnesium acetate, 7mM 2-mercaptoethanol, and 10mM Hepes, pH7.6, followed by dounce homogenisation. The final concentration of potassium chloride and magnesium acetate were increased to 90mM and 8.5mM respectively before centrifugation at 10,000 x g for 10min to give the post-mitochondrial supernatant fractions (S10s).

Assay for the 2-5A binding protein. The S10s (5µl or 10µl) were incubated for 1.5hr at 0°C with ppp(A2'p)$_3$A [^{32}P]pCp (3,000cpm, 1 to 3 x 10^6 Ci mol^{-1}) in a final volume of 18µl essentially under the conditions described for the radiobinding assay (9). The reaction mix was spotted onto nitrocellulose filters which were washed in water, dried and counted.

Ribosomal RNA cleavage assay for 2-5A-dependent nuclease activity. S10s (25µl) were incubated ± 70nM 2-5A for 2hr at 30°C under protein synthesis conditions (13) without creatine phosphokinase. The incubations were diluted 20-fold with 50mM sodium acetate pH5, 10mM EDTA, and 0.5% sodium dodecylsulphate and extracted twice with an equal volume of H_2O-saturated phenol/chloroform (1/1). Samples (3µg) of the ethanol precipitated RNA were denatured by incubation for 1hr at 50°C with 5µl of 10M urea 15µl of dimethylsulphoxide and 5µl of deionised glyoxal in a final volume (30µl) containing 65mM sodium phosphate pH7.0 and analysed by electrophoresis on 1.8% agarose gels (14,15). The ethidium bromide stained gels were photographed on a 302nm ultraviolet light box (U.V. Products Inc., Irvine, California).

Covalent binding of ppp(A2'p)$_3$A [^{32}P]pC to the 2-5A binding protein. The probe ppp(A2'p)$_3$A [^{32}P]pCp 1-3 x 10^6 Ci mol^{-1}) was digested with bacterial alkaline phosphatse (25u/mg) to remove the 3'-phosphate and yield a mixture of the 5'-terminally phosphorylated molecules as described in the legend to Fig.2. The digests were heated to 90°C for 5 min with 2mM EDTA to inactivate the phosphatase and oxidised with 10mM sodium periodate for 1hr at 20°C. Samples (50,000 cpm) were added to 5µl aliquots of S10, incubated for 2hr at 4°C and reduced with 10mM cyanoborohydride. The labelled protein was precipitated with acetone and analysed by electrophoresis in the presence of SDS on a 7.5% polyacrylamide gel.

RESULTS AND DISCUSSION

The radioactive 2-5A analogue ppp(A2'p)$_3$A [^{32}P]pCp binds to a protein in cell extracts from which it can be displaced by nanomolar levels of 2-5A (9). The same level of 2-5A is required to activate the 2-5A dependent nuclease (1,8,13,16) and to produce specific limited cleavage of rRNA in intact ribosomes in such extracts (10 and Fig.1B). The 2-5A binding and nuclease activities do not separate through a number of purification steps (17 and D.H.Wreschner, R.H.Silverman, C.S.Gilbert and I.M.K. unpublished results). For simplicity, therefore, it will be assumed that they are associated with the same protein molecule. In accord with this both activities are lost progressively after EMC virus infection of L-cells: by 4.5hr very little activity remains (Figs.1A & B). The inactivation, therefore, occurs relatively early in the virus growth cycle and is essentially complete at the time of maximal viral RNA and protein synthesis. A similar loss of both 2-5A binding and nuclease activities is observed in EMC-infected EAT and HeLa cells (Fig.1A and our own unpublished results). The loss of 2-5A-dependent nuclease activity is not restricted to its action on rRNA. For example, a similar reduction in activity on added ^{32}P-labelled mRNA has also been observed in extracts from EMC virus-infected HeLa cells.

