PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

The Antiviral Action of Interferon [and Discussion]

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The antiviral action of interferon

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On interferon treatment cells develop an antiviral state. This requires time and RNA and protein synthesis. At least six polypeptides and two enzymes have been reported to be synthesized in increased amounts in response to interferon and a multiplicity of effects have been attributed to it. Interferon has been reported to inhibit virus growth at the level of the uncoating of the virus, virus RNA and protein synthesis and virus maturation. This has led to the acceptance of a multisite model for interferon action. The evidence for this and for the role of two known interferon-mediated enzymes, the 2-5A synthetase and protein kinase, are reviewed.

Introduction

The title 'Interferon twenty-five years on' invites retrospection. In this paper we shall therefore give a very brief outline of how work on the antiviral action of interferon has developed, survey the current state of knowledge and review our own work on the unusual oligonucleotide ppp(A2′p)$_n$A (referred to as 2-5A), its mode of action, role in interferon-treated, virus-infected cells and possible wider significance in the control of cell metabolism.

Early work on the mechanism of action

Early work in Alick Isaacs's laboratory at the National Institute for Medical Research at Mill Hill demonstrated that on interferon treatment cells develop an antiviral state in which the replication of a wide spectrum of viruses is inhibited. The work of others in Isaacs's laboratory, notably Joyce Taylor and R. M. Friedman, showed that both RNA and protein synthesis are required for the development of the antiviral state. Elsewhere it was shown that interferon inhibits virus growth in cells infected with infectious RNA, establishing that, in these circumstances at least, inhibition must be occurring at a point beyond the entry and uncoating of the virus. Consistent with this, all stages in the replication of the positive-strand RNA viruses, for example Semliki Forest virus (SFV), were found to be inhibited, least effect being seen early with a cumulative inhibition through the growth cycle. Both viral RNA and protein synthesis were inhibited: because of their interdependent nature, however, it was impossible to distinguish upon which interferon was having the primary effect. Meanwhile, work had also begun on the DNA viruses. With the SV40 system it was shown that interferon could inhibit viral transformation but had no obvious effect once transformation was established. Moreover, although interferon inhibited the expression of SV40 T-antigen in a lytic infection, the expression of this antigen in the transformed cell was not affected. Thus production of the same antigen was being recognised in a lytic infection as 'viral' but in the transformed cell as 'host'. This was taken to reflect the integration of the SV40 DNA into the host genome in the transformed cell and the ability of the interferon-treated cell to distinguish between information of host and viral origin. In 1966 Joklik & Merigan showed that in the interferon-treated, vaccinia virus-infected

cell early viral messenger RNA was made but did not combine with ribosomes to form polysomes and was presumably not translated. That protein synthesis is indeed inhibited in this system was demonstrated some years later by Metz & Esteban (1972). On the other hand the work of several groups with the negative-strand RNA virus VSV and of others with SV40 indicated that it was the transcription of early viral RNA that was inhibited. In the former case this implies the inhibition of RNA-dependent RNA-transcription by a viral enzyme, in the latter, DNA-dependent RNA transcription by a host enzyme. Despite considerable progress, particularly over the last few years, we still have no clear view of the relative importance of these different apparent effects on viral RNA and protein synthesis. In fact, work on interferon action has focused mainly on the effects on protein synthesis and changes in the cell membrane. The induction of a number of new proteins in response to interferon has been demonstrated and at least two induced enzymes (the 2-5A synthetase and protein kinase) identified. (Comprehensive reviews of early work on interferon action are provided in Finter (ed.) (1973) and Vilček (1969).)

The mechanism of action of interferon: an overview

Interferon is not directly antiviral. Interferon treatment is always of the cell. It is active in extremely small amounts: only a few molecules per cell appear to be required to trigger the response. It is not yet certain whether interferon has to get into the cell, but the current consensus is that it works from the membrane. After interferon treatment it takes several hours for the antiviral state to develop, during which time there is a requirement for RNA and protein synthesis. The simplest interpretation of the data is that interferon induces cells to produce proteins that directly or indirectly inhibit virus growth. In fact, interferon treatment may switch on the expression of a whole bank of genes.