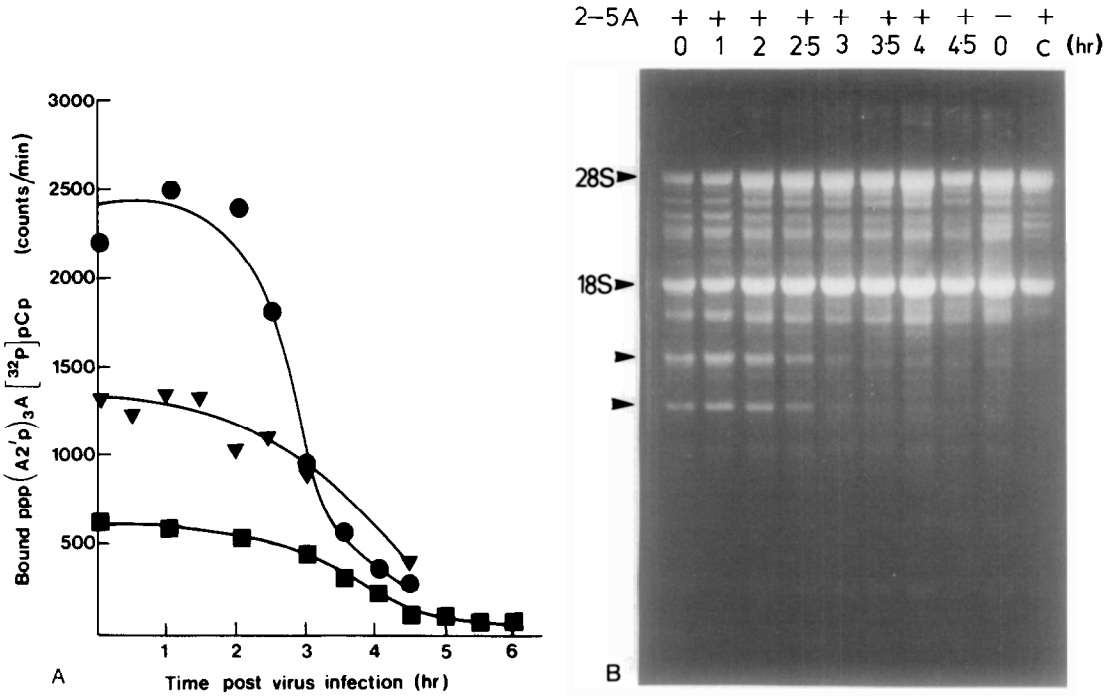

Figure.1. A. Binding of ppp(A2'p)$_3$A$[^{32}P]$ pCp to the 2-5A binding protein in post mitochondrial supernatant extracts (S10s:5, 5 and 10µl respectively) from L-(●), EAT (▼) and HeLa (■) cells with time (abscissa) after infection with EMC virus (Methods). B. Loss of 2-5A mediated rRNA cleavage activity from L-cell extracts with EMC infection. Extracts (25µl) prepared at the times post infection indicated at the top of the Figure were incubated with 70nM ppp(A2'p)$_2$A where indicated (2-5A+) for 2hr at 30°C. Lane C: the extract (0 hr after infection) with added 2-5A was not incubated (zero time control). The RNA was extracted, denatured, and analysed by electrophoresis on an agarose gel. A photograph of the ethidium bromide stained gel taken under UV light (302nm) is presented. The 18S and 28S rRNAs and the two most prominent 2-5A-mediated cleavage products are indicated by arrows to the left of the Figure. In both A and B the protein content of the S10s was adjusted to a constant value throughout.

The high affinity of the binding protein for 2-5A allows it to be labelled covalently and specifically in crude cell extracts using the radioactive analogue ppp(A2'p)$_3$A $[^{32}P]$ pC after periodate oxidation (D.H. Wreschner unpublished results). With this technique an almost complete loss of binding activity was observed in extracts of EAT cells 4.5hr post infection with EMC virus (Fig.2 arrowed). With extracts from interferon-treated, EMC virus-infected cells, however, the presence of relatively high levels (>20nM) of naturally occurring 2-5A (which competes with the radioactive analogue) obviates the use of this technique. Qualitatively similar results were obtained using the non-phosphorylated core of the molecule (A2'p)$_3$A $[^{32}P]$ pC (Fig. 2, 8hr time points: lanes 6 and 11). The labelling of the same protein with the 'core' as with the 2-5A analogue (although at much lower affinity) argues perhaps against the possibility of its having a separate function.