There are at least three antigenically distinct types of human interferon: leucocyte, fibroblast, and immune (in the old terminology; now IFNs α, β and γ). Indeed, the results of gene analysis and protein sequencing have shown that there are at least eight human leucocyte interferon genes, none of which have introns and all of which can be expressed in *Escherichia coli* (Allen & Fantes 1980; Nagata *et al.* 1980). Until recently it has been possible to say that to the limited extent investigated the different interferons appear to have the same mechanism of action. With IFN-γ, however, there are indications of differences early in the sequence of events, for example in the cell surface receptor and initial stages in the development of the antiviral state (Ankel *et al.* 1980; Branca & Baglioni 1981; Dianzani *et al.* 1980; Hovanessian *et al.* 1980; Rubin & Gupta 1980). In addition, the different IFN-α's are differently active on heterologous cells (see, for example, Weck *et al.* 1981). The diversity of interferon molecules may therefore be of importance in conferring tissue specificity. The differences may ultimately go beyond this and we may find ourselves contending with a true diversity of action. For the moment, however, there are no more than hints of this (Weck *et al.* 1981). It is therefore generally accepted that the fundamental biochemical mechanisms are likely to be the same and we shall make that assumption here.

Over the years, the work of a number of groups has indicated that in different cell virus systems the antiviral effect of interferon can be expressed at a number of levels. In addition to the effects on viral RNA and protein synthesis already mentioned, interferon has been reported to affect the methylation of viral and host RNA (Kahana *et al.* 1981; Sen *et al.* 1977) and the

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

maturation and release of enveloped viruses (reviewed in Friedman (1979)). In addition, despite the results with infectious RNA, it may yet turn out that interferon can affect uncoating. For example, the effects on early messenger RNA synthesis in the SV40-infected cell may ultimately be attributed to a phenomenon of this type. Multisite models for interferon action are therefore now accepted in which any one, or more than one, of these different possible mechanisms may play a role in a given cell–virus situation. In accord with this a cell can be in an antiviral state for one virus but not for another (Nilsen *et al.* 1980).

Moreover, it is now accepted that interferon is not just an antiviral agent. It induces changes in the cell membrane, in cell size and the cytoskeleton and it inhibits the growth of many cell lines (reviewed in Friedman (1979) and Taylor-Papadimitriou (1980)). For example, Pfeffer *et al.* (1980) have shown that pure human fibroblast interferon, as well as inhibiting the growth of human fibroblasts, affects cell size and the cytoskeleton, there being a marked increase in actin fibres and in the fibronectin network and a decrease in cell motility and ruffling. There also appears to be a multiplicity of changes in the cell membrane. These include an increase in the expression of HLA antigens and net negative charge, an inhibition of cap formation and of membrane fluidity, decreased expression of carbohydrate moieties and changes in the ratio of protein to lipid and in the fatty acid composition of the phospholipid (reviewed in Friedman (1979), Pfeffer *et al.* (1981) and Taylor-Papadimitriou (1980)). In accord with the last of these, inhibitors of fatty acid cyclooxygenase and superoxide dismutase can inhibit development of the antiviral state (Pottathil *et al.* 1980, 1981).

Consistent with the changes in the cell membrane is the observation that the maturation of the enveloped viruses is inhibited. This was first observed by a number of groups working with RNA tumour viruses and has now been extended to VSV (Friedman 1979; Maheshwari *et al.* 1980; Pitha *et al.* 1980). It seems that the replication of RNA tumour viruses can be inhibited at at least two levels, one very early before the integration of the provirus and the second at the level of maturation or release. In the latter, virus particles build up at the cell membrane and those that are released have reduced infectivity. In this connection, it appears that there is a defect in glycoprotein synthesis in interferon-treated cells at the same initial step in the pathway as is inhibited by tunicamycin.

The answer to interferon action is therefore not a simple one. More and more one is hearing the analogy with hormone action being drawn and that we should be thinking of the interferons as hormones or growth regulatory factors that also happen to have the ability to induce the antiviral state rather than as purely antiviral agents (for a recent comprehensive review of interferon action see Lengyel (1982)).