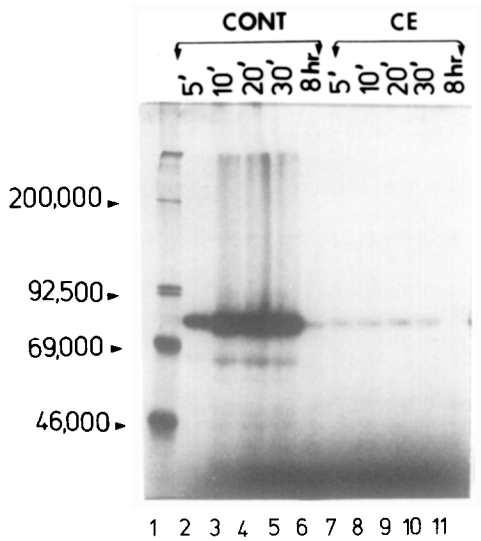

Figure. 2. Covalent binding of oxidised ppp(A2'p)$_3$A [^{32}P] pC to the 2-5A binding protein in EAT cell extracts from control (CONT) and 4.5hr EMC virus-infected (CE) EAT cells. Prior to covalent binding (Methods) ppp(A2'p)$_3$A [^{32}P] pCp (specific activity 1 to 3 Ci mol^{-1}) was partially (5, 10, 20 and 30 min lanes 2 to 5 and 7 to 10) or completely (8 hr lanes 6 and 11) digested with bacterial alkaline phosphatase to remove the 3'-phosphate (to permit oxidation) and yield a mixture of the 5'-terminally phosphorylated molecules p$_n$(A2'p)$_3$A [^{32}P] pC (n = 1 to 3) all of which have high affinity for the binding protein (9) or the non-phosphorylated core (A2'p)$_3$A [^{32}P] pC (8 hr) which binds with much lower affinity (9). Anlaysis of the covalently labelled protein was by SDS polyacrylamide gel electrophoresis. An autoradiograph of the dried gel is presented. The arrow indicates the affinity labelled binding protein and the numbers to the left the molecular weights of marker proteins. The covalently labelled band in lane 6 does not represent carry over from the adjacent lane. Similar results were obtained when samples of this type were electrophoresed separately.

A number of control experiments were carried out to exclude trivial explanations for the loss of nuclease activity observed in Figs. 1 and 2. It does not appear to reflect removal of the nuclease, bound for example to a virus replication complex, during the preparation of the extract (S10). Essentially identical results were obtained in the binding assay (Fig. 1A) with unfractionated extracts after NP40 lysis of the cells. Nor was it caused by an increase in the rate of degradation of the ppp(A2'p)$_3$A [^{32}P] pCp (probe) or by the presence of naturally occurring 2-5A in the extracts at later times after infection. For example, assay of heat inactivated material or TCA ether extracts for naturally occurring 2-5A, or any other low-molecular-weight compound capable of competing for binding with the probe (9), showed that there was insufficient material of this kind present to affect the assays used in Figs.1 and 2.In addition, the nuclease from extracts at late times post infection could not be activated in the presence of 1,000nM 2-5A. Even higher concentrations of a competitive inhibitor such as p(A2'p)$_n$A (18, 9, and R.E.

Brown and I.M.K. unpublished) would have been required to prevent its activation. Such concentrations would have been easily detected in TCA ether extracts by the radiobinding assay (9). Hence it seems most improbable that a low molecular weight competitor is responsible for the inhibition observed. Mixing experiments have also failed to provide any evidence for the presence of an inhibitory factor(s) in extracts from virus-infected cells capable of producing a significant reduction in the binding activity of control extracts.

The presence of rRNA cleavages which have already occurred in the intact cell (10) complicates the analysis of nucleolytic activity in extracts from interferon-treated cells at later times after infection with EMC virus. Accordingly, the effect of interferon treatment prior to infection was monitored by incubating such extracts with ^{32}P-labelled ribosomes from control cells: there was no loss of nuclease activity after infection (Fig.3 lanes 13 to 19). Interferon pretreatment, therefore, prevents the virus-mediated loss of the 2-5A dependent nuclease. This result is in marked contrast to those obtained with extracts from cells not treated with interferon whether assayed in parallel (Fig.3, CE lanes 2 to 9) or as described in Fig.1B.