The 2-5A and protein kinase systems

Against this rather complex background we decided to investigate the clearcut inhibition of protein synthesis observed in some interferon-treated, virus-infected cells and cell-free systems isolated from them (Friedman *et al.* 1972; Joklik & Merigan 1966; Metz & Esteban 1972). Our involvement with the 2-5A and protein kinase systems arose from our proposal that this type of inhibition might be triggered by the production of viral double-stranded RNA (dsRNA). In accord with this we showed that protein synthesis in cell-free systems from interferon-treated cells is unusually sensitive to inhibition by dsRNA (Kerr *et al.* 1974). Most importantly the dose–response curve for the effect in the cell-free system is identical to that for the antiviral

effect of interferon in the intact cell (Kerr *et al.* 1974). It seemed worth while, therefore, to examine the basis for this phenomenon.

When one incubates an extract from interferon-treated cells with ATP and dsRNA one activates a kinase which by analogy with the original observations in rabbit reticulocyte lysates is thought to phosphorylate the α subunit of eIF2, one of the initiation factors involved in protein synthesis, this phosphorylation contributing to a resultant inhibition of protein synthesis. In addition, one activates an enzyme (2-5A synthetase) that synthesizes 2-5A, which is effective at nanomolar concentrations in the activation of a nuclease with consequent inhibition of protein synthesis (these enzyme systems are reviewed in Baglioni (1979) and Williams & Kerr (1980)).

The kinase has been observed by a number of groups in a variety of different systems and we think it impossible to overemphasize its potential significance in mediating the multiplicity of effects attributed to interferon. The difficulty, as always with a kinase, is to know which phosphorylations to look for and the significance of those that one finds. For this and a variety of other reasons, relatively little work has been done on the kinase and we shall not discuss it further here, beyond pointing out that, from what is known of the requirements for the activation of the two systems, evidence for the operation of the 2-5A system makes it probable that the kinase will also be active. Evidence for the operation of the 2-5A system therefore automatically provides indirect evidence for activation of the kinase. For the remainder we shall be concerned with the 2-5A system, its mechanism of action through the 2-5A-dependent nuclease, the specificity of that nuclease, the wider possible significance of the system, the natural occurrence of 2-5A and its role in interferon-treated and control cells.

First you should know that 2-5A is not a single compound but an oligomeric series in which the trimer to pentamer, at least, are equivalently biologically active at nanomolar concentrations (Kerr & Brown 1978; Martin *et al.* 1979). Its structure has been confirmed by chemical synthesis and n.m.r. analysis (Martin *et al.* 1979) and it is clear from the results of a number of groups that it activates a nuclease. The nuclease is not yet available in a pure state but we have been able to obtain some indication of its probable specificity. The enzyme from rabbit, mouse, or human cells cleaves RNA preferentially on the 3′ side of UpN sequences to yield UpNp terminated products. Cleavage is observed predominantly at UA and UU sequences (Floyd-Smith *et al.* 1981; Wreschner *et al.* 1981*b*). Interestingly, both in the intact cell and cell-free system 2-5A is unstable and the activation of the nuclease is transient in the absence of a 2-5A generating system (Hovanessian *et al.* 1979; Minks *et al.* 1979; Williams *et al.* 1978). Nevertheless, when deliberately introduced into intact cells 2-5A can inhibit protein and DNA synthesis and virus growth (Hovanessian *et al.* 1979; Williams *et al.* 1979*b*).

Wider significance of the 2-5A *system*

We have not yet detected the 2-5A synthetase in *E. coli*, yeast, *Dictyostelium* or *Physarum*, or in the *Drosophila* or *Xenopus* tissues so far examined. It is widespread, however, in animal and avian cells and tissues (Stark *et al.* 1979). In addition, we have recently obtained evidence for the presence of a 2-5A binding protein (presumptive nuclease) in a variety of mammalian and reptilian tissues. There was very low binding activity in amphibian tissues but this remains to be rigorously characterized. None was detected in fish, plants, or lower orders (P. J. Cayley, unpublished). The basal levels of the synthetase present in different cells and tissues is very variable. This basal level can also vary with growth and hormone status, suggesting that the

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

2-5A system may be of wider significance in the control of cell growth development or differentiation (Stark *et al.* 1979). In addition, as was first shown by A. Ball, an enzyme which is almost certainly the synthetase can add AMP in 2′–5′ linkage to a variety of interesting metabolites including NAD, A5′p$_4$5′A, and ADP-ribose. Accordingly we have developed radioimmune (r.i.) and radiobinding (r.b.) assays for 2-5A and these related derivatives (Knight *et al.* 1980).