It was of interest to determine whether there is a similar virus-mediated inactivation of the other enzyme system known to be elevated in response to interferon. No evidence, however, was obtained for any such inactivation in response to EMC virus infection of the dsRNA-mediated protein kinase capable of phosphorylating the α subunit of the protein synthesis initiation factor eIF2.

The 2-5A system is,therefore, rendered ineffective in control but not in interferon-treated cells by inactivation of the 2-5A-dependent nuclease in response to EMC virus infection. This may provide an explanation for the fact that EMC virus replication can occur in control HeLa and L-cells in the presence of low levels of 2-5A and the related paradox that EMC virus grows well in control HeLa cells despite their unusually high levels of 2-5A synthetase (9 and R.H.Silverman, P.J.C., M.K. and I.M.K. in preparation). It now appears that it may be the prevention of the virus-mediated inhibition of the 2-5A-dependent nuclease rather than the induction of the 2-5A synthetase which is crucial for interferon to be effective in activating the 2-5A system in these cells.

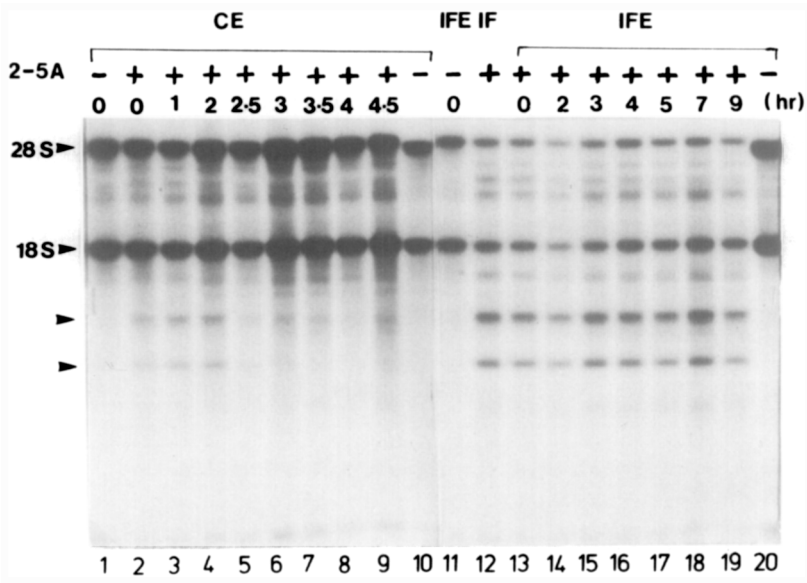

Fig.3. 2-5A-dependent nuclease activity in extracts from interferon-treated and control L-cells with time post infection with EMC virus. Extracts (S10s) were prepared at the times post infection indicated on the Figure : CE, control EMC infected; IF, interferon treated; IFE interferon-treated, EMC-infected. Samples (25µl) were incubated for 2hr at 30°C with ^{32}P-labelled L-cell ribosomes (0.2µg, 140,000 cpm) and 70nM ppp(A2'p)$_2$A where indicated (2-5A+) and the RNA extracted, denatured and analysed on agarose gels (Methods). An autoradiograph of the dried gel is presented. The 18S and 28S rRNAs and the two most prominent 2-5A mediated rRNA cleavage products are indicated on the left of the Figure. Residual cleavage observed late in virus infection (lanes 6-9) reflects carry over of a low level of 2-5A-dependent nuclease with the added ^{32}P-labelled ribosomes from control cells. No such cleavage was observed if ^{32}P-labelled ribosomes from infected cells were used in which the nuclease had been inactivated. The use of the latter type of ribosomes gave identical results to those presented in lanes 12-19 with extracts from interferon-treated EMC virus-infected cells. Interferon treatment consistently results in a higher level of cleavage in response to 2-5A (compare lanes 2 and 13).

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