Radioimmune and radiobinding assays for 2-5A *and related oligonucleotides*

The r.i. assay is based on the affinity of antibody to 'core' (A2′p5′A2′p5′A), the r.b. on the use of what is presumed to be the 2-5A-dependent endonuclease as a 2-5A binding protein. We use a nitrocellulose filter binding assay with 2-5A or 'core' labelled with [^{32}P]pCp at the 3′-terminus as probe. With ppp(A2′p)$_n$A3′-[^{32}P]pCp (n = 2 or 3) and (A2′p)$_2$A3′-[^{32}P]pC at specific activities of 1–3 MCi mol^{-1} the assays are sufficiently sensitive to detect 10 µl of nanomolar (i.e. 10 fmol) 2-5A or 'core'. The r.b. assay has almost the specificity of the nuclease assay. It falls short of this by detecting the 5′-monophosphate (pA2′p5′A2′p5′A) at 1–10 nM and 'core' at about 300 nM. All other nucleotides and oligonucleotides tested were ineffective at concentrations less than 1 mM. Accordingly the r.b. assay appears to have the specificity required to screen crude extracts for 2-5A. Subsequent fractionation of positive extracts is, however, required to determine which of the 2-5A or related components are present. The r.i. assay is not quite as good. Accordingly it is best employed after prior h.p.l.c. (or alternative) fractionation. Under these circumstances it has proved invaluable in the detection of low levels of naturally occurring 'core' (Knight *et al.* 1980).

Natural occurrence of 2-5A *and core*

We have analysed trichloroacetic-acid–ether extracts from both mouse L- and human HeLa cells with and without prior interferon treatment and encephalomyocarditis (EMC) virus infection, using either nuclease activation or the r.b. and r.i. assays for 2-5A (Knight *et al.* 1980; Silverman *et al.* 1982; Williams *et al.* 1979*a*). A comparison of the results obtained before and after h.p.l.c. analysis confirmed that most of the material registering in the r.b. assay of crude extracts was indeed 2-5A (Knight *et al.* 1980). From these results we can conclude that 2-5A occurs naturally in cells in amounts sufficient for it to be playing a part in the antiviral action of interferon. The r.b. assay can also detect nanomolar concentrations of NAD–2-5A, A5′p$_4$5′A–2-5A and ADP-ribose–2-5A. These are distinguishable, however, from 2-5A and 'core' after h.p.l.c. analysis and they were not detected even in experiments in which relatively high levels of 2-5A (more than 200 nM) were found (Cayley & Kerr 1982; Knight *et al.* 1980). Accordingly we can conclude that none of these derivatives is present in the cells examined at concentrations more than 5 nM.

2-5A-*mediated cleavage of ribosomal RNA in interferon-treated cells*

2-5A can therefore occur naturally in interferon-treated cells in amounts sufficient for it to play a part in the antiviral activities of interferon. In addition we now have evidence that the 2-5A-dependent nuclease is indeed active in such cells (Silverman *et al.* 1982; Wreschner *et al.* 1981*a*). On addition to a cell-free system, 2-5A induces a limited cleavage of ribosomal RNA in intact ribosomes yielding a highly characteristic pattern of products. These highly characteristic products are also observed in intact interferon-treated, EMC virus-infected L- or HeLa cells. The identity of the products was originally based on the pattern obtained on

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

electrophoresis on agarose gels (Wreschner *et al.* 1981 *a*). More recently, sequence analysis of the termini of the major products has confirmed the identity of the cleavages occurring in the intact cell and in response to 2-5A in the cell-free system (R. H. Silverman, J. J. Skehel & I. M. Kerr, unpublished). It therefore appears that not only is 2-5A present but the 2-5A-dependent nuclease is also active in these interferon-treated, EMC virus-infected cells.

Inactivation of the 2-5A*-dependent nuclease in response to EMC infection, and prevention of this inactivation by interferon*

HeLa cells have an unusually high level of 2-5A synthetase. In accord with this, 2-5A and limited 2-5A-mediated ribosomal RNA cleavage occur naturally in response to EMC infection in control as well as in interferon-treated cells (Silverman *et al.* 1982). Despite this, in the absence of interferon treatment, EMC grows well in these cells. An explanation for this paradox is provided by the fact that in the absence of interferon treatment the 2-5A-dependent nuclease is lost or inactivated at later times after infection (Silverman *et al.* 1982). Thus in cells that have not been pretreated with interferon the activation of the nuclease in response to infection is transient; limited RNA cleavage occurs but the inactivation of the ribonuclease presumably allows viral RNA replication to take over at later times after infection. Loss or inactivation of the nuclease was not observed in interferon-treated cells where virus replication is inhibited. It therefore seems that in the HeLa cell system, at least, it is the prevention of the virus-mediated inhibition of the 2-5A-dependent nuclease rather than the induction of the 2-5A synthetase that is crucial for interferon to be effective in controlling the activity of the 2-5A system. This emphasizes the more general point that, provided that sufficient synthetase is present for it to be in excess of its activator (presumably dsRNA), its absolute level, and whether or not it is significantly induced in response to interferon, may be irrelevant in determining the overall activity of the system.

The switch-off or inactivation of the 2-5A-dependent nuclease in response to infection (and its prevention by interferon) is not peculiar to HeLa cells, it also occurs in mouse L- and EAT cells (Cayley *et al.* 1982). Nor is it peculiar to EMC: it is also observed, for example, with SFV (P. J. Cayley, unpublished results).

Importance of the 2-5A *system in the antiviral action of interferon*

Putting these data together we have very good evidence that 2-5A is present and the 2-5A-dependent nuclease is active in interferon-treated, EMC virus-infected cells. On the other hand, in the absence of interferon treatment, the 2-5A system appears to be switched off in response to infection. It would be extraordinary if the 2-5A system were not in some way involved in the interferon-mediated inhibition of picornavirus growth in these cells. It must be re-emphasized, however, that the 2-5A system is only one of the mechanisms through which interferon is thought to act, and that evidence for its operation provides indirect evidence that the interferon and dsRNA-mediated protein kinase system is also likely to be active. The relative importance of the 2-5A system in the antiviral action of interferon in different cell–virus systems, therefore still remains to be established.

Conclusion

Concerning interferon action in general, multisite models and analogies with hormone action are currently favoured. The effects on picornavirus replication are thought to involve the 2-5A

system in part at least, those on RNA tumour viruses and VSV, in part, changes in the cell membranes. The roles of the interferon and dsRNA-mediated protein kinase or alternative mechanisms remain to be established. More particularly, with respect to the 2-5A system, the structure of 2-5A has been confirmed; we know that in the cell-free system and intact cell it is unstable and in the absence of a regenerating system transiently activates a nuclease. There is specificity in the degradation of the RNA observed. Cleavages occur on the 3′ side of UpNp doublets and show a high preference for UpA or UpU sequences. When deliberately introduced into intact cells, 2-5A inhibits protein and DNA synthesis and virus growth. It occurs naturally in amounts sufficient for it to play a part in the antiviral activity of interferon and we now have evidence that the 2-5A-mediated nuclease is indeed active in the interferon-treated, EMC virus-infected cell. In addition, in the absence of interferon treatment, the 2-5A system is switched off in response to EMC virus infection. Finally, the wide distribution of the 2-5A-dependent nuclease and 2-5A synthetase and the variation in the level of the latter with growth and hormone status are in accord with a wider possible significance for the 2-5A system in the control of cell growth, differentiation or development.

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Discussion

In discussion several points were made. Non-hydrolysable analogues for 2-5A are being investigated, but it is difficult to find any that are both more stable and sufficiently active. In response to questions from Professor Burke who asked how 2-5A could play a role in non-infected cells since no dsRNA would be present. Dr Kerr said that dsRNA has to have 50 base

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

pairs to be active, but RNA sequences of this size can occur in heterogeneous nuclear RNA as hairpin loops, and even in messenger RNA of normal uninfected cells. Furthermore, low concentrations of 2-5A can be found in cells treated with interferon only. It has been found by Nilsen and Baglioni that RNA with a double-stranded tract was degraded in a cell in which a single-stranded RNA was not; this suggests that there may be local activation of the enzyme, which adds a further complication to analysing the results and looking for correlations.

There was apparently no good correlation between the presence of 2-5A and whether normal or malignant cells are interferon-resistant or not. Some interferon-negative cell lines may be receptor-negative. Dr Kuwata drew attention to his group's poster on the subject.

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